Identification et caractérisation de nouveaux outils pharmacologiques sélectifs pour les différents récepteurs à peptides RF-amides

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IDENTIFICATION ET CARACTERISATION DE NOUVEAUX OUTILS PHARMACOLOGIQUES SELECTIFS POUR LES DIFFERENTS RECEPTEURS A PEPTIDES RF-AMIDES

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RESUME

Les RF-amides (RFRPs, NPFF, QRFP, PrRPs, Kisspeptines) sont une famille de peptides caractérisée par une signature Arg-Phe-NH_2 à l'extrémité C-terminale très conservée au cours de l'évolution. Ils exercent leurs effets *via* 5 récepteurs couplés aux protéines G (NPFF1R, NPFF2R, QRFPR, PrRPR, Kiss1R), et plusieurs études soutiennent leur implication dans la modulation de nombreuses fonctions physiologiques telles que la douleur et la nociception, la reproduction, ou encore le métabolisme. Néanmoins, l'étude de ces systèmes est entravée par l'absence d'outils pharmacologiques tels que des antagonistes sélectifs. C'est pourquoi mon travail de thèse a consisté au développement d'outils pharmacologiques permettant de répondre à ces attentes, particulièrement sur les récepteurs NPFF1R, NPFF2R et Kiss1R. Des études de relation structure-activité nous ont amenés à considérer l'importance de la signature Arg-Phe-NH_2 dans l'activité des peptides RF-amides sur leurs récepteurs. L'introduction de modifications au niveau N-terminal ou C-terminal du dipeptide Arg-Phe-NH_2 nous a conduit à l'identification d'un antagoniste hautement affin et sélectif pour le récepteur NPFF1R *in vitro* et *in vivo*. Ce dernier nous a permis d'identifier le rôle du récepteur NPFF1R dans les effets secondaires liés à l'administration d'opioïdes, tels que l'hyperalgésie et la tolérance morphinique. La responsabilité du récepteur NPFF1R dans ces phénomènes a été par la suite confirmée chez des souris KO NPFF1R. De plus, des données plus récentes suggèrent l'importance de ce dernier au niveau de l'axe hypothalamo-hypophyso-gonadotropique (HHG) des animaux saisonniers, et en particulier du hamster. Notre antagoniste nous a permis de déterminer le rôle du récepteur NPFF1R dans la libération de LH induite par le RFRP-3. Pour la première fois, nous avons également mis en évidence des molécules hautement affines et sélectives pour le récepteur NPFF2R, pour lequel nous avons révélé une activité agoniste partielle *in vitro*. Mon travail a également mené à la caractérisation *in vitro* d'un antagoniste sélectif du récepteur Kiss1R, qui vient compléter l'ensemble des outils disponibles pour l'étude des fonctions physiologiques modulées par ce récepteur et son ligand, la kisspeptine.

Dans l'ensemble, mon travail de thèse a permis de caractériser plusieurs ligands affins et sélectifs des récepteurs à peptides RF-amides, et de mettre en lumière le rôle du système RFRP-3/NPFF1R dans les effets à long-terme liés aux opioïdes.

Mots-clés : peptides RF-amides, RFRP-3, NPFF, NPFF1R, NPFF2R, Kiss1R, nociception, hyperalgésie, antagoniste, axe HHG

ABSTRACT

RF-amides (RFRPs, NPFF, QRFP, PrRPs, Kisspeptins) belong to a family of peptide characterized by a Arg-Phe-NH_2 C-terminus highly conserved throughout the evolution. They target 5 G-protein coupled receptors (NPFF1R, NPFF2R, QRFPR, PrRPR, Kiss1R) and most studies highlight their roles in the modulation of various functions as pain and nociception, reproduction or metabolism. Nonetheless, the study of these systems is severely limited by the absence of pharmacological tools as selective antagonists. Thus, my PhD project consisted in the development of selective ligands to answer these questions, notably on NPFF1R, NPFF2R and Kiss1R receptors. Structure-Activity relationship studies highlighted the importance of Arg-Phe-NH_2 signature for the activity of RF-amide peptides on their receptors. Introduction of modifications at N or C-terminus of Arg-Phe-NH_2 dipeptide led us to the identification of a compound displaying high affinity, selectivity and antagonist activity for NPFF1R both *in vitro* and *in vivo*. This compound allowed us to identify the critical role played by NPFF1R in the secondary effects related to opiates administration, as hyperalgesia and analgesic tolerance. The involvement of NPFF1R was then confirmed in KO NPFF1R mice. Moreover, recent data suggest the importance of NPFF1R on hypothalamo-pituitary gonadotropic (HPG) axis of seasonnal animals, and particularly of hamsters. Our antagonist allowed us to decipher the role of NPFF1R in RFRP-3-induced-LH release. For the first time, we also have characterized high affinity and selective compounds for NPFF2R that display partial agonist activity *in vitro*. Moreover, our work led to the *in vitro* characterization of a selective antagonist for Kiss1R, that complete the available tools to study the physiological functions modulated by this receptor and its ligand, the kisspeptin.

Overall, my PhD thesis led to the characterization of several selective ligands for RF-amide receptors, and highlight the role of RFRP-3/NPFF1R system in the long-term effects associated to opiates.

Key-words: RFamide peptides, RFRP-3, NPFF, NPFF1R, NPFF2R, Kiss1R, nociception, hyperalgesia, antagonist, HPG axis

LISTE DES ABREVIATIONS

AC :	adénylyl cyclase
ACTH :	hormone corticotrope
AGH :	aminoguanidine hydrazone
AMPA :	acide 2-amino-3-(5-méthyl-3-hydroxy-1,2-oxazol-4-yl)propanoïque
AMPc :	adénosine monophosphate cyclique
ANOVA :	*analyse of variance*
cAR :	récepteurs de l'AMPc
ARC :	noyau arqué
ARNm :	acide ribonucléique messager
ATP :	adénosine triphosphate
AUC :	*area under the curve*
β_2AR :	récepteur β_2-Adrénergique
BDNF :	*brain-derived neurotrophic factor*
BHE :	barrière hémato-encéphalique
BRET :	*bioluminescence resonance energy transfer*
CAMKII :	*calcium/calmodulin-dependant protein kinase II*
CHO :	*chinese hamster ovary*
CPP :	préférence de place conditionnée
CRH :	corticolibérine
aCSF :	*artificial cerebrospinal fluid*
CTX :	*cholera Pertussis Toxin*
CxCR :	*C-X-C chemokine receptor*
CXCL12 :	*C-X-C chemokine ligand type 12*
DAG :	diacylglycerol
DAMGO :	[D-Ala^2, N-MePhe^4, Gly-ol]-*enkephalin*
Dio3 :	déiodinase 3
DMH :	*dorsomedial hypothalamus*
DRG :	*dorsal root ganglia*
EAAT3 :	*excitatory amino acid transporter 3*
EPSC :	*excitatory postsynaptic current*

hERG : *human Ether-à-go-go-related gene*

ERK : *extracellular signal-regulated kinase*

FAK : *focal adhesion kinase*

FaRPs : *FMRFamide-related peptides*

FRET : *förster resonance energy transfer*

FSH : *follicle stimulating hormone*

GABA : *gamma-aminobutyric acid*

GDP : guanine diphosphate

GIRK : *G-protein-coupled-inwardly-rectifying potassium channel*

GLAST : *glutamate aspartate transporter*

GLT-1 : *glutamate transporter 1*

GnIH : *gonadotropin inhibitory hormone*

GnRH : *gonadotropin releasing hormone*

GRK : *G-protein coupled receptor kinase*

GTP : guanine triphosphate

H : hydrogène

HEK : *human embryonic kidney*

HIO : hyperalgésie induite par les opioïdes

HHG : hypothalamo-hypophyso-gonadotropique

ICV : intracérébroventriculaire

IL1-β : interleukine 1-β

IP3 : inositol triphosphate

ISH : *in situ hybridization*

KCC2 : *K^+/Cl^- cotransporter 2*

KO : *knock out*

Kp : kisspeptine

LH : *luteinizing hormone*

LP : *long photoperiod*

LTP : *long-term potentiation*

MAP kinase : *mitogen-activated protein kinase*

MMP-9 : *matrix metallopeptidase 9*

Mrgs : *mas-related genes*

α-MSH : *α-melanocyte stimulating hormone*

NMDA : acide N-méthyl-D-aspartique

NOP : *nociceptin/orphanin* FQ (N/OFQ)

dNPA : D.Asn-Pro-(*N*-Me)-Ala-Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2

NPFF : neuropeptide FF

NPFF1R : *neuropeptide FF receptor 1*

NPFF2R : *neuropeptide FF receptor 2*

NPY : neuropeptide Y

Orn : ornithine

OT : ocytocine

PACAP : *pituitary adenylate cyclase-activating peptide*

PAG : *periaqueductal grey*

PENK : *proenkephalin*

PFA : paraformaldéhyde

Pip : pipéridine

PIP2 : phosphatidylinositol 4,5-biphosphate

PKA : protéine kinase A

PKC : protéine kinase C

PLC : phospholipase C

POA : *preoptic area*

POMC : *proopiomelanocortin*

PRL : prolactine

PrRP : *prolactine-releasing peptide*

PrRPR : *prolactine-releasing peptide receptor*

PTH : parathormone

PTX : *pertussis toxin*

QRFP : *pyroglutamylated RF-amide peptide*

QRFPR : *pyroglutamylated RF-amide peptide receptor*

RCPG(s) : récepteur(s) couplé(s) aux protéines G

RFRP-3 : *RF-amide related peptide 3*

RGS : *regulator of G protein signaling family*

RhoGEF : *Rho guanine nucleotide exchange factor*

RVM : medulla rostrale ventro-médiale

SAR : *structure activity relationship*

s.c. :	sous-cutanée
SNC :	système nerveux central
SP :	*short photoperiod*
TH :	hormone thyroïdienne
TM :	domaine transmembranaire
TNFα :	*tumor necrosis α*
mTOR :	*mammalian target of rapamycin*
TRH :	Hormone thyréotrope
TrkB :	*tropomyosin receptor kinase B*
TRPV1 :	*transient receptor potential vanilloide 1*
TTX :	tétrodotoxine
VFT :	*venus flytrap*
VIP :	*vasoactive intestinal peptide*
WDR :	*wide dynamic range*
WT :	*wild Type*

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INTRODUCTION

Mes travaux de thèse s'articulent autour de la recherche de ligands spécifiques des récepteurs NPFF1R, NPFF2R et Kiss1R, qui sont des récepteurs couplés aux protéines G (RCPGs) dont de nombreuses études révèlent l'implication dans différentes modalités physiologiques, telles que la nociception, la douleur et la reproduction. C'est pourquoi j'aborderai dans l'introduction les propriétés relatives aux RCPGs, et le rôle des RF-amides et de leurs récepteurs aux niveaux moléculaire, cellulaire et physiologique ; ce point étant illustré par une revue que nous avons écrite au laboratoire (Quillet *et al.*, 2016). Je décrirai également les mécanismes neuronaux impliqués dans la douleur, les systèmes opioïdes et les effets iatrogènes des opioïdes, *via* notamment leurs interactions avec les systèmes RF-amides. Enfin, j'insisterai sur les études de relations structure-activité effectuées sur les différents peptides RF-amides, et le développement de ligands résultant de ces travaux.

1. Les récepteurs couplés aux protéines G

Afin d'être informés des besoins nécessaires à leur propre survie, et dans le but de perpétuer la descendance de l'espèce, les échanges et la communication entre les cellules et leur environnement sont essentiels aux organismes pluricellulaires. Ce dialogue entre cytosol et milieu extracellulaire est réalisé grâce à des protéines présentes à la surface membranaire, telles que les canaux ioniques, les récepteurs à activité enzymatique intrinsèque ou encore les récepteurs couplés aux protéines G (RCPGs). Parmi les molécules présentes à la surface des cellules, les RCPGs sont les plus riches en termes d'interaction avec des ligands et de réponse fonctionnelle intracellulaire. Chez des espèces primitives comme les levures ou les amibes, la famille des RCPGs est pionnière en matière de transduction du signal, et plus de 1% du génome des Vertébrés y est consacré (Devreotes, 1994; Dohlman *et al.*, 1991). En effet, ils sont capables d'être reconnus par différents signaux tels que les photons, les nucléotides, les lipides ou encore les protéines. Dans le génome humain, il existe plus de 800 gènes codant pour des RCPGs, impliqués dans une variété de fonctions telles que les fonctions cardio-vasculaires, la neurotransmission, la douleur ou encore les régulations hormonales (Fredriksson *et al.*, 2003). Par ailleurs, plus de la moitié des médicaments cible un élément engagé dans les voies de signalisation activées par les RCPGs. C'est pourquoi ils représentent aujourd'hui un enjeu majeur, à la fois dans le domaine fondamental, mais également en clinique. C'est notamment le cas des récepteurs aux peptides RF-amides et des récepteurs aux opioïdes qui nous intéressent dans cette étude.

1.1. Les différentes familles de RCPGs

Il existe trois principales familles de RCPGs, qui ont en commun un domaine central constitué de sept domaines transmembranaires (TM-I à TM-VII), reliés entre eux par trois boucles intracellulaires (i1, i2, i3) et trois boucles extracellulaires (e1, e2, e3). De manière générale, les domaines extracellulaires 1 et 2 sont connectés entre eux par un pont disulfure reliant deux cystéines, important pour la stabilisation d'un certain nombre de conformations. En revanche, ces différentes familles de RCPGs diffèrent par leur séquence, leur longueur et la fonction de leur domaine N-terminal extracellulaire et leur domaine C-terminal intracellulaire, ainsi que par leurs boucles intracellulaires, ce qui leur confère des propriétés spécifiques (Bockaert et Pin, 1999 ; Heifetz *et al.*, 2015). Alors que les RCPGs de la famille 1a et 1b sont activés par de petits ligands interagissant respectivement avec une cavité formée entre le TM-III et le TM-VI (ex : catécholamines, opioïdes, enképhalines, anandamide) ou les boucles extracellulaires et le domaine N-terminal (ex : peptides, cytokines, thrombine), les récepteurs de la famille 1c et 2 sont activés par de plus gros peptides (ex : les hormones ou les glycoprotéines pour la famille 1c et la PTH, PACAP, VIP, GnRH pour le groupe 2) *via* leur interaction avec e1 et e3 et possèdent un plus long domaine N-terminal. La troisième famille, dont les récepteurs phares sont les récepteurs métabotropiques au glutamate (mGluR) et $GABA_BR$, sont constitués au niveau extracellulaire de deux lobes séparés par une région charnière, baptisés « *Venus Flytrap* », en référence à cette plante carnivore décrite par le naturaliste irlandais John Ellis. Par ailleurs, la famille 4 regroupe les récepteurs aux phéromones (Dulac et Axel, 1995), et la famille 5 réunit les récepteurs de type « *smoothened* » et « *frizzled* », impliqués dans le développement embryonnaire de la drosophile. Enfin, les récepteurs de l'AMPc (cAR) constituent une sixième famille et n'ont été retrouvés que chez l'amibe *Dictyostelium discoideum* (Figure 1).

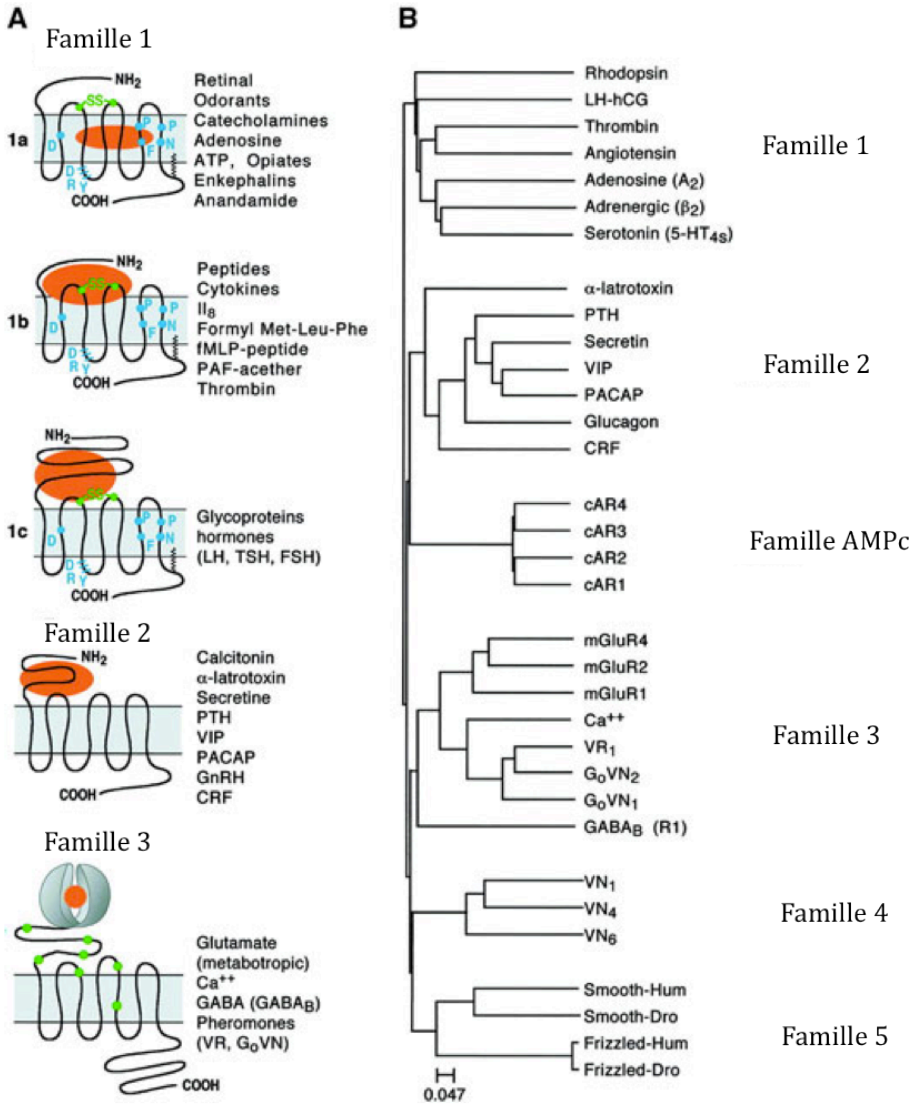

Figure 1 : Classification et diversité des RCPGs*. (A) Les RCPGs sont classés en 3 principales familles et se distinguent notamment par leur structure et la nature des ligands dont ils sont les cibles. (B) Dendrogramme illustrant l'évolution indépendante des différentes familles de RCPGs. D'après Bockaert et Pin, 1999.*

1.2. Dynamique des RCPGs

Bien qu'ancrés au sein de la bicouche lipidique des membranes cellulaires, les récepteurs couplés aux protéines G sont dynamiques et se meuvent en fonction des signaux captés dans l'environnement extracellulaire, ou intracellulaire (Latorraca *et al.*, 2016). Ils peuvent adopter plusieurs conformations, qualifiées d'actives ou inactives selon les fonctions qui en découlent. Cette transformation est associée à un changement d'orientation entre les domaines transmembranaires III et VI, engendré par une rotation du TM-VI provoquant sa séparation du TM-III, et démasquant ainsi le site

de liaison de la protéine G. En effet, les boucles intracellulaires i2 et i3, qui sont directement associées à ces deux TMs sont des sites clés dans la reconnaissance et l'activation des protéines G, initiatrices des voies de signalisation intracellulaires. La conformation active de ces récepteurs entraîne la liaison au niveau intracytoplasmique de différentes protéines dont notamment les protéines G, les GRKs (*G-protein coupled receptors kinases*) et les arrestines, qui se fixent préférentiellement sur les récepteurs phosphorylés. Des études biophysiques ont montré qu'il existait plusieurs états de conformations actives et inactives des récepteurs, notamment grâce à l'étude du récepteur β_2-adrénergique (β_2AR, Manglik *et al.*, 2015, figure 2).

Le monde des RCPGs est une sphère dynamique qui offre une richesse et une complexité de conversations entre les milieux extra- et intracellulaire. Les ligands canoniques ne suffisent plus pour décrire leur fonctionnement, et la notion de ligands biaisés émerge depuis ces dernières années. Le modèle classique d'activation d'un récepteur par son ligand consiste en la genèse d'une cascade de signalisation *via* l'intermédiaire de la protéine G, suivie d'une phosphorylation par les GRKs et d'un recrutement des arrestines en vue d'un recyclage et de la désensibilisation des récepteurs. Mais d'une manière plus complexe, certains ligands sont capables d'activer un récepteur de telle façon que le recrutement de la protéine G n'induise pas de désensibilisation, alors que d'autres pourraient induire une cascade de signalisation *via* le recrutement direct des arrestines.

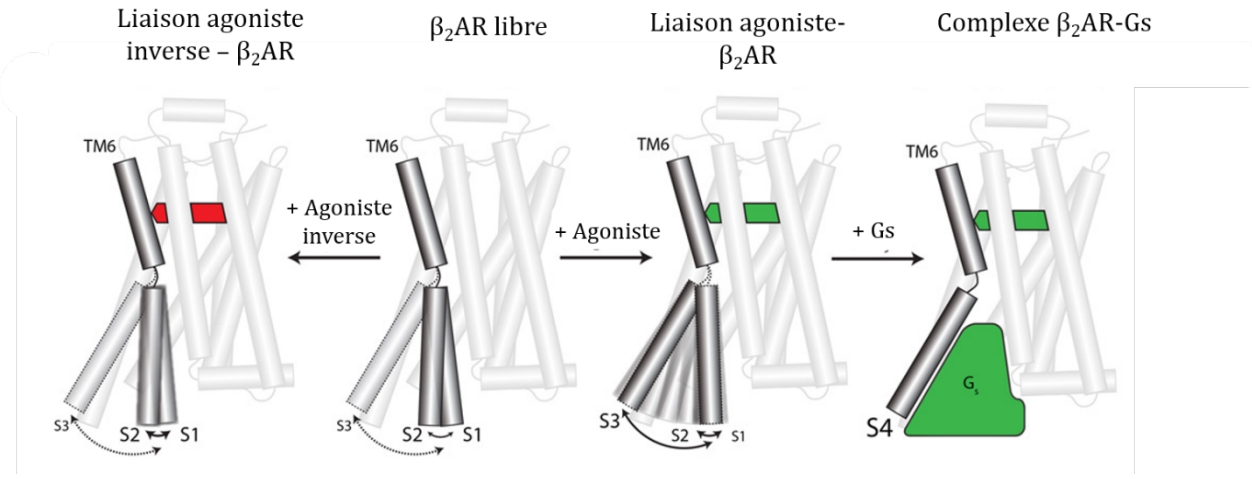

Figure 2 : Dynamique du récepteur β_2AR. *Sous forme libre, le récepteur est dans un état inactif (S1-S2) mais dynamique. La liaison d'un agoniste entraîne un changement de conformation, impliquant un mouvement du TM-VI vers un état intermédiaire (S3). La liaison de la protéine G engage l'état S3 pour stabiliser la conformation totalement active (S4). Un agoniste inverse favorise les permutations conformationnelles S1-S2. D'après Manglik et al., 2015.*

1.3. Cascade de signalisation intracellulaire des RCPGs

De manière classique, l'état actif d'un RCPG promeut le recrutement des protéines G hétérotrimériques, composées d'une sous-unité Gα et du dimère Gβγ (Furness et Sexton, 2017). Dans son état inactif, la protéine Gα est liée au GDP. Son interaction avec un récepteur activé entraîne un échange de nucléotide, *via* le remplacement du GDP par le GTP. La liaison de la molécule de GTP induit un changement de conformation de la protéine Gα, ce qui modifie l'orientation entre la sous-unité α et le dimère βγ, permettant à chacun d'agir sur d'autres messagers intracellulaires en aval (Figure 3). Gα est capable d'interagir avec l'adénylyl cyclase (AC), la phospholipase C (PLC) ou les RhoGEFs, qui modifient à leur tour l'activité d'autres molécules cibles. Les protéines Gβγ peuvent recruter des protéines à la membrane plasmique telles que les GRKs ou directement moduler l'activité de canaux ioniques, kinases, ou phospholipases afin de produire des réponses cellulaires. Leur fonction est surtout remarquable au niveau du système nerveux central, où elles stimulent les canaux potassiques de la rectification entrante (GIRK) (Yamada *et al.*, 1998) et inhibent l'ouverture des canaux calciques voltage dépendants de type N (Dascal, 2001). Cet enchaînement conduit à la libération de neurotransmetteurs au niveau des synapses, et concerne notamment le système opioïde, en particulier dans les phénomènes d'analgésie.

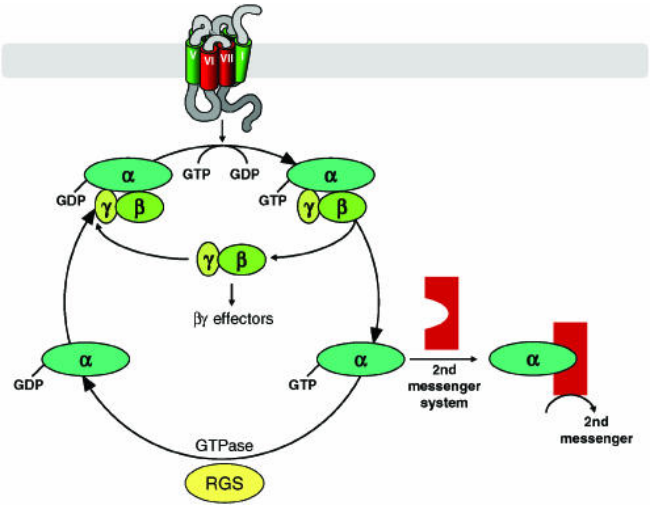

Figure 3 : Cycle d'activation des protéines G*. Sous forme inactive, la protéine G hétérotrimérique est liée au GDP. Son activation permet l'échange du GDP en GTP. La liaison du GTP entraîne un changement de conformation qui provoque la séparation de la sous-unité α liée au GTP et du complexe βγ. L'activité GTPase intrinsèque de la sous-unité α hydrolyse le GTP pour clore le signal. Les RGS (regulators of G-proteins) permettent d'accélérer ce processus. La réassociation de Gα-GDP et du complexe βγ met un terme à l'activation des effecteurs régulés par les sous-unités βγ et complète le cycle. D'après (Milligan et Kostenis, 2006).*

Il existe chez l'Homme 21 types de sous-unités α qui peuvent être classées en 4 familles en fonction de leur homologie de séquence : $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ et $G\alpha_{12/13}$. La liaison du GTP à la protéine Gα provoque une amélioration de l'affinité pour ses effecteurs. Parmi eux, l'AC catalyse la conversion de l'ATP en AMP cyclique (AMPc), qui peut à son tour agir comme un second messager et permettre l'activation d'enzymes telles que la protéine kinase A (PKA). Les sous-unités $G\alpha_s$ et $G\alpha_i$ régulent de manière opposée l'AC, la première en l'activant, et la seconde en inhibant son effet catalytique. Ces deux fonctions contraires ont été mises en évidence grâce à l'utilisation de toxines responsables du choléra (*cholera toxin*, CTX) et de la coqueluche (*pertussis toxin*, PTX). La CTX bloque la protéine $G\alpha_s$ dans un état actif suite à l'ADP-ribosylation d'un résidu arginine, alors que l'ADP-ribosylation d'un résidu cystéine par la PTX bloque la sous-unité $G\alpha_{i/o}$ dans un état inactif (Bokoch et Gilman, 1984 ; Huff *et al.*, 1985). D'autre part, la sous-unité $G\alpha_q$ active la phospholipase C (PLC), responsable de l'hydrolyse du PIP_2 (phosphatidylinosito 4,5 bisphosphate) en IP3 (Inositol 1,4,5 triphosphate) et DAG (diacylglycerol). A leur tour, l'IP3 et le DAG stimulent respectivement la libération de calcium à partir des réserves intracellulaires, ainsi que différents isoformes de la protéine kinase C (PKC). Enfin, les sous-unités Gα et Gβγ peuvent aussi indirectement activer la voie des MAP Kinases (*Mitogen Activates Protein Kinases* ; Milligan et Kostenis, 2006). Par ailleurs, certains récepteurs couplés aux protéines G sont capables d'interagir physiquement avec un même récepteur ou un autre RCPG, pour induire une réponse fonctionnelle. C'est ce que l'on appelle la dimérisation. Elle est soit alternative à l'activation d'un récepteur, soit nécessaire, comme c'est le cas pour les RCPGs de classe C (Geng *et al.*, 2013).

1.4. Pharmacologie des RCPGs

Au début du XX^{ème} siècle, le scientifique allemand Paul Ehrlich est le premier à donner naissance au concept d'interaction entre substances chimiques et récepteurs spécifiques présents à la surface des cellules. A la même époque à Cambridge, John Langley développe le terme de « *receptive substance* ». Les différents pères de la pharmacologie des RCPGs, que sont Alfred Joseph Clark, John Gaddum, Robert Stephenson et Heinz Schild, introduisent quant à eux les concepts de puissance et d'efficacité. Ils développent notamment des méthodes permettant la mesure et la quantification des réponses fonctionnelles générées par les agonistes et des effets des antagonistes au sein de tissus isolés (Arunlakshana et Schild, 1959 ; Stephenson, 1956). Ils transposent le concept de la loi d'action de masse développée par les deux chimistes norvégiens Cato Guldberg et Peter Waage en 1867, aux réponses fonctionnelles doses-dépendantes consécutives à la liaison ligand-récepteur, pour en déduire ainsi l'activité agoniste ou antagoniste. Lorsque les vitesses de réactions directes ou inverses (association/dissociation) sont égales, le système est dit « à l'équilibre ». Dans ce cas, la loi d'action des masses énonce que les concentrations des réactifs de départ et des produits formés sont reliées par une constante à une température donnée. Il peut exister plusieurs sites de reconnaissance des ligands sur le

récepteur. Les sites sur lesquels se fixent les ligands endogènes ou canoniques sont appelés sites primaires ou orthostériques, à la différence de sites allostériques qui sont reconnus par d'autres ligands, et localisés sur une autre partie du récepteur. La constante d'un système réactionnel à l'équilibre s'écrit :

$$L + R \underset{}{\overset{K_1}{\rightleftharpoons}} LR$$

Un récepteur R et son ligand L s'associent en complexe LR à une vitesse K_1.

Les différentes nomenclatures utilisées pour décrire les multiples rôles que peuvent endosser les ligands sont répertoriées dans la revue de Richard Neubig et collaborateurs (Neubig *et al.*, 2003). La liaison d'un agoniste entraîne un changement de l'état du récepteur vers une conformation active pour induire une réponse biologique, proportionnelle à la dose appliquée. En revanche, un agoniste inverse réduit la réponse biologique d'un récepteur constitutif, c'est-à-dire activé en l'absence de ligand. Lorsqu'un composé est dépourvu de tout effet propre, mais empêche la liaison de l'agoniste, bloquant ainsi son effet, on l'appelle antagoniste. Il est compétitif si son site de liaison est le même que le ligand dont il bloque l'action, et non-compétitif si ce site est différent (allostérique). L'effet d'un agoniste peut être modifié de manière positive ou négative grâce à la fixation d'un modulateur allostérique, qui peut jouer soit sur l'affinité du ligand, soit sur son efficacité. La relation entre la concentration d'un agoniste et son effet, une fois lié au récepteur, se traduit par l'équation de Hill :

$$\frac{E}{E_{max}} = \frac{[A]^{nH}}{[A]^{nH} + [A]_{50}{}^{nH}}$$

E est l'effet produit par un agoniste A, à une concentration [A]. Emax est l'effet maximal de A, nH est la constante de Hill, et $[A]_{50}$ est la concentration qui produit 50% de Emax.

La puissance d'un agoniste est décrite expérimentalement par son EC_{50}, la concentration à laquelle la moitié de l'effet maximal (efficacité) est obtenue. Si l'agoniste induit la réponse maximale que peut fournir une cellule ou un tissu, il est dit total. En revanche, un agoniste partiel est incapable de provoquer un effet aussi grand que le maximum produit par un agoniste total. La qualité d'un agoniste

dépend du système considéré, et un agoniste complet dans un contexte peut devenir partiel dans un environnement différent. La puissance d'un antagoniste est quant à elle représentée par le pA_2, le logarithme décimal négatif de la concentration molaire de l'antagoniste (K_B) qui nécessite de doubler la concentration d'agoniste pour obtenir le même effet (Schild, 1947, 1949). Pour appliquer cette équation, certaines conditions doivent être respectées, comme l'équilibre de la réaction (afin d'appliquer la loi d'action des masses), l'uniformité de la population de récepteurs cibles, ou encore une compétitivité entre l'agoniste et l'antagoniste (*i.e.* un site de reconnaissance identique sur le récepteur). Dans ces conditions, la pente de la droite permettant d'obtenir le pA_2 est égale à 1, et le K_B doit être égal à la constante de dissociation de l'antagoniste à l'équilibre (K_i). L'obtention d'un pA_2 passe par la détermination des EC_{50} de l'agoniste seul, et en présence d'une concentration croissante d'antagoniste. Dans une situation d'antagonisme compétitif, la liaison de l'agoniste exclut celle de l'antagoniste, et *vice versa*, car ils entrent en compétition pour le même site de liaison. Par contre, lorsque l'agoniste et l'antagoniste ciblent deux sites de liaison différents sur un même récepteur et peuvent ainsi s'y lier de manière simultanée, nous avons affaire à de l'antagonisme non-compétitif. Dans ce cas de figure, la pente de la courbe représentant le pA_2 est différente de 1, et l'efficacité maximale entre la réponse engendrée par l'agoniste en présence d'antagoniste n'atteint pas celle obtenue avec l'agoniste seul.

Les RCPGs sont la cible d'environ 30 % de médicaments commercialisés actuellement. C'est pourquoi ils représentent un centre d'intérêt commun dans plusieurs domaines de la recherche fondamentale et clinique. Le développement de ligands des récepteurs couplés aux protéines G est une des stratégies utilisées dans la compréhension des mécanismes fins des systèmes moléculaires associés à leur activation. De par la multiplicité et la complexité des fonctions qu'ils modulent, les récepteurs à peptides RF-amides, en sont de bons candidats.

2. Les RF-amides dans le règne animal

Les peptides RF-amides sont constitués d'une extrémité C-terminale Arg-Phe-NH_2 très conservée au cours de l'évolution, des Invertébrés les plus primitifs, aux Vertébrés les plus évolués. Cette conservation est un témoignage de l'importance de ces systèmes dans la biologie évolutive des espèces.

2.1 Les systèmes RF-amides chez les Invertébrés

L'histoire des RF-amides débute avec le tétrapeptide Phe-Met-Arg-Phe-NH_2 (FMRF-amide), purifié à partir d'extraits de ganglion de la palourde *Macrocallista nimbosa* (Price et Greenberg, 1977). Depuis cette découverte, des peptides RF-amides ont été identifiés au sein du système nerveux d'un grand nombre de divers Invertébrés (Walker *et al.*, 2009). Les découvertes concernant les RF-amides

chez les Invertébrés deutérostomiens permettent de mieux comprendre la distribution phylogénétique et les origines des neuropeptides RF-amides chez les Vertébrés (Elphick and Mirabeau, 2014). En effet, les études réalisées chez les Invertébrés suggèrent un rôle ancestral de ces peptides dans le développement du système nerveux, et des tissus périphériques, ainsi que dans la régulation centrale de la balance énergétique, le comportement alimentaire ou encore la reproduction (Sandvik *et al.*, 2014). Chez les insectes, les *FMRFamide-related peptides* (FaRPs) interviennent dans les contractions du cœur, du tube digestif et de l'oviducte (Merte et Nichols, 2002).

2.2 Les systèmes RF-amides chez les Vertébrés

Quelques années après la découverte du FMRF-amide chez le mollusque, une équipe identifiait le premier RF-amide de Vertébrés –le LPLRF-amide- dans le cerveau du poulet (Dockray *et al.*, 1983). Plus tard, ce peptide fut considéré comme un fragment de la *Gonadotropin-inhibitory hormone* (GnIH). En 2002, le dernier des RF-amides que l'on connaisse aujourd'hui chez les Vertébrés, le QRFP (alias 26RFa ou 43RFa), est identifié chez l'amphibien (Chartrel *et al.*, 2002).

Chez les Vertébrés, les peptides RF-amides sont divisés en 5 groupes :

i) Le groupe des neuropeptides FF (NPFF, NPAF, NPSF)

ii) Les *Prolactin-Releasing peptides* (PrRP20, PrRP31, *crucian carp RFamide* (C-RFa), *salmon RFa*)

iii) Le groupe des RFRPs (RFRP-1, RFRP-3, *frog growth hormone releasing peptide* (fGRP), *goldfish LPXRFamide peptide*)

iv) Le groupe des kisspeptines, traduits à partir du gène *kiss1* (Kp-10, Kp-13, Kp-14, Kp-54 chez l'Homme et Kp-52 (RYamide) chez la sourit et le rat) et ceux issus du gène *kiss2* uniquement chez les vertébrés non placentaires.

v) La famille des QRFPs (26RFa, 43RFa)

2.2.1. Les systèmes RF-amides chez les mammifères

Depuis la découverte du premier peptide RF-amide mammalien (NPFF ; Yang *et al.*, 1985), plus de trente années de recherche ont mené à l'identification d'une grande famille de neuropeptides caractérisés par leur extrémité C-terminale Arg-Phe-NH_2 et de leurs récepteurs. Une revue à laquelle j'ai contribuée, publiée dans le journal *Pharmacology and Therapeutics* en 2016 et intitulée « *RF-amide neuropeptides and their receptors in Mammals : Pharmacological properties, drug development and main physiological function* » (Quillet *et al.*, 2016) s'intéresse à la synthèse des connaissances actuelles concernant les différents systèmes RF-amides chez les mammifères. En premier lieu, cette revue

propose une description détaillée des peptides RF-amides et de leurs précurseurs, ainsi que des récepteurs aux peptides RF-amides. Cet article aborde également la pharmacologie *in vitro* de ces systèmes, avec un aperçu des propriétés de liaison entre ligands endogènes et leurs récepteurs, ainsi qu'avec les radiotraceurs développés pour les récepteurs à peptides RF-amides. De plus, nous nous sommes attardés sur les différentes voies de signalisation intracellulaires mises en jeu après l'activation des cinq récepteurs, sur les études de relation entre structure et activité (SAR), et enfin sur le rôle de tous ces systèmes dans différentes fonctions physiologiques. Plus particulièrement, notre revue décrit l'expression des protéines et de leurs messagers dans les régions du cerveau impliquées dans le métabolisme et le contrôle de l'appétit, la reproduction et la douleur et la nociception. Pour résumer, il existe 5 familles de neuropeptides RF-amides, dont les principaux représentants sont le neuropeptide FF (NPFF), les *RF-amide related peptide* (RFRPs), la *prolactin releasing peptide* (PrRP), la kisspeptine et le *pyroglutamylated RF-amide peptide* (QRFP). Ces différentes familles présentent des ligands endogènes dont les récepteurs sont respectivement NPFF2R (GRP74), NPFF1R (GPR147), PrRPR (GPR10), Kiss1R (GPR54), QRFPR (GPR103). En plus de posséder une très haute affinité sur leurs récepteurs endogènes, le PrRP, la kisspeptine et le QRFP présentent une affinité submicromolaire pour NPFF1R et NPFF2R. Ces cinq récepteurs sont des RCPGs appartenant à la famille de la rhodopsine. Ils sont principalement couplés à la protéine G_i (NPFF1R et NPFF2R) ou G_q (PrRPR, QRFPR, Kiss1R). Des analyses phylogénétiques ont révélé la filiation de NPFF1R, NPFF2R, PrRPR et QRFPR au sous-groupe β, alors que Kiss1R se rapproche plutôt du sous-groupe γ, comme les récepteurs à la somatostatine, aux opioïdes ou à la galanine (Elphick and Mirabeau, 2014 ; Fredriksson *et al.*, 2003 ; Yun *et al*., 2014). Cette origine distante d'un point de vue évolutif se retrouve aussi pour les ligands, la kisspeptine partageant un ancêtre commun avec la galanine et la spexine (Figure 4 ; Kim *et al.*, 2014; Yun *et al.*, 2014). Il est intéressant de relever que les récepteurs à la galanine et leurs ligands, ont aussi été décrits comme impliqués dans l'homéostasie énergétique, la douleur et la reproduction (Fang *et al.*, 2015 ; Lang *et al.*, 2015), ce qui suggère une proximité entre le système galaninergique et les peptides RF-amides et leurs récepteurs.

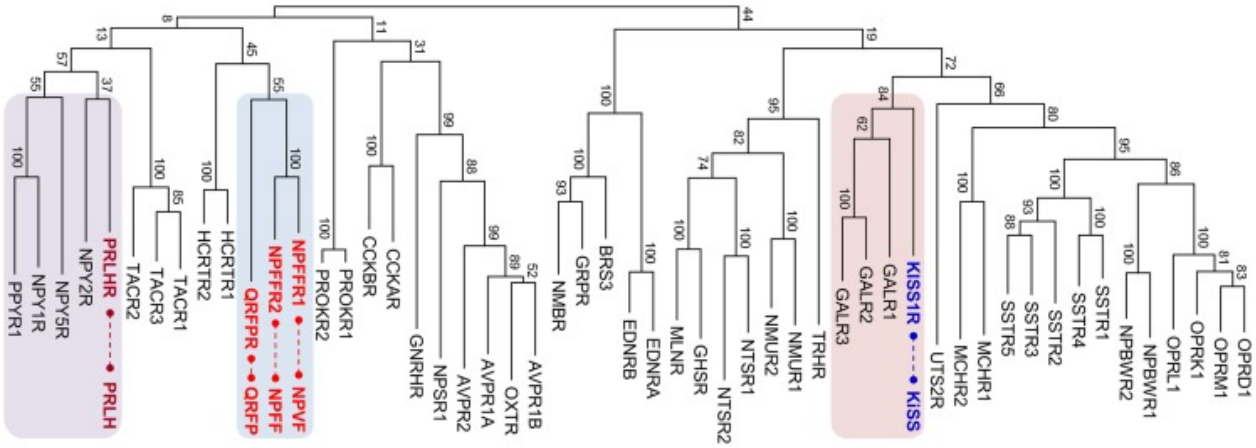

Figure 4 : Proposition d'arbre phylogénétique pour les RCPGS appartenant à la famille de la rhodopsine chez l'Homme*. Les branches concernant les peptides RF-amides sont colorées. Les récepteurs NPFF1R, NPFF2R et QRFPR sont très proches d'un point de vue phylogénétique. En revanche, PrRPR (PRLHR) et KISS1R sont distants des autres récepteurs à peptides RF-amides, et sont plutôt liés aux récepteurs à Neuropeptide Y (NPY) et galanine (GALR), respectivement. D'après (Kim et al., 2014).*

La distribution des 5 familles de peptides et de leurs récepteurs à des niveaux spinaux et supraspinaux aussi bien qu'en périphérie traduit clairement leur implication dans des fonctions fondamentales. Notre revue détaille la localisation et le rôle des 5 systèmes RF-amides dans la régulation de la prise alimentaire et de la balance énergétique chez les mammifères. Bien que leur implication dans le contrôle de la prise alimentaire soit très conservée à travers l'évolution (Bechtold et Luckman, 2007; Dockray, 2004), il est difficile d'affirmer que cela soit leur fonction primaire, et les RF-amides sont aujourd'hui plutôt considérés comme de subtils modulateurs de l'homéostasie énergétique, au niveau central comme périphérique. Nous y évoquons par exemple les mécanismes par lesquels le statut énergétique peut réguler l'expression de ces derniers, et leurs rôles dans le contrôle de l'homéostasie énergétique et le comportement alimentaire. En ce qui concerne la modulation de la reproduction, tous les systèmes, mis à part NPFF/NPFF2R ont été décrits comme impliqués. Les couples kisspeptine/Kiss1R et RFRPs/NPFF1R sont considérés comme les principaux régulateurs du contrôle central de la reproduction, agissant surtout sur les neurones à GnRH, à la base de l'axe HHG. Notamment, nous développons une partie exhaustive sur les rôles de la kisspeptine et du récepteur Kiss1R dans la stimulation de la sécrétion des hormones gonadotropes, de la boucle de rétrocontrôle stéroïdienne, de la transition vers la puberté, ainsi que dans la régulation de l'activité des organes sexuels. Ces neuropeptides et leurs récepteurs sont sensibles aux signaux environnementaux comme la photopériode ou les stéroïdes sexuels, pour permettre une fine régulation de l'axe reproducteur. Une description du contrôle de la saisonnalité chez des animaux comme la brebis ou le hamster, qui se reproduisent exclusivement durant les jours courts pour les premiers, et les jours longs pour les

seconds, est également abordée pour les systèmes kisspeptines et RFRPs. Néanmoins, bien que le rôle des kisspeptines en tant que puissants stimulateurs de l'axe reproducteur soit bien établi, celui des RFRPs reste controversé, car ils agissent d'une manière opposée chez les mammifères saisonniers selon leur saisonnalité, sexe et espèce. D'autre part, de nombreuses études mettent en évidence la responsabilité de tous les systèmes RF-amides dans la modulation de la nociception, à la fois dans des conditions basales ainsi que dans les douleurs chroniques. De plus, de nombreux peptides RF-amides et leurs récepteurs appartiennent au système anti-opioïde/pro-nociceptif, responsable entre autre, des effets secondaires liés à l'administration d'opioïdes.

Dans une dernière partie, les différents peptides et leurs récepteurs sont abordés d'un point de vue thérapeutique, comme cibles importantes dans les traitements des douleurs, de l'obésité, ou encore du diabète de type 2.

2.2.2. *RF-amide neuropeptides and their receptors in Mammals : Pharmacological properties, drug development and main physiological functions*

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RF-amide neuropeptides and their receptors in Mammals: Pharmacological properties, drug development and main physiological functions

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ABSTRACT

RF-amide neuropeptides, with their typical Arg-Phe-NH_2 signature at their carboxyl C-termini, belong to a lineage of peptides that spans almost the entire life tree. Throughout evolution, RF-amide peptides and their receptors preserved fundamental roles in reproduction and feeding, both in Vertebrates and Invertebrates. The scope of this review is to summarize the current knowledge on the RF-amide systems in Mammals from historical aspects to therapeutic opportunities. Taking advantage of the most recent findings in the field, special focus will be given on molecular and pharmacological properties of RF-amide peptides and their receptors as well as on their implication in the control of different physiological functions including feeding, reproduction and pain. Recent progress on the development of drugs that target RF-amide receptors will also be addressed.

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Abbreviations: AHA, anterior hypothalamic area; Amd, amygdala; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP, area postrema; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; BST, bed nucleus of stria terminalis; CCK, cholecystokinin; CHO, Chinese hamster ovary cells; COS, fibroblast-like cell line derived from monkey kidney tissue; CPu, caudate putamen; CREB, cAMP response element-binding protein; DMH, dorsomedial hypothalamic nucleus; DMR, dorsal or median raphe nuclei; DMX, dorsal motor nucleus of vagus; DRG, dorsal root ganglia; ERK, extracellular signal-regulated kinase; FSH, follicle stimulating hormone; GnIH, GnRH inhibitory hormone; GnRH, gonadotropin-releasing hormone; GPCR, G protein-coupled receptor; Hab, habenula; HEK, human embryonic kidney cells; HPG, hypothalamic–pituitary–gonadal axis; IHC, immunohistochemistry; ir, immunoreactive; ISH, in situ hybridization; JNK, c-Jun N-terminal kinase; Kiss, kisspeptin; Kiss1R, kisspeptin receptor; KO, knockout; LC, locus coeruleus; LH, luteinizing hormone; LHA, lateral hypothalamic area; LSN, lateral septal nucleus; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; α-MSH, melanocyte-stimulating hormone; NAc, nucleus accumbens; NPAF, neuropeptide AF; NPFF, neuropeptide FF; NPSF, neuropeptide SF; NPFF1R, neuropeptide FF receptor 1; NPFF2R, neuropeptide FF receptor 2; NPY, neuropeptide Y; NTS, nucleus tractus solitarius; OB, olfactory bulb; OFC, orbitofrontal cortex; OVX, ovariectomized; PAG, periaqueductal gray; PB, parabrachial nucleus; PC12, rat pheochromocytoma cells; PeV, periventricular hypothalamic nucleus; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; POA, preoptic area; POMC, proopio-melanocortin; PRL, prolactin hormone; PrRP, prolactin-releasing peptide; PT, pars tuberalis; PTX, pertussis toxin; PVN, paraventricular hypothalamic nucleus; QRFP-R, QRFP receptor; QRFP-R1, rodent QRFP receptor isoform 1; QRFP-R2, rodent QRFP receptor isoform 2; RFRP, RF-amide related peptide; RFRP-R, RFRP receptor; SAR, structure–activity/affinity relationship; 7TM, seven transmembrane; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; STN, spinal trigeminal nucleus; SuM, supramammillary nucleus; TM, tuberomammillary nucleus; VLM, ventrolateral medulla; VMH, ventromedial hypothalamic nucleus; VPL, ventral postlateral thalamic nucleus; VTA, ventral tegmental area.

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1. Introduction

The isolation in 1977 of the cardio-excitatory peptide Phe-Met-Arg-Phe-NH_2 (FMRF-NH_2) from the ganglia of the *Macrocalista nimbosa* clam (Price & Greenberg, 1977) and the subsequent identification of FMRF-NH_2-like immunoreactivity in insect, fish, amphibian, avian and mammalian tissues (Boer et al., 1980; Dockray et al., 1981; Weber et al., 1981; Dockray et al., 1983) paved the way to the discovery of a wide family of neuropeptides present in all major animal phyla (Walker et al., 2009; Jékely, 2013). This family is currently referred to as FMRFamide-like peptides or RF-amide peptides, a definition broad enough to encompass a vast repertoire of peptides that display a great sequence diversity but share a typical C-terminal Arg-Phe-NH_2 (RF-amide) sequence (Espinoza et al., 2000; Walker et al., 2009).

Although available information varies notably between phyla, bioinformatic analyses of genome and transcriptome databases, cDNA libraries screening and peptidomic strategies enabled the prediction and the identification of numerous RF-amide peptides and receptors in Invertebrates and Vertebrates. The most recent studies indicate that the RF-amide motif is both an ancient and convergent feature of neuropeptides that underwent various evolutionary scenarios (retention, modification or loss of the RF-amide signature) across the animal kingdom, in parallel or independently from their receptors (Elphick & Mirabeau, 2014).

In Invertebrates, RF-amides represent a large peptide family. For example, in the nematode *Caenorhabditis elegans*, up to thirty-one genes encoding more than seventy RF-amide peptides have been identified (Walker et al., 2009; Peymen et al., 2014). Functional studies also evidenced a broad spectrum of biological activities for RF-amides in Invertebrates, including regulation of the cardiovascular, respiratory, reproductive and sensory systems as well as learning and feeding-related behaviors, key functions for these peptides that emerged consistently throughout evolution (Walker et al., 2009; Nässel & Wegener, 2011; Krajniak, 2013; Elphick & Mirabeau, 2014; Peymen et al., 2014).

In Vertebrates, only five RF-amide peptide subfamilies encoded by five precursor genes have been identified: (1) neuropeptide FF (NPFF), (2) RF-amide-related peptide (RFRP), (3) prolactin-releasing peptide (PrRP), (4) kisspeptin and (5) pyroglutamylated RF-amide peptide (QRFP). These neuropeptides signal through five distinct G protein-coupled receptors (GPCRs) that belong to the rhodopsin family. Phylogenetic analyses of GPCRs in the human genome revealed the clustering of NPFF (GPR74), RFRP (GPR147), PrRP (GPR10) and QRFP (GPR103) receptors within the β group for neuropeptide receptors and the location of kisspeptin (GPR54) receptors within the γ group for somatostatin/opioid/galanin receptors (Fredriksson et al., 2003; Elphick & Mirabeau, 2014; Yun et al., 2014). Such a distant evolutionary origin of kisspeptin receptors also concerns their endogenous ligand as kisspeptins were proposed to arise from an ancestor common to galanin and spexin (Kim et al., 2014). Although current data support both parallel and independent evolutionary histories for kisspeptins and their receptors in Vertebrates (Pasquier et al., 2012), the kisspeptin system clearly separates from the other RF-amide peptide/receptor pairs (Yun et al., 2014).

In Mammals, the different RF-amide systems have been involved in the modulation of numerous functions including reproduction, cardiovascular function, pain and nociception, feeding and energy homeostasis, and reward, as well as in peripheral functions and tumor progression. In this review, we address the current knowledge on RF-amide systems in Mammals from historical aspects to therapeutic opportunities, with a particular focus on their prominent roles in feeding and metabolism, reproduction and nociception.

2. RF-amide neuropeptides and their precursors in Mammals

Mammalian RF-amide neuropeptides subdivide into five groups (Table 1): the neuropeptide FF (NPFF) group, the RF-amide-related peptide (RFRP) group, the prolactin-releasing peptide (PrRP) group, the kisspeptin group and finally the QRFP group (Fukusumi et al., 2006; Findeisen et al., 2011a; Parhar et al., 2012).

2.1. Mammalian RF-amide neuropeptides

2.1.1. The neuropeptide FF and neuropeptide AF subgroups

An octapeptide (NPFF) and an 18 amino acid-long peptide (NPAF), both harboring a typical carboxy-terminal RF-amide signature (Table 1), were first detected in bovine brain using antibodies directed against FMRF-NH_2, then purified and sequenced (Yang et al., 1985). These neuropeptides were subsequently isolated from human serum (Sundblom et al., 1995) and cerebrospinal fluid (Sundblom et al., 1997; Burlet-Schiltz et al., 2002), rat brain (Yang & Martin, 1995) and from rat and mouse spinal cords (Yang & Martin, 1995; Bonnard et al., 2001).

The existence of NPFF-containing peptide with three additional N-terminal amino acids was reported in rat (NPA-NPFF; Bonnard et al., 2001) and mouse (SPA-NPFF; Bonnard et al., 2003) spinal cords as well as in SH-SY5Y cells (SQA-NPFF; Bonnard et al., 2003). Conversely, a shorter NPAF form (SLAAPQRF-amide; often referred to as NPSF), has been isolated in rodent brain and spinal cord (Yang & Martin, 1995; Bonnard et al., 2001) as well as in human cerebrospinal fluid (Burlet-Schiltz et al., 2002) and SH-SY5Y cells (Bonnard et al., 2003).

All these peptides contain a common PQRF-amide C-terminal end but may vary in length and sequence depending on mammalian species (Table 1).

2.1.2. The RF-amide related peptides (RFRPs)

Gene database searches for additional peptides pointed to human ESTs encoding two putative RF-amide-related peptides (Hinuma et al., 2000; Liu et al., 2001; Ukena & Tsutsui, 2005), termed RFRP-1 (or NPSF) and RFRP-3 (or NPVF). Endogenous RFRP-1 and RFRP-3 peptides have been subsequently isolated as mature peptides in bovine (Fukusumi et al., 2001; Yoshida et al., 2003), Siberian hamster (Ubuka et al., 2012a) and in human brain hypothalamic extracts (Ubuka et al., 2009b) and cerebrospinal fluid (Burlet-Schiltz et al., 2002). Yet, only RFRP-3 was biochemically characterized in rat (Ukena et al., 2002) and macaque (Ubuka et al., 2009a).

According to species, RFRP peptides exhibit short or longer N-terminal portions but all of them display a well-conserved C-terminal LPXRF-amide (with X = L in RFRP-1 and Q in RFRP-3) motif (Table 1), as does the gonadotropin-inhibitory hormone (GnIH) identified in quail brain (Tsutsui et al., 2000). RFRP-1 displays closer structural homology with avian GnIH but functional studies tend to indicate that RFRP-3 is most likely the GnIH homolog in Mammals (Clarke et al., 2008; Pineda et al., 2010a; Smith & Clarke, 2010; Tsutsui et al., 2012; Ubuka et al., 2012b). A consensus view now considers mammalian

Table 1
RF-amide precursor proteins, RF-amide neuropeptides and their receptors in four mammalian species.

	Peptide	Species	Sequence	References	R
Pro-NPFF$_A$		Human, bovine	FLFQPQRF-NH_2	Sundblom et al, 1995; Yang et al, 1985	NPFF2R (GPR74, HLWAR77)
		Rat, mouse		Bonnard et al, 2001	
	NPFF	Human	SQAFLFQPQRF-NH_2	Bonnard et al, 2003	
		Rat	NPAFLFQPQRF-NH_2	Bonnard et al, 2001	
		Mouse	SPAFLFQPQRF-NH_2	Bonnard et al, 2003	
	(NPSF)	Human, rat, mouse	SLAAPQRF-NH_2	Burlet-Schiltz et al, 2002; Bonnard et al, 2001	
		Human	AGEGLNSQFWSLAAPQRF-NH_2	Bonnard et al, 2003	
	NPAF	Bovine	AGEGLSSPFWSLAAPQRF-NH_2	Yang et al, 1985	
		Rat (P)	EFWSLAAPQRF-NH_2	Roumy et al, 2000	
		Mouse (P)	QFWSLAAPQRF-NH_2	Roumy et al, 2000	
Pro-NPFF$_B$		Human	MPHSFANLPLRF-NH_2	Ubuka et al, 2009	NPFF1R (GPR147, OT7T022)
		Rat, mouse (P)	VPHSAANLPLRF-NH_2	Hinuma et al, 2000	
	RFRP-1 (GnIH,) NPSF)	Human (P)	SLNFEELKDWGPKNVIKMSTPAVNKMPHSFANLPLRF-NH_2	Fukusumi et al, 2001; Liu et al, 2001	
		Bovine	SLTFEEVKDWAPKIKMNKPVVNKMPPSAANLPLRF-NH_2	Fukusumi et al, 2001	
		Rat (P)	SVTFQELKDWGAKKDIKMSPAPANKVPHSAANLPLRF-NH_2	Fukusumi et al, 2001; Liu et al, 2001	
		Mouse (P)	SVSFQELKDWGAKKDIKMSPAPANKVPHSAANLPLRF-NH_2	Liu et al, 2001; Ukena & Tsutsui, 2005	
		Human	VPNLPQRF-NH_2	Ubuka et al, 2009	
	RFRP-3 (NPVF)	Bovine	AMAHLPLRLGKNREDSLSRWVPNLPQRF-NH_2	Yoshida et al, 2003	
		Rat	ANMEAGTMSHFPSLPQRF-NH_2	Ukena et al, 2002	
		Mouse (P)	VNMEAGTRSHFPSLPQRF-NH_2	Ukena & Tsutsui, 2005	
Prepro-PrRP		Human (P)	TPDINPAWYASRGIRPVGRF-NH_2	Hinuma et al, 1998	PrRP-R (GPR10, hGR3, UHR-1)
	PrRP-20	Bovine	TPDINPAWYAGRGIRPVGRF-NH_2	Hinuma et al, 1998	
		Rat, mouse (P)	TPDINPAWYTGRGIRPVGRF-NH_2	Hinuma et al, 1998; Tachibana et al, 2014	
		Human (P)	SRTHRHSMEIRTPDINPAWYASRGIRPVGRF-NH_2	Hinuma et al, 1998	
	PrRP-31	Bovine	SRAHRHSMEIRTPDINPAWYAGRGIRPVGRF-NH_2	Hinuma et al, 1998	
		Rat, mouse (P)	SRAHQHSMETRTPDINPAWYTGRGIRPVGRF-NH_2	Hinuma et al, 1998; Tachibana et al, 2014	
KiSS-1 protein	**Kp-10**	Human	YNWNSFGLRF-NH_2	Bilban et al, 2004	Kiss1R (GPR54, OT7T175, AXOR12)
	Kp-13	Human	LPNYNWNSFGLRF-NH_2	Kotani et al, 2001a; Bilban et al, 2004	
	Kp-14	Human	DLPNYNWNSFGLRF-NH_2	Kotani et al, 2001a; Bilban et al, 2004	
	Kp-52	Rat (P)	TSPCPPVENPTGHQRPPCATRSRLIPAPRGSVLVQREKDMSAYNWNSFGL**RY**-NH_2	Terao et al, 2004	
		Mouse (P)	SSPCPPVEGPAGRQRPLCASRSRLIPAPRGAVLVQREKDLSTYNWNSFGL**RY**-NH_2	Terao et al, 2004	
	Kp-54 (Metastin)	Human	GTSLSPPPESSGSRQQPGLSAPHSRQIPAPQGAVLVQREKDLPNYNWNSFGLRF-NH_2	Ohtaki et al, 2001; Kotani et al, 2001a; Bilban et al, 2004	
Prepro-QRFP		Human	TSGPLGNLAEELNGYSRKKGGFSFRF-NH_2	Jiang et al, 2003; Bruzzone et al, 2006	QRFP-R (GPR103, P518, AQ27, SP9155)
	QRFP-26 (26RFa)	Bovine (P)	VGGLLGTLAEELNGYSRKKGGFSFRF-NH_2	Chartrel et al, 2004	
		Rat (P)	ASGPLGTLAEELSSYSRRKGGFSFRF-NH_2	Chartrel et al, 2003	
		Mouse (P)	ASGPLGTLAEELSSYSRRKGGFSFRF-NH_2	Jiang et al, 2003	
	QRFP-43 (43RFa, P518)	Human	<EDEGSEATGFLPAAGEKTSGPLGNLAEELNGYSRKKGGFSFRF-NH_2	Fukusumi et al, 2003; Bruzzone et al, 2006	
		Rat	<EDSGSEATGFLPTDSEKASGPLGTLAEELSSYSRRKGGFSFRF-NH_2	Takayasu et al, 2006	
		Mouse (P)	<EDGSSEAAGFLPADSEKASGPLGTLAEELSSYSRRKGGFSFRF-NH_2	Jiang et al, 2003	

Mammalian RF-amide precursors (left boxes), RF-amide peptides (central panels) and RF-amide receptors (right boxes) of human, bovine and rodent origin are listed according to current nomenclature. Previous or alternative denominations are given under parentheses. RF-amide peptide sequences are from purified material or predicted (P) from putative cleavage of precursor proteins, as indicated in the original publications. The typical RY-NH^2 C-terminal sequence of mouse and rat kisspeptins is indicated in bold character.

RFRPs and avian GnIHs as RF-amide peptide orthologs (Kriegsfeld et al., 2006; Smith & Clarke, 2010).

2.1.3. The prolactin-releasing peptides (PrRPs)

Using a reverse pharmacological approach, novel RF-amide peptide members referred to as prolactin-releasing peptides (PrRPs) were identified as ligands of the orphan hGR3 receptor (Hinuma et al., 1998). Their purification from bovine hypothalamic extracts revealed the existence of 20- (PrRP-20) and 31- (PrRP-31) amino acid-long isoforms (Table 1), derived from a common PrRP precursor protein. Highly conserved mature PrRP peptides were also predicted to arise from human, mouse and rat precursors (Hinuma et al., 1998; Tachibana & Sakamoto, 2014).

2.1.4. The Kisspeptin peptides (Kisspeptins)

Independent studies aiming at defining the endogenous ligands of GPR54 (Kotani et al., 2001a), OT7T175 (Ohtaki et al., 2001) and AXOR12 (Muir et al., 2001) orphan receptors, led to the discovery of the kisspeptin RF-amide peptide group (Table 1). Three peptides of 54, 14 and 13 amino-acids were isolated from human placenta, characterized as potent GPR54/OT7T175 agonists and defined as translation products of the *KiSS-1* gene (Kotani et al., 2001a; Ohtaki et al., 2001). Accordingly, these endogenous peptides were defined as kisspeptin-54 (Kp-54; or metastin), kisspeptin-14 (Kp-14) and kisspeptin-13 (Kp-13). Additional detection of kisspeptin-10 (Kp-10) in human trophoblasts (Bilban et al., 2004) allowed one to confirm that all kisspeptins derive from the *KiSS-1* gene, a metastasis suppressor gene (Lee et al., 1996).

2.1.5. The pyroglutamylated RF-amide peptides (QRFPs)

The discovery of the last RF-amide peptide group started with the isolation of 26RFa, a neuropeptide from frog brain, and the observation of homologous sequences in human genome and rat ESTs (Chartrel et al., 2003). 26RFa rapidly turned out to correspond to the putative human P518 and mouse P550 peptidic ligands for the orphan SP9155 receptor identified from a series of peptides predicted to originate respectively from human and mouse precursor proteins (Jiang et al., 2003). Following a similar bioinformatic approach, Fukusumi and collaborators identified a human gene candidate encoding a precursor for a 43 amino-acid long peptide, starting from N-terminal pyro-Glu and ending at C-terminal Arg-Phe-NH_2, and named it QRFP (Fukusumi et al., 2003). Full-length QRFP was isolated from the supernatant of CHO cells transfected with human QRFP cDNA and found to behave as a potent agonist of the orphan human AQ27 receptor (QRFP-R).

Endogenous QRFP-26 and QRFP-43 peptides (Table 1) were subsequently identified in rat brain (Takayasu et al., 2006), in human hypothalamus and spinal cord extracts and in PC12 cell lysates (Bruzzone et al., 2006).

2.2. Precursors for RF-amide neuropeptides

The five groups of RF-amide peptides derive from sequential proteolytic/maturation processing of five distinct prepro-proteins encoded by five distinct precursor genes that contain a single coding exon giving rise to one mRNA transcript (no splice variants). RF-amide precursor proteins share a common structure: a hydrophobic signal sequence at their N-terminal end, paired basic amino acids (R and/or K) as recognition sites for endoproteases and a C-terminal consensus sequence (RXGR/K) for peptide processing, with Gly as an amide donor (Eipper et al., 1992; Southey et al., 2006). Cleavage (pro-protein convertases) and maturation (carboxypeptidase E and peptidyl α-amidating monooxygenase) enzymes are likely involved in the processing of RF-amide precursor proteins (Bonnard et al., 2001; Fukusumi et al., 2006; Southey et al., 2006).

2.2.1. The precursor for neuropeptide FF and neuropeptide AF

The gene encoding the proNPFF$_A$ precursor has been cloned in Mammals (Perry et al., 1997; Vilim et al., 1999) and contains three coding exons. Human, bovine, murine and rat proNPFF$_A$ precursors (113–115 amino acids long), as deduced from their cDNAs, are highly related polypeptides (40% overall sequence identity). They encompass two putative peptides (NPFF and NPAF) flanked at their C-termini by RFGR (NPFF) or RFGK (NPAF) motifs and by single or paired Arg residues as putative N-terminal cleavage sites (Perry et al., 1997; Vilim et al., 1999; Bonnard et al., 2001, 2003).

According to general processing rules, only N-terminal extended NPFF undecapeptides (i.e. NPA-, SQA- or SPA-NPFF, depending on species) and N-terminal extended NPAF-derived peptides (11 amino-acid long peptides, such as EFW-NPSF in rat or QFW-NPSF in mice (Table 1), which have not been isolated yet, and 18 amino-acid long peptides which were only found in human and bovine tissues) are predicted from proNPFF$_A$ processing (Vilim et al., 1999; Bonnard et al., 2001, 2003). Thus, the precursors apparently lacked consensus N-terminal cleavage sites for NPFF and the NPAF-derived octapeptide (SLAAPQRF-amide) and displayed species-dependent N-terminal processing mechanisms. Several hypotheses have been proposed to explain these inconstancies: i) in rodents, and similarly to human and bovine NPAF generation, proNPFF$_A$ precursor cleavage at a single Arg residue upstream the 'NPSF' sequence in NPAF could yield 11-amino acids long peptides such as EFW-NPSF in rat or QFW-NPSF in mice (Roumy et al., 2000; Bonnard et al., 2001; Table 1), ii) cleavage of three amino acid-extended peptides by tripeptidyl peptidases could produce NPFF and the NPAF-derived octapeptide (Perry et al., 1997; Bonnard et al., 2001, 2003) and iii) NPFF and NPSF may represent degradation products of longer peptides (Roumy et al., 2000; Talmont et al., 2010).

2.2.2. The precursor for RF-amide related peptides

The gene encoding the proNPFF$_B$ precursor contains three coding exons. The alignment of mammalian proNPFF$_B$ precursor polypeptides (189–203 amino acids depending on species) indicates a well conserved position of two distinct RFGR motifs defining RFRP-1 and RFRP-3 peptides C-termini (Hinuma et al., 2000; Fukusumi et al., 2001; Liu et al., 2001). With the exception of Siberian hamster, N-terminal cleavage for RFRP-1 occurs at a single basic R (or K in human) residue. Despite the fact that proNPFF$_B$ sequences upstream RFRP-3 are enriched in basic residues, cleavage of mature RFRP-3 peptides occurs at species-specific Arg residues, indicating that primary sequence information but also three-dimensional conformation might control N-terminal cleavage selectivity (Yoshida et al., 2003).

Processing of human, macaque and bovine proNPFF$_B$ precursors may produce in addition to RFRP-1 (a LPLRF-amide-containing peptide) and RFRP-3 (a peptide sharing with NPFF a C-terminal PQRF-amide motif), a putative RFRP-2 peptide, with an RS- or RL-amide C-terminal sequence depending on species (Ubuka et al., 2012b). However, the physiological relevance of RFRP-2 in Mammals is unlikely as this peptide has never been isolated from native tissues, lacks activity at OT7T022 receptors (Hinuma et al., 2000) and appears to be included in the N-terminal RFRP-3 sequence (Ubuka et al., 2012b). Rodent proNPFF$_B$ precursors lack the RFRP-2 sequence, suggesting that it retrogressed during the evolution of Mammals (Yoshida et al., 2003).

Although mammalian RFRPs and avian GnIH precursors exhibit significant conservation, GnIH precursor polypeptide in birds encodes one GnIH and two GnIH related peptides (GnIH-RP-1 and GnIH-RP-2), all harboring the typical C-terminal LPXRF-amide motif (Liu et al., 2001; Ubuka et al., 2012b). Two of them (GnIH and GnIH-RP-2) were characterized as isolated peptides (Satake et al., 2001). While GnIH-RP-1/RFRP-1 and GnIH/RFRP-2 are encoded at conserved positions in avian and mammalian precursor polypeptides (only RFRP-1 in rodents), the positions of GnIH-RP-2 and RFRP-3 were totally different (Ubuka et al., 2012b).

2.2.3. The prolactin-releasing peptide precursor

In Mammals, the PrRP precursor gene contains two coding exons (Yamada et al., 2001; Lin, 2008). Structurally similar human, bovine, rat and mouse prepro-PrRP proteins (82–98 amino acids depending on species) generate highly conserved mature PrRP peptides (Hinuma et al., 1998; Fukusumi et al., 2006; Lin, 2008). PrRP-20 and PrRP-31 derive from the same precursor protein, with the 20 amino acid-long sequence of PrRP-20 arising from N-terminal cleavage of the larger PrRP-31 peptide at the level of a conserved Arg residue.

2.2.4. The KiSS-1 protein

The *KiSS-1* gene that contains two coding exons was first identified as a metastasis suppressor gene in human melanoma (Lee et al., 1996). Its primary translation product, the KiSS-1 protein (130–145 amino acids depending on species) displays a moderate overall sequence homology in Mammals (Stafford et al., 2002; Terao et al., 2004; Roa & Tena-Sempere, 2007). Endowed with typical features for a precursor of secreted neuropeptides sharing a RF-amide signature at their C-terminus, the KiSS-1 protein turned out to be the precursor of the entire kisspeptin family (Kotani et al., 2001a; Muir et al., 2001; Ohtaki et al., 2001; Kirby et al., 2010; Pinilla et al., 2012).

Human kisspeptin-54 (metastin), with its sequence surrounded by basic residue pairs, is predicted to result from the cleavage of KiSS-1 by furin or prohormone convertases (Kotani et al., 2001a). The shorter kisspeptin isoforms (Kp-14, -13 and -10), which are all biologically active (Ohtaki et al., 2001), are generated according to processes (differential proteolysis of the KiSS-1 precursor or degradation of the N-

terminal part of Kp-54) that remain to be clarified as no obvious cleavage sites were identified.

The comparison of predicted sequences for kisspeptins from many mammalian species pointed to a highly conserved kisspeptin-10 sequence and, interestingly, suggested the existence of two kisspeptin subfamilies bearing either RF-amide (human and primates) or RY-amide (felines, ruminants, murine, dolphin) C-termini (Roa & Tena-Sempere, 2007; d'Anglemont de Tassigny & Colledge, 2010). In rat and mouse, the largest kisspeptin peptide is 52 amino acids long (Kp-52) with Arg-Tyr-NH_2 at its C terminus instead of Arg-Phe-NH_2 as in humans (Terao et al., 2004).

2.2.5. The prepro-QRFP precursor for QRFP-26 and QRFP-43 peptides

Molecular cloning of the QRFP precursor revealed its presence in all Vertebrates and a conserved organization (Chartrel et al., 2011; Ukena et al., 2013). In Mammals, precursor genes were characterized in mouse, rat, bovine and human; three coding exons encode similar (124–136 amino acids long depending on species) prepro-QRFP proteins (Chartrel et al., 2003; Fukusumi et al., 2003; Jiang et al., 2003). They contain a typical RFGR motif for C-terminal cleavage and amidation and several monobasic residues as putative, though nonconventional, N-terminal processing sites (Chartrel et al., 2011). Yet, efficient precursor cleavage occurred at a single conserved Lys residue, as assessed from the isolation of mature QRFP-26 (or 26RFa) peptides from frog (Chartrel et al., 2003) and human (Bruzzone et al., 2006) brains. As well, a highly conserved Arg residue, located upstream the QRFP-26 region in the precursor, represents a potential cleavage site for a prohormone convertase, possibly furin (Bruzzone et al., 2006), to generate the N-terminal extended QRFP-43 (or 43RFa) peptide (Bruzzone et al., 2006; Takayasu et al., 2006).

3. Mammalian RF-amide receptors

In parallel to the isolation of RF-amide neuropeptides and the characterization of their precursors, five orphan G protein coupled receptors (GPCRs) were successively identified as the natural targets of endogenous mammalian RF-amides in the late 90s and early 2000s.

3.1. The neuropeptide FF1 (GPR147) and neuropeptide FF2 (GPR74) receptors

Bonini and collaborators cloned rat and human orthologs of two G protein coupled receptors differing in their primary sequences and their anatomical distribution in rat and human tissues (Bonini et al., 2000). As they exhibited preferential binding affinities and activities, in the (sub)-nanomolar concentration range, towards NPFF and some RF-amide derivatives, they were defined as NPFF1 (rBN6 and hBO102) and NPFF2 (rBO119 and hBO89) receptors. Their pharmacological characterization was however incomplete. Following the identification of the proNPFF$_B$ genes, Hinuma and colleagues used a reverse pharmacology approach to provide convincing evidence for RFRPs to be the cognate ligands of the rat and human orphan OT7T022 receptor (Hinuma et al., 2000). Independently, Elshourbagy and coworkers identified the HLWAR77 receptor as the target of NPFF and NPAF peptides for which it displayed high affinity and potency (Elshourbagy et al., 2000). Meanwhile, Parker and collaborators cloned another putative receptor, called GPR74, structurally related to the neuropeptide Y (NPY) receptor family (Parker et al., 2000).

The systematic comparison of the pharmacological properties of proNPFF$_A$- (NPFF and NPAF derivatives) and proNPFF$_B$- (RFRPs) derived peptides at these diverse RF-amide receptors unambiguously demonstrated the molecular and functional identity of OT7T022 with the NPFF1/GPR147 receptor and of HLWAR77 with the NPFF2/GPR74 one (Liu et al., 2001; Mollereau et al., 2002; Yoshida et al., 2003).

3.2. The prolactin-releasing peptide (PrRP-R, GPR10) receptor

The orphan GPR10 receptor (Marchese et al., 1995) and its rat ortholog UHR-1 (Welch et al., 1995) turned out to correspond to the human PrRP receptor (first designated as orphan hGR3) targeted by the endogenous prolactin releasing peptides PrRP-20 and PrRP-31 (Hinuma et al., 1998). A highly homologous receptor has been subsequently cloned in mice (Gu et al., 2004).

3.3. The Kiss1 (GPR54) receptor

In 1999, Lee and coworkers cloned a rat orphan receptor (named GPR54) encoding a 396 amino acid polypeptide with significant homology with the galanin receptor within its putative transmembrane (TM) domains (Lee et al., 1999). In 2001, Ohtaki and collaborators found a rat orphan receptor (named rOT7T175), which was nearly identical to GPR54 (Ohtaki et al., 2001). Using its human counterpart hOT7T175 in a reverse pharmacology approach, they identified metastin (kisspeptin-54) as its cognate ligand. Meanwhile, Muir and colleagues cloned the human AXOR12 gene encoding for a 398 amino acid protein also analogous to rat GPR54 (Muir et al., 2001). Using again a reverse pharmacology strategy, they identified a series of peptides derived from the *KiSS-1* gene as potent agonists for AXOR12. Thus, OT7T175 and AXOR12 receptors were definitively catalogued as the Kiss1R or GPR54 receptor which is efficiently activated by all kisspeptins, including its shortest kisspeptin-10 member (Kotani et al., 2001a; Muir et al., 2001; Ohtaki et al., 2001).

3.4. The QRFP (GPR103 or P518) receptor

Along with the identification of the mouse and human prepro-QRFP gene, Jiang and coworkers (Jiang et al., 2003) identified, among possible RF-amide peptides encoded by the precursor, the P518 peptide (yet QRFP-26) as a putative endogenous ligand for the orphan SP9155 receptor. The QRFP (yet QRFP-43 or 43RFa) peptide, generated by the same precursor protein, was also isolated and found to be a high affinity and potent agonist at the human orphan AQ27 receptor (Fukusumi et al., 2003). Altogether, both SP9155 and AQ27 were identical to the orphan GPR103 receptor previously reported in cattle (Lee et al., 2001). The IUPHAR/BPS Guide to Pharmacology (http://www.guidetopharmacology.org) adopted the P518 receptor terminology according to the endogenous P518 peptide (or 26RFa) isolated by Jiang and colleagues (Jiang et al., 2003).

Humans have only one receptor for QRFP peptides while rodents have two receptor isoforms. Indeed, two orthologs of human GPR103 have been found in mouse and rat genomes and designated as GPR103A and GPR103B (Takayasu et al., 2006) and as QRFP-r1 and QRFP-r2 (Kampe et al., 2006), respectively. In this review, we will use the terminology QRFP-R1 and QRFP-R2 for both mouse and rat receptor subtypes.

3.5. Structural information

3.5.1. The neuropeptide FF1 (GPR147) and neuropeptide FF2 (GPR74) receptors

In humans, the NPFF1R and NPFF2R genes are located on chromosomes 10 (locus 10q21-22) and 4 (locus 4q21), respectively. Both contain 4 coding exons; no (NPFF1R) or up to three (NPFF2R) splice variants have been identified (further information, including other mammalian species, is available on IUPHAR and NCBI websites). As deduced from their cDNAs, rat and human NPFF1 receptors are proteins of 432 and 430 amino acids, respectively, which have 86% identity with each other (Bonini et al., 2000; Hinuma et al., 2000). Rat and human NPFF2 receptors (417 and 420 amino acids, respectively) share 78% identity and are nearly 50% identical to rat and human NPFF1 (Bonini et al., 2000; Elshourbagy et al., 2000). Receptor- and species-linked sequence divergences essentially concern the receptor N-terminus and

the second extracellular loop (domains that might be critical for ligand recognition and binding selectivity) as well as the third intracellular loop and the C-terminal tail known to interact with various signaling partners.

Comparing the primary sequence of human NPFF1 with that of other 7TM receptors (for which ligands have been identified) showed high homology (31–37% identity) with human orexin receptors 1 and 2, NPY1, 2 and 4 receptor subtypes, cholecystokinin (CCK) receptor A and the prolactin-releasing hormone (PRL) receptor (Bonini et al., 2000; Hinuma et al., 2000). The human NPFF2/GPR74 receptor has been also shown to exhibit high amino acid identity (33%) to the human NPY2 receptor subtype (Parker et al., 2000).

The existence of variants of the NPFF2 receptor has been reported in human and mouse (Cikos et al., 1999; Parker et al., 2000; Laemmle et al., 2003; Anko et al., 2006). The first description of a novel putative GPCR, termed NPGPR (Cikos et al., 1999), received poor attention as it was rapidly coined as an aberrant variant of the human NPFF2 receptor with an extended N-terminus (Elshourbagy et al., 2000) and a cloning artifact (Parker et al., 2000). Taking advantage of the determination of the exon–intron structure of the GPR74 gene (Parker et al., 2000), Laemmle and colleagues (Laemmle et al., 2003) identified three different forms of the human NPFF2 receptor resulting from alternative exon splicing: a full length NPGPR, most identical to the sequence published by Cikos and collaborators (Cikos et al., 1999), differs from HLWAR77 by an additional exon (exon 1) encoding 102 additional N-terminal residues. The GPR74 sequence differs from hNPFF2R both at 5′- and 3′- ends, hence it contains 3 additional aa and lacks 15 aa at the receptor N- and C-termini, respectively. Finally, a very short form of NPGPR, lacking exon 2 and encoding for a putative soluble protein of 132 amino acids, was also identified. Both NPGPR transcripts differed in their distribution in human tissues while binding studies indicated that the affinity of the full-length NPGPR for NPFF was nearly 100-fold lower than that of NPFF2 receptors (Laemmle et al., 2003). NPGPR forms were not found in mouse unlike NPFF2R and GPR74 which were considered as alternatively spliced receptor variants (Anko et al., 2006).

3.5.2. The prolactin-releasing peptide (GPR10) receptor

In humans, the PrRP-R gene is located on chromosome 10 (locus 10q26.13). It contains three coding exons that give rise to two splice variants (further information, including other mammalian species, is available on IUPHAR and NCBI websites). Human, rat and mouse PrRP receptors are 370 amino acid long proteins with a sequence identity close to 90% (Gu et al., 2004). They are closely related to the Neuropeptide Y receptor family (31% overall identity and 46% identity in TM domains) but they do not bind NPY-related peptides (Marchese et al., 1995).

3.5.3. The Kiss1 (Kiss1R, GPR54) receptor

In humans, the Kiss1R gene is located on chromosome 19 (locus 19p13.3). It contains five coding exons with no known splice variants (further information, including other mammalian species, is available on IUPHAR and NCBI websites). Rat (396 amino acids) and human (398 amino acids) kisspeptin receptor proteins share 85% sequence identity (98% identity in TM domains) whereas mouse and human proteins display 82% identity (Kirby et al., 2010). The N-terminus, the third extracellular loop and the C-terminal intracellular domain of rat and human receptor orthologs are less conserved (Kotani et al., 2001a).

The human Kiss1R displays a significant homology with the human GalR1, GalR2, and GalR3 (Muir et al., 2001; Ohtaki et al., 2001) although there is some discrepancy whether the highest sequence identity is to GalR3 (30%; Muir et al., 2001) or to GalR1 (45%; Kirby et al., 2010). It is clear however that Kiss1R does not interact with galanin (Lee et al., 1999).

3.5.4. The QRFP (QRFP-R, GPR103 or P518) receptor

In humans, the QRFP-R gene is located on chromosome 4 (locus 4q27). It contains eight coding exons with multiple possibilities of alternative splicing (further information, including other mammalian species, is available on IUPHAR and NCBI websites). As inferred from cDNA analyses, the mammalian QRFP receptor proteins vary in amino-acid (aa) length and content depending on their human (431 aa), mouse (GPR103A: 433 aa; GPR103B: 425 aa) or rat (QRFP-r1: 433 aa; QRFP-r2: 427 aa) origin (Fukusumi et al., 2003; Kampe et al., 2006; Takayasu et al., 2006). Rodent GPR103 isoforms share 75% and 78% identity in mouse and rat, respectively. Human QRFP-R exhibits a slightly higher homology with GPR103A (85% identity) and QRFP-r1 (84% identity) subtypes, as compared to GPR103B (79% identity) or QRFP-r2 (82%) receptors (Kampe et al., 2006; Takayasu et al., 2006).

QRFP receptors display some sequence homology with several GPCRs, including the orexin, galanin GalR1, NPY2 and CCK receptors, but NPFF1 and NPFF2 receptors are their closest known parents, with amino acid identity close to 50% (Jiang et al., 2003; Chartrel et al., 2011).

4. RF-amides in vitro pharmacology: ligand–receptor binding properties

Binding properties of RF-amide receptors are essentially from standard radioligand binding studies using iodine-labeled peptides and membrane preparations from brain tissues or from heterologous cells transiently or stably expressing recombinant RF-amide receptors. Owing to their high specific radioactivity, [^{125}I]-labeled RF-amide peptides allow the detection of minute amounts of receptors and the reliable characterization of binding sites endowed with nano- or subnanomolar affinity properties. Specific binding was usually defined as part of total binding displaceable by micromolar concentrations of unlabeled peptides. Most studies are performed under equilibrium binding conditions in order to determine receptor affinities (apparent equilibrium dissociation constants) for [^{125}I]-tracers (K_d values) or unlabeled compounds (K_i values) from saturation or competition experiments, respectively.

An overview of the characteristics of available tracers and of the binding affinity properties of the five RF-amide receptors for a representative set of peptides is presented in Tables 2 and 3. For a sake of clarity, only K_d or K_i values determined on recombinant RF-amide receptor transfected in heterologous cell lines are listed.

4.1. Radiolabeled tracers for RF-amide receptors

4.1.1. NPFF1R (GPR147) and NPFF2R (GPR74) radioligands

A iodinated analog of NPFF (with Phe^1 substituted for a Tyr residue), known as [^{125}I]Tyr-NPFF or [^{125}I]Y8Fa, was first introduced to characterize putative binding sites for this morphine modulating peptide (Allard et al., 1989). Thereafter, [D.Tyr^1, (NMe)Phe^3]NPFF (with substitution of L-Phe^1 for D-Tyr and N-methylation of the second peptidic bond) was selected out of a series of NPFF analogs for its high affinity and greater stability than Y8Fa (Gicquel et al., 1992, 1994). Its radio-iodinated derivative (Devillers et al., 1994), referred to as [^{125}I]-[D.Tyr^1, (NMe)Phe^3]NPFF, [^{125}I]-(1DMe)NPFF, [^{125}I]-(1DMe)Y8Fa or even [^{125}I]-1DMe, became a popular tracer for NPFF receptors, especially in autoradiographic studies (Gouarderes et al., 1997, 2000). [^{125}I]Y8Fa and [^{125}I]-(1DMe)Y8Fa display comparable affinities for both NPFF1 and NPFF2 receptors (Table 2).

The identification of $NPFF_A$ and $NPFF_B$ precursors and of two NPFF receptor subtypes prompted the search for R1- and R2-selective radioligands. Iodinated derivatives of RFRP-1 (YANLPLRFa) and RFRP-3 (YVPNLPQRFa) peptides, respectively referred to as [^{125}I]-Tyr-hNPSF (Liu et al., 2001) and [^{125}I]-Tyr-hRFRP-3 (Hinuma et al., 2000) or [^{125}I]-YVP (Gouarderes et al., 2002), were introduced as NPFF1-selective tracers. Conversely, [^{125}I]-EYWSLAAPQRFa or [^{125}I]-EYF, derived from a peptide present in the rat $NPFF_A$ precursor (Table 1), was characterized as a high affinity NPFF2-selective probe (Gouarderes et al., 2001).

Table 2
Binding affinities of human NPFF1 and NPFF2 receptors for various RF-amide peptides as afforded from binding studies using tritiated and iodinated radioligands.

Tracer		NPFF1R				NPFF2R				
		^{125}I-DMe	^{125}I-YVP	^{3}H-RFRP-3	^{3}H-FFRF	^{125}I-Y8Fa	^{125}I-DMe	^{125}I-EYF	^{3}H-EYF	^{3}H-FFRF
Tracer K_d (nM)		1.13[a]	0.18[b]–0.14[c] 0.27[d]–0.19[e]	2.65[f]	1[g]	0.24[d]–0.5[h]	0.37[a]–0.1[i]	0.06[b,k]–0.07[c]	0.54[f]	0.2[g]
Pro-NPFF$_A$-derived peptides (length, aa; K_i values, nM)										
NPFF	8 aa		2.5[b]–2.8[c]–2.4[d]	20[f]	0.3[g]	1[d]–1.14[h]	5.2[i]	0.21[b,c]–0.3[k]	0.63[f]	0.8[g]
I-Y-NPFF (I-Y8Fa)	8 aa		0.68[d]							
1DMe-NPFF	8 aa	8[a]	2[b]–1.1[c]	4.2[f]			3.2[a]–5.3[i]	0.18[b,c]–0.31[k]	0.38[f]	
hSQA-NPFF	11 aa		7[b]–4.1[c]–4.2[d]	25[f]	0.37[g]	0.34[d]–0.11[h]		0.3[b,k]–0.16[c]	0.14[f]	0.1[g]
mSPA-NPFF	11 aa		2.6[b]–4.0[d]	7.6[f]		0.36[d]–		0.07[b]–0.05[c]	0.04[f]	
rNPA-NPFF	11 aa		15[b]–3.4[c]					0.11[b]–0.03[c]		
r,m,h NPAF (NPSF)	8 aa		48[b]–32[c]	109[f]			37[i]	12[b,k]–20[c]	19[f]	
mQFW-NPAF	11 aa		8.8[b]	37[f]				0.35[b,k]–0.19[c]	0.29[f]	
rEFW-NPAF	11 aa		45[b]–21[c]					0.21[c]		
EYW-NPAF (EYF)	11 aa		15[b]–18[c]					0.32[b,k]–0.24[c]		
hNPAF	18 aa		12[b]–13[c]		3.1[g]	0.64[h]		0.22[b,k]–0.14[c]		0.9[g]
bNPAF	18 aa	63[a]	10[b]			1.8[h]	1.3[a]	0.16[c]		
Pro-NPFF$_B$-derived peptides (length, aa; K_i values, nM)										
hRFRP-1	12 aa		0.4[j]–1.3[c]–2.7[d]			3.8[d]	15[i]	3.9[c]–2.8[j]		
hRFRP-1	37 aa		15[d]		0.58[g]	17[d]				15[g]
hRFRP-3	8 aa		0.5[b]–0.6[c]–1.2[d]	3.4[f]	0.2[g]	150[d]	55[i]	17.4[b,c]–23[j]	32[f]	67[g]
hY-RFRP-3 (YVP)	9 aa		0.4[b]–0.7[c]					9[b,c]		
bRFRP-3	28 aa		1.6[d]			7[d]				
Miscellaneous peptides (length, aa; K_i values, nM)										
FMRF amide	4 aa	0.8[a]	2.1[b]–1.8[c]				4[a]–13[i]	6.6[b,c]–7[j]–10[k]		
PQRF amide	4 aa		23[b]–8.7[c]–15[d]			26[d]		6.8[b,c]		
Frog Pancreatic Peptide	36 aa	1,260[a]	4,470[b]–>1,000[c]				40[a]	7[c]–5.1[j]		

Human (h), bovine (b), rat (r) and mouse (m) RF-amide peptides are listed according to their precursor origin along with their amino-acid length.
K_i-values are from competition binding studies performed on NPFF1R- or NPFF2R-transfected cell lines as reported in the associated references.
Notes to Table 2:
a) Bonini et al. (2000); b) Gouarderes et al. (2002); c) Mollereau et al. (2002); d) Yoshida et al. (2003); e) Hinuma et al. (2000); f) Talmont et al. (2009); g) Elhabazi et al. (2013); h) Elshourbagy et al. (2000); i) Engstrom et al. (2003); j) Gouarderes et al. (2007); k) Kotani et al. (2001b).

Table 3
Summary of binding affinities and functional potencies of various RF-amide peptides at the five human RF-amide receptors.

Peptide	NPFF1R		NPFF2R		PrRP-R		Kiss1R		QRFP-R	
	Binding affinity	cAMP response	Binding affinity	cAMP response	Binding affinity	Calcium response	Binding affinity	Calcium response	Binding affinity	Calcium response
	K_i, nM	IC_{50}, nM	K_i, nM	IC_{50}, nM	K_i, nM	EC_{50} nM	K_i, nM	EC_{50} nM	K_i, nM	EC_{50}, nM
NPFF	2.5*	31–510[a,d–g]	0.25*	0.6–7[a,d,f,g,i]	>20,000[a,h]	>10,000[a]	>20,000[a]	>10,000[a]	>20,000[a]	>10,000[a]
1DMe-NPFF	1.5*	71[f]	0.25*	2.7[f]						
hSQA-NPFF	5*	47–153[d,f]	0.23*	0.25–0.9[d,f,i,r]	>20,000[a]	>10,000[a]	>20,000[a]	>10,000[a]	>20,000[a]	>10,000[a]
r,m,h NPSF	40*	876[f]	16*	222[f]						
mQFW-NPAF	8.8*		0.27*	1.5[f]						
hNPAF (18a)	12.5*	324–1350[a,f]	0.18*	0.18–3[a,f,i]	>20,000[a]	>10,000[a]	>20,000[a]	>10,000[a]	>20,000[a]	>10,000[a]
bNPAF (18a)			0.16*	0.4–1.5[f,i]						
hRFRP-1 (12a)	1.5*	4.5–9.6[d–f]	3.3*	21–330[d,f]	>100,000[h]					
hRFRP-1 (37a)	15*	12–20[a,d,e]		150–470[a,d]	>20,000[a]	>10,000[a]	>20,000[a]	>10,000[a]	>20,000[a]	>10,000[a]
hRFRP-3 (8a)	0.8*	3–12[a,d–g]	20*	74–1,000[a,d,f,g]	>20,000[a,h]	>10,000[a]	>20,000[a]	>10,000[a]	>20,000[a]	>10,000[a]
hPrRP-20	4.7[a]	9280[a]	0.7–23[a,h]	466[a]	0.06–1[a,h,j,k]	0.2–1[a,j,k]	>20,000[a]	>10,000[a]	>20,000[a]	>10,000[a]
hPrRP-31	11–45[a,b]	>10,000[a]	0.4–19[a,b,h]	240[a]	0.07–1[a,h,j,k]	0.3–1.5[a,k,l]	>20,000[a]	>10,000[a]	>20,000[a]	>10,000[a]
Kp-10	0.5–4.7[a,c]	380–10,000[a,c]	1.6–76[a,c]	292–1,000[a,c]	9,200[a]	>10,000[a]	0.04–2.3[a,m,n]	0.1[a,m]	8,020[a]	>10,000[a]
Kp-13	0.7[a]	830[a]	1.4[a]	475[a]	5,800[a]	>10,000[a]	0.9–4.2[a,n]	0.17[a]	>20,000[a]	>10,000[a]
Kp-54	0.5–15[a,c]	>10,000[a,c]	10–400[a,c]	>5,000[a,c]	>20,000[a]	>10,000[a]	0.3–3.7[a,m,n]	0.3–0.4[a,m]	>20,000[a]	>10,000[a]
hQRFP-26	24–38[a,b]	>10,000[a]	3–10[a,b]	>10,000[a]	10,500[a]	>10,000[a]	>20,000[a]	>10,000[a]	3.2–21[a,o]	0.2–7[a,q]
hQRFP-43	84–330[a,b]	>10,000[a]	53–131[a,b]	>10,000[a]	>20,000[a]	>10,000[a]	>20,000[a]	>10,000[a]	0.1–8[a,o,p]	0.3–23[a,p]

RF-amide peptides of rat (r), mouse (m), bovine (b) or human (h) origin have been considered, with amino acid lengths indicated under parentheses when needed. Affinity (K_i) and potency (EC_{50} and IC_{50}) values are min–max for the set of values selected from the literature.* Reported affinity constants are means of K_i values taken from Table 2, using [^{125}I]-YVP (hNPFF1) or [^{125}I]-EYF (hNPFF2) as the radioligand.
Notes to Table 3:
a) Elhabazi et al. (2013); b) Gouarderes et al. (2007); c) Oishi et al. (2011); d) Yoshida et al. (2003); e) Fukusumi et al. (2001); f) Mollereau et al. (2002); g) Liu et al. (2001); h) Engstrom et al. (2003); i) Elshourbagy et al. (2000); j) Roland et al. (1999); k) Langmead et al. (2000); l) Wang et al. (2012); m) Ohtaki et al. (2001); n) Kotani et al. (2001a); o) Fukusumi et al. (2003); p) Takayasu et al. (2006); q) Jiang et al. (2003); r) Kotani et al. (2001b).

[^{3}H]-FFRFa, a tritiated analog of a high affinity tetrapeptide at endogenous NPFF receptors (Payza et al., 1993) has been used in some binding studies (Gealageas et al., 2012; Elhabazi et al., 2013; Bihel et al., 2015) although it exhibits a poor NPFF1/2 receptor selectivity (Table 2). More recently, [^{3}H]-hRFRP-3 and [^{3}H]-EYF have been obtained by tritiation of the corresponding dehydro-Leu peptide precursors and characterized as interesting alternatives to radio-iodinated compounds (Talmont et al., 2009). More anecdotal probes were also reported such as rhodamine-labeled NPFF (TAMRA-NPFF) that might be useful in fluorescence polarization-based binding assays (Do et al., 2004).

The data presented in Table 2 deserve additional comments. Indeed, most saturation binding studies performed on membrane preparations from HEK or CHO cells transfected with individual receptors indicated an apparent homogenous population of high affinity binding sites. K_d values for most radioligands are within the 0.1–1 nM concentration range indicating a very high affinity of all these radiolabeled agonists for NPFF1 and NPFF2 receptors. Similar saturation studies, mostly focusing on NPFF2 receptors in native tissues, were performed on membrane preparations of rat spinal cord using [^{125}I]Y8Fa (K_d: 0.09 nM; Allard et al., 1989) or [^{125}I]-(1DMe)NPFF (K_d: 0.07 nM; Devillers et al., 1994) or of olfactory bulb using [^{3}H]-EYF (K_d: 0.4 nM; Talmont et al., 2010).

K_d values for [^{125}I]-(1DMe)NPFF (0.1 nM; Gouarderes et al., 1997) and [^{125}I]-EYF (0.02–0.04 nM; Gouarderes et al., 2001) were also determined from quantitative autoradiographic binding studies on rat and mouse brain tissue sections. It remains that true saturation was rarely achieved using radio-iodinated tracers (probably due to cost-effective manipulations and prohibitive radioactivity levels at high concentration) and that the maximal density in receptor sites (B_{max}) was in general extrapolated. Moreover, when compared to their iodinated analogs (Table 2), [^{3}H]-hRFRP-3 and [^{3}H]-EYF tracers displayed a 10- to 20-fold lower affinity for NPFF1 and NPFF2 receptors, respectively (Talmont et al., 2009, 2010). As well, [^{3}H]-EYF was reported to label a much greater amount of NPFF2R sites than [^{125}I]-EYF (Talmont et al., 2009). These observations tend to indicate that iodinated peptides are probably used under experimental conditions favoring the selection of a high affinity receptor site subpopulation (Zajac & Roques, 1989; Talmont et al., 2009).

4.1.2. Prolactin-releasing peptide-receptor, Kiss1R and QRFP-R radioligands

The binding properties of these receptors have been studied using a limited set of radio-iodinated peptides. [^{125}I]-hPrRP-20 (Langmead et al., 2000) and [^{125}I]-PrRP-31 of human (Roland et al., 1999), bovine (Hinuma et al., 1998) or rat (Maixnerova et al., 2011) origin were obtained through introduction of a ^{125}I atom at the level of the highly conserved Tyr residue in the PrRP sequence (Table 1). They display similar K_d values at the PrRP receptor (Table 3). Noteworthy, the introduction of the long lifetime europium lanthanide to a Lys residue added to the N-terminus of hPrRP-31 led to Eu-(Lys)hPrRP-31, an original tracer prone to time-resolved fluorescence measurements (Boyle et al., 2005).

Similarly to the labeling of PrRPs, the single conserved Tyr residue in human kisspeptins was iodinated to give [^{125}I]-Kisspeptin-10 (Kotani et al., 2001a), [^{125}I]-Kisspeptin-13 (Mead et al., 2007) and [^{125}I]-Kisspeptin-15 (Ohtaki et al., 2001) tracers. All of them display very similar binding affinities at the Kiss1 receptor (Table 3). Iodinated kisspeptin-54 was essentially used in radioimmunoassays (Dhillo et al., 2005).

To our knowledge, binding studies performed on QRFP receptors are few and only [^{125}I]-QRFP-43 has been used as a radiotracer (Fukusumi et al., 2003; Takayasu et al., 2006; Elhabazi et al., 2013).

4.2. Binding affinity properties of RF-amide receptors for their endogenous peptides

Values reported in Tables 2 and 3 are from competition studies performed on membrane preparations from CHO or HEK cells expressing recombinant receptors, using fixed concentrations of tracers (usually close or below their respective K_d values). K_i values are taken from a selection of papers that examined the binding affinities of the five human RF-amide receptors for different RF-amide peptides.

4.2.1. Neuropeptide FF1 (GPR147) and neuropeptide FF2 (GPR74) receptors

Table 2 summarizes K_i values for agonists derived from the proNPFF$_A$ (NPFF, NPA-, SPA- and SQA-NPFF, and NPAF peptides from various species including the 'NPSF' octapeptide) and the proNPFF$_B$ (RFRP-1 and RFRP-3) precursors as well as for other RF-amide peptides such as FMRFa, PQRFa and the frog pancreatic peptide. This list also includes K_i values for unlabeled tracer analogs regarded here as competitors.

RFRP-1 and RFRP-3 peptides display highest affinity for human NPFF1 receptors (K_i range: 0.4–2.7 nM), followed by NPFF derivatives (K_i range: 2.4–15 nM) and finally by the NPAF peptide group (K_i range: 10–50 nM). Affinity ranking of the same series of compounds at the human NPFF2 receptor indicates a very modest (if no) preference for NPFF-type agonists (K_i range: 0.03–1.1 nM) over NPAF-type peptides (K_i range: 0.15–1.8 nM), with the exception of the octapeptide NPSF (K_i: 12–37 nM), and a lower affinity for RFRP-1 and RFRP-3 (K_i range: 3–150 nM). Thus, RF-amide peptides resulting from the cleavage of proNPFF$_B$ preferentially bind the NPFF1 receptor while the reverse is true for proNPFF$_A$-derived peptides at the NPFF2 receptor.

As most K_i values (as afforded from various studies using identical or distinct radioligands) are in overall agreement, one may assume that RF-amide peptides compete with tracers for binding to an identical and homogenous population of NPFF1 or NPFF2 receptor sites. Some differences have been noted depending on the type of radioligand (Table 2), such as a 5- to 6-fold lower binding affinity of peptides when assessed using [^{3}H]-hRFRP-3 (Talmont et al., 2009) or a 10- to 20-higher affinity of several peptides when using [^{3}H]-FFRF (Elhabazi et al., 2013) instead of [^{125}I]-YVP at the NPFF1 receptor. Affinity rank order was however preserved.

Binding selectivity of RF-amide peptides at NPFF1 and NPFF2 receptors is rather difficult to express in absolute quantitative terms as it hardly depends on the radioligand pairs which are considered (Table 2). Taking mean K_i values for the whole set of available data for each peptide, selectivity profiles for NPFF1- or NPFF2-preferring compounds may be proposed as follows. hRFRP-1 (37 aa), hRFRP-1 (12 aa), FMRFa, hY-RFRP-3 (or YVP; 9 aa) and hRFRP-3 (8 aa) display a 2-, 3-, 5-, 20- and 50-fold higher affinity for NPFF1R than for NPFF2R, respectively. Conversely, while the octapeptides (NPFF, 1-DMe and NPSF) display a very poor selectivity towards NPFF2 receptors (close to 2-fold), the undecapeptides derived from the NPFF$_A$ precursor exhibit a much greater preference for NPFF2 receptors. Indeed, their selectivity indexes rise from 30, 40 to 130 for human SQA-, mouse SPA- and rat NPA-NPFF derivatives, and from 60, 85 to 160 for EYW- (or EYF), mouse QFW- and rat EFW-NPAF derivatives, respectively. The frog pancreatic peptide (fPP) is the most NPFF2 selective peptide (Table 2). It proved to be an efficient NPFF2R blocker to reinforce the NPFF1 nature of [^{125}I]-(1DMe)NPFF (Bonini et al., 2000) or [^{125}I]-YVP (Gouarderes et al., 2002, 2004b) binding on native rat tissues.

Overall, ligand binding properties of NPFF receptors are essentially from studies performed on membrane preparations from recombinant HEK (Bonini et al., 2000; Gouarderes et al., 2002) or CHO cells (Kotani et al., 2001b; Gouarderes et al., 2002; Mollereau et al., 2002; Engstrom et al., 2003; Yoshida et al., 2003; Gouarderes et al., 2007; Talmont et al., 2010), with special focus on receptors of human origin. Given the low density of receptor sites in native tissues and possible mismatches due to co-expression of various receptor subtypes and to low radioligand binding selectivity, only a few experiments addressed the pharmacological properties of endogenous NPFF receptors. It remains that binding studies performed on rat spinal cord slices (Roumy et al., 2000; Gouarderes et al., 2001; Mazarguil et al., 2001) or membrane preparations (Gouarderes et al., 2002; Engstrom et al., 2003) and on mouse olfactory bulb membranes (Gherardi & Zajac, 1997; Gouarderes et al., 2001; Talmont et al., 2010) adequately reported on ligand binding

properties of endogenous NPFF2R. Indeed, these CNS areas are highly enriched in this receptor subtype (Bonini et al., 2000; Gouarderes et al., 2001, 2002, 2004a), are easy to dissect and provide sufficient material. Although the overall binding profiles of human and rodent NPFF2 receptors were comparable, the recurrent observation of a slightly higher affinity of rodent receptors for most RF-amide peptides was further investigated in a systematic comparative study by Talmont et al. (2010). The octapeptides NPSF and NPVF (or RFRP-3), respectively derived from the pro-$NPFF_A$ and pro-$NPFF_B$ precursors, as well as the NPFFR antagonist RF9, were found to display a tenfold higher affinity for the mouse NPFF2 receptor than for the human one. Thus, species-related differences in NPFF2R binding properties might have a repercussion on the evaluation of drug pharmacological properties in vivo.

4.2.2. PrRP (GPR10), Kiss1 (GPR54) and QRFP (GPR103) receptors

In contrast to the fairly high affinities of most RF-amides at both NPFF receptors (see below), the PrRP, the Kiss1 and QRFP receptors are very strong peptide discriminators (Table 3). Subnanomolar binding affinities and functional potencies are exclusively limited to their endogenous ligands while all other peptides display no significant interaction, even in the micromolar range. Noteworthy, the existence of receptor subtypes in mouse and rat is not associated with appreciable differences in their pharmacological properties (Kampe et al., 2006; Takayasu et al., 2006).

4.3. Promiscuous binding properties of NPFF1 and NPFF2 receptors

Several independent observations suggested the possibility for PrRPs (Engstrom et al., 2003; Ma et al., 2009), Kisspeptins (Lyubimov et al., 2010) and QRFPs (Bruzzone et al., 2007) to signal through NPFF receptors in vivo, in addition to their classical effects mediated by their respective PrRP, KiSS-1 and QRFP receptors. As an example, the pivotal role of NPFF receptors in the hyperalgesic and anti-morphine effects of PrRP-20, kisspeptin-10 and QRFP-26 was demonstrated in a systematic in vivo study, taking advantage of the selective NPFF receptor antagonist RF9 (Elhabazi et al., 2013).

More direct evidence for a low level of discrimination of NPFF1 and NPFF2 receptors towards most RF-amide peptides was provided by a set of in vitro binding and functional studies performed on heterologous cell lines expressing either NPFF receptor.

Data summarized in Table 3 indicate that PrRPs (Engstrom et al., 2003; Gouarderes et al., 2007; Elhabazi et al., 2013), Kisspeptins (Oishi et al., 2011; Elhabazi et al., 2013) and QRFPs (Gouarderes et al., 2007; Elhabazi et al., 2013) bind with nanomolar affinity to both NPFF1 and NPFF2 receptors. PrRPs and QRFPs exhibit a moderate NPFF2 selectivity whereas Kisspeptins exhibit a slight preference for NPFF1 receptors. The affinity of kisspeptins for the NPFF1 receptor was nearly equivalent to that of RFRPs while prolactin releasing peptides bound in a concentration range similar to that of proNPFF$_A$-derived peptides to NPFF2 receptors.

Functional studies (Table 3) also indicated that all RF-amide peptides were able to mediate a typical $G_{i/o}$ response through the stimulation of NPFF1 and NPFF2 receptors, i.e. a concentration-dependent inhibition of forskolin-induced cAMP accumulation. In general, PrRPs, kisspeptins and QRFPs potencies were lower than those of typical NPFF agonists. It remains that PrRPs at NPFF2R as well as kisspeptin-10 and kisspeptin-13 at both NPFF receptors promoted significant responses in the submicromolar concentration range, as did several proNPFF$_A$ and proNPFF$_B$-derived peptides at NPFF1 and NPFF2 receptors, respectively.

Thus, in contrast with the three other RF-amide receptors, NPFF1 and NPFF2 receptors exhibit a complex pharmacology, at least in vitro, as they are targeted by most RF-amide peptides. Only careful evaluation of the nature of the receptors involved in PrRPs, Kisspeptins and QRFPs in vivo effects, using for example highly selective antagonists or knockout mice for the different RF-amide receptors, will avoid misleading conclusions especially in tissues enriched in NPFF receptors.

5. RF-amides in vitro pharmacology: intracellular signaling pathways

Following their discovery, RF-amide receptors have been rapidly assigned to G-protein coupled receptors according to their typical 7TM signatures, their modulation by guanine nucleotides and, more recently, for their ability to promote the binding of non-hydrolysable GTP analogs following agonist stimulation. Several studies have been performed to characterize the nature of the G proteins involved in RF-amide receptor function as follows.

5.1. NPFF1R and NPFF2R signaling pathways

$G\alpha_{i/o}$ coupling of NPFF1 and NPFF2 receptors is now well established. It has been verified using various procedures aiming at quantifying a dose-dependent inhibition of forskolin-stimulated accumulation of cAMP by RF-amide peptides in various heterologous cell lines (CHO, COS, HEK, SH-SY57) expressing either NPFF1 (Hinuma et al., 2000; Fukusumi et al., 2001; Liu et al., 2001; Mollereau et al., 2002; Kersante et al., 2006; Oishi et al., 2011; Elhabazi et al., 2013) or NPFF2 (Elshourbagy et al., 2000; Liu et al., 2001; Mollereau et al., 2002; Yoshida et al., 2003; Talmont et al., 2010; Oishi et al., 2011; Elhabazi et al., 2013) receptors. These cAMP responses were typically abolished by cell pre-treatment with the pertussis toxin PTX. All RF-amide peptides had a similar maximal efficacy and behaved as full agonists (Mollereau et al., 2002).

Table 3 summarizes potencies values of the five groups of RF-amide peptides for inhibiting cAMP production (IC_{50}) and allows their comparison with binding affinity constants (K_i). Listing lower and upper limits for IC_{50} values reported in the literature allows one to appreciate great variations for some agonists that may be explained by differences in receptor expression levels or assay conditions.

ProNPFF$_A$- and proNPFF$_B$-derived peptides are the most potent ones at NPFF2 and NPFF1 receptors, respectively, in agreement with their binding affinity properties. The NPFF1 receptor appears a much better peptide discriminator in terms of functional potency than binding affinity: most RF-amide peptides exhibit a nanomolar affinity while PrRPs, Kisspeptins and QRFPs show only very modest potencies in the low micromolar range. Such an exclusion of surrogate agonists for promoting a robust cAMP response was less marked with the NPFF2 receptor as RFRPs, PrRPs and Kisspeptins were active in the submicromolar range. Another interesting observation (Table 3) is the apparent lack of concordance between affinity and functional parameters. This may arise from assay conditions (radioligand nature, buffer composition, incubation time) and functional contexts (recombinant cells, receptor expression levels, modified G-proteins and/or reporter systems) that probably do not reproduce native physiological ones. However, one cannot exclude that it may also meet predictions of the so-called ligand paradox (Rosenkilde & Schwartz, 2000; Kenakin & Onaran, 2002), revisited into the more recent concept of biased agonism (Galandrin et al., 2007; Kenakin & Christopoulos, 2013; Wisler et al., 2014). Indeed, they both describe the K_i value as a macroscopic binding affinity constant for an ensemble of possible receptor conformations whereas potency is linked to the ability of an agonist to stabilize the active receptor state (s) relevant to the type of response which is monitored.

Except the mention of a small NPFF-induced increase in inositol phosphate release in NPFF1-transfected CHO cells, which most likely resulted from activation of the G_q/PLC pathway secondary to that of PTX-sensitive G proteins (Bonini et al., 2000), no mobilization of intracellular calcium nor release of arachidonic acid metabolites could be detected upon stimulation of NPFF receptors (Hinuma et al., 2000). Conversely, several studies took advantage of the existence of promiscuous $G\alpha_{q/z5}$ and $G\alpha_{q/i3}$ (Bonini et al., 2000), $G\alpha_{q/i4}$ (Findeisen et al., 2011b) and

$G\alpha_{q/i5}$ or $G\alpha_{q/o5}$ (Elshourbagy et al., 2000; Liu et al., 2001) proteins — with $G\alpha_i$, $G\alpha_o$ and $G\alpha_z$ belonging to the inhibitory class of G proteins (Wettschureck & Offermanns, 2005) — to redirect NPFF receptors towards a robust and easy to monitor intracellular calcium release in response to RF-amide peptides. Similarly, the human $G\alpha_{16}$ protein and its murine counterpart $G\alpha_{15}$ — which belong to the $G\alpha_q$ class of subunits (Wettschureck & Offermanns, 2005) — were employed to artificially couple NPFF1R (Kim et al., 2015) and NPFF2R (Kotani et al., 2001b) to phospholipase C activation.

A few data in the literature suggested a possible coupling between NPFF receptors and $G\alpha_s$. NPFF and 1-DMe were reported to stimulate, though at a very high concentration, adenylate cyclase in mouse olfactory bulb and spinal cord membranes (Gherardi & Zajac, 1997). In isolated rat dorsal raphe neurons which express essentially NPFF2 receptors (Gouarderes et al., 2002), NPFF was found to partially block the inhibition exerted by nociceptin on $[Ca^{2+}]_i$ transients triggered by depolarization (Roumy & Zajac, 1999); such an effect was prevented by the cholera toxin CTX, indicating that these receptors may couple to stimulatory G_s proteins (Roumy & Zajac, 2001). As well, co-transfection of NPFF1 receptors with the $G\alpha_{q/s}$ chimera allowed NPFF to promote a weak but significant intracellular calcium mobilization in COS cells (Bonini et al., 2000). No responses were observed with the NPFF2 receptor, again suggesting coupling differences between the two NPFF receptors.

It is now well admitted that GPCRs may couple with a variety of G proteins, or even bypass these canonical partners, to initiate an array of intracellular signaling cascades (Defea, 2008; Maurice et al., 2011). The selection of particular pathways is primarily dependent on the agonist nature, a phenomenon often referred to as biased agonism (Galandrin et al., 2007; Kenakin & Christopoulos, 2013; Wisler et al., 2014). In order to investigate the repertoire of endogenous G-proteins involved in NPFF-mediated signal transduction, several synthetic peptides corresponding to the last ten carboxy-terminal residues of various Gα subunits were tested for their ability to alter NPFF receptor binding or function. Following their delivery in SH-SY5Y cells stably expressing the NPFF2 receptor, the $G\alpha_{i1,2}$, $G\alpha_o$ and $G\alpha_{i3}$ peptides inhibited specific [^{125}I]-EYF binding to various extents, probably by promoting R–G uncoupling (Mollereau et al., 2005a). Surprisingly, the $G\alpha_s$ peptide was as efficient as $G\alpha_{i1,2}$ in reducing [^{125}I]-EYF binding and was found to potentiate the anti-opioid effect of 1-DMe in Ca^{2+} conductance measurements. Using the [^{35}S]-GTPγS binding experimental paradigm and various blocker peptides, Gouarderes and colleagues showed that $G\alpha_{i3}$ and $G\alpha_s$ are the main transducers for NPFF1 receptors in CHO cells while NPFF2 may couple to $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$ and $G\alpha_s$ (Gouarderes et al., 2007).

Many GPCRs exploit the release of the Gβγ heterodimer, consecutive to G protein activation (essentially the $G_{i/o}$ class), to enrich their signaling repertoire (Clapham & Neer, 1997; Wettschureck & Offermanns, 2005). Consistent with this observation, short application (15–30 s) of various NPFFR agonists was found to inhibit voltage-gated (N-type) Ca^{2+} channels in dissociated DRG neurons (Roumy & Zajac, 1996; Gelot et al., 1998) and in SH-SY5Y neuroblastoma cells transfected with NPFF1 (Kersante et al., 2006) or NPFF2 (Mollereau et al., 2007) receptors and to enhance G_q-triggered IP_3-gated Ca^{2+} channel activity in NPFF1R- (Kersante et al., 2006) and NPFF2R- (Mollereau et al., 2005a) expressing SH-SY5Y cells. Both responses, which were sensitive to PTX treatment, most likely involved the mediation of Gβγ subunits released from $G_{i/o}$ proteins. Indeed, inhibition of $[Ca^{2+}]_i$ transients is known to rely on direct Gβγ binding to the α_1 subunit of Ca_V2 calcium channels (Clapham & Neer, 1997; Zamponi & Currie, 2013) whereas coincident signaling between $G_{i/o}$ and G_q coupled receptors arises from a physical interaction between Gβγ and PLC, leading to a potentiation of Gq-triggered Ca^{2+} release from intracellular stores (Philip et al., 2010; Lyon & Tesmer, 2013).

Interestingly, the attenuation of $[Ca^{2+}]_i$ transients in response to acute NPFFR activation vanished with prolonged agonist pre-incubation time (20–30 min), probably due to receptor desensitization (Kersante et al., 2006; Mollereau et al., 2007). Such a condition (lack of direct effect of NPFF derivatives on the magnitude of $[Ca^{2+}]_i$ rise evoked by cell depolarization) was selected in a number of studies to evidence the anti-opioid effects of NPFF receptors, in recombinant cells and in isolated neurons (Mollereau et al., 2005b; Mouledous et al., 2010b, for reviews)

Consistent with a recurrent observation for many GPCRs, NPFF2R activation by NPFF was found to recruit beta-arrestin 2 at the plasma membrane level of HEK cells (Elshourbagy et al., 2000). Finally, it is worth mentioning that in differentiated SH-SY5Y cells, which express endogenous NPFF2 receptors, NPFF rapidly and transiently stimulated the ERK pathway, in a cAMP-dependent manner, and activated components of the NF-κB system in an ERK-independent manner (Sun et al., 2012). NPFF was also found to stimulate the ERK pathway in SK-N-MC neuroblastoma cells (Anko & Panula, 2006) while RFRPs inhibited gonadotropin gene transcription in gonadotrope LβT2 cells via inhibition of AC/cAMP/PKA-mediated ERK activation (Son et al., 2012).

5.2. PrRP-R signaling pathways

Along with the discovery of the PrRP receptor, arachidonic acid metabolite release was first taken as a biological output for screening putative endogenous ligands on transfected CHO cells (Hinuma et al., 1998). Such a PrRP-mediated response was linked to the lipoxygenase pathway. Further examination of PrRP effects on primary or adenoma-derived pituitary cells (with endogenous PrRP-R expression) pointed to their ability to stimulate prolactin hormone (PRL) secretion (Hinuma et al., 1998).

There is now an overall consensus to consider functional G_q coupling and IP_3-induced calcium mobilization from intracellular stores as a major transduction pathway for PrRP receptors (Hinuma et al., 1998; Roland et al., 1999; Langmead et al., 2000). Both PrRP-20 and PrRP-31 behaved as full agonists and activated the receptor with equal potencies (Langmead et al., 2000; Engstrom et al., 2003; Elhabazi et al., 2013), suggesting a pivotal role for the amidated C-terminal part of these peptides (Hinuma et al., 1998; Roland et al., 1999; Engstrom et al., 2003). As shown in Table 3, PrRPs potencies in promoting a calcium response through PrRP-R activation well agreed with binding affinities.

PrRPs were first reported to have no relevant effects on either basal or forskolin-stimulated cAMP levels (Langmead et al., 2000). However, other studies pointed to the ability of these peptides to partially suppress cAMP production in pituitary cells (Hinuma et al., 1998) and of PTX to abolish by more than 80% the stimulation of [^{35}S]-GTPγS binding to membranes of PrRP-R expressing CHO cells in response to PrRP-20 and -31 (Engstrom et al., 2003). The latter data thus suggested a possible coupling of PrRP-R to the $G_{i/o}$ class of G proteins in these cells.

PrRPs (0.1–10 nM) were also found to significantly increase cAMP levels both in rat PC12 cells (Nanmoku et al., 2003; Samson & Taylor, 2006) and in PrRP-R expressing CHO cells (Wang et al., 2012), supporting G_s coupling. Addition of PrRPs to rat PC12 cells stimulated dopamine release as well as activity and expression of tyrosine hydroxylase, the enzyme responsible for the biosynthesis of catecholamines (Nanmoku et al., 2003, 2005). These effects were blocked by calcium chelators and reduced by PKA and PKC inhibitors, suggesting the involvement of G_s/AC/PKA and G_q/PLC/PKC pathways.

Possible regulation by PrRPs of the mitogen-activated protein kinase (MAPK) family has been investigated in rat RC-4B/C or GH3 pituitary tumor cells and in PC12 pheochromocytoma cells, which all express endogenous PrRP receptors (Hinuma et al., 1998; Takahashi et al., 2002; Nanmoku et al., 2003; Maixnerova et al., 2011). In RC-4B/C cells, PrRP-20 and -31 increased ERK1/2 and CREB phosphorylation and induced prolactin release (Maixnerova et al., 2011). In PC12 and GH3 cells, the activation of ERK and JNK by PrRPs was found to primarily involve $G_{i/o}$ coupling, with a prominent role exerted by Gβγ heterodimers (Kimura et al., 2000; Nanmoku et al., 2005). p38 MAPK activity remained unaffected (Nanmoku et al., 2005). In GH3 cells, both ERK

and JNK cascades were necessary to stimulate rat prolactin (rPRL) promoter activity, probably via the phosphorylation of an Ets transcription factor (Kimura et al., 2000). In PC12 cells, PrRP stimulation of ERK activity has been proposed to regulate tyrosine hydroxylase transcription, possibly through formation of an AP-1 complex (Nanmoku et al., 2005).

PrRP-R signaling also included Akt activation, a serine/theonine kinase known to play a central role in regulating key functions such as protein synthesis, glucose homeostasis, cell survival and death. In GH3 cells, PrRPs-induced Akt activation was mediated by $G_{i/o}$ proteins, in a $G_{\beta\gamma}$-dependent manner, and was partially dependent on PKC and extracellular calcium (Hayakawa et al., 2002). Blockade of Akt activation by wortmannin and examination of the effects of dominant-negative Akt or CREB proteins or of constitutively active Akt on rPRL promoter activity allowed the authors to suggest that PrRPs activation of a PI3Kinase/Akt cascade is necessary to induce rPRL promoter activity via a CREB-dependent mechanism. Recently, the PrRP receptor has been shown to play a key role in the pathogenesis of uterine fibroids, with PrRPs promoting specifically the proliferation of leiomyoma cells through activation of a PI3K-Akt-mTOR pathway (Varghese et al., 2013). Moreover, the aberrant expression of PrRP-R by leiomyoma cells was linked to the loss of transcriptional repression exerted by the REST tumor suppressor in the normal myometrum.

Further information on down-stream signaling pathways for the PrRP-R was provided by Lin and coworkers who identified a typical motif at the receptor C-terminus prone to elicit an interaction with PDZ domain proteins (Lin et al., 2001). Co-immunoprecipitation studies pointed the glutamate receptor interacting protein (GRIP), AMPA binding protein (ABP) and protein that interacts with C-kinase (PICK1) as potential interacting partners for both PrRP and AMPA receptors, suggesting that these receptors could be scaffolded together at the synapse level. More recently, PICK1 has been found to play a regulatory role in PrRP-R clustering and recycling (Madsen et al., 2012).

5.3. Kiss1R signaling pathways

In various Kiss1R-transfected cell models, kisspeptins promote a robust calcium signal as the result of receptor coupling to the $G_{q/11}$ protein, activation of phospholipase C (PLC), cleavage of phosphatidylinositol 4,5 biphosphate (PIP_2), and IP_3-mediated calcium release from intracellular stores (Kotani et al., 2001a; Muir et al., 2001; Ohtaki et al., 2001). Kisspeptin-induced calcium mobilization was not affected by PTX (Kotani et al., 2001a) nor were basal or forskolin-elevated cAMP levels modulated by kisspeptins (Muir et al., 2001), supporting a lack of coupling of Kiss1R to $G_{i/o}$ or G_s proteins. Thus, and although kisspeptins may also trigger the release of arachidonic acid (Kotani et al., 2001a), monitoring of calcium mobilization became a popular functional assay to evaluate the agonist nature and potency of peptide derivatives at the Kiss1 receptor. As shown in Table 3, kisspeptins were active in the low nanomolar concentration range while NPFF- and NPAF-related peptides, as well as RFRPs, PrRPs and QRFPs, did not bind to nor stimulated the Kiss1R. The N-terminally truncated peptides, kisspeptin-10 and -13 were three to ten times more active than kisspeptin-54 (metastin). Non-amidated metastin was weakly active (Ohtaki et al., 2001), again pointing to the importance of the C-terminally amidated sequence in receptor activation. To note an overall good correlation between peptide affinity and agonist potency.

On the other hand, the rise in intracellular calcium and diacylglycerol levels, as the result of enhanced PIP_2 hydrolysis by Kiss1R-stimulated phospholipase C, leads to protein kinase C (PKC) activation. In turn, activated protein kinase C initiates an array of intracellular phosphorylation events that appear to involve various subsets of kinases, depending on the cellular context. Indeed, in Kiss1R/CHO cells, kisspeptins induced a strong and sustained phosphorylation of ERK1/2 MAP kinases and a weak stimulation of p38 MAP kinase (Kotani et al., 2001a), similar to the pathway found in hypothalamic GnRH neurons (Liu et al., 2008). In a human placental cell line, kisspeptin-10 transiently activated ERK1/2 and p38 MAP kinases and inhibited GSK3 beta activity through its phosphorylation (Roseweir et al., 2012). In anaplastic thyroid cancer cells with endogenous Kiss1R expression, metastin activated only ERK (Ringel et al., 2002) while it stimulated ERK and PI3K/Akt pathways in Kiss1R-null thyroid cancer cells stably transfected with Kiss1R (Stathatos et al., 2005). In a pancreatic beta cell line, kisspeptin-54 was reported to inhibit the expression of the transcriptional factor Isl-1 and insulin secretion via Ca^{2+}/PKC/ERK1/2 pathways (Chen et al., 2014). In a breast cancer cell line, the recruitment of GRK2 and β-arrestin-2 in response to kisspeptin-10 was found a critical step for Kiss1R signaling towards ERK1/2 (Pampillo et al., 2009). In MEF fibroblasts, Kiss1R was reported to positively regulate ERK1/2 activity, using $G\alpha_{q/11}$ and β-arrestin-2 pathways in a co-dependent and temporally overlapping manner. While β-arrestin-2 potentiated signaling to ERK, downstream of Kiss1R, β-arrestin-1 had the opposite effect (Szereszewski et al., 2010). The possibility for β-arrestins to mediate Kiss1R signaling in their own right was further investigated in two recent studies. In mice lacking either β-arrestin-1 or -2, Kp-54-triggered GnRH secretion was significantly reduced as assessed from serum gonadotropin levels (Ahow et al., 2014). Conversely, following selective ablation of $G\alpha_{q/11}$ in GnRH neurons, mice exhibited a milder reproductive phenotype as compared to wild-type but kept significant hormonal responses to Kp-54 administration (Babwah et al., 2015). These observations clearly indicated that Kiss1R remains signaling competent in the absence of $G\alpha_{q/11}$ and that it may employ the β-arrestin pathway to mediate GnRH secretion in a $G\alpha_{q/11}$-independent manner.

The physiological relevance of the Kiss1R/PLC/Ca^{2+} pathway was confirmed by pharmacological experiments performed on rat hypothalamic explants (Castellano et al., 2006a) as well as by electrophysiological recordings and calcium imaging studies in GnRH neurons from GnRH-GFP mice (Dumalska et al., 2008; Liu et al., 2008; Zhang et al., 2008). The $G\alpha_q$ signaling pathway was clearly involved in the stimulatory effect of kisspeptins on GnRH secretion (Castellano et al., 2006a) and in the ability of these peptides to evoke a potent and sustained depolarizing response in GnRH neurons (Dumalska et al., 2008; Liu et al., 2008; Pielecka-Fortuna et al., 2008; Zhang et al., 2008). Long-lasting depolarization and increased firing rate of GnRH neurons mainly resulted from the concerted closure of inwardly rectifying K^+ (Kir) channels, opening of transient receptor potential (TRPC) channels, and inhibition of a slow afterhyperpolarization (sAHP) current. Kisspeptin-induced PIP_2 hydrolysis inhibits Kir channels (K_{ATP} and GIRK channels) opening, a way to overcome the inhibition of GnRH neurons exerted by several $G\alpha_{i/o}$ coupled receptors, such as $GABA_B$ (Zhang et al., 2009) and μ-opioid (Lagrange et al., 1995) receptors. In turn, activation of TRPC channels requires PIP_2 depletion and cSrc kinase activation and is probably facilitated by calcium influx through low voltage-activated T-type calcium channels (Zhang et al., 2008; Rønnekleiv & Kelly, 2013; Zhang et al., 2013a). Kisspeptin inhibition of calcium-activated slow afterhyperpolarization (sAHP) currents was reported to involve protein kinase C activity and to facilitate a sustained firing of GnRH neurons (Zhang et al., 2013b). Once cells are depolarized, the opening of high voltage-activated Ca^{2+} (VGCCs) channels participates, together with TRPC channel activation and Ca^{+2} release from IP_3- and ryanodine-sensitive stores, to the overall rise in intracellular Ca^{2+} concentration that leads to GnRH secretion (Zhang & Spergel, 2012; Rønnekleiv & Kelly, 2013).

Following the first description of *KiSS-1* as a tumor suppressor gene (Lee et al., 1996), the effects of kisspeptins on cell mobility and chemotaxis, proliferation and cytoskeletal organization have been evaluated, together with underlying signaling processes. Among noticeable effects, metastin inhibited the chemotaxis of CHO cells towards fetal calf serum and of B16-BL6 melanoma cells towards fibronectin, only when Kiss1R was expressed in these cells (Ohtaki et al., 2001). Kisspeptin-10 inhibited trophoblast migration and their invasion of the uterine extracellular matrix, a process essential for fetal development (Bilban et al.,

Table 4
Binding affinities of NPFF-related peptides for endogenous RF-amide receptors expressed in the dorsal horn of rat spinal cord.

NPFF derivatives	Sequence									K_i values (nM)	
	1	2	3	4	5	6	7	8		[^{125}I]Tyr^1-NPFF[a]	[^{125}I]-1DMe[b]
NPFF	F	L	F	Q	P	Q	R	F	$-NH_2$	0.21 ± 0.03	0.34 ± 0.07
NPFF-OH	F	L	F	Q	P	Q	R	F	**–OH**		5,178 ± 2,195
NPFF(2–8)		L	F	Q	P	Q	R	F	$-NH_2$	0.20 ± 0.02	
NPFF(3–8)			F	Q	P	Q	R	F	$-NH_2$	0.43 ± 0.06	
NPFF(4–8)				Q	P	Q	R	F	$-NH_2$	20.9 ± 3.1	
NPFF(5–8)					P	Q	R	F	$-NH_2$	15.5 ± 2.3	
NPFF(6–8)						Q	R	F	$-NH_2$	300 ± 45	
[Tyr^8]NPFF	F	L	F	Q	P	Q	R	**Y**	$-NH_2$		34 ± 10
[Ala^8]NPFF	F	L	F	Q	P	Q	R	**A**	$-NH_2$		312 ± 73
[Lys^7]NPFF	F	L	F	Q	P	Q	**K**	F	$-NH_2$		245 ± 90
[Ala^7]NPFF	F	L	F	Q	P	Q	**A**	F	$-NH_2$		2,359 ± 617
[Glu^6]NPFF	F	L	F	Q	P	**D**	R	F	$-NH_2$		307 ± 87
[Asn^6]NPFF	F	L	F	Q	P	**N**	R	F	$-NH_2$		7.3 ± 1.7
[Gly^4]NPFF	F	L	F	**G**	P	Q	R	F	$-NH_2$		10± 1
[Glu^4]NPFF	F	L	F	**D**	P	Q	R	F	$-NH_2$		1.15 ± 0.26
[Asn^4]NPFF	F	L	F	**N**	P	Q	R	F	$-NH_2$		2.34 ± 1.09
[Tyr^3]NPFF	F	L	**Y**	Q	P	Q	R	F	$-NH_2$		0.22 ± 0.03

Residue changes in the original NPFF sequence are highlighted in bold character.
[a] K_i values are from competition studies performed on rat spinal cord membrane preparations Gicquel et al. (1994).
[b] K_i values are from quantitative autoradiography performed on rat spinal cord sections Mazarguil et al. (2001).

2004; Roseweir et al., 2012). Metastin induced also an excessive formation of focal adhesions and stress fibers in Kiss1R/B16-BL6 cells — thereby explaining the observed reduction in cell motility — which was linked to the phosphorylation of focal adhesion kinase (FAK) and paxillin (Ohtaki et al., 2001). In Kiss1R/CHO cells, kisspeptin-10 stimulated stress fiber formation, in a C3 exoenzyme-dependent manner, suggesting a possible intervention of the Rho subfamily of G proteins (Kotani et al., 2001a). Kisspeptins severely reduced the proliferation rate of various Kiss1R-expressing cell lines (Kotani et al., 2001a; Becker et al., 2005), at variance with expected effects of MAPK pathways in the control of cell cycle and proliferation.

A number of mechanisms have been postulated for kisspeptin/Kiss1R anti-metastatic and tumor-suppressor effects in numerous human cancers, including a role on matrix metalloproteinases and an interplay of Kiss1R with chemokine and EGF receptors (Makri et al., 2008; Castano et al., 2009; Ji et al., 2013). It is important to mention here that, in breast cancer, elevated KiSS-1/Kiss1R expression was paradoxically correlated with worsened clinical outcome (Marot et al., 2007; Zajac et al., 2011; Cvetkovic et al., 2013).

5.4. QRFP-R signaling pathways

Pioneering work in the QRFP receptor field pointed to intracellular calcium mobilization as a major signal triggered by QRFP peptides in transfected CHO and HEK cells (Fukusumi et al., 2003; Jiang et al., 2003; Takayasu et al., 2006). These peptides also inhibited forskolin-induced cAMP accumulation in QRFP-R/CHO cells (Fukusumi et al., 2003). Thus, QRFP receptors may couple to both G_q and $G_{i/o}$ proteins. QRFP-26 has been reported to stimulate, though at high concentrations, cAMP production in rat pituitary cells pretreated with forskolin (Chartrel et al., 2003); the underlying mechanism remains to be clarified.

As shown in Table 3, QRFP-26 and QRFP-43 elicit a calcium response in the (sub) nanomolar concentration range, but they also inhibit cAMP production (Fukusumi et al., 2003). They are equipotent agonists in both assays, in agreement with their binding affinity for the human QRFP-R. Regardless the human, mouse or rat origin of QRFPs, the two mouse receptor isoforms were slightly less efficient than the human receptor in triggering intracellular calcium release, with EC_{50} values ranging from 73 to 194 nM and from 42 to 70 nM, for QRFP-R1 and QRFP-R2 receptors respectively (Takayasu et al., 2006).

In the rat perfused pancreas, QRFP-26 was found to reduce the insulin secretion induced by either glucose, arginine or exendin-4, a GLP1 (glucagon-like peptide 1) analog that stimulates cAMP production. Such a response to QRFP-26 was suggested to involve QRFP-R/$G_{i/o}$ coupling as it was absent in PTX-treated rats (Egido et al., 2007).

Activation of the QRFP receptor in human adrenocortical H295R cells also led to a decrease in cAMP production and to an increase of intracellular Ca^{2+} levels. QRFP treatment of these cells induced the secretion of aldosterone and cortisol which was found to involve PKC, MAPK and/or T-type Ca^{2+} channel-dependent pathways (Ramanjaneya et al., 2013). As discussed by the authors, future studies should delineate which particular G protein inhibitory α-subunits couple to the QRFP receptor and how they may lead to an increase in $[Ca^{2+}]_i$ levels. Whether T-type calcium channels modulate QRFP-induced aldosterone secretion via an interaction with Gβγ subunits remains an interesting question. Indeed, their activity was found to be inhibited by certain βγ dimers, independently of voltage, in a channel subtype-specific manner unique to the Ca_v3 family (Wolfe et al., 2003).

6. Structure–activity relationships studies on mammalian RF-amide peptides and non-peptidic ligands for RF-amide receptors

Structure–activity/affinity relationship (SAR) studies are a key step towards a better comprehension of ligand features underlying high activity/affinity at their cognate receptors. They are also essential for the development of highly selective pharmacological tools (agonist or antagonist compounds) prone to unravel in vivo functional properties of given receptor subtypes under physio-pathological conditions. In the RF-amide field, several SAR studies have been conducted to define the minimal peptide sequence and the critical amino acids responsible for a high affinity and selective interaction with each RF-amide receptor. These studies, together with high-throughput screening campaigns, provided valuable information to develop several non-peptidic

Table 5
Comparison of potency (EC_{50}) and efficacy (E_{max}) at human NPFF1 and NPFF2 receptors of NPFF analogs with variations in their RF-amide motif.[a]

NPFF derivatives	Sequence	EC_{50} (nM); E_{max} (%)			
		NPFF1R		NPFF2R	
NPFF	F-L-F-Q-P-Q-Arg-Phe-NH_2	12	100%	3	100%
Modification of the phenylalanine at position 8					
[Ala^8]NPFF	F-L-F-Q-P-Q-Arg-**Ala**-NH_2	ND	–	ND	(25%)
[Nle^8]NPFF	F-L-F-Q-P-Q-Arg-**Nle**-NH_2	659	93%	287	(83%)
[Cha^8]NPFF	F-L-F-Q-P-Q-Arg-**Cha**-NH_2	44	91%	17	(98%)
[Tyr^8]NPFF	F-L-F-Q-P-Q-Arg-**Tyr**-NH_2	301	91%	70	(83%)
[Trp^8]NPFF	F-L-F-Q-P-Q-Arg-**Trp**-NH_2	3,410	55%	205	(99%)
[His^8]NPFF	F-L-F-Q-P-Q-Arg-**His**-NH_2	2,180	29%	2,750	67%
[D-Phe^8]NPFF	F-L-F-Q-P-Q-Arg-**Phe**-NH_2	200	84%	132	115%
[Hph^8]NPFF	F-L-F-Q-P-Q-Arg-**Hph**-NH_2	1,760	61%	1,331	98%
[Phg^8]NPFF	F-L-F-Q-P-Q-Arg-**Phg**-NH_2	1,780	29%	1,070	98%
Modification of the arginine at position 7					
[Ala^7]NPFF	F-L-F-Q-P-Q-**Ala**-Phe-NH_2	7,610	26%	1,228	(60%)
[Cit^7]NPFF	F-L-F-Q-P-Q-**Cit**-Phe-NH_2	3,170	66%	2,469	(59%)
[Lys^7]NPFF	F-L-F-Q-P-Q-**Lys**-Phe-NH_2	1,290	83%	565	(81%)
[Orn^7]NPFF	F-L-F-Q-P-Q-**Orn**-Phe-NH_2	3,510	57%	1,692	74%
[Agb^7]NPFF	F-L-F-Q-P-Q-**Agb**-Phe-NH_2	1,390	31%	1,219	96%
[Agp^7]NPFF	F-L-F-Q-P-Q-**Agp**-Phe-NH_2	3,200	17%	1,524	54%

Residue changes in the original NPFF sequence are highlighted in bold character.
Values are from dose-response curves using an inositol phosphate accumulation assay on COS-7 cells transfected with human NPFF receptors and a chimeric $G\alpha_q$ protein. EC_{50} values refer to agonist concentrations that evoked a half-maximal response. E_{max} values were defined at the highest agonist concentration tested, usually at 100 μM or at 10 μM (when indicated under parentheses). ND: the EC_{50} value was not determined as full receptor activation could not be ascertained at the agonist concentration which was tested (10 μM).
[a] Data are taken from Findeisen et al. (2011b).

compounds, exhibiting together greater stability, agonist or antagonist activities and therapeutic potential.

6.1. Structure–activity/affinity relationship on the neuropeptide FF peptide scaffold

Several modifications (amino acid deletions or substitutions) were introduced within the NPFF sequence (both at its N- or C-terminus) and the binding affinities of the resulting peptides were examined using [^{125}I][Tyr^1]NPFF (Gicquel et al., 1994) or [^{125}I]1DMe-NPFF (Mazarguil et al., 2001) as the radioligands. Despite the lack of R1/R2 selectivity of these tracers (see Table 2), data presented in Table 4 most likely refer to the NPFF2 receptor as binding studies were performed on rat spinal cord preparations highly enriched in NPFF2R (Liu et al., 2001; Gouarderes et al., 2002, 2004b). First of all, NPFF-OH (with a carboxylic acid group at its C-terminus) displayed no significant affinity, highlighting the common property of the RF-amide receptor family to bind C-terminally amidated neuropeptides. While deletion of the first two amino acids at the NPFF N-terminus did not alter their affinity towards NPFF receptors, NPFF(4–8) and NPFF(5–8) showed a reduction in affinity of 2 orders of magnitude. Further shortening of the NPFF sequence led to the tripeptide NPFF(6–8) with submicromolar affinity. Conservative substitution of Phe^8 for another aromatic amino acid such as tyrosine ([Tyr^8]NPFF), or of Arg^7 for another basic residue ([Lys^8]NPFF), both led to a marked decrease in affinity. The replacement of Arg^7 or Phe^8 residues with alanine, such as in [Ala^7]NPFF and [Ala^8]-NPFF, was much more deleterious. While substitution of the Gln^6 residue for its acid counterpart ([Glu^6]NPFF) also led to a significant reduction in affinity, modifications of the four first N-terminal NPFF amino acids were much better tolerated, highlighting the primary importance of the C-terminal RF-amide motif for high affinity binding to NPFF receptors.

Whether the typical Arg-Phe-NH_2 signature also dictates functional activity for NPFF was further investigated by replacing Arg^7 or Phe^8 with a series of aliphatic and aromatic residues (Table 5). The potency and efficacy of NPFF analogs were determined from IP accumulation assays performed on COS-7 cells co-transfected with a chimeric G protein and human NPFF1 or NPFF2 receptors (Findeisen et al., 2011b). In agreement with former binding data (Table 4), substitution of Phe^8 for Ala abolished NPFF agonist activity at NPFF1 and NPFF2 receptors. Interestingly, its replacement with non-aromatic cyclohexylalanine ([Cha^8]-NPFF) preserved significant activity at both receptors. Conversely, the substitution of Phe at the C-terminus by other aromatic amino acids such as Tyr, and especially Trp or His residues, led to a marked loss of peptide activity especially towards NPFF1R. Substitution of the natural L-Phe enantiomer for the D-Phe one resulted in a significant drop of agonist potency at both receptor subtypes. Elongation or shortening of the Phe side chain, such as in [Hph^8]NPFF or [Phg^8]NPFF, severely impaired peptide potency at both receptors and impacted agonist efficacy especially at the NPFF1 receptor. Substitution of Arg^7 by non-protonable side chains, such as in [Ala^7]NPFF Ala or [Cit^7]NPFF, led to a drastic loss of activity, suggesting the requirement of a cationic group at this position. However, its replacement by charge-conserving residues (such as Lys or Orn) or by Arg analogs with shorter side chains (such as Agp and Apg), also markedly reduced peptide potencies at both receptors. Nevertheless, many of these compounds retained a full agonist activity towards NPFF2R, while exhibiting partial agonism at NPFF1 receptors.

Selectivity is also a critical point to evaluate the role of each individual receptor subtype. NPFF per se exhibits a modest selectivity towards NPFF2 receptor (Table 2). As the C-terminus part was considered as crucial for affinity and functional potency, Mazarguil and coworkers (Mazarguil et al., 2012) introduced an alanine-based sequence at the N-terminus of NPFF to obtain the dA(NMe)AAFLFQPQRF-NH_2 peptide that displayed a better NPFF2R selectivity (K_i values 0.16 nM and 12.1 nM at NPFF2R and NPFF1R, respectively) essentially due to a decrease in affinity at the NPFF1R subtype.

6.2. Dipeptidic and peptidomimetic agonists and antagonists of neuropeptide FF receptors

A dipeptide derivative as small as dansyl-RF-amide was reported to suppress morphine-induced analgesia in vivo after peripheral administration, suggesting that the minimal Arg-Phe-NH_2 sequence was essential for anti-opioid activity (Brussaard et al., 1989). Indeed, this compound was later found to bind NPFF receptors in the rat spinal cord with fairly high affinity (Payza et al., 1993). Taking the Arg-Phe-NH_2 motif as the minimal sequence required for peptide activity, Simonin and colleagues synthesized the dipeptide **RF9** (Fig. 1), with a bulky adamantoyl moiety at its N-terminus, and provided evidence for its antagonist nature and similar binding affinity constants at human NPFF1 (58 nM) and NPFF2 (75 nM) receptors (Simonin et al., 2006). More recently, independent studies reported on weak partial to full agonist activity of **RF9** at NPFF1R and/or NPFF2R expressed in heterologous cell lines (Talmont et al., 2010; Findeisen et al., 2012; Kim et al., 2015). Overall, these findings raise the question of the relevance of the various in vitro assays built to study NPFFR functional properties. Such

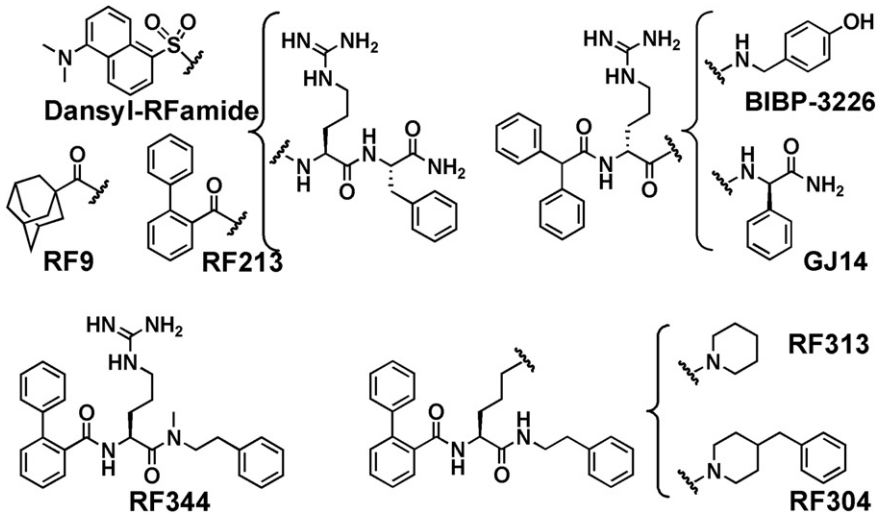

Fig. 1. Dipeptides, arginine and non-natural amino acids as ligands of NPFF receptors. See text for further details on structural and pharmacological properties of the compounds.

an important issue deserves further consideration. As **RF9** interacted with other RF-amide receptors only in the low micromolar range, it was introduced as the first selective NPFF antagonist available (Simonin et al., 2006). In contrast with the former dansyl-RF-NH_2 agonist, **RF9** was able to prevent opioid-induced hyperalgesia and associated analgesic tolerance following its peripheral administration in rats (Simonin et al., 2006). **RF9** was also found to display a strong gonadotropin-releasing activity in vivo in several mammalian species (Pineda et al., 2010a,b; Caraty et al., 2012a; Rizwan et al., 2012) that was suspected to involve an off-target action on kisspeptin receptors (Garcia-Galiano et al., 2012; Glanowska et al., 2014; Liu & Herbison, 2014; Sahin et al., 2015). Very recently, its Kiss1R agonistic component was clearly evidenced both in vitro and in vivo (Kim et al., 2015; Min et al., 2015), thereby questioning **RF9** reliability as a pharmacological tool to delineate the role of NPFF receptors in the modulation of reproductive function.

An extensive structural exploration of the N-terminus of the Arg-Phe-NH_2 sequence led to the identification of **RF213**, a novel dipeptide bearing a 2-phenylbenzoyl moiety (Gealageas et al., 2012). **RF213** exhibited a subnanomolar affinity for NPFF1 receptors, similar to that of RFRP-3 (NPVF), and an appreciable selectivity for NPFF1R (K_i = 0.3 nM) versus NPFF2R (K_i = 590 nM), and the ability to oppose fentanyl-induced hyperalgesia in rats.

Both NPFF receptors exhibit a high structural homology with NPY receptors (Section 3.5.1) and a significant affinity for the NPY Y1-selective **BIBP3226** antagonist (Bonini et al., 2000; Mollereau et al., 2002). Inspired from previous works (Aiglstorfer et al., 2000; Mollereau et al., 2002), Kim and collaborators (Kim et al., 2015) reported on a NPFF antagonist structurally related to BIBP3226 (Fig. 1). Based on a D-Arg-D-Phg backbone, this compound called **GJ14** showed preferential binding affinity for the NPFF1 receptor subtype (K_i values 21 nM and 387 nM at NPFF1R and NPFF2R, respectively). Interestingly, **GJ14** was found to exhibit anxiolytic properties, supporting NPFF receptors as novel potential targets for treating anxiety disorders. As a single but highly functionalized D-arginine residue, such as in **BIBP3226** (Fig. 1), may efficiently drive affinity and antagonistic properties at NPFF receptors (Mollereau et al., 2001, 2002; Kim et al., 2015), the C-terminus of the Arg-Phe-NH_2 analog **RF213** was deleted to afford the corresponding L-arginine **RF344** derivative (Fig. 1). This compound was found to bind with high affinity (K_i close to 20 nM) to both NPFF receptors (Bihel et al., 2015).

The significant affinity of the arginine **BIBP3226**, **GJ14** and **RF344** derivatives for NPFF receptors highlights the requirement of a basic moiety (guanidine of arginine) combined with a hydrophobic group (phenyl ring). To go further with this simple pharmacophore pattern, the guanidine moiety was replaced with various secondary or tertiary amines, following a peptidomimetic approach. A new set of non-natural ornithine derivatives was developed and tested on both NPFF receptors (Bihel et al., 2015). The most interesting compounds (shown on Fig. 1) were **RF313** (NPFF1R: K_i = 172 nM; NPFF2R: K_i = 563 nM) and **RF304** which exhibited a modest selectivity towards the NPFF2R subtype (NPFF1R: K_i = 400 nM; NPFF2R: K_i = 34 nM). As non-natural ornithine derivatives are much less polar (RF313: Log D = 3.4, PSA = 63 $Å^2$) than arginine derivatives, the probability they cross the blood–brain-barrier through a passive transport should be increased (Hitchcock, 2008). Indeed, **RF313**, despite its lower affinity for NPFF receptors when compared to **RF9** or **RF344**, was able to prevent opioid-induced hyperalgesia in rats after oral administration (Bihel et al., 2015).

6.3. Non-peptidic agonists and antagonists of neuropeptide FF receptors

Dipeptides analogs were developed following a rational approach starting from the natural NPFF sequence. Alternatively, small non-peptidic ligands of NPFF receptors were mainly identified from various chemical libraries following high throughput screening (HTS) campaigns. **Benzoyl Gomisin Q** and **Gomisin G** (Fig. 2), two natural compounds isolated from the Korean herb *Schizandra chinensis*, showed micromolar agonist potency at NPFF2R (Do et al., 2006). Interestingly, these compounds do not have any cationic moiety, suggesting that they bind to NPFF2R in a completely different way than arginine derivatives, although still keeping agonist activity.

Several pharmaceutical companies also discovered novel NPFF ligands (Fig. 2). In 2003, Synaptic Pharmaceuticals patented quinolino- and quinazolino-guanidine derivatives (Forray et al., 2003; WO/2010/026657). The quinazoline series showed submicromolar and micromolar affinities for hNPFF1R and hNPFF2R, respectively, and were described as hNPFF1 antagonists and hNPFF2 partial agonists. The quinoline series bound with higher affinity to both NPFF receptors, showing a modest selectivity for the NPFF1R subtype, and were described as antagonists. However, despite their drug-like character, quinazoline and its derivatives suffered from selectivity limitations, as they also displayed nanomolar affinities for a variety of receptors (NMDA, α_2-adrenergic, NPY1) and transporters (serotonin and norepinephrine). In 2004, Actelion Pharmaceuticals patented a series of guanidinothiazole compounds (Caroff et al., 2004; WO/2004/083218) as hNPFF1R antagonists in the low nanomolar affinity range. However, no data were provided regarding the interaction of these compounds with the hNPFF2R and their antagonistic character at hNPFF1R was not documented. In 2009, Acadia Pharmaceuticals reported the discovery and the characterization of the first non-peptidic NPFF2R agonists in

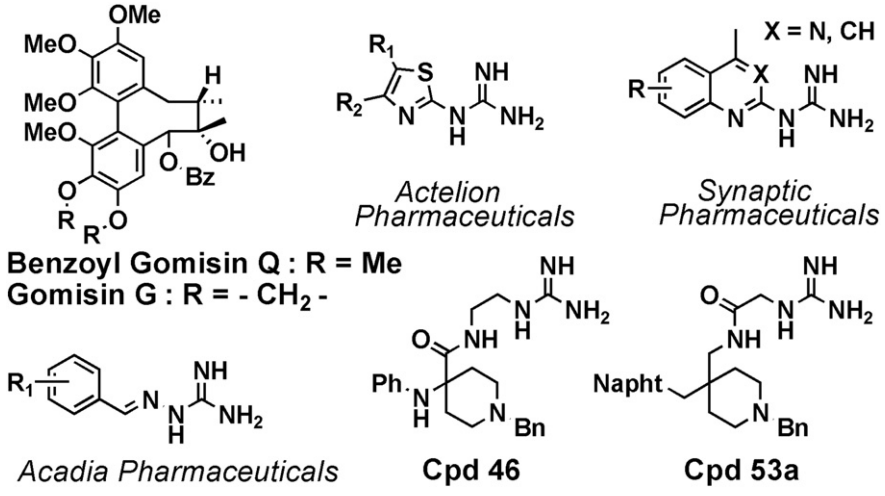

Fig. 2. Non-peptidic small molecules as ligands of NPFF receptors. See text for further details on structural and pharmacological properties of the compounds.

the micromolar range (Gaubert et al., 2009). Tested in vivo in rats, these aminoguanidines dose-dependently attenuated hypersensitivity in acute inflammatory and neuropathic pain models. Although structurally-related to Guanabenz, a well-known agonist of the α_2-adrenergic receptor used to treat hypertension, these compounds did not show any agonistic activity at the α_{2A}, α_{2B} or α_{2C} adrenergic receptors (Nguyen et al., 2014). Very recently, Journigan and collaborators described a novel series of ligands based on a generic guanidine–piperidine scaffold (Journigan et al., 2014). By simply modifying the functional groups linking the piperidine ring to the guanidine moiety and the aromatic platform, they were able to modulate the NPFF1/NPFF2 selectivity of ligands. While compound **53a** (Fig. 2) exhibited a modest preference towards the NPFF2R subtype (NPFF1R: K_i = 112 nM; NPFF2R: K_i = 30 nM), compound **46** was described as a NPFF1-selective antagonist (NPFF1R: K_i = 81 nM; NPFF2R: K_i = 1426 nM). Both antagonists, at high concentration, only partially reversed the cAMP responses of NPFF1R or NPFF2R expressing cells to agonists. Noteworthy, compound **46** was able to prevent opioid-induced hyperalgesia (tail withdrawal test) following i.c.v. administration in mice, suggesting that NPFF1R is critically involved in this phenomenon.

6.4. Structure–activity/affinity relationship on prolactin-releasing peptides and their analogs

The prolactin-releasing peptides PrRP-20 and PrRP-31 (Table 1) were much less studied than NPFF or Kisspeptins. In 2005, Boyle and coworkers reported an extensive SAR of the N-truncated peptide (19–31)-PrRP (Table 6; Boyle et al., 2005). In agreement with the prominent role of the RF-amide motif, PrRP with a C-terminal carboxylic acid was inactive. The systematic replacement of amino acids at different positions of (19–31)-PrRP pointed to a crucial role for the C-terminal heptapeptide. This SAR study confirmed previous work from Roland and collaborators who identified the heptapeptide (25–31)-PrRP as the shortest PrRP sequence preserving agonistic activity (Roland et al., 1999). However, a more recent study reported that Phe^{31} in PrRP-20 could be replaced with naphtylalanine or tyrosine, leading to compounds endowed with in vitro pharmacological properties comparable to those of PrRP-20 and able to elicit long-lasting anorexigenic effects after central administration in fasted mice (Maletinska et al., 2011). From a structural point of view, PrRP-20 exhibits an amphipathic α-helical secondary structure with the residues Ile^{14}, Val^{17} and Phe^{20} located on the same face of the helix (D'Ursi et al., 2002; DeLuca et al., 2013).

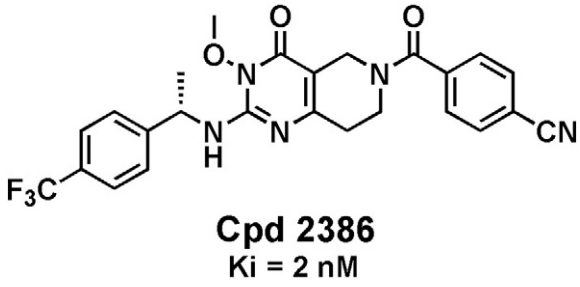

Fig. 3. Non-peptidic PrRP-R antagonist. The binding affinity constant K_i for Compound **2386** was derived from competition studies conducted on membranes from PrRP-R-expressing HEK cells using [^{3}H]-PrRP as the tracer (WO/2010/137738A1 patent). The IC_{50} value (the drug concentration that inhibits half of specific radioligand binding) was converted into a K_i value using the Cheng–Prusoff formula.

To date, no PrRP-R antagonists were described in peer-reviewed articles, but Otsuka Pharmaceuticals recently patented heterocyclic antagonists built on a tetrahydropyrido[4,3-d]pyrimidinone scaffold (Takahashi et al., 2010; WO/2010/137738). These compounds display, like compound **2386** (Fig. 3), a high affinity for the PrRP receptor and were developed for the treatment of stress-related diseases.

6.5. Structure–activity/affinity relationship on Kisspeptins and their analogs

Since the discovery of the kisspeptin receptor (Kiss1R or GPR54) and of its endogenous ligands (Table 1), numerous structure-activity studies focused on the N-terminally truncated sequences of kisspeptins (especially kisspeptin-10 and -13) and confirmed the C-terminus amide as a signature of this family of ligands (Table 7). Ala-scan experiments on kisspeptin-10 highlighted the critical role of ^{6}Phe and ^{10}Phe residues in terms of affinity and activity. Phe^{10} could be replaced by another aromatic residue (Trp, Tyr) without any loss of activity. Arg^9 may be replaced by a basic amino acid such as lysine, but more surprisingly, its substitution for Ala preserved a potent agonist activity (EC_{50} = 88 nM) despite the loss of the electrostatic interaction of the guanidine residue. Finally, Phe^6 appeared to be one of the most important residues

Table 6
Binding affinity and functional potency of PrRP-(19–31) peptide analogs at PrRP receptors.[a]

PrRP-related peptides	Sequence														PrRP-R	
															K_i (μM)	EC_{50} (μM)
(19–31)PrRP	W	Y	A	S	R	G	I	R	P	V	G	R	F	$-NH_2$	0.0053	0.02
(19–31)PrRP acid	W	Y	A	S	R	G	I	R	P	V	G	R	F	**–OH**	5.0000	–
[Trp^{31}](19–31)PrRP	W	Y	A	S	R	G	I	R	P	V	G	R	**W**	$-NH_2$	0.0245	0.05
[Nle^{31}](19–31)PrRP	W	Y	A	S	R	G	I	R	P	V	G	R	**Nle**	$-NH_2$	0.0195	0.05
[Leu^{31}](19–31)PrRP	W	Y	A	S	R	G	I	R	P	V	G	R	**L**	$-NH_2$	0.395	2.06
[Gln^{31}](19–31)PrRP	W	Y	A	S	R	G	I	R	P	V	G	R	**Q**	$-NH_2$	0.0635	0.17
[Met^{31}](19–31)PrRP	W	Y	A	S	R	G	I	R	P	V	G	R	**M**	$-NH_2$	0.0955	0.35
[Lys^{30}](19–31)PrRP	W	Y	A	S	R	G	I	R	P	V	G	**K**	F	$-NH_2$	–	Inactive
[Pro^{30}](19–31)PrRP	W	Y	A	S	R	G	I	R	P	V	G	**P**	F	$-NH_2$	–	Inactive
[Ala^{29}](19–31)PrRP	W	Y	A	S	R	G	I	R	P	V	**A**	R	F	$-NH_2$	0.0945	0.24
[Phg^{28}](19–31)PrRP	W	Y	A	S	R	G	I	R	P	**Phg**	G	R	F	$-NH_2$	0.0047	0.01
[Ala^{27}](19–31)PrRP	W	Y	A	S	R	G	I	R	**A**	V	G	R	F	$-NH_2$	0.1870	nd
[Ala^{26}](19–31)PrRP	W	Y	A	S	R	G	I	**K**	P	V	G	R	F	$-NH_2$	0.0220	0.12
[Ala^{25}](19–31)PrRP	W	Y	A	S	R	G	**A**	R	P	V	G	R	F	$-NH_2$	0.0505	0.13
[Phg^{25}](19–31)PrRP	W	Y	A	S	R	G	**Phg**	R	P	V	G	R	F	$-NH_2$	0.0082	0.01
[Ala^{24}](19–31)PrRP	W	Y	A	S	R	**A**	I	R	P	V	G	R	F	$-NH_2$	0.0115	0.01
[Pro^{23}](19–31)PrRP	W	Y	A	S	**P**	G	I	R	P	V	G	R	F	$-NH_2$	0.0115	0.62
[Gly^{22}](19–31)PrRP	W	Y	A	**G**	R	G	I	R	P	V	G	R	F	$-NH_2$	0.0078	0.01
[Thr^{21}](19–31)PrRP	W	Y	**T**	S	R	G	I	R	P	V	G	R	F	$-NH_2$	0.0068	0.02

Residue changes in the original PrRP sequence are highlighted in bold character.
K_i values are from competition binding studies, with Eu-(Lys)PrRP31 as a tracer, while EC_{50} values were determined in a calcium mobilization assay using HEK293-ASR-1 cells.
[a] Data are taken from Boyle et al. (2005).

Table 7
Binding affinity (K_i) and functional potency (EC_{50}) at Kiss1R of kisspeptin-10 and kisspeptin-13 derived peptides.[a]

Kisspeptin derivatives	Sequence														Kiss1R	
															K_i (nM)	EC_{50} (nM)
Kp-13	L	P	N	Y	N	W	N	S	F	G	L	R	F	$-NH_2$	1.1 ± 0.4	23 ± 11
Kp-13 acid	L	P	N	Y	N	W	N	S	F	G	L	R	F	**–OH**	552 ± 54	1070 ± 116
Kp-10				Y	N	W	N	S	F	G	L	R	F	$-NH_2$	1.3 ± 0.6	17.3 ± 10.7
[Trp^{10}] Kp-10				Y	N	W	N	S	F	G	L	R	**W**	$-NH_2$	1.6 ± 0.4	33.8 ± 14.4
[Tyr^{10}] Kp-10				Y	N	W	N	S	F	G	L	R	**Y**	$-NH_2$	2.2 ± 0.1	46.3 ± 11.3
[Ala^{10}] Kp-10				Y	N	W	N	S	F	G	L	R	**A**	$-NH_2$	240 ± 57	347 ± 111
[Leu^{10}] Kp-10				Y	N	W	N	S	F	G	L	R	**L**	$-NH_2$	81 ± 7	393 ± 107
[Gln^{10}] Kp-10				Y	N	W	N	S	F	G	L	R	**Q**	$-NH_2$	495 ± 63	2100 ± 300
[Lys^{9}] Kp-10				Y	N	W	N	S	F	G	L	**K**	F	$-NH_2$	9.4 ± 6.0	70 ± 12
[Cit^{9}] Kp-10				Y	N	W	N	S	F	G	L	**Cit**	F	$-NH_2$	88.1 ± 7.9	214 ± 84
[Ala^{9}] Kp-10				Y	N	W	N	S	F	G	L	**A**	F	$-NH_2$	83 ± 11	87.8 ± 3.3
[Ala^{8}] Kp-10				Y	N	W	N	S	F	G	**A**	R	F	$-NH_2$	30 ± 8	109 ± 38
[Ala^{7}] Kp-10				Y	N	W	N	S	F	**A**	L	R	F	$-NH_2$	5.0 ± 1.4	22.4 ± 10.4
[Ala^{6}] Kp-10				Y	N	W	N	S	**A**	G	L	R	F	$-NH_2$	199 ± 38	285 ± 76
[Trp^{6}] Kp-10				Y	N	W	N	S	**W**	G	L	R	F	$-NH_2$	7.7 ± 0.8	25.0 ± 9.5
[Tyr^{6}] Kp-10				Y	N	W	N	S	**Y**	G	L	R	F	$-NH_2$	16.2 ± 1.6	701 ± 30
[Leu^{6}] Kp-10				Y	N	W	N	S	**L**	G	L	R	F	$-NH_2$	142 ± 27	290 ± 85
[Gln^{6}] Kp-10				Y	N	W	N	S	**Q**	G	L	R	F	$-NH_2$	298 ± 27	215 ± 66
[Ala^{5}] Kp-10				Y	N	W	N	**A**	F	G	L	R	F	$-NH_2$	0.8 ± 0.3	29.4 ± 15.7
[Ala^{4}] Kp-10				Y	N	W	**A**	S	F	G	L	R	F	$-NH_2$	33.4 ± 9.5	39.5 ± 6.6
[Ala^{3}] Kp-10				Y	N	**A**	N	S	F	G	L	R	F	$-NH_2$	1.8 ± 0.4	11.3 ± 2.9
[Ala^{2}] Kp-10				Y	**A**	W	N	S	F	G	L	R	F	$-NH_2$	21.1 ± 4.7	30.3 ± 14.9
[Ala^{1}] Kp-10				**A**	N	W	N	S	F	G	L	R	F	$-NH_2$	10.5 ± 3.3	33.0 ± 14.8

Residue changes in the original Kp-10 sequence are highlighted in bold character.
For a sake of clarity, kisspeptin-10 and kisspeptin-13 derivatives are abbreviated as Kp-10 and Kp-13, respectively. K_i values are from competition experiments performed on Kiss1R-(GPR54) containing membranes, using [^{125}I]-metastin as the tracer. EC_{50} values are from a functional calcium mobilization assay performed on Kiss1R-expressing HEK293 cells.
[a] Data are taken from Orsini et al. (2007).

of the kisspeptin-10 sequence: this position did not tolerate many alterations and only aromatic residues preserved nanomolar binding affinity. NMR experiments on kisspeptin-13 in a membrane-like environment (SDS micelles) suggested a relatively stable helical conformation of the C-terminal part of the peptide (Orsini et al., 2007) while molecular modeling studies located the two important Phe^6 and Phe^{10} residues on the same face of the helix (Gutierrez-Pascual et al., 2009). Taking into account these results highlighting the importance of the C-terminus sequence of kisspeptin-10, Niida and coworkers proposed a series of N-terminus acylated pentapeptides (FGLRWa) and identified the **FM053a** compound (Fig. 4) as a full agonist (Niida et al., 2006; Tomita et al., 2006). A tryptophan residue at the C-terminus was preferred over phenylalanine as it slightly improved agonist activity at Kiss1R (Tomita et al., 2006). Further studies on this N-acylated pentapeptide series led to the agonist **FTM080** (Fig. 4), which was nearly as potent as kisspeptin-10 (Tomita et al., 2007, 2008a). However, **FTM080** was found to be a substrate of matrix-metalloproteinases (MMPs) and to be cleaved between glycine and leucine residues in murine serum (Tomita et al., 2008b). To bypass such an enzymatic degradation, the authors inserted an appropriate dipeptide isoster into the pentapeptide sequence and found **FTM145** (Fig. 4) to be more stable (murine serum: $t_{1/2}$ = 38 h) while keeping potent agonism at Kiss1R (Tomita et al., 2008b). Other modifications of kisspeptin-10 were also found to enhance its in vivo stability. In 2010, Curtis and coworkers reported that the [dY]1KP-10 analog, with a ^{D}Tyr at position 1, kept an in vitro bioactivity at Kiss1R similar to that of kisspeptin-10 while displaying an improved efficacy to increase plasma LH and testosterone levels in mice (Curtis et al., 2010). N^w-methylation of Arg^9, replacement of Gly^7 with azaglycine, and substitution of Trp^3 either by Ser, Thr, β-(3-pyridyl)alanine or by ^{D}Trp, led to a series of Kp-10 analogs that showed excellent Kiss1R agonist activity as well as higher stability in mouse serum (Asami et al., 2012a,b). All these modifications led to the disclosure by Takeda of compounds TAK-448 and TAK-683 (Matsui et al., 2012; Asami et al., 2013, 2014). Both pseudopeptides (Fig. 4), when administered subcutaneously for several days to healthy men or prostate cancer patients, were well-tolerated and induced a sustained down regulation of testosterone into the castration range (Scott et al., 2013; MacLean et al., 2014). These two phase-1 studies indicated that the metastin analogs TAK-448 and TAK-683 may be promising candidates in clinical trials for the treatment of prostate cancer. Very recently, the triazololipopeptide **17** (Fig. 4) that included a 1,2,3-triazole as a proteolysis-resistant amide bond mimic and an albumin-binding motif at its N-terminus, was introduced as a highly potent kisspeptin analog able to trigger a prolonged increase of both LH and FSH plasma concentration in the ewe during the anoestrus season (Beltramo et al., 2015).

In 2009, the first Kiss1R antagonist, the peptide 234, was synthesized on the basis of the substitution in kisspeptin-10 of Leu^8 for D-Trp and of Ser^5 for Gly (Roseweir et al., 2009). This peptide was able to reduce the pulsatile GnRH secretion in female pubertal monkeys and to inhibit the firing of GnRH neurons in murine brain. However, peptide 234 suffered from enzymatic degradation. In 2010, the Takeda pharmaceutical company isolated the first non-peptidic Kiss1R antagonists from a high-throughput screen of its internal chemical library. The antagonists **9I** (IC_{50} = 3.7 nM) and **15a** (IC_{50} = 3.6 nM) belong to a new family of compounds (Fig. 5) bearing a tetra-substituted pyridine scaffold (Kobayashi et al., 2010a,b; Doebelin et al., 2013). As a proof of concept, intravenous administration of **15a** to castrated male rat suppressed plasma LH level, indicating the possibility for Kiss1R antagonists to act as a novel drugs for sex-hormone dependent diseases.

6.6. Structure–activity/affinity relationship on QRFP peptides and their analogs

Along with the discovery of QRFP-43 (or 43RFa), Fukusumi and collaborators presented the first SAR study for QRFP neuropeptides (Fukusumi et al., 2003). QRFP-43 (with its typical N-terminal pyroglutamic acid) and the shorter QRFP-26 peptide (Table 1), which result from differential processing of the same precursor protein (see Section 2.2.5), display highest affinity and activity towards QRFP receptors (Table 3). As expected for RF-amide peptides, the carboxylic acid form of QRFPs was inactive. Moreover, serial deletion of the N-terminal sequence of QRFP-26 was found to gradually attenuate its affinity and potency (Fukusumi et al., 2003). The C-terminus decapeptide QRFP-(34–43) exhibited a submicromolar affinity for QRFP-R and very

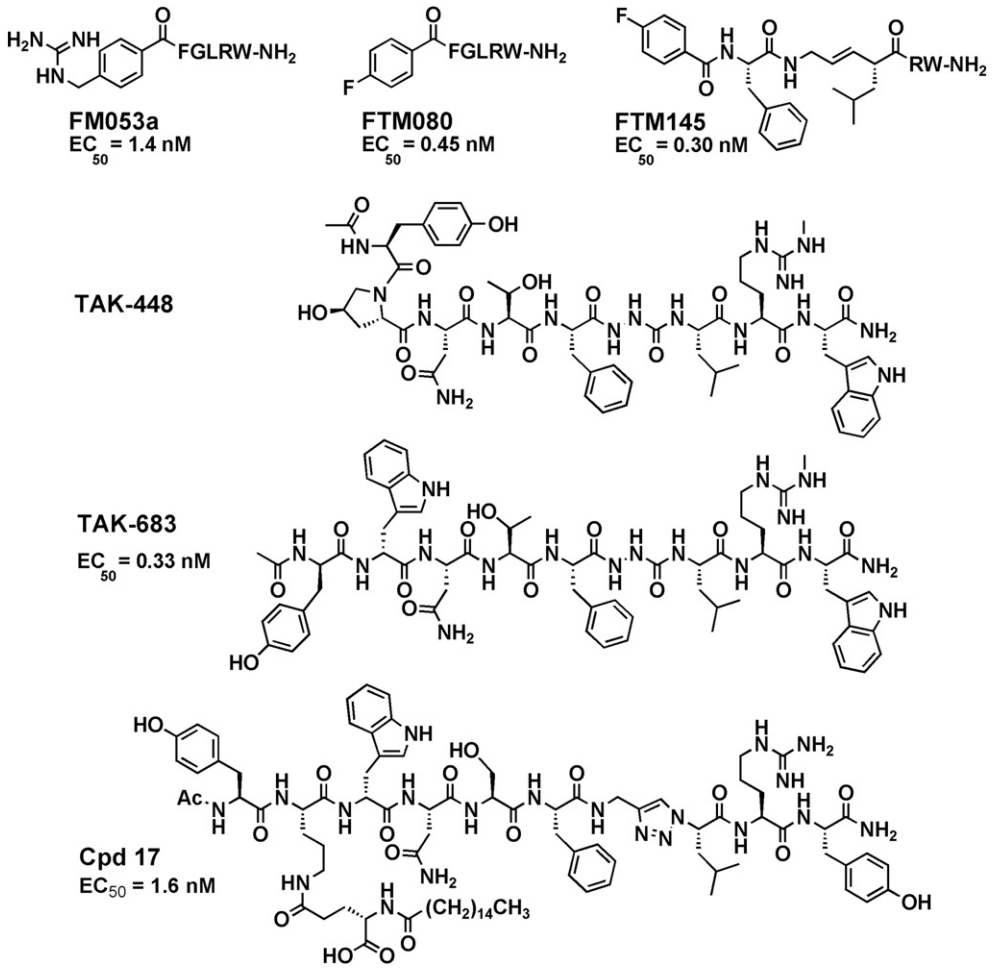

Fig. 4. Pentapeptides and peptidomimetics as Kiss1R agonists. The agonist activity of the compounds was evaluated through their ability to induce an intracellular calcium mobilization in Kiss1R-transfected CHO or HEK cells. EC_{50} values for **FM053a** (Tomita et al., 2006), **FTM080** (or compound 1 in Tomita et al., 2008b), **FTM145** (or compound 19 in Tomita et al., 2008b), **TAK-683** (or compound 22 in Asami et al., 2014) and Compound **17** (Beltramo et al., 2015) were derived from dose-response curves to define the concentrations of peptides leading to half-maximal effects.

weak (if no) agonist activity; thus, QRFPs appeared to be much more sensitive to sequence shortening than other RF-amide peptides such as NPFF or kisspeptins. This observation is consistent with structural studies indicating a well-defined conformation of QRFP-26, with an amphipathic α-helical structure (Pro^4-Arg^{17}) flanked by N- and C-terminal disordered regions (Thuau et al., 2005). Both the C-terminal sequence and the amphipathic helix seem important for QRFP-26 affinity and activity. However, a systematic Ala-scan and D-scan performed on the QRFP-(20–26) heptapeptide (GGFSFRF-NH_2) showed that the last three C-terminal residues were very sensitive to substitution, while position 23 rather well tolerated both modifications (Le Marec et al., 2011). Most importantly, replacement of ^{23}Ser by a norvaline led to the agonist [Nva^{23}](20–26)26RFa that was 3-fold more potent (EC_{50} = 233 nM) than the native heptapeptide (Le Marec et al., 2011). Using a peptidomimetic approach applied to QRFP-(20–26), the same authors designed a series of aza-β^3 analogs and identified the [$Cmpi^{23}$, aza-β^3-Nva^{23}](21–26)RFa pseudopeptide as a stable and potent GPR103 agonist (**LV-2172**; Fig. 6) with a long lasting orexigenic effect in mice (Neveu et al., 2012). To increase the metabolic stability of the QRFP-(20–26) heptapeptide, another peptidomimetic strategy targeted the N-terminal Gly-Gly pair which is prone to undergo enzymatic degradation (Pierry et al., 2013). The peptide bond was replaced by a fluorin–olefin moiety, which exhibits isosteric and isoelectronic properties similar to those of the native amide bond. The resulting [$\Psi^{20,21}$(CF = CH)](20–26)26RFa compound (**LV-2098**; Fig. 6) displayed a five-fold longer half-life (53 min as compared to 11 min for the heptapeptide) in human serum and an activity similar to that of the parent QRFP-(20–26) peptide (Pierry et al., 2013).

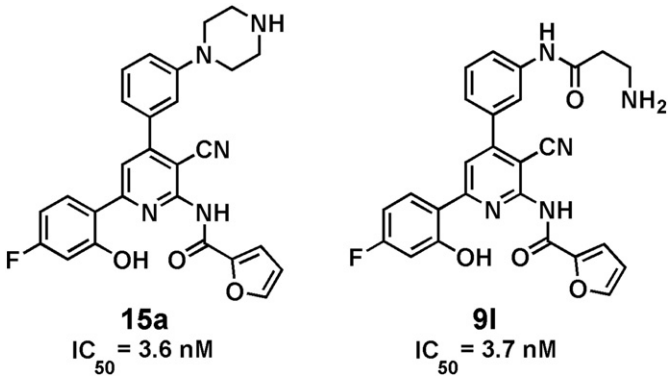

Fig. 5. Non-peptidic Kiss1R antagonists. IC_{50} values for compounds **15a** (Kobayashi et al., 2010b) and **9l** (Kobayashi et al., 2010a) were defined as the concentrations that displaced half specific [^{125}I]-Kp14 binding on membranes of Kiss1R-expressing CHO cells.

Only a few QRFP-R antagonists were disclosed (Fig. 6). In 2010, Banyu Pharmaceuticals patented a series of indole derivatives (Haga et al., 2010; WO/2010/126164). More recently, AstraZeneca developed

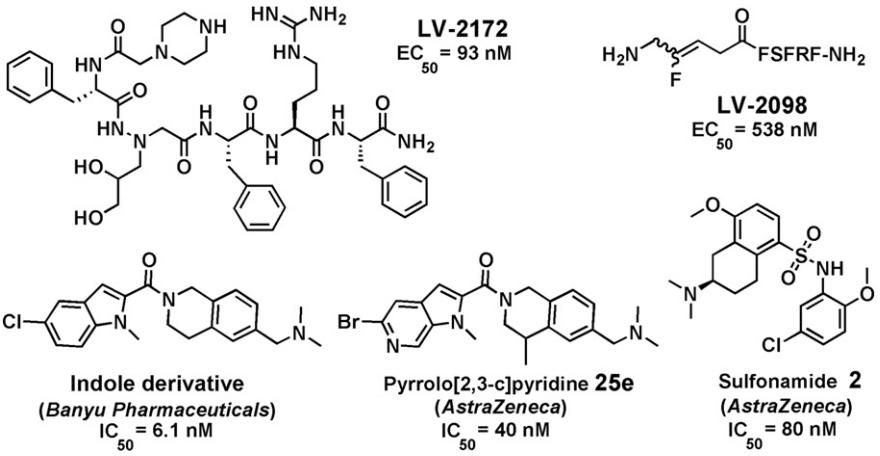

Fig. 6. Pseudopeptidic agonists and antagonists of the QRFP receptor. EC_{50} values for **LV-2172** (Neveu et al., 2012) and **LV-2098** (Pierry et al., 2013) were defined as their potencies, as agonists, to promote an increase in intracellular calcium levels in $G\alpha_{16}$/Kiss1R transfected CHO cells. IC_{50} values for the indole derivative (compound 7 in WO/2010/126164 patent), compound **25e** (Georgsson et al., 2014) and the sulfonamide **2** (Nordqvist et al., 2014) were from [^{125}I]-QRFP43 binding inhibition experiments performed on membranes of QRFPR-expressing cells.

non-peptidic antagonists based on a pyrrolo[2,3-c]pyridine scaffold. The lead compound (**25e**; Fig. 6) has an IC_{50} of 40 nM and better drug metabolism, pharmacokinetics and safety properties than the former indole derivatives (Georgsson et al., 2014). Using NMR in combination with conformational studies, these authors claimed that the peptidic (20–26)RFa agonist and the pyrrolo[2,3-c]pyridine could share overlapping pharmacophores, the antagonist mimicking the C-terminal Arg^{25}-Phe^{26} residues of the endogenous peptide (Georgsson et al., 2014). At the same time, AstraZeneca published the results of a high throughput screening performed on 900,000 compounds which led to the identification of three chemical families, and in particular of the compound **2** (Fig. 6) with an IC_{50} of 80 nM in a binding assay (Nordqvist et al., 2014).

In summary, most SAR studies in the RF-amide field have been conducted starting from endogenous peptides. Although novel compounds exhibited interesting pharmacological properties, most of them are of peptidic nature and thus display a limited 'druggability'. Many of these compounds have been optimized for their affinity/potency towards a given receptor but their selectivity profile for the entire RF-amide receptor family has rarely been reported. This is a critical issue if one remembers the propensity of NPFF receptors to bind most endogenous RF-amide peptides. Off-target actions and possible intervention of other receptors involved in the biological question of interest should also be considered when using novel pharmacological tools and interpreting functional data. Only a few non-peptidic compounds were described, probably owing to the difficulty to develop heterocyclic compounds from peptidic ligands. However, using rational design or high throughput screening, some heterocyclic structures are emerging, but none of them reached clinical studies yet.

7. Mammalian RF-amide peptides and their receptors in feeding and metabolism

7.1. Overview on energy homeostasis and regulation of food intake

Feeding behavior is intimately linked to energy homeostasis, a finely tuned equilibrium between energy intake (food consumption), energy expenditure (metabolism, thermogenesis, muscle activity) and energy storage (adipogenesis). Many endocrine and metabolic signals, in combination with genetic, cognitive, emotional and environmental factors, provide nutritionally-relevant information to a central homeostatic regulator that generates, via efferent neural circuits, appropriate responses to meet caloric needs and maintain energy balance.

Peripheral signals (neuropeptides and nutrients) released from the gastrointestinal tract, such as cholecystokinin (CCK) and ghrelin, from the pancreas (insulin, glucagon) and the adipose tissue (leptin, adiponectin) target specialized hypothalamic nuclei, either directly through the blood circulation and/or indirectly, via vagal and sympathetic input to relaying brain stem nuclei.

The hypothalamic network includes several interconnected nuclei, such as the arcuate (ARC), paraventricular (PVN), ventromedial (VMH), dorsomedial (DMH) and supraoptic (SON) nuclei and the lateral hypothalamic (LHA) and preoptic (POA) areas, that integrate peripheral and central information and exert, via a variety of orexigenic (such as NPY, Agouti-related peptide, galanin or orexin) and anorexigenic (such as proopio-melanocortin (POMC), melanocyte-stimulating hormone (α-MSH) or oxytocin) neuropeptides and neurotransmitters, a bi-directional control of food intake and metabolism. As a nodal homeostatic center, the hypothalamus communicates with the dorso–vagal complex (nucleus tractus solitarius (NTS), area postrema (AP), dorsal motor nucleus of the vagus (DMX)) in the brainstem and the lateral parabrachial nucleus (PB) in the midbrain to regulate consummatory aspects of feeding (including taste aversion and satiety), with the cortico-limbic system (ventral tegmental area (VTA), nucleus accumbens (NAc), amygdala (Amd), orbitofrontal cortex (OFC)) to integrate the reward and hedonic valuation of food, and with the hypothalamic–pituitary–gonadal (HPG) axis to coordinate appetite and energy metabolism with reproductive state. It also responds to circadian influences and interacts with neurons involved in thermoregulation.

Deeper insights and expert comments have been published in several recent reviews devoted to the homeostatic control of food intake and energy metabolism (Lenard & Berthoud, 2008; Parker & Bloom, 2012; Williams & Elmquist, 2012; Yeo & Heisler, 2012; Schwartz & Zeltser, 2013; Morton et al., 2014), to the dysregulation of reward systems in eating disorders (Volkow et al., 2011; Avena & Bocarsly, 2012; Meye & Adan, 2014; Singh, 2014) and to the link between nutrition and reproduction (Evans & Anderson, 2012; Hirschberg, 2012; Navarro & Tena-Sempere, 2012; Schneider et al., 2013).

More specifically, current knowledge on the involvement of the RF-amide system in feeding behavior and energy balance will be presented hereafter.

7.2. RF-amide related peptides and the NPFF1 receptor

7.2.1. Distribution of RF-amide related peptides and their receptor in peripheral organs and brain areas involved in energy homeostasis

The localization of RFRP-containing neuronal cell bodies and of NPFF1 receptors, in relation with a possible implication in the control

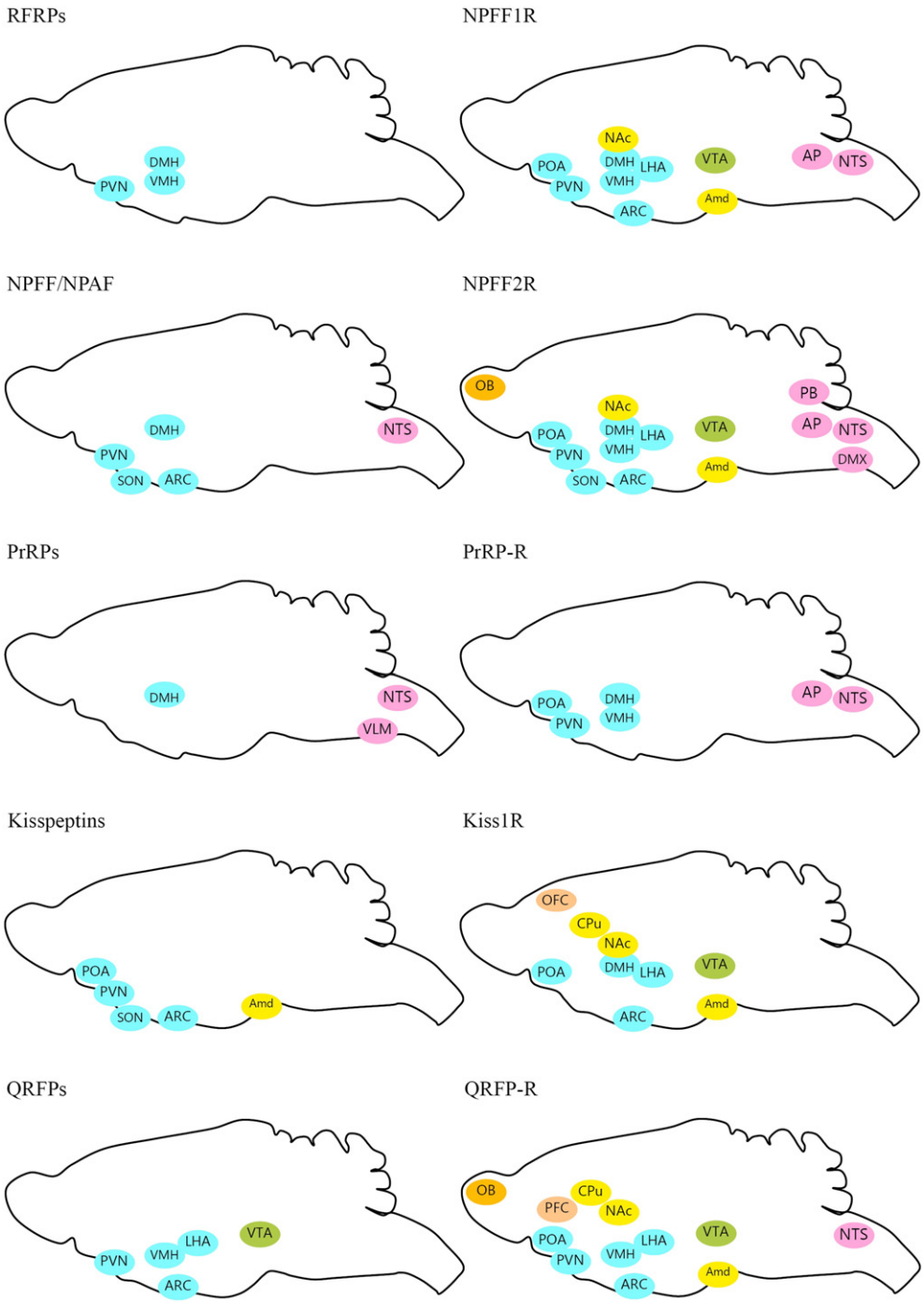

Fig. 7. Anatomical distribution of RF-amide peptides and their cognate receptors in rodent (rat and mouse) brain areas involved in energy homeostasis. Highlighted brain structures have been selected on the basis of their known or putative roles in feeding behavior and regulation of energy homeostasis in Mammals and of their enrichment in RF-amide peptides (left panel) and their cognate receptors (right panel), as afforded from distribution studies in mouse brain. When specified in the literature, the localization of RF-amide peptide-containing cell bodies is privileged. Colored areas refer to distinct anatomical structures according to MeSH database: hypothalamic nuclei (light blue); pons/medulla oblongata nuclei (pink); basal ganglia (yellow); tegmentum mesencephali nuclei (green); telencephalon nuclei (orange); cortical areas (light orange). Details on the preferential distribution of QRFP-R1 and QRFP-R2 receptor isoforms are provided in Section 7.6.1. Abbreviations are listed in the corresponding section.

of food intake, is presented on schematic sagittal sections of rodent brain (Fig. 7). Further details on the localization of RFRP peptides and of NPFF1R in Mammals are available in dedicated reviews (Fukusumi et al., 2006; Smith & Clarke, 2010; Parhar et al., 2012).

7.2.1.1. Localization of RF-amide related peptide neuronal cell bodies. As assessed from in situ hybridization (ISH) and immunohistochemistry (IHC) studies performed on mouse (Ukena & Tsutsui, 2001; Kriegsfeld et al., 2006), rat (Hinuma et al., 2000; Fukusumi et al., 2001; Yano et al., 2003; Kriegsfeld et al., 2006; Johnson et al., 2007; Legagneux et al., 2009; Rizwan et al., 2009), hamster (Kriegsfeld et al., 2006), ovine (Clarke et al., 2008; Dardente et al., 2008; Smith et al., 2008a) and macaque (Ubuka et al., 2009a) brains, RFRP-positive neuronal cell bodies locate within the hypothalamus, essentially in the paraventricular (PVN) and dorsomedial (DMH) nuclei and between the DMH and ventromedial (VMH) nuclei.

7.2.1.2. Localization of RF-amide related peptide fibers. No significant levels of immunoreactive (ir) RFRPs nor RFRPs mRNA were detected in peripheral tissues. In contrast, RFRP-ir neuron projections and nerve terminals have been visualized in a range of brain areas in rat, mouse, hamster, ovine and primate (Ukena & Tsutsui, 2001; Yano et al., 2003; Gibson et al., 2008; Qi et al., 2009; Ubuka et al., 2009a). They include major structures involved in energy balance, such as the arcuate (ARC), dorsomedial (DMH) and paraventricular (PVN) nuclei as well as the preoptic (POA) and lateral (LHA) areas of the hypothalamus, the amygdala (Amd) in rat, mouse and hamster, and more caudal areas such as the lateral parabrachial nucleus (PB) and the nucleus tractus solitarius (NTS). In the ovine brain, retrograde tracer labeling further demonstrated that RFRP-3 neurons in the PVN and DMH nuclei project onto the ARC, LHA, VMH and POA (Qi et al., 2009) where the NPFF1 receptor is expressed.

Double-label immunohistochemistry in ovine brain allowed to identify a subset of RFRP-3 neurons in close apposition with NPY- or POMC-producing cells in the ARC and with orexin or melanin-concentrating hormone (MCH) neurons in the LHA (Qi et al., 2009). Moreover, RFRP neuronal cell bodies in the DMH were found to project onto GnRH neurons in the preoptic area of human (Ubuka et al., 2009b), hamster (Kriegsfeld et al., 2006), sheep (Smith et al., 2008a) and monkey (Ubuka et al., 2009a) brains, suggesting that RFRPs are involved in the regulation of GnRH neurons.

7.2.1.3. Distribution of NPFF1R (GPR147) in mammalian tissues. As assessed from RT-qPCR analyses, NPFF1 receptors are expressed in the rat testis (and to a much lower extent in the ovary, placenta and retina) and show a broad distribution in the human and rat CNS (Bonini et al., 2000; Hinuma et al., 2000). NPFF1R localization in rat brain and human hypothalamus was also examined using ISH (Liu et al., 2001) and IHC (Goncharuk et al., 2004) approaches. More quantitative insights on the anatomical distribution of the NPFF1 receptor in the CNS of various mammalian species were obtained from autoradiography, a technique aiming at localizing specific radioligand binding sites on brain tissue sections. First studies were performed on rat brain, using [^{125}I]-Y8Fa (Allard et al., 1992) and [^{125}I]-1DMe (Dupouy & Zajac, 1996; Dupouy et al., 1996), two tracers that do not discriminate among NPFF receptors subtypes (Table 2 and Section 4.1.1). To increase labeling specificity, subsequent autoradiographic studies included the frog pancreatic peptide (fPP) to abolish an undesirable NPFF2 binding component (Bonini et al., 2000) and took advantage of the more NPFF1-selective [^{125}I]-YVP radioligand to investigate NPFF1 receptor distribution (Gouarderes et al., 2002, 2004a, 2004b). Collectively, and within the context of feeding regulation and energy homeostasis, all these data pointed to the presence of the NPFF1R in the ARC and PVN hypothalamic nuclei, its low to moderate expression in other hypothalamic regions (DMH and VMH nuclei, LHA and POA areas), in the amygdala (Amd), and in brainstem (AP, NTS) regions (Liu et al., 2001; Gouarderes et al., 2002; Goncharuk et al., 2004; Gouarderes et al., 2004b).

It is worthwhile mentioning the existence of discrepancies in NPFF1R density profiles between ISH, IHC and autoradiography studies, but also between the different mammalian species studied so far (Goncharuk et al., 2004; Gouarderes et al., 2004b) and even between mouse or rat strains (Gouarderes et al., 2004a). For example, in Sprague–Dawley rats, [^{125}I]-YVP autoradiography showed the presence of the NPFF1R in the nucleus accumbens (NAc) and its absence in the ventral tegmental area (VTA), with the reverse observation being true in Wistar rats (Gouarderes et al., 2004a). Conversely, immunoblotting on extracts from the same regions of Sprague–Dawley rats indicated abundant NPFF1R in the VTA and a few in the NAc (Wu et al., 2010). This emphasizes the importance of comparing receptor (and peptide) distribution patterns afforded from various techniques and the great caution that should be taken when analyzing IHC data that used antibodies that have not yet been fully validated. Nevertheless, the possibility for NPFF receptors to localize on dopaminergic (and/or GABAergic) neurons in the rat VTA should not be excluded as double immunohistochemical staining suggested the expression of NPFF1R and NPFF2R on tyrosine hydroxylase- (or glutamic acid decarboxylase-) positive neurons (Wu et al., 2010).

7.2.2. Effect of RF-amide related peptides on food intake

Despite the considerable interest RFRP peptides and their NPFF1 (GPR147) receptor received as members of an opponent system to kisspeptin/Kiss1R in reproduction (Section 8) and the prominent link between the reproductive and metabolic status, less studies addressed their contribution in the control of food intake and energy balance in Mammals.

Similarly to effects of GnIH originally reported in birds (Tachibana et al., 2005), RFRP-3 (i.c.v.) was found to increase food intake within 2 h during photophase in male rats fed ad libitum (Johnson et al., 2007; Johnson & Fraley, 2008; Murakami et al., 2008) as well as in male mice, ewes and non-human primates (Clarke et al., 2012). The i.c.v. administration of RFRP-3 to rats also caused a rapid and strong expression of the c-Fos immediate early gene (taken as a marker of neuronal activity) in several brain areas involved in the regulation of food intake and energy balance, such as the ARC, SON and rostral portion of the NTS (Yano et al., 2003). In sheep, RFRP-3 infusion increased c-Fos labeling in orexigenic (NPY) as well as in anorexigenic (POMC) cells of the arcuate nucleus (Clarke et al., 2012). This paradoxical activation of POMC cells by RFRP-3 remains to be clarified since other studies reported a negative effect of this peptide on the firing of POMC neurons in mouse brain (Fu & van den Pol, 2010).

In marked contrast with former findings, anorectic effects of RFRP-1 (Kovacs et al., 2012) and RFRP-3 peptides (Kovacs et al., 2014) have been reported in rats on a liquid diet, following micro-injection of the peptides into the amygdala. These effects were suppressed by the NPFF1/2 receptor antagonist RF9. The reasons for such striking discrepancies regarding the effects of RFRPs on food intake remain unclear but a possible link between an RFRP-3-elicited anxiogenic behavior in rats (Kaewwongse et al., 2011) and the hypophagic effect of this peptide deserves further investigation.

7.2.3. Effect of RF-amide related peptides on energy homeostasis

I.c.v. injection of RFRP-3 in mice induced a significant hypothermia which was fully prevented by co-administration of the R1/R2 selective NPFF antagonist RF9 (Quelven et al., 2005; Mouledous et al., 2010a). RFRP-mediated decrease in body temperature was consistent with the presence of NPFF1R in several hypothalamic regions involved in thermoregulation in rodents (Ishiwata et al., 2001; Romanovsky, 2007). A more recent study pointed to a lack of effect of RFRP-3 on overall energy expenditure in male rats and on thermogenesis in female sheep that contrasted with the general rule for 'appetite-regulating' peptides that predicts opposite effects on food intake and energy expenditure (Clarke et al., 2012). Interestingly, the authors suggested that RFRP-3 may function as a molecular switch between feeding and reproductive behavior, with high RFRP-3 activity favoring feeding over reproduction.

7.2.4. Effect of feeding status on RF-amide related peptides/NPFF1R expression

The number of RFRP-immunoreactive neuronal cell bodies in the DMH of Syrian hamster females was found to decrease following food restriction while their activity increased, as assessed from c-Fos labeling (Klingerman et al., 2011). Food intake remained similar to that of animals fed ad libitum but food hoarding significantly increased.

The impact of extreme metabolic changes was examined in wild-type and NPFF1 receptor null mice (Leon et al., 2014). Although both wild-type and NPFFR1 KO animals displayed a similar body weight in adulthood, complete food deprivation was found to accelerate weight loss in KO mice, in agreement with the expected function of RFRPs as orexigenic factors. High-fat diet evoked a similar increase in body

weight in both strains but one cannot exclude that such modest alterations might be due to compensatory mechanisms.

Food availability and photoperiod have been shown to differentially impact RFRPs (in the DMH) and *KiSS-1* (in the ARC) genes expression in male Syberian hamsters (Paul et al., 2009). Shorter photoperiods increased RFRP mRNA levels — although this point has been disputed (Revel et al., 2008) — while they remained unaffected by food restriction. At the opposite, KiSS-1 mRNA markedly decreased in animals exposed to reduced food availability whereas they were insensitive to the photoperiod. Such responses, which are unlikely to be driven by altered gonadal steroid production, suggest a specific recruitment of these RF-amide systems in the integration of photic and metabolic cues for the control of reproduction.

Light-induced neuronal activity of the suprachiasmatic nucleus (SCN) signals rest in nocturnal rodents and activity in diurnal primates (Houben et al., 2009). Such a circadian control is overridden when food access is restricted to a predictable episode during the rest phase, so that animals become active and anticipate the feeding event. Food anticipatory activity (FAA) has been recently found to be modulated by the interaction of DMH and SCN nuclei, within a hypothalamic network comprising RFRP fibers (Acosta-Galvan et al., 2011). Interestingly, this study demonstrated the activation of some RFRP neurons in the DMH during FAA and their projection onto vasoactive-intestinal and gastrin-releasing peptides neurons in the SCN, in close association with afferent fibers arriving from the retina.

7.3. Neuropeptide FF/neuropeptide AF and the NPFF2 receptor

7.3.1. Distribution of neuropeptide FF and neuropeptide AF and of their receptor in the brain and in peripheral organs involved in energy homeostasis

The localization of NPFF/NPAF-containing cell bodies and of NPFF2R in rodent brain, in relation with their known or putative roles in the control of food intake, is presented in Fig. 7.

7.3.1.1. Localization of neuropeptide FF/neuropeptide AF-containing neuronal cell bodies. The distribution of NPFF, NPAF and of their proNPFF$_A$ precursor is essentially from ISH and IHC studies performed on rat brain.

Highest levels of NPFF-containing neurons were found in the nucleus tractus solitarius (NTS), significant expression also occurred in the supraoptic (SON) and paraventricular (PVN) hypothalamic nuclei, while the existence of NPFF-ir neuronal cell bodies in the dorsomedial (DMH), ventromedial (VMH) and arcuate (ARC) nuclei was more ambiguous (Kivipelto et al., 1989; Boersma et al., 1993; Lee et al., 1993; Panula et al., 1996; Vilim et al., 1999; Goncharuk et al., 2006). A comparative semi-quantitative immunohistochemical analysis of the distribution of NPFF in rat and human forebrain revealed a similar pattern in both species, with a noticeable difference in the human SON which exhibited very low NPFF-ir levels (Goncharuk et al., 2006).

7.3.1.2. Localization of neuropeptide FF/neuropeptide AF-containing neuronal fibers and terminals. Immunoreactive NPFF fibers and nerve terminals exhibit a wide distribution in mammalian brain, including the hypothalamus (POA, ARC, DMH, PVN and VMH nuclei), limbic areas (NAc, amygdala), and the lateral parabrachial nucleus (PB) in the midbrain (Kivipelto et al., 1989; Panula et al., 1996; Vilim et al., 1999; Goncharuk et al., 2006).

7.3.1.3. Localization of NPFF2R. The distribution of NPFF2R in mammalian tissues has been examined using RT-qPCR, ISH, IHC and autoradiography, often in parallel with NPFF1R.

As shown from RT-qPCR performed on peripheral tissues, significant NPFF2R mRNA levels were detected in rat (Bonini et al., 2000) and human (Elshourbagy et al., 2000) adipose tissue. Low to moderate NPFF2R expression was also reported in rat retina, salivary gland, stomach, intestine and liver (Bonini et al., 2000).

Mapping of NPFF2R mRNA in rat brain areas showed a limited pattern of expression, including the medulla (NTS, dorsal motor nucleus of the vagus), the hypothalamus (LHA) and the amygdala (Bonini et al., 2000; Liu et al., 2001). Immunohistochemical detection of NPFF2 receptors in the human brain provided a more precise profile: in the forebrain, highest receptor density was found in the amygdala and DMH and moderate to low expression in the VMH, LHA, PVN and VTA; within the medulla oblongata, the dorsal motor nucleus of the vagus nerve (DMX) and the region of catecholaminergic cell groups (A1/C1) were the most enriched areas in NPFF2R (Goncharuk & Jhamandas, 2008).

Autoradiographic studies using the NPFF2R selective [^{125}I]-EYF tracer under controlled conditions (Gouarderes et al., 2001, 2002, 2004b), indicated a wider distribution and a considerably greater density of NPFF2R in the rat brain, as compared to NPFF1R. Brainstem (NTS and PB) and hypothalamic (VMH, LHA, POA and ARC) nuclei as well as areas belonging to the dopaminergic mesolimbic system (VTA, NAc, caudate putamen) exhibited significant levels in NPFF2 binding sites. It is noteworthy that some NPFFergic neurons from the NTS project within the lateral parabrachial nucleus (Kivipelto & Panula, 1991; Panula et al., 1996; Jhamandas et al., 2001) where mu-opioid receptors are expressed (Chamberlin et al., 1999; Nicklous & Simansky, 2003).

Autoradiographic studies also pointed to marked differences in the distribution of NPFF2 receptor binding sites according to species (Dupouy & Zajac, 1996; Gouarderes et al., 2004b) and rodent strain (Gouarderes et al., 2004a). No NPFF2R sites were detectable in the central nervous system of the diurnal *Octodon degus* rodent nor in the olfactory bulb (OB) of rats, in marked contrast with mice that exhibited a high density of binding sites in this latter region. As the olfactory bulb is known to contribute to the evaluation of hedonic properties of food and to contain a circadian clock that participates to feeding anticipation (Nolasco et al., 2012; Caba et al., 2014), a direct participation of NPFF2R in olfactory bulb-linked feeding behavior in rats is unlikely.

Although available data revealed some species- and methodological-dependent variations in the relative density of endogenous proNPFF$_A$ peptides or NPFF2R within brain areas, they provided an overall distribution pattern that is consistent with their role in feeding regulation.

7.3.2. Effect of neuropeptide FF and neuropeptide AF on food intake

As an introductory remark, it is important to keep in mind that proNPFF$_A$-derived peptides poorly discriminate between NPFF1 and NPFF2 receptors in terms of binding affinity. Although such a drawback might be compensated for by a greater functional selectivity (Table 3), high local peptide concentrations may be reached when injected into the brain. Therefore, data need to be interpreted with much caution unless highly selective NPFF1 or NPFF2 receptor antagonists or genetic invalidated mice are used.

NPFF is involved in the modulation of diverse feeding-related processes, including intestinal motility (Decker et al., 1997; Fang et al., 2005), osmoregulation (Majane & Yang, 1991; Sunter et al., 2001; Kalliomaki & Panula, 2004) and food intake (Murase et al., 1996; Sunter et al., 2001; Nicklous & Simansky, 2003; Dockray, 2004).

Initial studies reported that i.c.v. injections of NPFF induce a rapid, transient and dose-dependent reduction in food intake in fasted rats (Murase et al., 1996; Sunter et al., 2001) and in ad libitum fed mice (Bechtold & Luckman, 2006). In rats, the hypophagic action of NPFF was first attributed to the concomitant large increase in water intake induced by the peptide (Sunter et al., 2001) but no significant dipsogenic effects of NPFF (Nicklous & Simansky, 2003) or NPAF (Newmyer & Cline, 2011) were detected in subsequent studies. NPFF-induced hypophagia is unlikely to result from an interference with satiety signaling sequences as the activity of NPFF neurons within the NTS was insensitive to the satiety factor CCK (Bechtold & Luckman, 2007). In turn, i.c.v. administration of NPFF was found to activate oxytocin neurons in the PVN (Jhamandas & MacTavish, 2003), suggesting the participation of the anorectic oxytocin peptide in NPFF-induced hypophagia.

The well-known anti-opioid effects of NPFF in nociception (Section 9) and orexigenic action of opioid agonists (Morley et al., 1983; Yim & Lowy, 1984), opposite to that of NPFF, raised the possibility for an interplay between both systems in the regulation of food intake. Indeed, the central administration of FMRF-amide peptides in mice or of NPFF in rats dose-dependently prevented hyperphagia induced by mu or kappa opioid receptor agonists (Kavaliers & Hirst, 1986; Nicklous & Simansky, 2003). At higher doses, NPFF infusion into the lateral parabrachial nucleus markedly increased food intake in freely feeding rats, with a delayed onset and a prevention by naloxone very similar to mu-opioid responses (Nicklous & Simansky, 2003). Thus, NPFF may exert pro- and anti-opioid effects on food intake, very similarly to its capacity to induce anti-nociception or to oppose opioid-induced analgesia (Section 9.2.2).

7.3.3. Effects of neuropeptide FF, neuropeptide AF and neuropeptide SF on energy homeostasis

EFW-NPAF or dNPA (two NPFF2R preferring peptides) reversed the hypothermia evoked by saline injection in mice when injected into the third ventricle (Quelven et al., 2005; Mouledous et al., 2010a). This response, which was blocked by the NPFF antagonist RF9, is probably due to NPFF2R-induced hyperthermia. However, given the bell-shape dose-response curves for these agonists and the decrease in body temperature consecutive to the administration of the poorly NPFF2R-selective NPFF and 1DMe peptides in mice (Desprat & Zajac, 1997; Zajac et al., 2000; Quelven et al., 2005; Fang et al., 2008), one cannot exclude that NPFF2R-driven hyperthermia might be dominated by the hypothermic NPFF1R response in case of co-stimulation of both NPFF receptors (Mouledous et al., 2010a).

NPFF and NPAF peptides were found to modulate the expression of a number of genes involved in adipose metabolism in 3T3-L1-differentiated rodent fat cells (Lefrere et al., 2002). Up-regulation of beta2 and beta3-adrenergic receptor genes was accompanied with an increase in receptor expression and potency of beta adrenergic agonists to stimulate adenylate cyclase activity in NPAF- and NPFF-treated cells. Thus, these RF-amide peptides may impact body energy storage and utilization via activation of beta-adrenergic receptors known to activate lipolysis and thermogenesis and to exert a negative control on lipogenesis and glucose transport (Lafontan & Berlan, 1993). To note however that NPFF, NPAF and NPSF failed to modify lipid accumulation in 3T3-L1 cells while they partially reduced adipogenesis in 3T3-F442A preadipose fat cells (Herrera-Herrera & Salazar-Olivo, 2008). As peptide potencies did not correlate with their affinities for NPFF2 receptors, the occurrence of alternative signaling pathways was suggested.

In marked contrast with former data obtained on rodent adipocytes, NPFF was found to inhibit catecholamine-induced lipolysis in human fat cells, via NPFF2R activation (Dahlman et al., 2007). Similar responses to NPFF and NPSF were observed in human adipocytes differentiated in vitro from adipocyte precursor cells, taken either from non-obese or obese donors, while the expression of key genes involved in lipolysis modulation (including beta-adrenergic receptor genes) remained unaffected (van Harmelen et al., 2010). Altogether, these data indicate that NPFF, NPAF and NPSF peptides may display marked species differences in their ability to regulate lipolysis and fat storage.

7.3.4. Metabolic context and genetic predisposition to obesity

Hyperosmolar stress of rats was reported to down-regulate NPFF gene transcription in the SON and PVN nuclei (two hypothalamic nuclei involved in body fluid homeostasis), independently from the vasopressin regulatory system, but underlying mechanisms remain to be clarified (Kalliomaki & Panula, 2004).

Chronic food restriction or diet-induced obesity in rats did not affect NPFF2R mRNA levels in the hypothalamus (Laemmle et al., 2003). Conversely, a clinical examination of the relative expression of NPFF1 and NPFF2 receptors in the adipose tissue from non-obese and obese individuals showed a significantly lower NPFF2R mRNA level in the obese cohort (van Harmelen et al., 2010).

Among candidate marker genes of obesity, MC4R (melanocortin-4 receptor) and FTO (fat mass and obesity associated protein) genes show frequent prevalence of mutations or polymorphisms related to obesity (Dina et al., 2007; Loos et al., 2008). Looking at single-nucleotide polymorphisms in the NPFF2R gene, some haplotypes have been associated with an increased risk of obesity whereas others were strongly protective against obesity, in association with higher lipolytic activity (Dahlman et al., 2007; Hunt et al., 2011). The authors concluded that NPFF2R may play an important role in obesity predisposition, independently of NPY2R, FTO and MC4R loci.

7.4. Prolactin-releasing peptides and the prolactin-releasing peptide receptor

7.4.1. Distribution of prolactin-releasing peptides and their receptor in peripheral organs and brain areas involved in energy homeostasis

The brain distribution of PrRP-containing cell bodies and of the PrRP receptor, in light with their known or putative roles in feeding behavior in Mammals, is presented in Fig. 7. Detailed localization of PrRPs and their receptor in peripheral and central tissues has been published in several reviews (Sun et al., 2005; Fukusumi et al., 2006; Lin, 2008; Dodd & Luckman, 2013).

7.4.1.1. Localization of prolactin-releasing peptide-containing neurons and cell bodies. PrRP mRNA was detected in a limited set of peripheral organs involved in feeding such as the pancreas, the stomach and possibly the intestine (Fujii et al., 1999; Roland et al., 1999; Nieminen et al., 2000). As assessed from RT-PCR, ISH and IHC studies performed in rat brain, PrRP cell bodies concentrate within the nucleus tractus solitarius (NTS), and to a lesser extent in the ventrolateral medulla (VLM) of the caudal brainstem and the dorsomedial hypothalamic (DMH) nucleus (Fujii et al., 1999; Iijima et al., 1999; Maruyama et al., 1999; Ibata et al., 2000; Lee et al., 2000). In the VLM and NTS, PrRP co-localizes with tyrosine hydroxylase within A1 and A2 noradrenergic neurons, respectively (Chen et al., 1999; Roland et al., 1999).

7.4.1.2. Localization of prolactin-releasing peptide fiber projections. In the rat brain, a dense network of PrRP-ir fibers was identified in the PVN, SON, DMH/LHA and POA regions of the hypothalamus, but also in the amygdala and area postrema (Iijima et al., 1999; Maruyama et al., 1999; Ibata et al., 2000; Lin, 2008). PrRP-positive neurons from the NTS project to the PVN (Onaka, 2004) where they contact anorexigenic oxytocin (Maruyama et al., 1999; Ibata et al., 2000; Yamashita et al., 2013) and corticotrophin-releasing hormone-containing neurons (Maruyama et al., 1999; Matsumoto et al., 1999; Lin et al., 2002). More than 90% of PrRP neurons are leptin receptor-positive (Ellacott et al., 2002).

7.4.1.3. Localization of the prolactin-releasing peptide receptor. The PrRP receptor is widely distributed in the brain, with high to moderate expression in the VMH, PVN, POA, DMH, AP and NTS consistent with a prominent role in the regulation of food intake and energy homeostasis (Fujii et al., 1999; Roland et al., 1999; Ibata et al., 2000; Lin, 2008). Although such a receptor localization was supported by ISH, RT-PCR and IHC studies, the existence of mismatches between the localization of PrRP-containing nerve fibers and of PrRP-R raised several hypotheses, among which the possibility for PrRP to signal via other receptors (Lin, 2008; Dodd & Luckman, 2013).

7.4.2. Effect of prolactin-releasing peptides on food intake

Accumulating evidence indicates a prominent role of the PrRP system in the stimulation of energy consumption and the reduction of food intake. In fasted rats, i.c.v. administration of PrRP reduces food intake (without impacting water intake) and weight gain, and increases

energy expenditure in brown adipose tissue (Lawrence et al., 2000, 2002; Ellacott et al., 2003; Gu et al., 2004). Compared to an i.c.v. application, intra-DMH injection of PrRP promoted an earlier, weaker and longer anorexia in rats while intra-PVN injection had no significant impact on feeding behavior (Seal et al., 2001).

PrRP-induced hypophagia is abolished in PrRP-R KO mice (Bechtold & Luckman, 2006) and in the 'OLETF' rat strain that expresses non-functional PrRP receptors (Watanabe et al., 2005). These rats, together with PrRP −/− and PrRP-R −/− mice, showed late-onset obesity and a hyperphagic phenotype, which depended on feeding experimental paradigms (Gu et al., 2004; Watanabe et al., 2005; Bechtold & Luckman, 2007; Bjursell et al., 2007; Takayanagi et al., 2008).

Genetic ablation of the PrRP system in mice abrogates CCK-mediated satiety (Lawrence et al., 2002; Bechtold & Luckman, 2006; Takayanagi et al., 2008). Several explanations have been proposed in light of the prominent role exerted by the NTS in satiety regulation, the privileged localization of PrRP neuronal cell bodies, fibers and receptors within this brainstem nucleus and their functional activation by the satiety factor CCK (Lawrence et al., 2002; Dodd & Luckman, 2013). To sum up, PrRP-induced hypophagia may originate from a reduction in meal size due to an acceleration of satiety signaling.

7.4.3. Effects of prolactin-releasing peptide on energy homeostasis

Pair-feeding studies suggest that PrRP effect on body weight cannot be attributed solely to its anorectic-like action. Indeed, acute PrRP injections in fasted and satiated rats cause a long lasting hyperthermia, an increase in oxygen consumption and in mRNA levels of the brown adipose tissue factor UCP-1, pointing to an increased energy expenditure (Ellacott et al., 2002, 2003; Lawrence et al., 2004). Noteworthy, animals became rapidly refractory to the anorectic and body weight action of PrRP upon its chronic administration (Ellacott et al., 2003). In marked contrast with the long term effects of leptin (Zhang et al., 1994), tolerance is a phenomenon encountered for many short term acting peptides such as CCK (Crawley & Beinfeld, 1983), galanin (Smith et al., 1994) or MCH (Rossi et al., 1997). Thus, although leptin and PrRP have similar and additive acute effects (Ellacott et al., 2002), the latter peptide may affect energy homeostasis only in the short to medium term.

PrRP −/− and PrRP-R −/− male mice show no significant differences in oxygen consumption or core body temperature in basal conditions, as compared to wild-type animals (Bjursell et al., 2007; Takayanagi et al., 2008). However, the increase in O_2 consumption was reduced in PrRP −/− mice after stressful stimuli (Takayanagi & Onaka, 2010). As former studies showed that emotional stress and nociceptive stimuli were able to activate medullary and hypothalamic neurons (Maruyama et al., 2001) and to up-regulate their content in PrRP mRNA (Mera et al., 2006), PrRP may regulate energy expenditure in response to stress conditions (Onaka et al., 2010; Maniscalco et al., 2012).

Finally, the possibility that PrRP mediates central anorectic effects through a receptor distinct from PrRP-R has been evoked (Ellacott et al., 2005; Onaka et al., 2010; Dodd & Luckman, 2013). The NPFF2 receptor is a putative candidate as it displays a high affinity for PrRPs (Table 3), a significant expression in hypothalamic areas, and mediates a similar response (reduction in food intake) following i.c.v. NPFF injection (Section 7.3).

7.4.4. Effects of metabolic signals on prolactin-releasing peptide/prolactin-releasing peptide receptor expression

PrRP-R null mice exhibit a reduced glucose tolerance and a significant increase in insulin and leptin levels (Gu et al., 2004). The anorectic response to leptin is impaired in PrRP and PrRP-R KO mice (Takayanagi et al., 2008; Dodd & Luckman, 2013), an observation which could be related to the fact that most PrRP-containing neurons in the hypothalamus and brainstem express leptin receptors (Ellacott et al., 2002). PrRP mRNA expression in the NTS, DMH and VMH is down regulated in situations of food restriction that correlate with reduced signaling of the adipose-derived hormone leptin (Lawrence et al., 2000; Ellacott et al., 2002). Although the functional interplay between leptin and PrRP has not been fully elucidated, both peptides may mediate satiety signaling from the adipose tissues.

7.5. Kisspeptins and the Kiss1 receptor

7.5.1. Distribution of kisspeptins and their receptor in peripheral organs and brain areas involved in energy homeostasis

A schematic presentation of the localization of Kiss-containing cell bodies and of Kiss1R in the rodent brain, which supports the involvement of this RF-amide system in feeding behavior, is shown Fig. 7. The neuroanatomy of the kisspeptin system in mammalian brain has been described in many papers (Clarkson et al., 2009; Herbison et al., 2010; Smith, 2013) along with potential detection artifacts (Mikkelsen & Simonneaux, 2009).

7.5.1.1. Localization of kisspeptin-containing neurons and cell bodies. KiSS-1 mRNA was detected in several peripheral organs involved in feeding processes such as mouse and human pancreatic cells (Ohtaki et al., 2001; Hauge-Evans et al., 2006), mouse and human liver (Ohtaki et al., 2001; Song et al., 2014) and rat fat tissue (Brown et al., 2008). KiSS-1 immunoreactivity was also detected in mouse and human plasma (Song et al., 2014).

Within the mammalian brain, kisspeptin-containing neuronal cell bodies essentially locate within two hypothalamic regions: the arcuate nucleus (ARC) and the periventricular area of the third ventricle (referred to as RP3V), a continuum of cells that extends from the anteroventral periventricular nucleus (AVPV) to the preoptic (POA) area. ISH and IHC studies have consistently highlighted these kisspeptin cell populations in every mammalian species examined to date, including mice (Gottsch et al., 2004; Clarkson & Herbison, 2006), rat (Kinoshita et al., 2005; Smith et al., 2006b; Mikkelsen & Simonneaux, 2009; Xu et al., 2012), hamster (Revel et al., 2006; Greives et al., 2007), ovine (Franceschini et al., 2006; Pompolo et al., 2006; Goodman et al., 2007), monkey and man (Shahab et al., 2005; Rometo et al., 2007; Ramaswamy et al., 2008). Kisspeptin-ir and KiSS-1 mRNA-expressing cell populations were also detected in the amygdala (Kim et al., 2011; Xu et al., 2012) and between the dorsomedial (DMH) and ventral (VMH) hypothalamic nuclei (Dun et al., 2003; Brailoiu et al., 2005; Clarkson & Herbison, 2006; Franceschini et al., 2006; Bosch et al., 2012). A broad distribution profile for KiSS-1 neurons was described using a Kiss1-Cre/β-galactosidase transgenic mouse model (Cravo et al., 2011) but the specificity of this reporter system awaits further confirmation.

One cannot exclude that early IHC data might have been limited by cross-reactivity of kisspeptin antibodies with other RF-amide peptides (Mikkelsen & Simonneaux, 2009). More recently, using a well-characterized kisspeptin antibody validated on brain tissue sections of Kiss1-KO mice, Clarkson and coworkers provided a detailed description of KiSS-1-ir cell bodies and fibers in female mouse brain and confirmed the existence of three main populations of kisspeptin-producing cells located in the RP3V, the ARC and in the DMH at a very low density (Clarkson et al., 2009). It is to note that the relative distribution of kisspeptin neurons within these hypothalamic regions vary between species and gender, with the AVPV nucleus displaying a marked sexual dimorphism in rodents (Section 8.5.1).

7.5.1.2. Localization of kisspeptin fiber projections. Abundant neuronal Kiss-ir fibers have been found in hypothalamic areas such as the ARC, POA, paraventricular (PVN) and the DMH nuclei in rodents, while low-density fibers were located in discrete extra-hypothalamic regions (Brailoiu et al., 2005; Kinoshita et al., 2005; Clarkson & Herbison, 2006; Franceschini et al., 2006; d'Anglemont de Tassigny et al., 2008; Ramaswamy et al., 2008; Clarkson et al., 2009). Using anterograde and retrograde tracing techniques to establish the projection pattern of kisspeptin cells in mouse brain, Yeo and Herbison confirmed the

existence of wide projections of neurons originating from the ARC and the RP3V regions onto DMH, PVN and POA hypothalamic nuclei (Yeo & Herbison, 2011). ARC projections to associated limbic structures such as the lateral hypothalamic area (LHA) were also identified.

7.5.1.3. Localization of Kiss1 receptors. Kiss1R transcripts are present in various peripheral tissues involved in energy homeostasis such as the pancreas, the adipose tissue and possibly the stomach and the intestine (Kotani et al., 2001a; Muir et al., 2001; Ohtaki et al., 2001; Hauge-Evans et al., 2006; Chen et al., 2014; Song et al., 2014).

In rat brain, Kiss1R mRNA was detected by ISH in several hypothalamic nuclei (ARC, DMH, LHA and POA) and in mesocorticolimbic areas such as the nucleus accumbens (NAc), ventral tegmental area (VTA), amygdala (Amd), striatum and orbito frontal (OFC) cortex (Lee et al., 1999). All latter structures have been involved in food rewarding processes (Gao & Horvath, 2008; Lenard & Berthoud, 2008; Dardeno et al., 2010; Volkow et al., 2011).

The use of a transgenic Kiss1R LacZ knock-in mouse provided a detailed mapping of Kiss1R gene expression that fairly well fitted the distribution of receptor mRNAs in rat brain (Herbison et al., 2010). Noticeable exceptions were the lack of X-gal labeling in the amygdala and VTA areas and especially in the ARC that contains abundant kisspeptin fibers in mouse and rat (Clarkson et al., 2009; Mikkelsen & Simonneaux, 2009; Yeo & Herbison, 2011) and expresses functional Kiss1 receptors as shown by electrophysiological studies performed on mouse brain (Fu & van den Pol, 2010).

7.5.2. Kisspeptin-mediated control of food intake and energy balance

Despite their 'strategic' location within hypothalamic areas and peripheral tissues (pancreas and adipose tissue) involved in energy homeostasis, endogenous kisspeptins and their receptor have long been considered as poorly effective in controlling body weight or food consumption. Indeed, initial studies failed to evidence central effects of kisspeptins on food intake in ad libitum-fed or pre-fasted rats (Navarro et al., 2004; Thompson et al., 2004; Castellano et al., 2005). This was later confirmed in ewes that received food and water ad libitum (Clarke et al., 2012). Kiss1R KO mice displayed no obvious phenotypes related to feeding behavior as well (Seminara et al., 2003). However, Kisspeptin-10 (i.c.v.) was reported to increase meal intervals in mice, while meal size remained unchanged (Stengel et al., 2011), and to slightly reduce food intake and body weight of female rats during the pubertal period (Sahin et al., 2015).

In contrast to former reports, convincing evidence was provided, at least in Kiss1R KO female mice up to 8–10 weeks old, for a regulatory role of kisspeptins in body weight, adiposity, energy balance and glucose homeostasis (Tolson et al., 2014). Both male and female Kiss1R −/− mice displayed an increased fat mass along with marked hyperleptinemia. Only females showed a significant increase in body weight, higher basal glucose levels and altered glucose tolerance relative to wild-type or heterozygous females. Surprisingly, KO females were hypophagic and their obesity likely resulted from reduced energy expenditure and locomotor activity. Thus, adult female Kiss1R KO mice display a metabolic and diabetic phenotype but further experiments are needed to dissect the contribution of strain, gender and dependency on kisspeptin signaling (ligand versus receptor). Indeed, a previous comparison of male and female Kiss1R KO mice pointed to a slight decrease in body weight of males at 9–12 weeks, without noticeable variation in females (Lapatto et al., 2007; Garcia-Galiano et al., 2012). KiSS-1 KO female mice were significantly smaller than their wild-type littermates at two months of age (d'Anglemont de Tassigny et al., 2007) while another study found no feeding-related phenotype for KiSS-1 KO mice of either sex (Lapatto et al., 2007).

The work by Tolson and collaborators (Tolson et al., 2014) raises also several questions regarding the interaction of metabolic/diabetic cues and the role of kisspeptin signaling in the brain and/or the periphery. Both in vitro and in vivo kisspeptin treatments have been found to enhance glucose-induced insulin secretion (Hauge-Evans et al., 2006; Wahab et al., 2011), supporting a role of kisspeptins in pancreatic β cell function. Such a possibility may underlie the impaired glucose tolerance of Kiss1R KO female mice (Tolson et al., 2014). However, several studies already reported an inhibition by kisspeptins of glucose-induced insulin secretion from β pancreatic MIN6 (Hauge-Evans et al., 2006) and NIT (Chen et al., 2014) cell lines, as well as from rat perfused pancreas (Silvestre et al., 2008) and mouse pancreatic islets (Vikman & Ahren, 2009; Chen et al., 2014). Chen and coworkers investigated into more detail the mechanisms by which kisspeptin affects insulin release and evidenced a cross-talk between Kiss1R signaling and the transcriptional factor Isl-1 (Chen et al., 2014). Kiss1R and Isl-1 were found to be coexpressed in mouse pancreatic islets and in NIT cells. Both in vitro and in vivo experiments, taking advantage of an Isl-1-inducible KO mouse model, revealed that Kisspeptin-54 inhibited insulin secretion by lowering Isl-1 expression and promotor activity of the insulin gene. Conversely, a feedback loop driven by insulin negatively regulated KiSS-1 mRNA levels and kisspeptin concentration in mouse NIT and pancreatic cells. This study also identified two distinct signaling pathways responsible for kisspeptin and insulin effects. Most interestingly, the discovery of a hormonal kisspeptin circuit between the liver and the endocrine pancreas highlighted a link between hyperglucagonemia, increased hepatic kisspeptin production and impaired glucose-stimulated insulin secretion from β pancreatic cells in type 2 diabetes (Song et al., 2014). Moreover, and in contrast with Tolson and coworkers' findings (Tolson et al., 2014), liver-specific KiSS-1 knockdown in mice submitted to various metabolic alterations was found to increase glucose-induced insulin secretion and to improve glucose tolerance (Song et al., 2014).

7.5.3. Impact of energy status on expression of the kisspeptin system

As the metabolic status is a known regulator of reproductive activity (Schneider, 2004; Schneider et al., 2013), much attention has been paid to the kisspeptinergic system as a link between energy balance and fertility (Castellano et al., 2010a; Navarro & Tena-Sempere, 2012; Parhar et al., 2012; Wahab et al., 2013; De Bond & Smith, 2014). Indeed, the expression and activity of kisspeptins and their receptor are highly sensitive to metabolic cues and regulatory factors. The next section will focus on the impact of negative (fasting, caloric restriction, lactation) and positive (high fat diet, obesity, diabetes) energy balance states on the KiSS-1/Kiss1R system as both under- and over-nutrition result in reproductive dysfunction.

7.5.3.1. Low energy balance. Acute fasting was consistently associated with a significant reduction of hypothalamic KiSS-1 mRNA levels in pre-pubertal male and female rats (Castellano et al., 2005) and in female (Brown et al., 2008; Kalamatianos et al., 2008; Matsuzaki et al., 2011) and male (Luque et al., 2007) adult rats. Similar responses to low food supply were reported in monkeys (Wahab et al., 2011). Chronic food restriction also down-regulated KiSS-1 expression in ovariectomized (OVX) ewes (Backholer et al., 2010) and in post-pubertal gilts (Zhou et al., 2014).

Other conditions of negative energy balance, such as lactation (Yamada et al., 2007; Xu et al., 2009), chronic blockade of mTOR in ovariectomized (OVX) rats (Roa et al., 2009), caloric restriction (True et al., 2011) or metabolic stress, such as streptozotocin-induced diabetes mellitus (Castellano et al., 2006b, 2009a) or acute inflammation evoked by systemic injection of lipopolysaccharide (Castellano et al., 2010b), also pointed to a close relationship between altered gonadotropic function and decreased hypothalamic KiSS-1 mRNA levels.

The reduction in hypothalamic KiSS-1 expression concerned both the ARC and AVPV nuclei in rodents (Castellano et al., 2005; Luque et al., 2007; True et al., 2011) and pig (Zhou et al., 2014) or the ARC and POA in ewe brain (Backholer et al., 2010). However, KiSS-1 expression was reported to be more severely impaired in the AVPV of fasted OVX rats (Kalamatianos et al., 2008) whereas KiSS-1 mRNA levels were selectively decreased in the ARC of gonadally intact adult female

rats (Matsuzaki et al., 2011) or of OVX rats following chronic mTOR blockade (Roa et al., 2009). Thus, these two KiSS-1 neuronal populations probably differentially contribute to the coupling of energy balance with gonadal function.

The impact of a low energy balance on hypothalamic Kiss1R expression appears also highly variable: an increase in Kiss1R expression was reported in rats (Castellano et al., 2005); a decrease was observed both in male adult rats (Luque et al., 2007) and in nutrient-restricted gilts, specifically in the ARC nucleus (Zhou et al., 2014), while no significant expression changes occurred neither in the ARC nor in the AVPV of fasted female rats (Matsuzaki et al., 2011).

The functional relevance of former observations was confirmed by studies on undernourished female rats (Castellano et al., 2005) and on uncontrolled diabetic male and female rats (Castellano et al., 2006b, 2009a) where kisspeptin-10 injections partially rescued hypogonadotropism and reproductive defects. Similarly, in women suffering from hypothalamic amenorrhea (often associated with negative energy balance), repeated administration of kisspeptin-54 substantially improved their gonadotropic function (Jayasena et al., 2010). Interestingly, acute fasting and caloric restriction in pubertal female rats not only decreased KiSS-1 mRNA in the ARC and AVPV but also inhibited the hypothalamic expression of the neurokinin B (NKB) gene and its NK3 receptor (Navarro et al., 2012). Given the coexpression of NKB and KiSS-1 in ARC neurons (Goodman et al., 2007; Ramaswamy et al., 2010) and the fact that delayed female puberty may also be partially prevented by repeated administration of NKB (Navarro et al., 2012), there is a possibility for NKB and KiSS-1 signaling to cooperate in the metabolic control of puberty (Navarro, 2013).

7.5.3.2. Positive energy balance. Obesity and diabetes are paradigms of altered energy homeostasis in which more than sufficient reserves of metabolic fuels are available, but the body cannot use them. Although they are frequently associated to lower fertility and altered puberty onset, the effects of persistent overweight on the expression and activity of the hypothalamic kisspeptinergic system have been much less investigated than those of energy deficit (Ahmed et al., 2009; Loret de Mola, 2009; Biro & Wien, 2010; Rachon & Teede, 2010).

In ob/ob mouse lacking the leptin gene, kisspeptin signaling is disrupted as revealed by a decrease in *KiSS-1* gene expression (Smith et al., 2006a), especially within the ARC nucleus (Quennell et al., 2011). Noteworthy, exogenous leptin (but not caloric restriction) is able to reverse adverse effects of obesity on reproduction in males and females (Mounzih et al., 1997; Cleary et al., 2001).

Studies on DBA/2J mice, prone to high fat diet-induced infertility, evidenced a marked reduction in KiSS-1 mRNA levels in the ARC and RP3V and of Kiss-ir neurons in the AVPV (Quennell et al., 2011). Conversely, pre-pubescent gilts fed a high fat diet showed an early attainment of puberty and increased KiSS-1 and Kiss1R mRNAs expression in the AVPV (Zhuo et al., 2014). Metabolic programming of puberty onset related to post-natal over/under feeding and differential hypothalamic KiSS-1 expression has recently been proposed (Castellano et al., 2011).

Streptozotocin (STZ)-treated rats is a widely used model of type 1 diabetes, with the destruction of pancreatic islets leading to hypoinsulinemia and hyperglycemia (Arison et al., 1967). Such diabetic animals are characterized by a hypogonadotropic hypogonadism (Spindler-Vomachka & Johnson, 1985; Sexton & Jarow, 1997), with normal LH responses to kisspeptin, low levels in KiSS-1 mRNAs but normal Kiss1R expression in the hypothalamus (Castellano et al., 2006b). Gonadotropin deficiency in this diabetic model most likely occurred upstream of GnRH neurons, with hypoleptinemia being a critical factor for reducing Kiss expression and subsequent excitatory input to GnRH neurons (Castellano et al., 2006b; Luque et al., 2007; Quennell et al., 2011).

Most interestingly, KiSS-1 was recently uncovered as a causal link between early dysregulated glucagon secretion by pancreatic α cells and impaired glucose-induced insulin secretion from β cells in type 2 diabetes (Song et al., 2014). Glucagon was found to stimulate KiSS-1 production in the liver from human diabetic patients and diabetic mouse models. Importantly, KiSS-1 knockdown in the liver of hyperglucagonemic, glucose-intolerant, high-fat-diet fed, and leptin insensitive db/db mice increased glucose-induced insulin secretion and improved glucose tolerance.

In the adipose tissue, the *KiSS-1* gene is regulated by sex steroids and the nutritional status (Brown et al., 2008). In contrast with findings in the hypothalamus, food restriction increased Kiss mRNA levels in the adipose tissue of male and female rats, while rats fed a high fat diet or obese Zucker rats showed a lower KiSS-1 expression in this tissue. Neonatal excitotoxic lesion of the ARC did not affect KiSS-1 mRNA levels in fat tissue, suggesting that kisspeptins may function as local regulators of adipocyte function.

Altogether, much data emphasize the importance of the kisspeptin neuronal network in the integration of metabolic signals to control puberty and reproduction. In the hypothalamus, the kisspeptin system may operate as a metabolic sensor that transmits nutritional relevant information to the HPG axis to adjust reproductive activity with available energy reserves. In fact, an array of metabolic signals and neuropeptides are involved in the maintenance of energy balance and reproductive processes (Schneider, 2004; Schneider et al., 2013) and the interplay of leptin, but also ghrelin and adiponectin, as well as of NPY/POMC neuropeptides with the kisspeptinergic system still receives considerable attention (Castellano et al., 2009b; True et al., 2011; Evans & Anderson, 2012; Garcia-Garcia, 2012; Sanchez-Garrido & Tena-Sempere, 2013; De Bond & Smith, 2014).

7.6. Pyroglutamylated RF-amide peptides and their QRFP receptor

7.6.1. Distribution of QRFPs and their receptor in peripheral organs and brain areas involved in energy homeostasis

A schematic presentation of the localization of QRFP-containing cell bodies and of QRFP-R1/R2 receptors in rodent brain, with reference to regions involved in the regulation of energy balance, is shown Fig. 7.

7.6.1.1. Localization of QRFP-containing neurons. The expression and localization of QRFP-26 and QRFP-43 peptides and of their transcripts have been assessed in human, rat and mouse tissues using RT-qPCR, ISH and IHC.

At the periphery, significant levels of QRFP transcripts were found in the human retina (Jiang et al., 2003), rat optic nerve (Fukusumi et al., 2003) and mouse eye (Takayasu et al., 2006). RT-qPCR and IHC experiments using antibodies against QRFP-26 confirmed the expression of QRFPs in human gut and pancreatic islets as well as in mouse gut, liver, endocrine pancreas and MIN6 insulinoma cells (Jiang et al., 2003; Prevost et al., 2015). QRFPs mRNA are also expressed in mouse adipose tissue and 3T3-L1 adipocytes (Zhang et al., 2007; Mulumba et al., 2010; Jossart et al., 2014) and in murine macrophages (Jossart et al., 2014). Circulating levels of QRFP-26 were also measured in human plasma by using a radio-immunoassay (Galusca et al., 2012; Prevost et al., 2015).

Within the CNS, ISH studies indicated that QRFP-26 and QRFP-43 were primarily expressed in the hypothalamus, within the LHA, the periventricular (PeV), VMH and ARC nuclei in rat (Chartrel et al., 2003; Kampe et al., 2006), mouse (Takayasu et al., 2006) and human (Bruzzone et al., 2006) brains. QRFP mRNA also displays a species-dependent distribution: in the human brain, it is abundant in the cerebellum and the medulla, with a moderate expression in the caudate putamen, VTA and hypothalamus while in mouse brain, it concentrates in the hypothalamus and the cortex (Jiang et al., 2003).

7.6.1.2. Localization of the QRFP receptor in the CNS and peripheral tissues. The distribution of QRFP-R in mammalian tissues is essentially from RT-qPCR, in situ hybridization and radioligand binding assays.

Fairly high levels of QRFP receptor mRNA were found in the human retina (Jiang et al., 2003) whereas lower levels were detected in rat and

mouse eye (Fukusumi et al., 2003; Takayasu et al., 2006). QRFP-R mRNA was also detected in human omental adipocytes and in murine 3T3-L1 adipocytes (Mulumba et al., 2010) as well as in mouse adipose tissue and MIN6 insulinoma cells (Prevost et al., 2015). The presence of QRFP-R immunoreactivity in human pancreatic sections has been recently reported (Prevost et al., 2015).

The QRFP receptor is primarily expressed in the CNS where it displays a much wider distribution than QRFP peptides. Within the human brain, QRFP-R mRNA is moderately enriched in the hypothalamus and the cortex, lower levels are found in the amygdala and VTA (Jiang et al., 2003). The localization of QRFP receptors in mouse (Jiang et al., 2003) and rat (Fukusumi et al., 2003) brains, first examined through RT-PCR and ISH experiments, was further detailed thanks to a comparative study based on ISH and autoradiographic mapping of [^{125}I]-26RFa binding sites in rat brain (Bruzzone et al., 2007). The existence of some regions enriched in 26RFa binding sites but deprived of QRFP mRNA as well as inconstancies in the distribution patterns of the QRFP-R in rat brain (Fukusumi et al., 2003; Bruzzone et al., 2007) were, at least in part, explained by the presence of two receptor orthologs QRFP-R1 and QRFP-R2 in rodent brain (mouse GPR103A/B; (Takayasu et al., 2006) and rat QRFP-r1/2, (Kampe et al., 2006)). Given the high homology and close pharmacological properties of these receptors (Section 3.5.4), the possibility that riboprobes and/or [^{125}I]-26RFa actually labeled a mixture of GPR103A/B receptors in rat brain could not be excluded.

Although a systematic comparison of the distribution of GPR103A/B receptor transcripts was undertaken in mouse brain (Takayasu et al., 2006), data from Fukusumi and coworkers (Fukusumi et al., 2003) were taken as the reference pattern for QRFP-r1 expression in rat brain (Kampe et al., 2006). Several qualitative and quantitative discrepancies regarding the distribution of the GPR103B (QRFP-r2) receptor mRNA in mouse and rat brain (Kampe et al., 2006) still merit further examination. With reference to regions regulating energy balance, a consensus view reconcilying both rodent species may be proposed as follows: QRFP-R1 mRNA is found in the olfactory bulb, the cortex–amygdala transition zone, in various hypothalamic areas (ARC, VMH, PVN), in the NTS (Fukusumi et al., 2003; Takayasu et al., 2006) and possibly in the parabrachial (PB) and dorsal motor vagal (DMX) nuclei (Bruzzone et al., 2007); QRFP-R2 mRNA is mainly detected in the nucleus accumbens (NAc) shell and caudate putamen (CP), in the VTA, and in some regions (POA, LHA, PVN) of the hypothalamus (Kampe et al., 2006; Takayasu et al., 2006). Thus, QRFP receptor subtypes differentially distribute in rodent brain, with only very little overlap.

7.6.2. QRFP-mediated control of food intake

First evidence for an orexigenic effect of QRFPs was provided by studies performed on mice, either freely fed (do Rego et al., 2006; Takayasu et al., 2006), submitted to a moderate high fat diet (Moriya et al., 2006) or to partial food restriction (Chartrel et al., 2003). Acute i.c.v. injection of QRFPs, during early light phase, induced a dose-dependent increase in food intake, in locomotor activity and in oxygen consumption (do Rego et al., 2006; Takayasu et al., 2006).

Chronic administration of QRFPs had no significant impact on body weight in mice under standard chow diet, in marked contrast with NPY which led to obesity (Moriya et al., 2006; Takayasu et al., 2006). The body weight of QRFP-R1 null mice — which suffer from a severe osteopenia — was indistinguishable from that of their wild-type littermates (Baribault et al., 2006), although no indication was given on how the animals were housed or fed. Thus, QRFP anabolic action may be counteracted by an increase in energy expenditure. In mice fed a moderately high fat diet, the continuous infusion of QRFPs caused a transient hyperphagia and a net increase in body weight (Moriya et al., 2006). However, pair-fed control and treated mice experienced a similar weight gain while QRFP-43 clearly increased fat mass. Thus, QRFPs may cause metabolic changes independently of their orexigenic effect.

The situation was more contrasted in rats, in which QRFPs were first reported to have no significant effects on the intake of standard low fat chow diet (Kampe et al., 2006; Patel et al., 2008). Subsequent studies evidenced an orexigenic effect of QRFP-26 and QRFP-43 only when rats were fed a high fat diet (Primeaux et al., 2008) or food-restricted (Lectez et al., 2009). Thus, QRFPs may increase the motivation to eat, drive preference for highly palatable food rich in fat and sucrose and/or regulate reward systems in the brain (Primeaux et al., 2013).

The possibility for QRFP-26 and QRFP-43 to mediate their orexigenic activity in part through the modulation of the hypothalamic NPY/POMC system (Takayasu et al., 2006) was confirmed by a series of converging observations in rats (Lectez et al., 2009). These data support the view that QRFP neurons originating from the VMH and LHA project onto NPY-ergic neurons within the ARC. QRFP-26 release and subsequent receptor activation increase food intake by stimulating the expression and release of NPY which in turn inhibits the firing of POMC neurons by activating Y1 and Y5 NPY receptors (Lectez et al., 2009).

Searching for putative interactions of QRFPs with other orexigenic peptide networks pointed to orexin as a plausible candidate. QRFP-promoted food intake in orexin −/− mice was similar to that found in wild-type mice (Takayasu et al., 2006). In genetically obese mice models with leptin insufficiency (ob/ob) or insensitivity (db/db), orexin expression was down-regulated (Yamamoto et al., 2000) while prepro-QRFP transcript was up-regulated (Takayasu et al., 2006). Thus, QRFP and orexin probably exert orexigenic effects through independent signaling pathways.

7.6.3. QRFPs effects on energy homeostasis and adipose metabolism

Acute i.c.v. administration of QRFP in mice significantly increased their locomotor activity during the light and dark periods (do Rego et al., 2006; Takayasu et al., 2006). Oxygen consumption, taken as an index of metabolic rate, was also enhanced (Takayasu et al., 2006). Pair-feeding studies conducted under moderate fat diet indicated that mice chronically treated with QRFPs did not display marked differences in locomotor activity. However, lowered rectal temperature and expression of the brown adipose tissue uncoupling protein-1 (UCP-1) indicated a decreasing energy expenditure (Moriya et al., 2006). These QRFP-treated mice also exhibited a higher fat mass and increased plasmatic concentrations of insulin, leptin, cholesterol, free fatty acids and triglycerides (Moriya et al., 2006).

The inhibition by QRFP-26 of glucose-induced insulin secretion in rat perfused pancreas (Egido et al., 2007) was observed in rat pancreatic INS-1E-β cells and in human pancreatic islets (Granata et al., 2014). In the latter study however, QRFP-26 and QRFP-43 were found to exert opposite effects on insulin secretion, mediated via $G_{i/o}$ inhibition or G_s stimulation of cAMP production, respectively. QRFP receptor silencing blocked the QRFP-43 insulinotropic effect in INS-1E-β cells, leaving the QRFP-26 insulinostatic response unaffected. In marked contrast with these findings, Prevost and collaborators showed that QRFP-26 was able to stimulate insulin release from MIN6 insulinoma cells, via the activation of endogenous QRFP receptors (Prevost et al., 2015). As MIN6 cells do not express the NPFF2 receptor, the possibility for QRFP-26 to mediate an inhibition of insulin release from INS-1E-β cells and pancreatic islets via activation of NPFF2R has been evoked (Prevost et al., 2015). Most interestingly, QRFP-26 was proposed to play an incretin-like role in the regulation of glucose homeostasis: following glucose ingestion, it is secreted from the gut into the blood to reach pancreatic β cells (where it stimulates insulin secretion) and insulin-sensitive tissues, such as the striated muscle and the liver (where it may possibly favor glucose uptake), in order to adjust glycemia levels (Prevost et al., 2015).

The role of QRFPs and of their QRFP receptor in adipogenesis and lipid metabolism has been investigated in differentiated murine 3T3-L1 adipocyte cells (Mulumba et al., 2010). These cells (which express the QRFP precursor and the QRFP-R2 receptor) responded to a QRFP-26 or QRFP-43 application by a pronounced increase in their triglyceride content and in fatty acid uptake and by up-regulating various genes

involved in lipid storage. These responses as well as the inhibition by QRFP-26 or QRFP-43 of isoproterenol-induced lipolysis were blocked following QRFP-R2 knockdown in 3T3-L1 cells. Thus, in addition to their central orexigenic effect, QRFPs may act as peripheral adipogenic factors to regulate fatty acid uptake and fat storage, via activation of the QRFP-R2.

7.6.4. Impact of energy status on the expression of QRFPs and their receptor

Obese individuals and human patients with type-1 and type-2 diabetes, under fasting conditions, show marked elevation in their QRFP-26 plasma levels (Prevost et al., 2015). In fasted mice, as compared to fed control animals, as well as in genetically obese mice characterized by leptin insufficiency (ob/ob) or leptin insensitivity (db/db) fed ad libitum, hypothalamic prepro-QRFP mRNA levels are up-regulated (Takayasu et al., 2006). On the other hand, mice fed a moderately high fat diet and chronically treated with QRFP-43 display a marked hyperleptinemia, including in QRFP/pair-fed groups (Moriya et al., 2006).

Rats consuming high fat diet for 3 weeks gained more weight and fat mass, as compared to animals fed a low fat diet, and showed increased prepro-QRFP mRNA levels in the VMH/ARC region of the hypothalamus while QRFP-R expression remained unchanged (Primeaux et al., 2008). Another study systematically examined the influence of dietary composition on hypothalamic QRFP-43 content (Beck & Richy, 2009). Rats fed ad libitum for 8 weeks with either standard or high-fat diet did not display differences in body weight or total energy intake, indicating that rats adapted their quantity of food to counterbalance the higher energy density of high fat diet. However, diet composition strongly impacted hypothalamic QRFP-43 expression, as it became undetectable in the VMH of rats submitted to high fat diet. Thus, a decrease in the QRFP orexigenic tone may contribute to the normalization of caloric intake in rats fed a high fat diet. Most interestingly, QRFP-43 levels in the VMH (but not in the LHA) inversely correlated with leptin plasma levels which in turn significantly correlated with diet adipogenic index, as expected (Barsh & Schwartz, 2002). Leptin exerts its anorexigenic activity mainly via the NPY/POMC system in the ARC (Satoh et al., 1997; Tang-Christensen et al., 1999) and recent studies already pointed to the importance of the QRFP/leptin balance in the regulation of that system (Lectez et al., 2009). The data from Beck and Richy confirmed the role of leptin in the VMH where it may regulate, according to its plasma levels, the expression of the orexigenic QRFP peptides (Beck & Richy, 2009).

The nutritional or energy status also affects QRFP expression in peripheral tissues. Indeed, in a mouse model of diet-induced obesity, the expression of QRFP-R2 was strikingly increased in metabolically active fat tissues whereas QRFP gene expression was decreased, most probably by pro-inflammatory cytokines such as TNF-α (Mulumba et al., 2010).

Jossart and collaborators uncovered the expression and secretion of QRFP by macrophages, and its paracrine role between macrophages and adipocytes with a pro-adipogenic effect (Jossart et al., 2014). Down-regulation of QRFP expression in adipocytes and macrophages was found to primarily depend on IFN-β levels which were up-regulated in macrophages following LPS stimulation and elevated in obese mice with metabolic endotoxemia. It is to note that endotoxins play an important role in the initiation of insulin resistance in obesity (Cani et al., 2008) and that macrophages participate to the development of obese adipose tissue inflammation (Anderson et al., 2010). More generally, this study highlighted an autocrine/paracrine function for QRFP and its contribution to a switch in the obese adipose tissue from a proadipogenic to a pro-inflammatory state. Therefore, QRFP was proposed as a potential biomarker for metabolic endotoxemia in adipose tissue (Jossart et al., 2014).

In line with consistent observations indicating that QRFPs had no effect on food intake when injected during the dark phase in mice (Takayasu et al., 2006) and rats (Kampe et al., 2006), QRFP-26 was recently identified as a circulating hormone submitted to circadian variations in humans (Galusca et al., 2012). In healthy women, QRFP-26 plasma levels were high and stable during the morning, dramatically felt at noon and remained low until next early morning. Anorectic patients revealed an alteration of the circadian rhythmicity and a marked increase in mean circulating QRFP-26 levels together with those of ghrelin. In addition to lower body mass index (BMI) and fat mass, these subjects had also lower leptin and 17β-estradiol levels than the control group. These data may reflect an adaptive mechanism of the organism to promote energy intake and to increase fat stores in response to undernutrition.

To conclude this section, all RF-amide neuropeptides and their receptors contribute in many ways to the regulation of food intake and energy balance in Mammals. Despite the fact that their implication in feeding behavior has been conserved throughout evolution (Dockray, 2004; Bechtold & Luckman, 2007), it is still difficult to ascertain whether it represents their primary function. Nowadays, RF-amides may be better regarded as one of the numerous peptidergic systems that cooperate to finely tune energy homeostasis, both at the central and peripheral levels. In addition to the difficulties associated to deciphering the proper role of each RF-amide/receptor pair in the regulation of complex physiological functions (appetite, energy metabolism or food reward, among others) as well as in pathological conditions such as obesity and diabetes, the promiscuous binding properties of NPFF1 and NPFF2 receptors still represent a major drawback that should be soon relieved with the development of highly selective pharmacological tools.

8. Mammalian RF-amide peptides and their receptors in reproduction

8.1. Control of reproduction and hypothalamic–pituitary–gonadal axis in Mammals

The control of reproduction in Mammals is governed by the hypothalamo–pituitary–gonadal (HPG) axis and by complex positive and negative steroidal feedback from the periphery. Identified in 1977 by the Medicine Nobel Laureates Roger Guillemin and Andrew V. Schally, the Gonadotropin-Releasing Hormone (GnRH) released by scattered hypothalamic cells represents the main positive regulator of the reproductive system (Matsuo et al., 1971; Burgus et al., 1972). GnRH neurons are mainly located in the preoptic area (POA) and massively project to the median eminence (Hahn & Coen, 2006). Triggered by gonadal steroids, the pulsatile secretion of GnRH into the portal blood enhances the synthesis and pulsatile secretion of LH (luteinizing hormone) and FSH (follicle stimulating hormone) gonadotropins from the anterior pituitary (Plant, 2015). In females, positive and negative steroidal feedbacks on GnRH neurons finely tune LH release. During the almost entire cycle period, negative feedback mechanisms manage the pulsatile secretion of LH allowing follicular development and steroidogenesis (Andreu et al., 1998; Jones & Hsueh, 1981). However, during proestrus, the LH surge preceding ovulation is triggered by a positive feedback (Moenter et al., 2003) in different rodent species but not in primates (Yamaji et al., 1971; Karsch et al., 1973; Knobil, 1974).

Among multiple modulators of reproduction, RF-amide peptides are involved in the control of the HPG axis. Notably, Kisspeptin and its receptor are now well described as potent positive regulators of mammalian GnRH neurons. On the other hand, although the precise role of the RFRP-3/NPFF1R system is not entirely understood, especially in Mammals, several studies highlight its subtle negative effect under special conditions.

8.2. The RF-amide related peptides/ NPFF1R system in the regulation of reproductive axis

It is important to keep in mind that depending on the publications, RFRP-1 or RFRP-3 are considered mammalian orthologs of GnIH (GnRH Inhibitory Hormone) which represents the main GnRH inhibitory system in birds (Tsutsui et al., 2000, 2010). RFRP-1 is now considered the structural ortholog, whereas RFRP-3 is considered the functional ortholog of avian GnIH (Clarke et al., 2008; Pineda et al., 2010a; Smith & Clarke, 2010; Tsutsui et al., 2012;

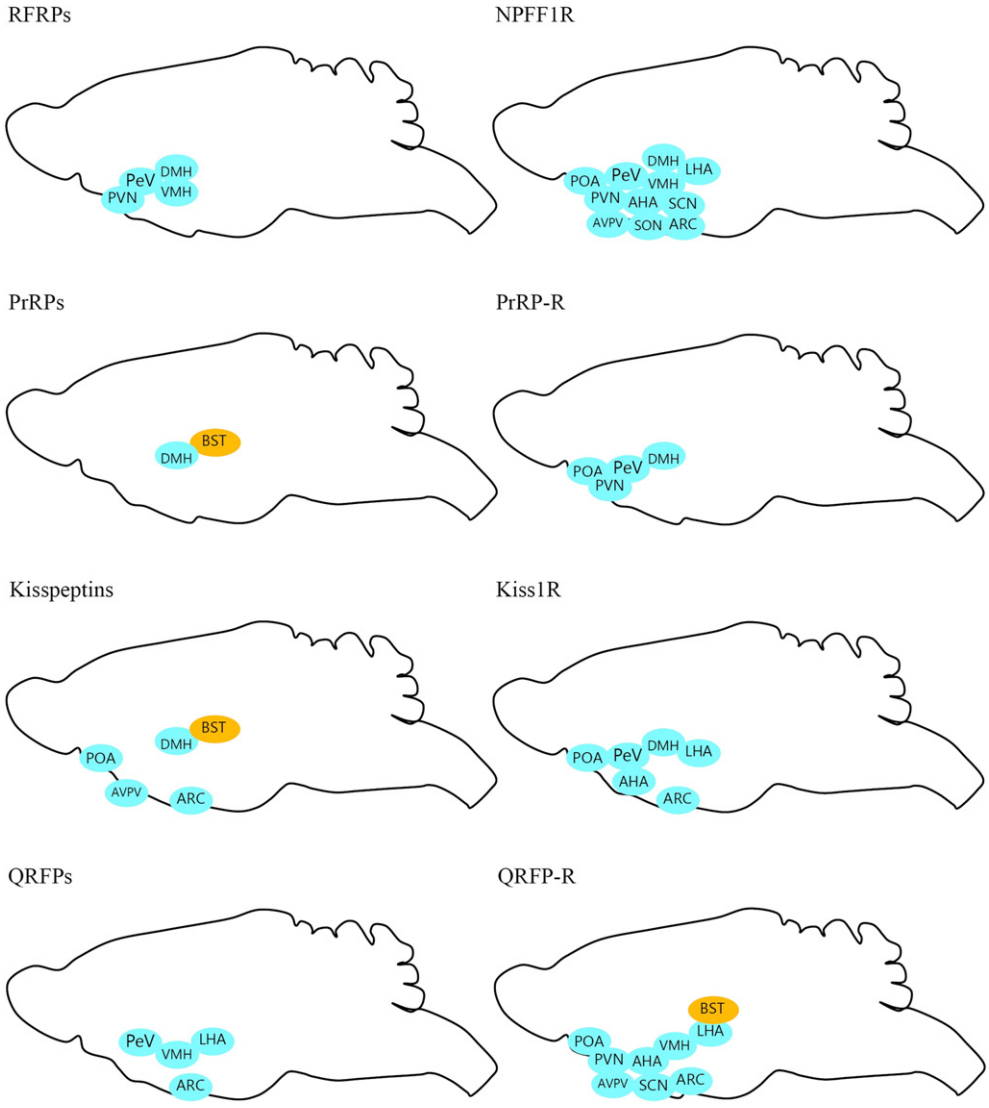

Fig. 8. Anatomical distribution of RF-amide peptides and their cognate receptors in rodent (rat and mouse) areas involved in reproduction. Highlighted brain structures have been selected on the basis of their known or putative roles in the control of reproduction in Mammals and of their enrichment in RF-amide peptides (left panel) and their cognate receptors (right panel), as afforded from distribution studies in mouse brain. When specified in the literature, the localization of RF-amide peptide-containing cell bodies is privileged. Colored areas refer to distinct anatomical structures according to MeSH database: hypothalamic nuclei (light blue); telencephalon nuclei (orange). Details on the differential distribution of QRFP-R1 and QRFP-R2 receptor isoforms are provided in Section 8.6.1. Abbreviations are listed in the corresponding section.

Ubuka et al., 2012b). This explains why most studies in Mammals focused on RFRP-3.

8.2.1. Localization of RF-amide related peptides and their NPFF1 receptor in reproductive pathways

The distribution of RFRP peptides and receptor in brain regions involved in the control of reproduction in rats and mice are highlighted in Fig. 8.

RFRP peptides are mainly expressed within the CNS (Chartrel et al., 2006; Tsutsui et al., 2009, 2010). In situ hybridization studies allowed to detect RFRP mRNA in the dorsomedial hypothalamic nucleus (DMH) in mouse and hamster brain (Kriegsfeld et al., 2006; Ubuka et al., 2012a) as well as in the periventricular nucleus (PeV) and in the region between dorsomedial (DMH) and ventromedial (VMH) hypothalamic nuclei in rat brain (Hinuma et al., 2000; Legagneux et al., 2009). RFRP-expressing neuronal cell bodies mainly locate within the intermediate periventricular nucleus (PeV) of the hypothalamus in macaque (Ubuka et al., 2009a) and in DMH and PVN in sheep (Clarke et al., 2008). On the other hand, immunohistochemistry pointed to very high levels of RFRP-1 in the thalamus and midbrain with cell bodies more precisely localized within PeV and VMH nuclei (Fukusumi et al., 2001). Using a rabbit antibody raised against white-crowned sparrow GnIH, immunoreactive cells were found to concentrate within the DMH in rats, mice and Syrian hamsters (Kriegsfeld et al., 2006). In the rat, Syrian hamster and sheep brain, many RFRP-3 fibers establish close contacts with GnRH neurons in the preoptic area (Kriegsfeld et al., 2006; Johnson et al., 2007; Smith et al., 2008a), suggesting a role for this RF-amide peptide in the control of reproductive axis. This peptide may also act upstream of GnRH neurons, as RFRP-ir fibers were found to project onto kisspeptin neurons that express NPFF1R in the rat (Rizwan et al., 2012).

NPFF1R mRNA expression was reported in the suprachiasmatic nucleus (SCN), PeV, supraoptic nucleus (SON), and pars tuberalis (PT) in the sheep (Dardente et al., 2008). In rats, NPFF1R mRNA was found in the medial preoptic nucleus, the anteroventral periventricular nucleus (AVPV), medial (MH), paraventricular and periventricular hypothalamic nuclei and in the SCN (Hinuma et al., 2000; Liu et al., 2001). In mice, NPFF1R is weakly expressed in GnRH neurons (Poling et al., 2012). In

vitro binding autoradiography in rats showed NPFF1R expression in several regions of hypothalamus, including the anterior (AHA) and lateral (LHA) hypothalamic area and the ARC, VMH, PVN and PeV nuclei (Gouarderes et al., 2004a). In mice, binding autoradiography displayed NPFF1R expression in AHA, ARC, LHA, MH, PVN and PeV (Gouarderes et al., 2004a).

8.2.2. The RF-amide related peptides/ NPFF1R system in the regulation of reproduction

Since the discovery of the inhibitory effect of GnIH in the Japanese quail (Tsutsui et al., 2000), RFRPs (the GnIH homologs in Mammals) have been proposed to counterbalance the positive regulation exerted by kisspeptin on GnRH secretion. Such a hypothesis was supported by the observation of their inhibitory effects both in vitro and in vivo, at the hypothalamic and pituitary levels, in various Vertebrate classes including Mammals. At the same time, Hinuma and collaborators provided the first demonstration for a pituitary role of a "mammalian GnIH" in rats by reporting a dose-dependent effect of RFRP-1 (i.c.v. injection) on plasma prolactin levels. However, no effect of this peptide was observed on LH and FSH levels (Hinuma et al., 2000). More recently, several studies showed that the central and peripheral administration of RFRP-3 was able to decrease the release of gonadotropins, notably LH, in various Vertebrates including Mammals (Kriegsfeld et al., 2006; Johnson et al., 2007; Clarke et al., 2008; Kadokawa et al., 2009; Sari et al., 2009; Pineda et al., 2010a).

8.2.2.1. RF-amide related peptides/NPFF1R effects on gonadotropin-releasing hormone and gonadotropin secretion. In Mammals, a majority of in vitro and in vivo studies reported an inhibitory effect of RFRPs on gonadotrophin secretion, both at hypothalamic and pituitary levels. However, it is important to mention here that most experiments in the field that took RF9 (see Fig. 1 and Section 6.2 for further details) as a highly selective NPFF1R antagonist probably led to inaccurate interpretation. Indeed, the rise in gonadotropin release consecutive to RF9 administration, first attributed to a decrease in the endogenous RFRP-3 tone due to NPFF1R blockade (Pineda et al., 2010a,b; Caraty et al., 2012a; Garcia-Galiano et al., 2012; Glanowska et al., 2014), is now clearly assigned to the ability of this compound to activate Kiss1 receptors. Recent studies indeed showed that RF9 promotes intracellular calcium mobilization, inositol phosphate accumulation and ERK phosphorylation in Kiss1R-expressing cell lines (Kim et al., 2015; Min et al., 2015). Moreover, in NPFF1R KO mice, RF9 (5 nmol/5 μL; i.c.v. administration) triggers a robust LH release, similar to that obtained in wild-type littermates, whereas it displays a very weak effect in Kiss1R KO mice (Min et al., 2015). Therefore, owing to its clear Kiss1R agonist component, we will not keep describing RF9 as a tool to modulate LH release via inhibition of NPFF1 receptors.

In vitro, RFRP-3 dose-dependently inhibited the release of LH and FSH induced by GnRH in cell cultures from sheep pituitary and this effect, which was more prominent on LH than on FSH release, strictly depended on the presence of GnRH (Clarke et al., 2008; Sari et al., 2009). RFRP-3 reduced, in a dose-dependent manner, the mobilization of intracellular calcium evoked by GnRH in ovine pituitary cells in vitro (Clarke et al., 2008). In anterior pituitary cells of cattle, RFRP-3 (0.1 nM to 1 μM concentration range) efficiently decreased LH levels in the presence of 1 nM GnRH (Kadokawa et al., 2009). In LβT2 cells, a mouse gonadotrope cell line, RFRP-1 and RFRP-3 also inhibited GnRH-induced LH release (Son et al., 2012). In ram pituitary cell cultures, RFRP-3 totally blocked the increase in expression of LHβ and FSHβ mRNA subunits and the stimulation of ERK-1/2 phosphorylation induced by GnRH (Sari et al., 2009; Son et al., 2012). Both RFRP-1 and RFRP-3 inhibited GnRH-induced cAMP/PKA activation in LβT2 cells (Son et al., 2012). In contrast with former findings, RFRP-3 treatment of isolated anterior pituitary cells from rats, for 3 h at doses ranging from 1 to 100 nM, did not induce any significant variation in LH levels, in the presence or absence of GnRH (Anderson et al., 2009). The study of RFRP-3 effects on mouse GnRH neurons evidenced three types of responses: 41% of cells underwent a suppression of firing rate, 12% displayed an increased firing rate, while no effect was seen in remaining cells (Ducret et al., 2009). The authors also reported that the impact of RFRP-3 on GnRH neurons firing did not significantly vary during the estrous cycle, nor with sex or localization of GnRH neurons.

In vivo, the central injection of RFRP-3 was found to only slightly inhibit gonadotropin release in female and male rats (Johnson et al., 2007; Pineda et al., 2010a) and to reduce GnRH levels in the pig only at a single effective dose of 0.1 nM (Li et al., 2013). Intravenous (i.v.) administration of RFRP-3 diminishes the response of ovariectomized (OVX) rats to GnRH (Rizwan et al., 2009). LH pulses (but not FSH) are suppressed in ewes following i.v. RFRP-3 injection (Clarke et al., 2008). Although RFRP-3 secreted from the median eminence into the hypophyseal portal blood inhibited GnRH-induced LH release from sheep pituitary, no correlation between RFRP-3 and LH pulses could be evidenced (Smith et al., 2012). I.c.v. and i.p. RFRP injections (500 and 600 ng, respectively) into the Syrian hamster caused a drastic reduction (more than 70%) in plasma LH levels within 30 min (Kriegsfeld et al., 2006). Significant differences in plasmatic LH and FSH levels were also detected in rats following i.c.v. injection of RFRP-3 (Johnson et al., 2007). More recently, the generation of the first mouse line with constitutive inactivation of the NPFF1 receptor allowed one to observe an increase in KiSS-1 expression in the ARC, elevated serum FSH levels and a greater LH response to GnRH, confirming the inhibitory effect of RFRP-3 on reproductive axis (Leon et al., 2014). RFRP-3 (i.c.v.) also impacted sex behavior in male rats (mounts, intromissions, ejaculations) during the photophase (Johnson et al., 2007), in sharp contrast with its lack of effect in mice, sheep and non-human primates (Clarke et al., 2012).

The site of action of RFRPs in Mammals remains controversial. For example, i.v. administration of RFRP-3 was found to promote a significant decrease in serum LH serum levels after 120 min while an i.c.v. injection of RFRP-3 had no effect on pulsatile LH secretion in OVX rats (Murakami et al., 2008). Moreover, and in contrast with the well-reported paradigm for an inhibitory effect of RFRP-3 on reproduction, RFRP-3 (i.c.v.) clearly stimulated the gonadotrophic axis via GnRH activation in male Syrian hamsters (Ancel et al., 2012) and short-day Siberian hamsters (Ubuka et al., 2012a), although an inhibitory effect has been shown in female hamsters (Kriegsfeld et al., 2006). Moreover, in the striped hamster, hypothalamic RFRPs mRNA levels were found to be higher in males during the breeding season (Zhao et al., 2014).

8.2.2.2. The RF-amide related peptide system and the positive feedback of estrogens on reproductive axis. In rodents, many studies indicate that inhibition of the RFRP system is necessary for estrogens to exert a positive feedback at hypothalamic and pituitary levels, in agreement with the observation of RFRP fibers in close contact with GnRH neurons in the hypothalamus of rats, Syrian hamsters and sheep (Kriegsfeld et al., 2006; Johnson et al., 2007; Smith et al., 2008a). RFRP-3 had a central inhibitory effect on GnRH neuronal activity during the GnRH/LH surge induced by estradiol, but not on tonic pulsatile LH secretion in rat (Anderson et al., 2009; Rizwan et al., 2009). RFRP-3 infusion within the AVPV — a region known to mediate the positive feedback of estrogen responsible for the preovulatory surge in rats — suppressed c-Fos cellular activation but only slightly decreased LH concentration at the surge preovulatory peak (Anderson et al., 2009). The targeting of RFRP-containing cells by gonadal steroids in the DMH is supported by the presence of the estrogen receptor α (ERα) on RFRP-ir cells and by an increase in their c-Fos labeling in response to estrogen (E2) administration (Kriegsfeld et al., 2006). In OVX mice, E2 down-regulated prepro-RFRP mRNA in the hypothalamus, suggesting that the attenuation of the RFRP endogenous tone by estrogen may allow the positive feedback responsible for preovulatory LH surge (Molnar et al., 2011). However, no effects of i.c.v. RFRP-3 injection, at either dose, have been

observed on LH pulse frequency or amplitude nor on mean plasma LH concentration in rats (Anderson et al., 2009).

8.2.2.3. RF-amide related peptides and gonadotropins during the estrus cycle. The different effects of RFRP-3 during the estrus phase in mice and hamsters are modulated by estrogen levels (Kriegsfeld et al., 2006; Gibson et al., 2008; Molnar et al., 2011). The female cycle divides into four stages (metaestrus, diestrus, proestrus and estrus), with different estrogen levels and variable cellular ratios (leucocytes, epithelial cells and cornified cells) within the vagina (Banks, 1993; Priedkalns & Leiser, 1998). During the estrus cycle, two patterns of GnRH and LH secretion are observed (Fox & Smith, 1985). The ARC drives a tonic pulsatile secretion of LH that is responsible for the negative feedback, which lasts during almost the entire cycle. The positive feedback is triggered by high concentrations of estrogens that stimulate GnRH release following AVPV activation. This mechanism only occurs at the proestrus stage to provoke the preovulatory LH surge. In the Syrian hamster, SCN fibers contact RFRP-containing neuronal cells. Moreover, during proestrus, the number and activity of RFRP-containing cells are reduced in the DMH compared to diestrus, where levels are maximal (Gibson et al., 2008). During the LH surge, early gene expression is reduced in RFRP-positive cells, under the dependence of estrogens (Gibson et al., 2008). As well, c-Fos-positive RFRP-1-ir neurons are significantly higher during diestrus than in proestrus or estrus stages (Jorgensen et al., 2014). RFRP-3 suppresses cellular activation within the AVPV, a region known to robustly express c-Fos, especially in kisspeptin-ir neurons during the preovulatory surge (Smith et al., 2006a). However, no effect of estrogens on RFRPs mRNA levels has been observed in female rats (Quennell et al., 2010).

8.2.2.4. The RF-amide related peptide system and the regulation of sexual organ activity. Central and direct actions of RFRPs on testicular activity have been extensively reviewed by Ubuka and coworkers (Ubuka et al., 2014). RFRPs and NPFF1R mRNAs are expressed in rodent (Zhao et al., 2010; Singh et al., 2011a,b), pig (Li et al., 2012) and human (Oishi et al., 2012) gonads, in agreement with the potential autocrine/paracrine control of testicular activity exerted by GnRH and RFRPs (Anjum et al., 2012). RFRPs and NPFF1R protein expression is observed in the testis of Syrian hamsters, and more particularly in myoid cells of all tubules and spermatocytes, round spermatids and elongated spermatids, with various patterns according to the phase of the seminiferous epithelial cycle (Zhao et al., 2010). ISH studies in Syrian hamster testis showed a strong expression of RFRPs and NPFF1R mRNAs in spermatocytes and spermatids (Zhao et al., 2010). A significant correlation between RFRPs, GnRH and GnRH-R immunostaining levels was observed in mouse testis from birth to senescence (Anjum et al., 2014). RFRP-ir levels decrease from birth to pre-puberty, whereas they significantly increase during pubertal and reproductive stages to senescence. Compared to control mice, RFRP treatment induced a significant and dose-dependent decrease in GnRH-R expression in the testis (Anjum et al., 2012) and the inhibition of testicular activities in adult mice, either directly or through GnRH (Anjum et al., 2014). RFRP-3 was also found to decrease the proliferation of germinal cells, to increase apoptotic markers and to inhibit LH receptor expression, 3β-hydroxysteroid dehydrogenase and P450 side-chain cleavage (enzymes involved in sexual steroid synthesis) and testosterone secretion from mice testes (Anjum et al., 2014).

8.3. The neuropeptide FF/NPFF2R system and the regulation of reproductive axis in Mammals

8.3.1. Localization of the neuropeptide FF/NPFF2R system in reproduction pathways

In rat and mouse brain (Fig. 8), NPFF-immunoreactive neurons, fibers and terminals mainly locate in the hypothalamus (Kivipelto et al., 1989). They massively project onto the neurohypophysis and establish synaptic contacts with pituitary cells (Kivipelto et al., 1989; Boersma et al., 1993). At the periphery, the NPFF2R protein was found to be expressed in late elongated spermatids of Syrian hamster testis (Zhao et al., 2010).

8.3.2. Function of the neuropeptide FF/NPFF2R system in reproduction

To our knowledge, no evidence for a function of NPFF in reproduction has been published to date. As reported by Pineda and collaborators, central injection of NPFF in rats had no effect on circulating LH and FSH levels (Pineda et al., 2010a).

8.4. The prolactin-releasing peptides/prolactin-releasing peptide-receptor system and the regulation of reproductive axis in Mammals

8.4.1. Localization of prolactin-releasing peptides and prolactin-releasing peptide-receptor in reproduction pathways

A schematic presentation of the localization of these peptides and their receptor in rodent brain areas involved in reproduction is provided in Fig. 8.

In the rat, PrRP mRNA is distributed throughout the CNS, with highest levels in the DMH and medulla oblongata and moderate expression in the hypothalamus (Hinuma et al., 1998). However, bioactive PrRPs have been found to be significantly enriched within the hypothalamus as compared to the medulla oblongata in rat brain (Fujii et al., 1999). Within the medulla oblongata, PrRPs are expressed in the ventrolateral reticular formation (VLRN), the commissural nucleus of the lateral NTS and the dorsal medullary of the reticular field where noradrenergic neurons are located (Chen et al., 1999). These noradrenergic neurons project onto the hypothalamus (Chen et al., 1999) where they stimulate GnRH neurons in the medial septum diagonal band and in the POA (Wright & Jennes, 1993). In the hypothalamus, PrRPs were mainly located in the bed nucleus of striaterminalis (BST; Matsumoto et al., 1999), in close proximity to the POA area where PrRP-R-ir and GnRH-ir neurons were shown to colocalize (Feng et al., 2007). Both PrRP and PrRP-R mRNA were present in rat testis and epididymis (Nieminen et al., 2000).

Expression of PrRPs in female rat brain varies during the estrus cycle and is linked to GnRH expression (Feng et al., 2007). Sex steroids, which are involved in the phase transition during the estrus cycle, have a direct influence on PrRP mRNA and peptide expression in rats (Kataoka et al., 2001). In ovariectomized rats, estrogen up-regulated PrRP mRNA levels in the NTS and VLRN while progesterone induced a significant increase in PrRP mRNA only in the NTS. In the latter region, double-label immunocytochemistry allowed to identify a majority (70%) of PrRP immunoreactive neurons that co-expressed ERα receptors (Kataoka et al., 2001).

PrRP-R mRNA was found in several hypothalamic regions such as the medial preoptic area and the periventricular, paraventricular and dorsomedial nuclei in rats (Roland et al., 1999) and was also detected in human and rat pituitaries (Welch et al., 1995; Hinuma et al., 1998; Fujii et al., 1999).

8.4.2. Function of the prolactin-releasing peptides/prolactin-releasing peptide-receptor system in the reproductive axis

There is some evidence for an action of PrRPs on the reproductive axis and gonadotropin secretion. Although PrRP-31 failed in promoting LH, FSH or PRL release from rat anterior pituitary cells in vitro (Kawamata et al., 2000), i.c.v. injection of PrRP-31 in rats led to a significant increase in plasma LH, FSH and testosterone levels, and stimulated the release of GnRH from male rats hypothalamic explants (Seal et al., 2000). Administration of anti-PrRP serum significantly reduced the amplitude of the LH surge in female rats, suggesting a role of PrRPs in the preovulatory signal (Hizume et al., 2000). The secretory profile of PrRPs in the rat hypothalamus, before and during LH surge, has been examined using push–pull perfusion of sex steroids (Watanobe, 2001). An early rise in PrRPs and a subsequent release of GnRH, followed by the LH surge, have been observed in the medial preoptic area (POA), a region

enriched in GnRH neurons. Thus, PrRPs may mediate the steroid-induced LH surge through the activation of GnRH neurons in POA (Watanobe, 2001).

8.5. The Kisspeptins/Kiss1R system in the regulation of reproductive axis

The pivotal role of Kisspeptins in reproduction was first apprehended with the isolation of Kiss1R mutations in idiopathic hypogonadotropic hypogonadism (de Roux et al., 2003; Seminara et al., 2003) and the observation of a similar phenotype in KiSS-1 KO mice (d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007). Kiss1R null mice were also reported to be infertile (Seminara et al., 2003). Altogether, these data demonstrated that the Kisspeptins/Kiss1R system is mandatory for the development of puberty and the control of reproduction. As a consequence, over the past decade, research on the involvement of this system in the control of reproduction of many different species has been very active and led to the publication of a considerable amount of literature.

In this review, we have tried to summarize the main findings in the field. Further details are available in recent reviews that specifically address this topic (Pinilla et al., 2012; Wahab et al., 2013; Clarke et al., 2015; Plant, 2015).

8.5.1. Localization of Kisspeptins and Kiss1R in reproductive pathways

The distribution of kisspeptins in the mammalian nervous system has been detailed in recent reviews (Mikkelsen & Simonneaux, 2009; Lehman et al., 2010). A schematic presentation of the localization of these peptides and their receptor within the rodent brain is provided in Fig. 8.

In the mammalian brain, two main populations of kisspeptinergic neurons are known to be involved in reproduction regulation and feedback signals. The presence of KiSS-1 mRNA and protein in the arcuate (ARC) and dorsomedial hypothalamic (DMH) nuclei and the preoptic region, including the preoptic area (POA) and the anteroventral periventricular nucleus (AVPV), was identified by in situ hybridization (Gottsch et al., 2004; Irwig et al., 2004; Han et al., 2005; Shahab et al., 2005; Smith et al., 2005a, Estrada et al., 2006, Revel et al., 2006, Smith et al., 2006b, Adachi et al., 2007, Kauffman et al., 2007a,b; Rometo et al., 2007; Smith et al., 2007, 2008b, 2009a; Takase et al., 2009; Ansel et al., 2010; Smith et al., 2010) or immunohistochemistry (Kinoshita et al., 2005; Clarkson & Herbison, 2006; Franceschini et al., 2006; Goodman et al., 2007; Greives et al., 2007; Mason et al., 2007; Decourt et al., 2008; Ramaswamy et al., 2008; Clarkson et al., 2009; Ohkura et al., 2009; Smith et al., 2009a; Takase et al., 2009; Cheng et al., 2010; Desroziers et al., 2010; Hrabovszky et al., 2010; Ramaswamy et al., 2010; Wakabayashi et al., 2010). However, within these main regions, each mammalian species displays a specific KiSS-1 distribution pattern.

KiSS-1 expression displays a marked sexual dimorphism in almost all mammalian species studied to date (Ansel et al., 2010; Cheng et al., 2010; Hrabovszky et al., 2010). In rodents, females express a significantly higher number of Kiss1R/Kiss-ir neurons than males in AVPV, in direct link with the absence of testosterone during a perinatal critical window (Gogan et al., 1980, 1981; Corbier, 1985; Connolly & Resko, 1994; Simerly, 1998, 2002; Smith et al., 2005a,b; Clarkson & Herbison, 2006; Adachi et al., 2007; Kauffman et al., 2007a; Ansel et al., 2010). This suggests that in adults, sex differences in the number of Kiss1R/Kiss-ir neurons in AVPV are not due to difference in circulating levels of gonadal steroids. In contrast, no sex differences were found in the ARC (Clarkson & Herbison, 2006; Adachi et al., 2007; Kauffman et al., 2007a). Kisspeptin immunoreactive fibers contact GnRH neuron cell bodies (Kinoshita et al., 2005; Clarkson & Herbison, 2006; Smith et al., 2008a) and their terminals in the median eminence (Smith et al., 2011). Kisspeptin neurons in the ARC, POA and AVPV regions of sheep, rat and mouse brain co-express steroid receptors (Lehman et al., 2010).

In all mammalian species, high levels of Kiss1 receptor mRNA and protein are found in GnRH neurons of the hypothalamus and in cells of the periventricular (PeV) region of the hypothalamus, suggesting that the effects of kisspeptins are mediated by these neurons (Irwig et al., 2004; Han et al., 2005; Colledge, 2009; Smith et al., 2009a; Herbison et al., 2010). In rat brain, Kiss1R mRNA was detected in ARC, DMH, LHA, POA and AHA hypothalamic regions (Lee et al., 1999). In the periphery, KiSS-1 and Kiss1R have been detected in rat ovary (Terao et al., 2004; Castellano et al., 2006b) and oviduct (Gaytan et al., 2007), as well as in human and marmoset ovaries (Gaytan et al., 2009). Although the functional role of this system in male gonads remains controversial, expression of KiSS-1 and Kiss1R has been reported in testis (Mei et al., 2013; Salehi et al., 2015).

8.5.2. Functions of the Kisspeptins/Kiss1R system in reproduction

The involvement of kisspeptin in different aspects of reproduction in Mammals has been recently reviewed (Pinilla et al., 2012; Wahab et al., 2013; Clarke et al., 2015; Plant, 2015). This peptide is involved in the stimulation of gonadotropin release, in the steroid feedback loop of the gonadal axis and in the pubertal switch at hypothalamic, pituitary and gonadal levels. Thus, the disruption of the KiSS-1/Kiss1R system is responsible for severe reproductive disorders.

8.5.2.1. Kisspeptins stimulate the secretion of gonadotropins. Kisspeptins induce a massive release of gonadotropins via its stimulatory action on GnRH neurons (Han et al., 2005). Kisspeptin-induced LH secretion is blocked by the GnRH antagonist, acyline (Gottsch et al., 2004; Irwig et al., 2004). In vitro, kisspeptin-10-mediated GnRH secretion from hypothalamic fragments is absent in Kiss1R null mice (d'Anglemont de Tassigny et al., 2008). Systemic or i.c.v. administration of kisspeptin-10 or -54 stimulates LH release in a large variety of mammalian species, including rodents (Gottsch et al., 2004; Irwig et al., 2004; Matsui et al., 2004; Kinoshita et al., 2005; Messager et al., 2005; Navarro et al., 2005; Patterson et al., 2006; Thompson et al., 2006; Tovar et al., 2006; Greives et al., 2007), sheep (Messager et al., 2005), cows (Kadokawa et al., 2008) and primates (Dhillo et al., 2005; Shahab et al., 2005; Plant et al., 2006; Dhillo et al., 2007). Similar effects of kisspeptins on FSH secretion have been described in rodents (Gottsch et al., 2004; Matsui et al., 2004; Navarro et al., 2005; Thompson et al., 2006; Roa et al., 2008), sheep (Caraty et al., 2007) and humans (Dhillo et al., 2005), with noticeable differences in the delay and sensitivity of the FSH response as compared to the LH one (Navarro et al., 2005). On cultured baboon pituitary cells, kisspeptin evoked a dose-dependent release of LH but not of FSH (Luque et al., 2011). Finally, there is also emerging evidence to consider kisspeptin as a good candidate for therapeutic outcomes in the field of human reproduction (Clarke et al., 2015). Indeed, kisspeptin increases LH pulsatility in healthy volunteers as well as in patients with defects in gonadotropin secretion (George et al., 2011; Chan et al., 2011; Jayasena et al., 2013; Jayasena et al., 2014). In addition, kisspeptin is able to trigger oocyte maturation and embryo implantation during in vitro fertilization, without side-effects encountered with standard molecules (Jayasena et al., 2014; Abbara et al., 2015).

8.5.2.2. Kisspeptins are involved in the steroidal feedback loop. The pulsatile release of GnRH into the hypophyseal portal blood stimulates the synthesis and secretion of gonadotropins in the peripheral circulation, allowing LH and FSH to reach the gonads and to stimulate the secretion of sex steroids. Subsequently, sex steroids initiate either positive or negative feedback loops, depending on tissues or brain regions that are targeted and on the presence of the kisspeptin system (Navarro et al., 2004; Smith et al., 2005a,b). For example, the anteroventral periventricular nucleus of the hypothalamus (AVPV), which is responsible for the stimulation of GnRH secretion and subsequent preovulatory LH surge and rise in FSH level, is submitted to an estrogen-positive feedback (Wiegand & Terasawa, 1982; Adachi et al., 2007). In rodents, the

decrease in kisspeptin mRNA expression and immunoreactivity observed in the AVPV following gonadectomy can be restored by estrogens (Smith et al., 2005a; Adachi et al., 2007). Estrogens up-regulate *KiSS-1* gene expression in AVPV through activation of estrogen ERα receptors (Adachi et al., 2007). Kisspeptin neurons in the AVPV mediate the positive feedback of estrogen by opposing the inhibitory action of GABA signaling in GnRH neurons (Zhang et al., 2009). The arcuate nucleus (ARC) can also mediate a positive feedback, at least in the sheep (Estrada et al., 2006). On the other hand, in rodents, sheep and primates, the negative feedback exerted by sex steroids is controlled at the ARC (infundibular nucleus in humans) level. There, estrogens suppress the frequency of GnRH pulses and reduce kisspeptin mRNA expression (Smith et al., 2005a; Adachi et al., 2007; Li et al., 2009). In addition, and in marked contrast with former observations in the AVPV, high levels of estrogens counteract the increase in kisspeptin mRNA and immunoreactivity induced by gonadectomy in rodents (Smith et al., 2005a; Adachi et al., 2007).

8.5.2.3. Kisspeptins as a switch for puberty. Kisspeptins are crucial for puberty onset. During the pubertal transition, an increase in hypothalamic kisspeptin mRNA expression was found to occur in rats (Navarro et al., 2004), mice (Han et al., 2005) and monkeys (Shahab et al., 2005). The release of kisspeptin-54 in the median eminence of female rhesus monkeys coincided with the pubertal increase in GnRH release (Keen et al., 2008). Kiss1R and KiSS-1 KO mice of both genders display striking reproductive abnormalities, such as underdevelopment of gonads and accessory reproductive organs, reduced levels of gonadotropins and steroid hormones, absence of estrous cycle or impaired gametogenesis and sexual maturation (Funes et al., 2003; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007; Dungan et al., 2007; Kauffman et al., 2007b; Lapatto et al., 2007). In humans, loss-of-function mutations in Kiss1R cause hypogonadotropic hypogonadism (de Roux et al., 2003; Seminara et al., 2003; Lanfranco et al., 2005; Semple et al., 2005) whereas gain-of-function mutations cause precocious puberty (Teles et al., 2008).

8.5.2.4. Kisspeptins as a regulator of sexual organ activity. In vitro, kisspeptins were found to favor oocyte maturation (Saadeldin et al., 2012) and to induce the production of progesterone in cultured rat luteal cells (Peng et al., 2013). Kisspeptins are expressed in Leydig cells in the testes, in the cumulus oocyte complex and in the epithelium of ovarian and oviductal tissues. Kiss1R is found in seminiferous tubules and more particularly in the acrosomal region of spermatids and mature spermatozoa (Hsu et al., 2014). Moreover, kisspeptin-10 challenge of Fluo-4 loaded sperm was found to promote an increase in intracellular calcium indicating that these highly specialized cells express a functional Kiss1R in these highly specialized cells, which suggests a possible direct role of this receptor in mammalian fertilization (Hsu et al., 2014).

8.6. The QRFPs/QRFP-R system and the regulation of reproductive axis in Mammals

8.6.1. Localization of the QRFPs/QRFP-R system in reproduction pathways

A schematic presentation of the localization of QRFPs and QRFP-R1/R2 in rodent brain areas involved in reproduction is provided in Fig. 8.

Prepro-QRFP mRNA was originally shown to be mainly expressed in discrete rat brain areas such as the ventromedial hypothalamic nucleus (VMH) and the lateral hypothalamic (LHA) area (Chartrel et al., 2003). Other studies evidenced also QRFP mRNA in ARC of rats (Fukusumi et al., 2003; Kampe et al., 2006) and PeV of mice (Takayasu et al., 2006). In adult female rat pituitary, maximal relative values of QRFP-R and QRFP transcripts were found during diestrus, while a lower expression was seen during the period preceding full pubertal development (Navarro et al., 2006).

In rodent brain, the distribution of QRFP-receptors markedly depends on the receptor isoform and the animal species which are considered. Within mouse brain areas involved in reproduction, QRFP-R1 mRNA was observed in the VMH while QRFP-R2 mRNA was detected in several hypothalamic regions such as AVPV, PVN and LHA and in the SCN (Takayasu et al., 2006). In rats, both QRFP-R1 and QRFP-R2 isoforms were found in the preoptic (POA) and anterior (AHA) hypothalamic areas, which contain the main populations of GnRH neurons, in the PVN and VMH nuclei as well as in the bed nucleus of stria terminalis (BST). In contrast with mice, rat QRFP-R1 mRNA preferentially located within the ARC and LHA (Fukusumi et al., 2003; Kampe et al., 2006). QRFP and QRFP-R mRNA were also found in the human pituitary (Jiang et al., 2003).

In peripheral tissues, QRFP and QRFP-R mRNAs were found in human (Jiang et al., 2003; Baribault et al., 2006) and rodent (Fukusumi et al., 2003; Jiang et al., 2003; Takayasu et al., 2006) testes. In mouse testis, the QRFP-R2 isoform appeared to predominate (Takayasu et al., 2006).

8.6.2. Function of the QRFPs/QRFP-R system in the reproductive axis

Known as a hypophysiotropic peptide (Chartrel et al., 2003), QRFP-26 emerged in the recent years as a new actor in the regulation of the reproductive axis (Navarro et al., 2006; Patel et al., 2008). Indeed, following its incubation with pituitary tissue from adult female rats at diestrus, QRFP-26 was found to induce the release of LH in a dose-dependent manner (10^{-10} to 10^{-6} M) and of FSH at micromolar concentrations. Moreover, the application of high doses of QRFP-26 on this tissue enhanced the GnRH-evoked secretion of LH and FSH, without any significant effect on PRL release (Navarro et al., 2006). The i.c.v. injection of QRFP-26 led to a significant increase in serum LH (but not FSH) level, in high and low estrogen environment, as well as during the afternoon preceding preovulatory surge in female rats. A systemic administration of QRFP-26 was also able to elicit a significant increase of LH (but not FSH) in cyclic female rats at diestrus. No effect has been observed in males, suggesting a sexual dimorphism in terms of gonadotropin activation by QRFP-26 (Navarro et al., 2006). However, in male rats, the i.c.v. administration of QRFP-43 promoted a significant increase in LH and FSH plasma levels, an effect which was blocked by a GnRH antagonist (Patel et al., 2008). The central action of QRFP-43 was also confirmed on hypothalamic explants from male rats, with a significant increase in GnRH release while no changes in LH or FSH levels were found in the anterior pituitary (Patel et al., 2008). No effect on circulating gonadotropin or sex steroid levels was observed after intraperitoneal administration of high doses of this peptide (Patel et al., 2008). Similarly, in the adult male rhesus monkey, peripheral administration QRFP-26 and QRFP-43 had no effect on testosterone and prolactin secretion (Wahab et al., 2012).

8.7. RF-amide peptides and control of reproduction in seasonal breeders

To ensure optimal conditions for their reproduction in temperate climates, some animals, called “seasonal breeders”, are sensitive to environmental changes and undergo a photoperiodic control of their reproductive activity. Photoperiod is an important physical factor that modifies sexual behavior and molecular activity in the brain. In the wild world, offspring are born during the most favorable season, usually spring. However, according to gestation duration, photoperiodic animal species may breed at different periods of the year, thereby defining ‘long-day’ (LD) and ‘short-day’ (SD) breeders. Given their short gestation, small animals (like the hamster) reproduce in springtime, when days are long. On the other hand, animals with a longer gestation period (about 4 months for the sheep), are called short-day breeders, or fall-breeders.

Time of breeding is directly related to gestation length and critically depends on environmental signals, among which the duration of light exposure (called photoperiod) is the main protagonist. Indeed, photoperiod is a reliable indicator of seasonal changes, with day length being translated at the brain level with melatonin, a neurohormone synthesized and secreted by the pineal gland during the

night, and inhibited by light. As a consequence, short days are associated with long nocturnal melatonin secretion, whereas long days correspond to short nocturnal melatonin secretion. Depending on the breeding animal, via a process that is not well understood, translation of the melatonin signal makes sheep sexually active in short-day (SD) and hamster sexually active in long-day (LD). The distribution of melatonin binding sites in the brain differs across species (Masson-Pevet et al., 1994), although a high density of melatonin receptors has been observed in the pars tuberalis of the adenohypophysis in many mammalian species (Masson-Pevet & Gauer, 1994). This region emerged as an important structure involved in melatonin effects on seasonal reproductive function (Klosen et al., 2002; Dardente et al., 2003; Johnston et al., 2006). Owing to their regulation by melatonin, much interest has been recently devoted to kisspeptin and RFRP-3 peptides, now regarded as major actors of the seasonal control of reproduction in Mammals (Simonneaux et al., 2013).

8.7.1. The Kisspeptin system and the seasonal control of reproduction

KiSS-1 appears to be the missing link between HPG and melatonin. In seasonal animals such as long-day (LD) and short-day (SD) breeders, *KiSS-1* gene expression is regulated by the photoperiod.

In the Syrian hamster (a long-day breeder), kisspeptin-10 challenge counteracts the suppressive effect of short-light exposure on reproduction and restores testicular activity, indicating that low kisspeptin levels signal an arrest of reproduction in short-days (Revel et al., 2006). Melatonin and sex steroids effects on kisspeptin neurons seem to play a crucial role in timing the neuroendocrine control of the gonadotropic axis in the Syrian hamster. Indeed, in SD compared to LD, KiSS-1 expression in the ARC and the AVPV of male and female hamsters is strongly reduced, as are circulating levels of testosterone in agreement with the stimulatory role of kisspeptins on the gonadotropic axis (Revel et al., 2006; Simonneaux et al., 2009; Ansel et al., 2010; Bartzen-Sprauer et al., 2014). Moreover, the increase in KiSS-1 mRNAs in the ARC observed after castration of LD Syrian hamsters (Revel et al., 2006; Ansel et al., 2010) is consistent with the inhibitory effect of sex steroids on KiSS-1 expression in this region. Thus, the decrease in KiSS-1 expression observed in short days in the ARC is not due to a diminution of testosterone levels, whereas in the AVPV, it likely results from the lowering of circulating testosterone (Ansel et al., 2010). On the other hand, pinealectomy prevents the down-regulation of KiSS-1 expression in SD Syrian hamsters (Revel et al., 2006) and increases the number of KiSS-1 neurons in the ARC, but not in the AVPV, of male or female SD hamsters (Ansel et al., 2010). Finally, some Kiss-1-positive cells were found in the mediobasal hypothalamus where melatonin target sites locate, providing additional support for a link between melatonin and kisspeptin systems (Hastings et al., 1988; Maywood and Hastings, 1995; Goldman, 2001; Lewis et al., 2002; Mikkelsen & Simonneaux, 2009). Thus, in the Syrian hamster, the long nocturnal peak of melatonin in SD seems to inactivate the reproductive state via an inhibition of KiSS-1 expression which is either sex steroid-dependent in the AVPV or sex steroid-independent in the ARC. However, in the Siberian hamster — which also belongs to long-day breeders — kisspeptin levels in the ARC were found to be higher under SD conditions than under LD ones (Greives et al., 2007; Mason et al., 2007; Simonneaux et al., 2009). Such unexpected findings raised several hypotheses and still merit further clarification (Simonneaux et al., 2013).

As in long-day breeders, kisspeptin expression in sheep (a short-day breeder) varies with the season and sex steroid levels. In ewe, as observed in the Syrian hamster, acute and chronic administration of kisspeptins during anestrus reactivates the gonadal axis and triggers ovulation (Caraty et al., 2007; Smith et al., 2009b). The expression of KiSS-1 mRNA in the ARC and the number of contacts between kisspeptin and GnRH neurons are higher during the breeding season, as compared to the non-breeding period. Such a differential expression is independent from sex-steroid feedback as it is preserved in OVX ewes, with or without estrogen replacement (Franceschini et al., 2006; Caraty et al., 2007; Smith et al., 2008b). Furthermore, estrogens have been found to exert a stronger inhibition of KiSS-1 expression in anestrus rather than during the breeding season, suggesting a change in sensitivity towards negative estrogen feedback according to the season (Smith et al., 2008a).

Altogether, data from Syrian hamster and sheep are consistent with a role for ARC kisspeptin neurons in the control of seasonal reproduction. They also indicate that the same melatonin message can lead to opposite reproductive states in different species, according to the breeding type (Karsch et al., 1984; Revel et al., 2008). In sheep brain, melatonin receptors have been localized in the pre-mammillary hypothalamic area, a structure anatomically closely related to ARC (Malpaux et al., 1998). However, a direct effect of melatonin on kisspeptin neurons has not been demonstrated yet, although the long melatonin peak in SD clearly suppresses sexual activity in hamsters and increases it in sheep.

8.7.2. The RF-amide related peptides/NPFF1R system and the seasonal control of reproduction

In Mammals, RFRP-expressing neurons are mainly located in the mediobasal hypothalamus (Ukena & Tsutsui, 2001; Kriegsfeld et al., 2006; Clarke et al., 2008; Dardente et al., 2008; Revel et al., 2008; Smith et al., 2008a; Rizwan et al., 2009). In this region, RFRPs expression was found to vary according to photoperiod in LD and SD breeders (Dardente et al., 2008; Revel et al., 2008; Smith et al., 2008a; Mason et al., 2010; Ubuka et al., 2012b; Janati et al., 2013), but not in non-photoperiodic animals, such as rats (Revel et al., 2008). This region was found to contain melatonin binding sites and its electrolytic lesion prevented the inhibitory effect of melatonin on reproduction of the long-day breeder Syrian hamster (Maywood et al., 1996).

In seasonal Mammals, RFRP-immunoreactivity decreases in short-day periods, whatever the LD or SD breeding type of the species considered. In Syrian and Siberian hamsters, RFRPs mRNA levels are higher in the long photoperiod than during the short one (Revel et al., 2008), an observation which is inconsistent with the inhibitory role of RFRPs on reproductive axis previously reported. In other long-day breeders, such as the Jerboa and European hamsters, RFRPs expression is also inhibited in SD-maintained animals (Janati et al., 2013). In SD-adapted Syrian and Siberian hamsters (no reproductive activity), hypothalamic RFRPs mRNA was strongly down-regulated, in association with a decrease in RFRP immunoreactivity in perikarya and fibers (Revel et al., 2008; Mason et al., 2010; Ubuka et al., 2012a). Remarkably, in short-day breeders, RFRPs mRNA and protein expression were also reduced in SD conditions when animals are sexually active (Dardente et al., 2008; Smith et al., 2008a). In sheep, hypothalamic RFRPs mRNA levels are about 20% higher under LP than SP conditions (Dardente et al., 2008). In the marsupial brain, there is a two-fold increase in RFRP-3 cell body number in the hypothalamus during the non-breeding season as compared to the breeding one (Harbid et al., 2013). In agreement with its inhibitory role on the gonadotropic axis, RFRP-3 input on GnRH neurons varied with the season (Clarke et al., 2008; Smith et al., 2008a). The analysis of the sequence of the goat RFRP gene highlighted a positive correlation between gene mutations and average daily sunshine duration, reproductive seasonality and litter size (Huang et al., 2012).

Testosterone treatment of SD-adapted Syrian hamsters (or testis ablation of LD-animals) did not alter RFRP mRNA levels (Revel et al., 2008). However, estrogens down regulates RFRP mRNA levels in OVX mice (Molnar et al., 2011) and ER-α is present in a subset of RFRP-positive neurons in the hypothalamus where they respond to estrogen administration (Kriegsfeld et al., 2006; Gibson et al., 2008; Molnar et al., 2011), suggesting a role for sex steroids in the regulation of RFRPs expression.

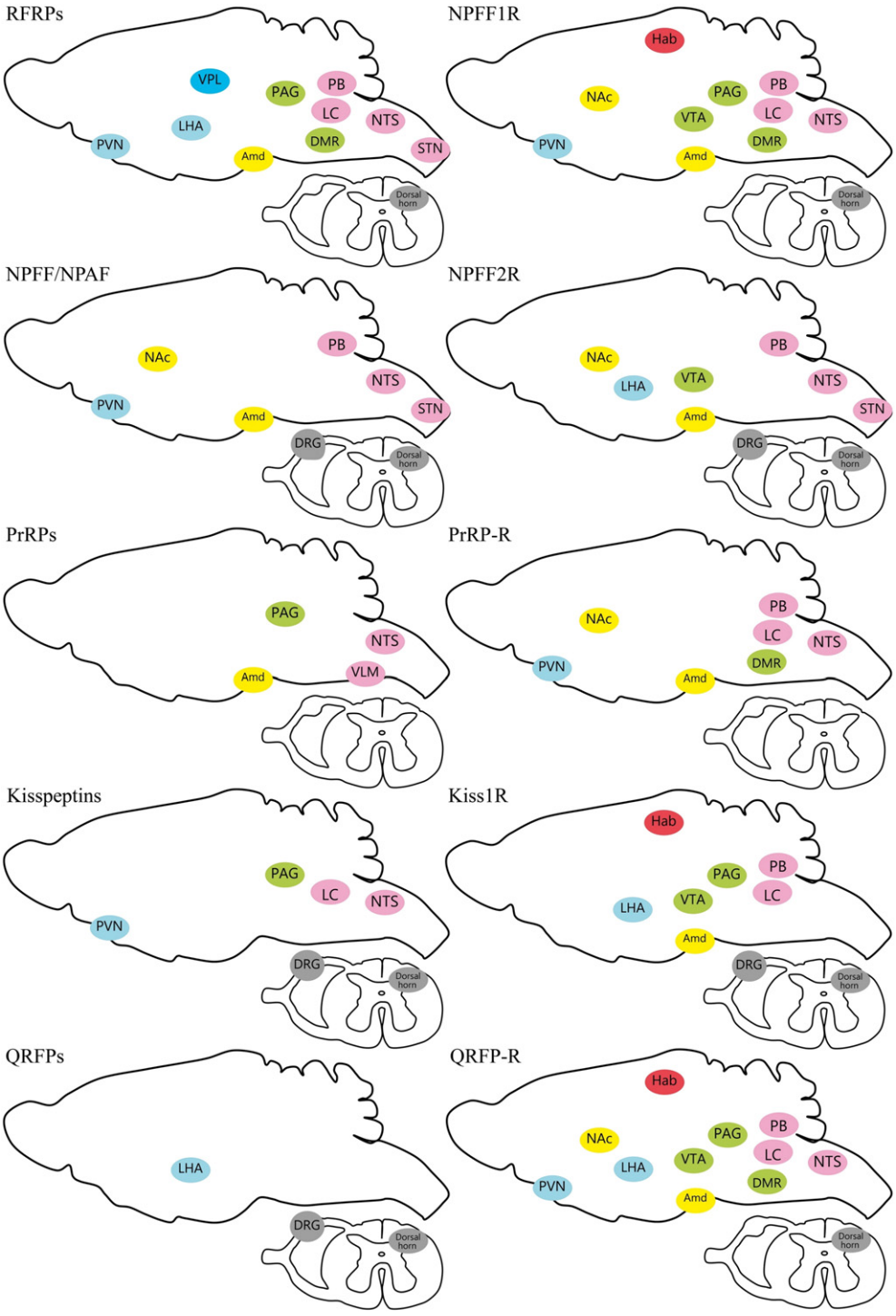

Fig. 9. Anatomical distribution of RF-amide peptides and their cognate receptors in rodent (rat and mouse) central and peripheral nervous system areas involved in nociception. Highlighted brain structures have been selected on the basis of their known or putative roles in nociception and pain modulation in Mammals and of their enrichment in RF-amide peptides (left panel) and their cognate receptors (right panel), as afforded from distribution studies in mouse brain. When specified in the literature, localization of RF-amide peptide-containing cell bodies is privileged. Colored areas refer to distinct anatomical structures according to MeSH database: hypothalamic nuclei (light blue); pons/medulla oblongata nuclei (pink); basal ganglia (yellow); tegmentum mesencephali nuclei (green); epithalamus (red); thalamic nuclei (blue); spinal cord and DRG (light gray). To note that the expression of NPFF1R in the dorsal horn of the spinal cord depends on rodent strains (Bonini et al., 2000; Liu et al., 2001; Gouarderes et al., 2004a). The expression of NPFF in the DRG is still a matter of controversy (Ayachi and Simonin, 2014, for a review). The distribution of QRFP-R1 and QRFP-R2 isoforms in pain-related areas is detailed in the original papers by Fukusumi et al., 2003; Kampe et al., 2006 and Takayasu et al., 2006. Abbreviations are listed in the corresponding section.

Melatonin modulates RFRPs mRNA levels according to the photoperiod (Revel et al., 2008; Ubuka et al., 2012a). For example, during the short-day period, melatonin was found to decrease RFRPs mRNA levels in the Siberian hamster (a long-day breeder), whereas the pineal hormone induced the expression of GnIH in quails which are sexually inactive during this period (Ubuka et al., 2005; Revel et al., 2008; Ubuka et al., 2012a). At the periphery, RFRP and NPFF1R mRNA expression in male Syrian hamster testis is impacted by photoperiod and reproductive conditions (Zhao et al., 2010).

Often referred to as an inhibitor of reproduction in Mammals, RFRPs cannot be reduced to this simple assertion as they exert a sexually and photoperiodically dimorphic action in hamsters (Ancel et al., 2012; Ubuka et al., 2012a). While an inhibitory effect on gonadotropin release was reported in OVX female Syrian hamsters (Kriegsfeld et al., 2006), the i.c.v. administration of RFRP-3 to male Syrian hamsters stimulated GnRH cell activity and increased LH production and circulating levels of testosterone. The i.c.v. injection of RFRP-3 in male Syrian hamsters under photo-inhibitory conditions, led to an increase in LH release and reactivated the reproductive axis after 5 weeks of continuous treatment, similarly to what was observed under LD conditions (Ancel et al., 2012), and stimulates gonadotropin secretion in LD Siberian hamster (Ubuka et al., 2012a).

No photoperiod-dependent effects have been reported in seasonal Mammals for the other members of the RF-amide peptide and receptor family. In contrast to kisspeptins, the roles of RFRPs in reproduction seem to be more difficult to analyze owing to their strong variations according to the LD or SD breeding type of the animal models.

Overall, all mammalian RF-amide peptides, except NPFF, have been implicated in the modulation of reproduction. Among them, special attention was paid to Kisspeptin/Kiss1R and RFRPs/NPFF1R systems as main regulators of central control of reproduction, acting on GnRH neurons. These neuropeptides integrate environmental signals as photoperiod and sexual steroids to permit a regulation of the reproductive axis with variable intensity. Nevertheless, although the role of kisspeptins as potent stimulators of the reproductive axis has been well-established, RFRPs function remains controversial in Mammals. They appear to regulate reproduction mainly in seasonal breeders, but in opposite way according to the reproductive state, sex and species. In the future, the identification of novel pharmacological tools, as highly selective NPFF1R antagonists, should be very helpful to clearly decipher the precise action of this system in the wide and complex field of reproduction.

9. Mammalian RF-amide peptides and their receptors in nociception and pain

Several reports indicate that mammalian RF-amide peptides and their receptors are involved in the modulation of nociception. Here, we will briefly present their distribution in pain pathways in mice, rats and humans and summarize current knowledge on their roles in nociception and in the modulation of opiates effects. These topics have been discussed into more details in a recent review (Ayachi & Simonin, 2014).

9.1. The RF-amide related peptides/NPFF1R system in nociception and pain

9.1.1. Localization in pain pathways

Fig. 9 presents the distribution of RFRPs and their NPFF1 receptor in the regions of rat and mouse central and peripheral nervous systems involved in the modulation of nociception.

RFRP-1 and RFRP-3 containing neuronal cell bodies and fibers are widely distributed in the brain, with high levels in the hypothalamus. RFRP-1/-3 immunoreactivity was detected in several regions known to be implicated in the modulation of nociception like the spinal trigeminal nucleus (STN), nucleus tractus solitarius (NTS), dorsal medial raphe nucleus (DMR), locus coeruleus (LC), parabrachial nucleus (PB), periaqueductal gray (PAG), amygdala (Amd), ventral postlateral nucleus (VPL) of the thalamus, and the paraventricular nucleus (PVN) of the hypothalamus (Ukena & Tsutsui, 2001; Yano et al., 2003). ISH studies also revealed the presence of RFRP mRNA in the lateral hypothalamic area (LHA) of rat brain (Hinuma et al., 2000). In addition, dense networks of RFRP-immunoreactive fibers and high levels of RFRP mRNA were found in the dorsal horn of mouse spinal cord (Ukena & Tsutsui, 2001; Yang & Iadarola, 2003). In rats, much lower levels or no RFRP expression at all were observed in this region (Yano et al., 2003; Pertovaara et al., 2005).

In rats or mice, the NPFF1 receptor mRNA locates within the raphe nucleus, locus coeruleus, parabrachial nucleus, periaqueductal gray, amygdala, ventral tegmental area (VTA), habenula (Hab), nucleus accumbens (NAc) and the paraventricular nucleus of the hypothalamus (Bonini et al., 2000; Hinuma et al., 2000; Liu et al., 2001). NPFF1R mRNA expression and binding sites in the spinal cord or in the nucleus tractus solitarius were reported to vary between rat and mouse strains (Bonini et al., 2000; Liu et al., 2001; Gouarderes et al., 2004a). In humans, NPFF1R mRNA was detected in several areas, including in pain- or nociception-associated regions such as the spinal cord, hypothalamus, and amygdala (Bonini et al., 2000).

9.1.2. Modulation of nociception

Under basal nociceptive conditions, RFRP-1 effect primarily depends on opioid receptor activation as the peptide had no effect when injected (i.c.v.) alone while it decreased or blocked morphine analgesia (Liu et al., 2001). Conversely, i.c.v. RFRP-3 administration in mice had no effect on morphine analgesia but significantly decreased thermal nociceptive threshold when injected alone (Elhabazi et al., 2013). A few studies examined RFRPs effects under chronic pain conditions; in a neuropathic model, RFRP-1 has been shown to induce an anti-nociceptive effect in rats (Pertovaara et al., 2005).

9.2. The neuropeptide FF/NPFF2 receptor system in pain and nociception

9.2.1. Localization in pain pathways

A schematic distribution of NPFF and NPAF peptides and of their NPFF2 receptor in regions of rat and mouse central and peripheral nervous systems involved in nociception and pain modulation is presented in Fig. 9.

NPFF is unevenly expressed in the rat CNS with highest levels localized to the superficial layers of the dorsal spinal cord (Yang et al., 2008). NPFF mRNA was found in the spinal trigeminal nucleus, nucleus tractus solitarius and paraventricular hypothalamus nucleus; NPFF-ir fibers were also detected in parabrachial nuclei, amygdala, and nucleus accumbens (Panula et al., 1996; Yang & Iadarola, 2006; Yang et al., 2008, for reviews). The presence of NPFF in dorsal root ganglia (DRG) remains controversial (Yang & Iadarola, 2006; Ayachi & Simonin, 2014). A few studies focused on NPAF which was also found in mouse and rat spinal cords (Bonnard et al., 2001; Yang et al., 2008). In humans, the presence of NPFF in the spinal cord was revealed by using a specific radioimmunoassay (Majane et al., 1988), while NPFF mRNA was detected both in the medulla and spinal cord (Nystedt et al., 2002). NPFF and NPAF peptides were also detected in human cerebrospinal fluid using a radioimmunoassay (Sundblom et al., 1997) and mass-spectrometry (Burlet-Schiltz et al., 2002).

The localization of NPFF2R mRNA and protein in rodents has been extensively reviewed (Panula et al., 1996; Roumy & Zajac, 1998; Fukusumi et al., 2006; Yang & Iadarola, 2006; Yang et al., 2008). NPFF2R mRNA was detected in various brain regions involved in the modulation of nociception such as thalamic nuclei, hypothalamus, and superficial layers of the spinal cord (Bonini et al., 2000; Elshourbagy et al., 2000; Liu et al., 2001; Yang & Iadarola, 2003). NPFF2R transcripts have also been detected in rat (Bonini et al., 2000; Yang & Iadarola, 2006) and human post-mortem (Yang et al., Neuropeptides 2008) DRGs, as well as in the spinal trigeminal nucleus (STN) of rat (Bonini et al., 2000; Yang & Iadarola, 2003) and primate (Zeng et al., 2003) brains. Human NPFF2R mRNA was also found in the spinal cord, medulla oblongata, hypothalamus, substantia nigra and amygdala (Bonini et al., 2000). Immunohistochemistry and western blotting provided evidence for the presence of NPFF2R in the rat ventral tegmental area (VTA), nucleus accumbens (NAc) and spinal cord (Wu et al., 2010) and for NPFF2R immunoreactivity in the lower brainstem and spinal cord of monkey CNS, with dense labeling in the spinal trigeminal nucleus and superficial layers of the dorsal horn

(Zeng et al., 2003). Binding experiments using radiolabeled tracers pointed to the presence of NPFF2R binding sites in the superficial layers of the spinal cord, lateral hypothalamus (LHA), parabrachial nucleus (PB), nucleus tractus solitarius (NTS) and spinal trigeminal nucleus (STN) in rats and mice (Allard et al., 1989, 1992; Dupouy & Zajac, 1996; Bonini et al., 2000; Gouarderes et al., 2000, 2002, 2004a,b). Interestingly, pharmacological (neonatal capsaicin treatment) and surgical (dorsal rhizotomy and peripheral axotomy) lesions induced a dramatic decrease in [^{125}I]-(1DMe)NPFF binding density in the superficial layers of the dorsal horn of rat spinal cord, suggesting that NPFF2R are partly associated with primary afferents (Gouarderes et al., 1996). Ligation of rat lumbar dorsal spinal roots promoted a net accumulation of receptor sites on the side peripheral to the ligature, as afforded from [^{125}I]-(1DMe)NPFF autoradiographic evaluation of NPFF2R sites along ligated root fibers (Gouarderes et al., Synapse 2000). Thus, a population of NPFF2 receptors is synthesized in DRGs and migrates through anterograde axonal transport towards primary afferent endings in the rat spinal cord. Finally, binding experiments conducted on human brain tissues pointed to high densities of [^{125}I]-Y8Fa binding sites in superficial layers of the spinal cord dorsal horn and in the spinal trigeminal nucleus (Allard et al., 1994), though the low NPFF1/2R binding selectivity of Y8Fa (Table 2) precluded a clear definition of the NPFF receptor subtype which was labeled. Noteworthy, within the CNS, highest levels of NPFF1R and NPFF2R mRNA were detected in the human and rat spinal cord, respectively (Bonini et al., 2000; Yang & Iadarola, 2003).

9.2.2. Modulation of nociception

9.2.2.1. Effect of peptide injection. The effects of NPPF and NPAF on nociception have been widely described in several reviews (Panula et al., 1996; Roumy & Zajac, 1998; Fukusumi et al., 2006; Simonin et al., 2006; Yang & Iadarola, 2006; Yang et al., 2008; Mouledous et al., 2010b; Ayachi & Simonin, 2014). NPFF injection was reported to induce two different types of responses depending on the site of injection. In rats or mice, intrathecal NPFF injection evoked analgesia and potentiated opioids action, whereas i.c.v. injection induced pro-nociceptive effects and reversal of morphine analgesia (Roumy & Zajac, 1998). Anti-morphine analgesic effects of NPFF were blocked by RF9, a selective antagonist of NPFF1/2 receptors, which potentiated opiate-induced analgesia and prevented the development of opiate-induced hyperalgesia and tolerance (Simonin et al., 2006; Elhabazi et al., 2012). Very similarly to NPFF, NPAF administration reversed or potentiated morphine analgesia depending on its route of administration (i.c.v. or i.t., respectively; Bonnard et al., 2001; Jhamandas et al., 2006). Altogether these data indicate that NPFF/NPAF and their NPFF2 receptor contribute to the homeostatic control of opiates action.

9.2.2.2. Anti-opioid effect. To explain tolerance to morphine analgesia and opioid-induced hyperalgesia, the existence of a homeostatic equilibrium between an anti-nociceptive opioid system and a pro-nociceptive anti-opioid system has been proposed (Rothman, 1992; Simonnet & Rivat, 2003). This hypothesis postulates that during opiate treatment, the stimulation of the opioidergic system by exogenous opiates will trigger the release of endogenous pro-nociceptive anti-opioid molecules which will counteract opiates action. Upon chronic opiate administration, anti-opioid molecules levels increase and counteract opiate action, thereby leading to the necessity to increase opiate dosage to reach the same analgesic effect. This may explain the loss of efficacy of opiates, i.e. tolerance to analgesia. Furthermore, when stopping the treatment, the endogenous tone of the anti-opioid system remains high, leading to the observation of a decrease in the basal nociceptive threshold and to so-called opioid-induced hyperalgesia. Several lines of evidence support this hypothesis. Particularly, morphine treatments have been shown to induce an increase in NPFF-like immunoreactivity in brain and spinal cord (Malin et al., 1990; Devillers et al., 1995; Stinus et al., 1995) whereas the blockade of NPFF1/2 receptors with an antagonist (RF9) potentiated opiate analgesia and prevented the development of analgesic tolerance and of opioid-induced hyperalgesia (Simonin et al., 2006; Elhabazi et al., 2012). The anti-opioid properties of the NPFF/NPAF system not only concern opiate nociceptive modulating properties but also other opiate effects such as reward, locomotor activity, feeding and drinking behavior (see Section 7.3.2) or intestinal motility (Mouledous et al., 2010b).

9.2.2.3. Persistent pain. The implication of NPFF and NPFF2R in persistent pain has been recently reviewed (Ayachi & Simonin, 2014). In inflammatory pain, several reports indicated that NPFF and NPFF2R were up-regulated at the level of their transcripts (Vilim et al., 1999; Yang & Iadarola, 2003; Nystedt et al., 2004), binding sites (Lombard et al., 1999) and protein expression, as assessed by NPFF and NPFF2R immunoreactivity (Yang & Iadarola, 2003; Ayachi & Simonin, 2014). Conversely, no up-regulation of NPFF and a modest up-regulation of NPFF2R were observed in neuropathic pain conditions (Vilim et al., 1999; Nystedt et al., 2004). It remains that NPFF displayed anti-allodynic and anti-hyperalgesic effects in both models of persistent pain in rodents (Ayachi & Simonin, 2014). In humans, the concentrations of NPFF in the plasma and in the cerebrospinal fluid of patients with a chronic pain disorder did not differ from those of healthy volunteers, suggesting a lack of up-regulation of this system under persistent pain conditions (Sundblom et al., 1997).

9.3. The prolactin-releasing peptides/prolactin-releasing peptide-receptor system in nociception and pain

9.3.1. Localization in pain pathways

Fig. 9 depicts the distribution of PrRPs and PrRP-R in various nociception and pain modulated areas from rat and mouse central and peripheral nervous system. More details on the distribution of PrRPs and PrRP-R in the central nervous system and on their localization in pain pathways are available in several reviews (Fukusumi et al., 2006; Lin, 2008; Dodd & Luckman, 2013; Ayachi & Simonin, 2014).

In rats, the presence of PrRPs has been demonstrated in several areas implicated in pain control or in processing of nociceptive inputs (Kalliomaki et al., 2004). Indeed, PrRP mRNA was found in the medulla oblongata, nucleus tractus solitarius (NTS) and ventrolateral medulla (VLM). PrRP-ir nerve fibers were detected in the VLM, periaqueductal gray (PAG) and basolateral amygdaloid nucleus. No signal was found in the spinal cord, suggesting that pain modulatory effects of PrRPs are probably of supraspinal origin (Kalliomaki et al., 2004). The PrRP receptor was also detected in various regions involved in pain processing including the nucleus tractus solitarius (NTS), lateral and medial parabrachial nuclei (PB), locus coeruleus (LC), raphe nucleus (DMR), amygdala (Amd), nucleus accumbens (NAc), and the paraventricular hypothalamic nucleus (PVN) (Fujii et al., 1999; Roland et al., 1999; Fukusumi et al., 2006).

In human brain, PrRP mRNA was found to be enriched in the hypothalamus, medulla oblongata and thalamus (Fujii et al., 1999; Takahashi et al., 2000), in agreement with the involvement of this peptide in the control of pain transmission. In contrast, human PrRP receptor mRNA was detected only in the pituitary gland.

9.3.2. Modulation of nociception

PrRPs modulate nociception under basal conditions but in opposite ways, depending on the site of injection. Indeed, when injected directly into the nucleus tractus solitarius (NTS), PrRP-20 induced analgesia (Kalliomaki et al., 2004). When injected in the caudal ventrolateral medulla (VLM) or the third ventricle in rats and mice, PrRP promoted hyperalgesia (Kalliomaki et al., 2004; Laurent et al., 2005; Elhabazi et al., 2013). Similarly, under neuropathic pain conditions, PrRPs attenuated tactile allodynia when injected into the PAG or the NTS, but had no effect when injected into the caudal ventrolateral medulla (Kalliomaki et al., 2004).

Likewise the NPFF/NPFF2R system, PrRPs and their receptor have been proposed to belong to an anti-opioid system. Indeed, PrRP-R KO mice displayed potentiated morphine-induced analgesia, reduced morphine tolerance, enhanced morphine-induced conditioned place preference acquisition and decreased severity of the naloxone-precipitated morphine-withdrawal syndrome, as compared to controls (Laurent et al., 2005). Moreover, the existence of a functional interplay between NPFF1/2 and PrRP receptors has been described. Indeed, the blockade of NPFF1/2 receptors by a selective antagonist (RF9) led to the suppression of the hyperalgesic and anti-morphine effects of PrRPs (Elhabazi et al., 2013), while the anti-opioid effect of NPFF was absent in the PrRP-R KO mice (Laurent et al., 2005).

9.4. The kisspeptins/Kiss1R system in nociception and pain

9.4.1. Localization in pain pathways

The distribution of kisspeptins and Kiss1R in several nociception and pain modulating areas of rat and mouse central and peripheral nervous systems is illustrated in Fig. 9. Further details on the localization of this peptidergic system in humans and rodents are available in Kirby et al. (2010).

Kisspeptins are expressed in various pain-related pathways in rodents. Thus, in mouse brain, kisspeptin-ir fibers have been located in the paraventricular hypothalamic nucleus, periaqueductal gray, locus coeruleus and nucleus solitarius (Dun et al., 2003; Clarkson et al., 2009). In the rat, both kisspeptin mRNA and immunoreactivity have been found in the dorsal horn of the spinal cord and in L4/L5 DRG wherein kisspeptin was probably expressed by nociceptors (Dun et al., 2003; Mi et al., 2009). In humans, low levels of KiSS-1 mRNA were found in the brain and more specifically in the hypothalamus and the basal ganglia (Muir et al., 2001), a region known to play a role in pain processing and analgesia.

In rat and mouse brain, Kiss1R was detected in several regions involved in pain control such as the locus coeruleus (LC), parabrachial nuclei (PB), periaqueductal gray (PAG), ventral tegmental area (VTA), amygdala (Amd), lateral hypothalamus area (LHA) and habenula (Hab) (Lee et al., 1999; Herbison et al., 2010). Kiss1R-positive immunostaining has also been found in rat DRG neurons and in lamina I and II of the dorsal horn of the spinal cord (Mi et al., 2009). In humans, Kiss1R mRNA has been detected in many areas implicated in pain and nociception like the amygdala, locus coeruleus, medulla oblongata and spinal cord (Kirby et al., 2010).

9.4.2. Modulation of nociception

Kisspeptin displays peripheral pro-nociceptive properties as its intraplantar injection induced a nocifensive response and decreases pain thermal threshold in the hot plate test (Spampinato et al., 2011). At the central level, kisspeptin-10 administration (i.c.v.) induced hyperalgesia as well as anti-morphine activity (Elhabazi et al., 2013). These pro-nociceptive properties also appear to be implicated in inflammatory pain as kisspeptin induced hyperalgesia in formalin test, enhanced TRPV1 phosphorylation at the site of injection and ERK1/2 phosphorylation in the dorsal horn in mice (Spampinato et al., 2011). Kisspeptins and Kiss1R transcripts and proteins were also upregulated in DRG and dorsal horn neurons of rats submitted to the Complete Freund Adjuvant (CFA) model of chronic inflammatory pain (Mi et al., 2009).

9.5. The QRFPs/QRFP-R system in nociception and pain

9.5.1. Localization in pain pathways

Fig. 9 displays the localization of QRFPs and of QRFP receptors in nociception and pain modulating areas in rats and mice. QRFP mRNA has been mainly detected in the lateral hypothalamic area (Chartrel et al., 2011) but several pieces of evidence also suggest the presence of QRFPs in the rodent spinal cord and DRG (Takayasu et al., 2006; Yamamoto et al., 2008, 2011). QRFP-R1/2 mRNA and binding sites are widely expressed in rodent CNS including in the nucleus solitarius tract (NTS), parabrachial nucleus (PB), locus coeruleus (LC), raphe nucleus (DMR), periaqueductal gray (PAG), ventral tegmental area (VTA), amygdala (Amd), habenula (Hab), lateral hypothalamic area (LHA), nucleus accumbens (NAc), paraventricular nucleus of the hypothalamus (PVN) and the spinal cord (Chartrel et al., 2011). In situ hybridization experiments showed that the expression patterns of QRFP-R1 and R2 isoforms in mouse brain did not overlap (Takayasu et al., 2006).

In humans, quantitative PCR analyses pointed to highest expression of QRFP-43 precursor mRNA in the brain, and more precisely in the medulla, retina and vestibular nucleus (Jiang et al., 2003). By using immunohistochemistry and in situ hybridization, Bruzzone and collaborators identified QRFP-26-immunoreactive fibers and QRFP-26-expressing neurons in human paraventricular hypothalamus nucleus and dorsal horn of spinal cord (Bruzzone et al., 2006). In human brain, QRFP receptor mRNA was highly enriched in the trigeminal ganglion, hypothalamus and vestibular nucleus (Jiang et al., 2003).

Overall, QRFP transcript and QRFP-R1/2 binding sites or mRNA were detected in several pain neuronal pathways, raising up their potential involvement in the transmission or integration of nociceptive inputs (Chartrel et al., 2011; Ayachi & Simonin, 2014).

9.5.2. Modulation of nociception

The effects of QRFP peptides on the modulation of nociception have been recently reviewed (Ayachi & Simonin, 2014). In naive rats or mice, depending on site of application and peptide concentration, QRFP-26 was reported to induce a pro-nociceptive response or no effect on the basal nociceptive threshold (Yamamoto et al., 2008, 2009, 2011; Elhabazi et al., 2013). Conversely to basal nociceptive state, QRFP injection in rats with inflammatory or neuropathic pain produced an anti-allodynic effect (Yamamoto et al., 2008, 2009, 2011).

To conclude this section, the distribution pattern and/or in vivo activity of all RF-amide peptides and their receptors attest of their involvement in the modulation of nociception, both under basal or chronic pain conditions. Moreover, several RF-amide peptides and their receptors belong to an anti-opioid pro-nociceptive system that is prone to modulate opiates action. All these data emphasize the importance of these RF-amide systems in the modulation of nociception and opiate analgesia and the need to develop alternative therapeutic approaches for pain treatment.

10. Conclusion and therapeutic perspectives

Since the discovery of the first mammalian RF-amide peptide NPFF (Yang et al., 1985), thirty years of research efforts have led to the identification of a rather large family of mammalian neuropeptides and their receptors. These neuropeptide signaling systems seem to be conserved during the course of evolution, not only at a structural level, but also functionally. Their well-established occurrence prior to the divergence of protostomes and deuterostomes emphasizes the importance of these systems in both Vertebrates and Invertebrates. In Mammals, functional studies support the evolutionary continuity of RF-amides as key regulators of energy balance, feeding behavior, reproduction, and sensory modulation. Moreover, their distribution both at the spinal and supraspinal levels as well as at the periphery clearly reflects their involvement in these functions. From a philogenetic point of view, kisspeptin and its receptor separate from the other RF-amide systems. However, it is interesting to note that galanin receptors and their ligands that cluster with kisspeptin ones have also been clearly involved in energy homeostasis, pain and reproduction (Lang et al., 2015; Fang et al., 2015), suggesting that the galanin system should also be considered when studying the function of RF-amide peptides and their receptors.

From a therapeutic perspective, although these different mammalian RF-amide systems have been involved in the regulation of the same functions, some of them are emerging as interesting targets for specific applications. NPFF/NPFF2R seems to be the most promising pair to target for pain treatment. Indeed, NPFF2R antagonists may be regarded as innovative tools to improve the efficacy and limit the undesirable side effects of opiates during chronic pain therapy (Elhabazi et al., 2012). Moreover, looking at single-nucleotide polymorphisms in the NPFF2R gene, some haplotypes have been associated with an increased risk of obesity whereas others were strongly protective against obesity, in association with higher lipolytic activity (Dahlman et al., 2007; Hunt et al., 2011), suggesting that NPFF2R may play an important role in obesity predisposition. Kisspeptin/Kiss1R and to a lower extent RFRP/NPFF1R represent the most promising members of the RF-amide family to control reproduction and fertility both in animals and humans. Kisspeptin receptor agonists could be helpful in improving the reproductive activity of domestic animals, including stimulation of quiescent gonadotropin axis and optimization of artificial insemination (Caraty et al., 2012b), as well as in the treatment of fertility disorders in women (Clarke et al., 2015). Furthermore, the recent discovery of a hormonal kisspeptin circuit between the liver and the endocrine pancreas highlighted a link between hyperglucagonemia, increased hepatic kisspeptin production and impaired glucose-stimulated insulin secretion from β pancreatic cells in type 2 diabetes mellitus (Song et al., 2014). These observations suggest that peripherally-acting kisspeptin receptor antagonists could represent interesting therapeutic tools for the treatment of type 2 diabetes (Song et al., 2014). Targeting QRFP/QRFPR and PrRP/PrRPR systems appears more promising in the control of feeding behavior. Indeed, QRFPs have been shown to be regulated by adiposity signals and nutrient status and to be orexigenic in several mammalian species. Therefore, elucidating the mechanisms by which QRFP receptor agonists increase high-fat diet intake could markedly influence the development of therapies for the treatment of obesity (Primeaux et al., 2013). The PrRP system has been shown to be an important point of integration in the control of energy metabolism and stress response, suggesting that this anorexigenic system might also represent an interesting target for the treatment of obesity (Onaka et al., 2010).

In conclusion, the mammalian RF-amide system shares many features for a family that includes not only peptides with a conserved Arg-Phe-NH_2 sequence at their carboxyl-terminal end but also receptors that display significant sequence homologies and regulate the same set of physiological functions. Strikingly, endogenous opioid peptides and their receptors play prominent roles in the same functions, suggesting a close link between opioid and RF-amide systems. Such a relationship has been pointed very early for NPFF, with the proposal of an anti-opioid function that has been largely characterized in subsequent studies. Further progress in the development of selective pharmacological tools for the different RF-amide receptors should greatly facilitate the study of their function in various mammalian species and confirm their potential as therapeutic targets.

Conflict of interest

The authors declare no conflicts of interest.

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2.2.3. Les autres fonctions régulées par les RF-amides et leur récepteurs

Avant toute chose, il est important de relever que les différentes formes de stress liés à l'environnement (*e.g.* variation de température, stress social) influencent fortement les systèmes physiologiques dont la régulation est liée au statut énergétique de l'individu, comme la reproduction ou la prise alimentaire. En effet, afin que ces différentes partitions s'accordent de manière idéale, les êtres vivants doivent être à l'écoute des ressources disponibles qu'ils détiennent. En stockant ou en consommant de l'énergie, elles tendent vers un équilibre pour s'adapter au mieux aux conditions extérieures. Les peptides RF-amides participent aux liens étroits qui les unissent et font le relai entre les informations environnementales et les réponses physiologiques qui en découlent. C'est pourquoi nous retrouverons un effet de chacun des systèmes RF-amides dans les voies neurohormonales relatives au stress.

La revue récente de Jérome Leprince et collaborateurs comporte un résumé détaillé des différentes fonctions dans lesquelles sont impliqués les peptides 26RFa et 43RFa (Leprince *et al.*, 2017). Chez la souris, ces peptides ont un impact sur la locomotion (26RFa, icv. ; Kampe *et al.*, 2006 ; do Rego *et al.*, 2006 ; Takayasu *et al.*, 2006). D'autre part, le 26RFa et le 43RFa sont exprimés dans des régions du cerveau impliquées dans les réponses au stress et à l'anxiété (Takayasu *et al.*, 2006). A la différence du 43RFa, le 26RFa (icv.) diminue l'anxiété *via* la transmission GABAergique et β-Adrénergique (Palotai et Telegdy, 2016). Au niveau central, le 26RFa et le 43RFa entraînent également une hausse à long-terme de la pression artérielle moyenne et du rythme cardiaque (Takayasu *et al.*, 2006). En périphérie, ils induisent des effets hypotensifs ou hypertensifs selon les voies de signalisation auxquelles ils font appel (Fang *et al.*, 2009). En périphérie, le 26RFa et son récepteur sont exprimés dans le cortex et la medulla de la glande surrénale (Fukusumi *et al.*, 2003 ; Ramanjaneya *et al.*, 2013), où les peptides agissent localement pour réguler la sécrétion des corticostéroïdes à travers des modes de communication paracrine et/ou autocrine. Ce système est aussi engagé dans les effets antiapoptotiques et la sécrétion d'insuline des cellules β pancréatiques. Enfin, même si peu d'études ont été réalisées à ce sujet, on remarque une expression du peptide et de son récepteur dans de nombreuses tumeurs (colorectale, testiculaire, pancréatique, du foie, du sein, des ovaires et de la prostate ; *Human Protein Atlas*).

La kisspeptine a été identifiée comme un suppresseur de métastase dans un cas de mélanome (Lee et Welch, 1997 ; Lee e*t al.*, 1996). Son action anti-cancéreuse a été révélée par la suite dans d'autres cancers comme celui de la thyroïde, des ovaires, de la vessie, de l'estomac, de l'œsophage, du pancréas, du poumon et de l'hypophyse (Tng, 2015). Son rôle dans la prévention du cancer est résumé dans plusieurs revues (Cho e*t al.*, 2012 ; Gottsch *et al.*, 2006 ; Kirby *et al.*, 2010 ; Makri *et al.*, 2008). Pour inhiber le caractère invasif d'une tumeur, la kisspeptine exerce ses effets sur des protéines engagées dans la plasticité matricielle des tissus. D'une part, en réprimant l'activité des protéines de la matrice (MMP-9, *matrix metalloproteinase* ; Makri *et al.*, 2008 ; Li *et al.*, 2012 ; Navenot *et al.*, 2009). D'autre part,

en promouvant la formation d'adhésions focales et de fibres *via* l'activation des kinases d'adhésion focale (FAK), ce qui contribue à la diminution de la motilité cellulaire nécessaire à la propagation des métastases (Kotani *et al.*, 2001 ; Cho et *al.*, 2009). En revanche, des travaux montrent qu'une surexpression de la kisspeptine et du récepteur Kiss1R est observée dans les cancers hépatocellulaires (Ikeguchi et Hirooka, 2003 ; Schmid *et al.*, 2007), et que ce système aurait un rôle délétère dans le cancer du sein (Cvetković *et al.*, 2013).

Le PrRP a été nommé de cette façon suite à la découverte de son implication dans la libération de prolactine (PRL, Kawamata *et al.*, 2000; Samson *et al.*, 1998). Mais il semble être moins puissant que la TRH (*Thyrotropin-releasing hormone*) *in vitro* et *in vivo* concernant la libération de PRL (Samson *et al.*, 1998 ; Jarry *et al.*, 2000 ; Curlewis *et al.*, 2002). Au niveau anatomique, les neurones à PrRP se situent dans la *medulla oblongata* et le noyau dorso-médial de l'hypothalamus (DMH) et projettent directement vers l'hypothalamus, où ils contactent des neurones à corticolibérine (CRH, *corticotropine-releasing hormone*), ocytocine (OT), vasopressine et somatostatine, qui libèrent à leur tour ces neuropeptides impliqués dans les réponses au stress (Maruyama *et al.*, 1999). Les neurones à PrRP sont activés par une diversité de stimuli anxiogènes (Onaka, 2004). On note aussi un rôle dans les fonctions cardiovasculaires, même si une étude propose que les actions cardiovasculaires centrales du peptide soient traduites par le récepteur NPFF2R (Ma *et al.*, 2009). Par ailleurs, on observe une forte expression de l'ARNm du PrRP dans le noyau thalamique réticulé qui joue un rôle dans la régulation du sommeil. De plus, une infusion de PrRP (icv.) augmente la durée du sommeil paradoxal et diminue celle du sommeil lent (Zhang *et al.*, 2000, 2001).

Une revue résume les différentes fonctions physiologiques dans lesquelles sont impliqués le NPFF et son récepteur, notamment au niveau des régulations cardiaques et neuroendocrines (Jhamandas et Goncharuk, 2013). Pour résumer, des injections de NPFF dans le tractus solitaire du tronc cérébral (la première terminaison des influx cardiovasculaires venant de la périphérie) entraînent une modification de la pression artérielle et du rythme cardiaque (Laguzzi, 1996 ; Jhamandas et MacTavish, 2002 ; Jhamandas et Mactavish, 2003 ; Fang et al., 2010). Dans les tissus cérébraux *post-mortem* d'individus présentant une hypertension artérielle, on observe une réduction significative du NPFF dans des noyaux hypothalamiques et du tronc cérébral impliqués dans les fonctions cardiovasculaires (Goncharuk *et al.*, 2011). Par ailleurs, le NPFF (icv.) diminue l'activité locomotrice des animaux et bloque celle induite par la morphine, renforçant le lien entre systèmes RF-amides et opioïdes. NPFF et NPFF2R participent à la régulation des réponses émotionnelles, et sont impliqués dans le développement des comportements dépressifs (Lin *et al.*, 2016), et pourraient être impliqués dans l'apparition de la migraine (Zhao *et al.*, 2016). Dans des cellules Neuro2A en culture, qui expriment de manière endogène le récepteur NPFF2R, un analogue de NPFF inhibe la production d'AMPc et prolonge la taille des neurites *via* une voie de signalisation impliquant ERK. Cet effet est bloqué par le RF9 (Yu *et al.*, 2016). Le NPFF a été aussi détecté

dans des sites centraux impliqués dans la régulation de la température (Allard *et al.*, 1992 ; Dupouy *et al.*, 1996), où il entraîne une modification significative de ce paramètre (Desprat *et al.*, 1997 ; Fang *et al.*, 2006, 2008 ; Frances *et al.*, 2001 ; Quelven *et al.*, 2005). Le NPFF entraîne une hyperthermie, alors que le RFRP-3 induit une hypothermie, et ces effets sont prévenus par le RF9 (Moulédous *et al.*, 2010 ; Quelven *et al.*, 2005). Des études démontrent que la neutralisation centrale du NPFF atténue la stimulation du circuit de la récompense activé par la morphine et la cocaïne (Marchand *et al.*, 2006 ; Kotlinska *et al.*, 2008).

A l'instar de ce qui est observé dans l'hypothalamus pour le PrRP, les neurones à RFRP projettent directement vers des cellules exprimant la CRH et l'OT, ce qui aboutit à la libération d'ACTH et d'OT dans la circulation périphérique (Qi *et al.*, 2009). Après un stimulus stressant, on observe une augmentation de l'activité des neurones RFRPs et de l'expression de leurs ARNm dans l'hypothalamus (Kaewwongse *et al.*, 2011 ; Kirby *et al.*, 2009). La première activité biologique reportée du RFRP-1 est la sécrétion de PRL consécutive à son administration icv. chez le rat, *via* un mécanisme différent de celui mis en jeu par PrRP (Hinuma *et al.*, 2000). Le RFRP-1 (icv.) stimule la libération de l'ACTH (*Adrenocorticotropic hormone*) et de l'ocytocine chez le rat (Kaewwongse *et al.*, 2011), et module les fonctions contractiles cardiaques (Nichols *et al.*, 2012). Le RFRP-1 est retrouvé dans les régions limbiques comme l'amygdale, qui est impliquée dans la régulation des fonctions mnésiques, l'apprentissage et la peur conditionnée (Lénard *et al.*, 2014). Chez les rongeurs, on note un effet anxiogènique du RFRP-3, bloqué par le GJ14, un antagoniste des récepteurs NPFFR, d'un point de vue cellulaire et comportemental (Kim *et al.*, 2015).

3. Aperçus anatomique, physiologique et cellulaire des voies de la douleur

Afin de nous protéger des dangers du monde extérieur, l'évolution et la sélection naturelle ont permis à l'être humain de disposer d'un système de détection des stimuli nociceptifs ou douloureux, néfastes pour le maintien de son intégrité. Lorsqu'un signal nociceptif atteint la peau, les viscères ou autres organes, celui-ci est converti en un message électrique se propageant le long du neurone sensitif jusqu'à la moelle épinière. Le corps cellulaire du neurone sensitif est contenu dans les ganglions dorsaux de la moelle épinière. Son prolongement dans la racine dorsale de la moelle épinière fait synapse avec un autre neurone dont la prolongation à partir de la racine ventrale de la moelle épinière jusqu'à la périphérie permet une réaction motrice adaptée du corps appelée réflexe. Ce message nerveux emprunte également un chemin ascendant jusqu'au cortex cérébral, où il est intégré et perçu comme une douleur (Mertens *et al.*, 2015). Trois régions du système nerveux sont principalement responsables de la sensation et de la perception de la douleur :

i) Les voies afférentes du système nerveux périphérique, qui remontent de la corne dorsale de la moelle épinière aux centres supraspinaux situés au sein du système nerveux central.

ii) Les centres d'intégration de la douleur, localisés dans le tronc cérébral, le mésencéphale, le diencéphale et le cortex cérébral.

iii) Les voies efférentes, qui descendent du système nerveux central jusqu'à la moelle épinière.

3.1. Les voies afférentes de la douleur

Les voies afférentes sont constituées de neurones appelés « nocicepteurs », dont les terminaisons sont présentes au sein du système périphérique, au niveau de la peau, des viscères ou des muscles, et dont le corps cellulaire est localisé dans les ganglions dorsaux de la moelle épinière (DRG). Ils répondent à des stimuli thermiques, mécaniques ou encore chimiques. L'information générée par un stimulus aversif intègre le système nerveux central en empruntant les fibres Aδ, de petit diamètre et myélinisées, ou des fibres non myélinisées, et donc à vitesse de conduction du signal plus lente, baptisées fibres C (Yaksh *et al.*, 2011). Ces nocicepteurs ont la particularité d'être facilement activés par des substances chimiques libérées dans le milieu par des cellules endommagées ou participant à la réponse inflammatoire, comme les cytokines, les prostaglandines ou encore la trypsine (Caterina *et al.*, 2005). La neurotransmission du signal des terminaisons nerveuses à la moelle épinière nécessite une dépolarisation de la cellule en périphérie, induite par des canaux calciques et sodiques voltage dépendants. La propagation du potentiel d'action tout au long de la fibre se fait *via* les canaux sodiques voltage dépendants sensibles à la tétrodotoxine (TTX), et aux canaux potassiques à rectification retardée (Caterina *et al.*, 2005). Au sein de la moelle épinière, nous trouvons les terminaisons centrales des afférences primaires ; des neurones dont les longs axones projettent jusqu'au cerveau ; des interneurones, et des axones venant de régions diverses du cerveau. Sur un plan transversal, la moelle épinière se divise en plusieurs lames (lames de Rexed, Rexed *et al.*, 1952). Les afférences primaires y entrent par la corne dorsale *via* le tractus de Lissauer. En majorité, les fibres Aδ se terminent dans la lamina I, la portion ventrale de la lamina II et dans la lamina V (Figure 5), alors que les fibres C prennent fin le plus souvent dans les lames I, II et X, autour du canal central.

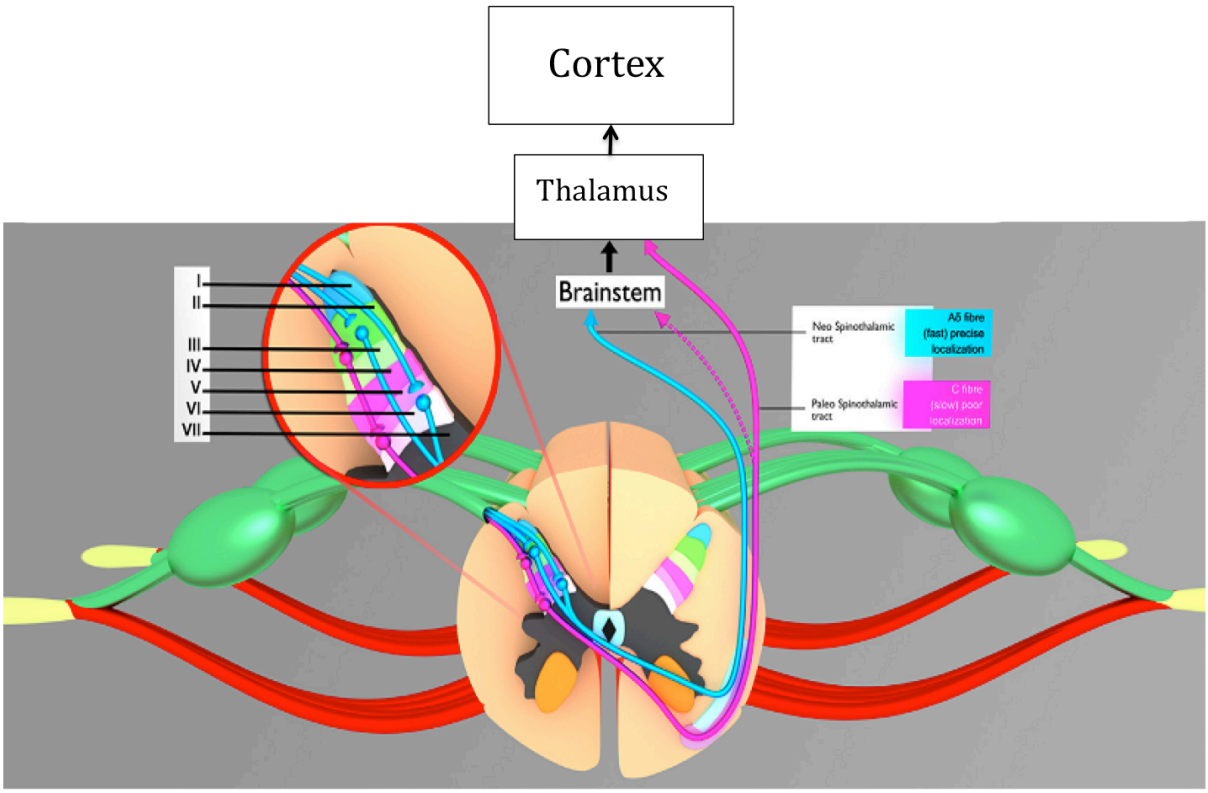

Figure 5 : Organisation anatomo-physiologique des différentes fibres et de leurs connexions au niveau de la corne dorsale de la moelle épinière. *Sur un plan transversal, la moelle épinière est divisée en lames, selon leur anatomie. A son entrée, les fibres afférentes primaires envoient leurs projections vers la corne dorsale où elles font synapse avec d'autres neurones au sein de lames bien spécifiques selon leur nature. Ces fibres poursuivent ensuite au niveau de la corne ventrale de la moelle épinière, pour remonter enfin vers le tronc cérébral, le thalamus et le cortex. En bleu, les fibres Aδ, fournissant la localisation précise de la douleur. En rose, les fibres C. D'après (Mertens et al., 2015).*

Lors de son arrivée au niveau spinal, le potentiel d'action issu des neurones afférents induit la libération de différentes classes de neurotransmetteurs. Les principaux neurotransmetteurs impliqués dans la transmission nociceptive sont le glutamate, l'aspartate, la substance P et d'autres neuropeptides. Les récepteurs glutamatergiques, tels que les récepteurs NMDA (N-méthyl-D-Aspartate) ou AMPA (*amino-3-hydroxy-5-methyl-4-isoxazole propionic acid*) sont connus pour initiés des réactions en chaîne intracellulaires pouvant modifier l'expression génomique des cellules en réponse à un stimulus nocif. Les interneurones, dont les axones résident dans la moelle épinière envoient à leur tour les informations vers les neurones de projections.

3.1.1 La théorie du portillon

Les synapses entre les neurones de premier et de second ordre localisés dans la *substantia gelatinosa* de la lame II et d'autres lames de la moelle épinière, fonctionnent comme un système constitué de portes, qui peuvent s'ouvrir ou se fermer et constituent un des dogmes majeurs avancé par la théorie du portillon. Cette porte, située dans la moelle épinière régulerait la transmission des influx douloureux jusqu'aux structures supra-spinales pour leurs futurs traitements et interprétations. Ce sont Melzack et Wall en 1965 qui proposent une première explication de cette théorie. Les stimulations non-nociceptives des fibres A (les fibres de gros diamètres), telles que le toucher, les vibrations ou les variations de température, peuvent entraîner la fermeture de cette porte, diminuant ainsi la perception de la douleur. Ce tonus inhibiteur pourrait être levé par l'arrivée de nouveaux signaux nociceptifs assez intenses pour pouvoir entraîner l'ouverture de la porte. Les voies efférentes du système nerveux central (SNC) peuvent également participer à la fermeture ou l'ouverture de cette barrière.

3.1.2. Anatomie des voies afférentes

Dans la moelle épinière, les neurones de second ordre, post-synaptiques, projettent leurs fibres dans le système antérolatéral de la moelle épinière, avant de remonter vers le système nerveux central. Ce système antérolatéral est composé des tractus spinomésencéphalique, spinoréticulaire, spinoparabrachial et spinothalamique. Dès leur arrivée dans la moelle épinière, les signaux douloureux empruntent deux voies pour aller jusqu'au cerveau (Figure 6) :

i) D'une part, le tractus néo-spinothalamique, pour les traitements rapides discriminants, correspond aux projections spinothalamiques.

ii) D'autre part, le tractus paléo-spinothalamique, un système plus primitif, correspond à l'association des projections spinoréticulaires, spinomésencéphaliques et spinoparabrachiales.

Le premier représente la composante discriminative de la douleur, et permet d'en fournir une localisation précise grâce à ses connexions avec les aires somatosensorielles (Carpenter *et al.*, 1991). Le second constitue la composante émotionnelle de la douleur.

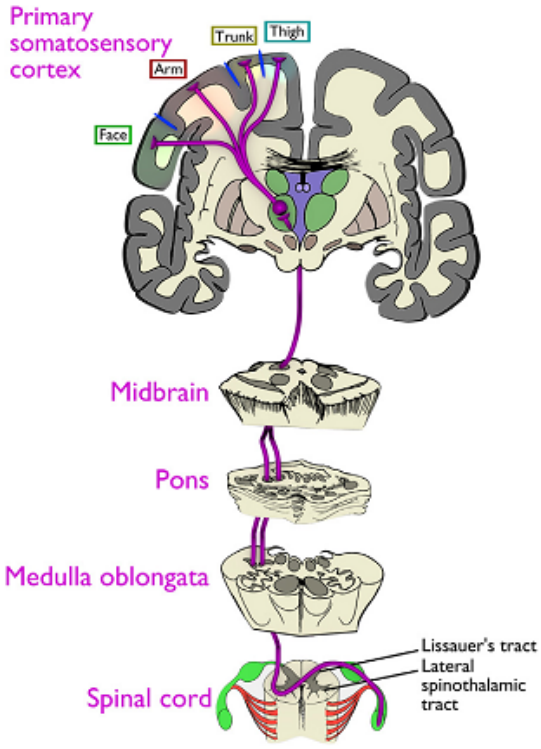

***Figure 6** : **Illustration du tractus spinothalamique et de ses projections corticales**. A partie de la moelle épinère, les fibres ascendantes passent par la medulla oblongata, le pons, et le mésencéphale pour atteindre le cerveau, et notamment le cortex comatosensoriel. D'après (Mertens et al., 2014).*

3.2. Les voies efférentes de la douleur

Une fois acheminés dans le cerveau et interprétés comme une douleur, les messages nerveux empruntent des voies descendantes du noyau du raphe magnus à la corne dorsale de la moelle épinière, pour moduler les réactions de l'organisme à la douleur. Ce noyau reçoit des messages nerveux de la PAG (substance grise périaqueducale), qui elle-même est connectée par diverses régions du SNC, telles que le cortex cérébral, la formation réticulée du tronc cérébral, l'hypothalamus et la moelle épinière. La PAG est connectée en bout de chaîne au système limbique, qui est associé à l'expérience émotionnelle. De fortes concentrations d'opioïdes endogènes (endorphines, enképhalines, dynorphines) sont retrouvées dans cette région, et sont responsables d'effets analgésiques importants. La PAG est décrit comme le centre endogène de l'analgésie car elle stimule différentes voies efférentes qui modulent ou inhibent les signaux afférents de la douleur au niveau de la moelle épinière (Hueter *et al.*, 2010). La voie néo-spinothalamique repose sur les neurones de gamme dynamique étendue (neurones WDR). Ces neurones sont capables de générer des *wind-up*, c'est-à-dire une hausse progressive et dépendante de la fréquence, et de la réponse des neurones consécutivement à des stimulations nociceptives répétées

identiques sur un même territoire. Des études montrent que les voies sérotoninergiques et noradrénergiques participent à la modulation de ce circuit neuronal de la douleur.

En conclusion, la douleur est définie par sa composante physique et son aspect émotionnel. Cette double perception est encodée par deux voies bien distinctes. D'une part, la voie néo-spinothalamique et ses neurones WDR, qui traitent des informations relatives à sa cartographie sensori-discriminative. D'autre part, les cellules de la voie paléo-spinothalamique, qui projettent vers des aires associées aux aspects émotionnels. Grâce à ces deux voies anatomiquement et fonctionnellement différentes, la perception et l'interprétation de la douleur dépendent de l'expérience de chaque individu, et la rendent ainsi unique. C'est pourquoi il est très difficile de juger de manière objective la douleur chez un patient, et d'en proposer un traitement adapté. Emergent alors aujourd'hui des questions sur l'existence de marqueurs de la douleur et la nécessité de son objectivation. Les opioïdes sont le traitement phare de différents types de douleur. Utilisés pour leur haut pouvoir analgésique, ils présentent néanmoins leurs limites et leur utilisation en clinique est fortement remise en cause.

4. L'ambivalence des opioïdes

A l'aube du XIX^{ème} siècle, le pharmacologue allemand Friedrich Wilhem Sertüner met en évidence un sel de cristallisation de l'opium aux propriétés analgésiques et hypnotiques puissantes qu'il appellera *morphium* (Schmitz *et al.*, 1985). Cette substance est au cours du siècle très utilisé par les médecins pour soigner les douleurs et en particulier celles subies par les blessés de guerre. Quelques années plus tard, en 1870, les médecins remarquent que les injections répétées de morphine entraînent une attirance pour cette substance, même après son arrêt. Dès 1875, le médecin allemand Edouard Levinstein propose de nommer ce symptôme *morphiumsucht*, ou morphinomanie (Vuillaume, 2015). Depuis plus de 200 ans, les adaptations physiologiques provoquées par les injections chroniques d'opioïdes sont connues, mais il n'existe toujours pas de traitements de substitution aussi efficaces en termes de pouvoir analgésiant et dépourvus d'effets secondaires. En effet, les opioïdes sont aujourd'hui la référence parmi les traitements des douleurs aigües et chroniques, en dépit des effets secondaires liés à leur administration, tels que la tolérance analgésique ou l'hyperalgésie induite par les opioïdes (HIO). La morphine demeure l'un des traitements les plus efficaces contre des douleurs sévères telles que les douleurs cancéreuses ou neuropathiques. De nombreux opioïdes utilisés en clinique provoquent une HIO, comme la morphine, le fentanyl, ou le remifentanil (Roeckel *et al.*, 2016). C'est pourquoi il est difficile de trouver un traitement adéquat visant à soulager les douleurs chroniques. L'HIO et la tolérance ne sont que deux effets secondaires de l'administration chronique de morphine parmi lesquels l'on compte la nausée, la constipation, la dépression respiratoire ou encore les troubles de l'humeur. Ces symptômes indésirables nécessitent donc son remplacement. Pour cela, il est indispensable

d'approfondir les études sur la complexité des opioïdes, leurs récepteurs, et les mécanismes impliqués dans leurs effets aversifs.

4.1. Les ligands et récepteurs opioïdes

A l'instar des récepteurs des peptides RF-amides, les récepteurs opioïdes sont des RCPGs partageant des similitudes avec ceux de la famille de la rhodopsine. Il existe trois principaux récepteurs aux opioïdes : le récepteur μ, dont le ligand sélectif est la morphine. Le récepteur δ, caractérisé dans le canal déférent de la souris, et le récepteur κ, activé sélectivement par la kétazocine (Snyder et Pasternak, 2003). Un quatrième récepteur, dénommé NOP (*nociceptin opioid peptide receptor*) a été plus récemment cloné. D'un point de vue pharmacologique, il se distingue des 3 récepteurs opioïdes précédemment cités car son activation ou blocage nécessite une très forte concentration de ligands (Mollereau *et al.*, 1994). En revanche, son rôle est clair dans la modulation des effets de l'activation de μ et dans l'analgésie morphinique et le développement de la tolérance (Mollereau *et al.*, 1994 ; Toll *et al.*, 2016). Il est fortement exprimé dans le cerveau ainsi que dans la moelle épinière et est couplé à la protéine Gi. Contrairement à l'étorphine qui active le récepteur NOP à fortes concentrations, les opioïdes classiques ne sont pas capables de l'activer ni de s'y fixer à faible concentration (Mollereau *et al.*, 1994). Ce n'est que deux ans après la mise en évidence du récepteur que son ligand endogène a été découvert, et nommé nociceptine ou orphanine FQ (N/OFQ). Le premier nom lui est donné en référence à sa capacité à diminuer le seuil nociceptif des souris lors d'une injection icv, (Meunier *et al.*, 1995). Le deuxième, car il a permis de désorphaniser NOP, et que son pentapeptide N-terminal est flanqué d'une Phe et d'une Gln.

Les récepteurs μ, δ, κ, et NOP partagent 60% d'homologie de séquence. Plus précisément, on note une homologie de 75% au niveau des séquences transmembranaires, et supérieure à 80% dans les boucles intracellulaires. En revanche, les différences les plus marquées sont observées aux extrémités N et C-terminales. Les ligands endogènes du système opioïde sont d'abord traduits sous forme de précurseurs inactifs, puis activés par des endo- ou exopeptidases. Il existe 4 précurseurs des peptides opioïdes. La pro-opio mélanocortine (POMC), de laquelle dérive (parmi d'autres neuropeptides non-opioïdes) la β-endorphine, sélective du récepteur μ. La pro-enképhaline (PENK) se clive en Met-enképhaline et Leu-enképhaline, qui se lient préférentiellement au récepteur δ. La prodynorphine est clivée en prodynorphine A et B, qui se lient préférentiellement au récepteur κ. Enfin, la pronociceptine donne lieu à la nociceptine, le ligand endogène du récepteur NOP. Ces neuropeptides partagent une séquence N-terminale commune composée des acides aminés Tyr-Gly-Gly-Phé-Met/Leu, nécessaire à leur fixation sur les récepteurs opioïdes. Seule la nociceptine diffère par le premier acide aminé de cette séquence pentapeptidique, présentant une phénylalanine à la place d'une tyrosine. Les endomorphines

sont des ligands endogènes sélectives du μ, mais dont le précurseur n'est pas encore connu. De plus, de nombreux ligands ont été développés pour ces récepteurs, comme les dérivés de l'opium (la morphine et l'héroine), ou encore le DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-*enkephalin)*, un dérivé d'enképhaline, qui est l'agoniste de synthèse le plus sélectif de μ (Waldhoer et *al.*, 2004).

Parmi les nombreux opioïdes exogènes, le laudanum a été découvert au XVI^{ème} siècle par le médecin suisse Paracelse, notamment connu pour sa célèbre réflexion : « Toutes les choses sont poisons, et rien n'est sans poison ; seule la dose détermine ce qui n'est pas un poison ». Cette préparation issue du pavot somnifère fut historiquement utilisée comme analgésique dans le cadre d'une thérapie, puis consommée pour sa dimension hédonique. Les siècles suivants ont vu naître la synthèse d'agonistes complets tels que la codéine et l'héroine (diacyle morphine), et d'autres analogues de la morphine comme l'oxycodone, l'acetaminophène ou l'hydromorphine. Les analogues de la morphine sont des agonistes partiels du récepteur mu aux opioïdes. C'est pourquoi ils apparaissent plus efficaces sur des voies de signalisation impliquant des phénomènes d'amplification comme celles des protéines G, que sur la voie des β-Arrestines, ce qui explique qu'ils n'induisent pas ou peu d'endocytose. Une revue récente présente un tableau récapitulatif des différents effets bénéfiques et de l'addiction et dépendance induites par la stimulation spécifique d'un récepteur opioïde (Badal et *al.*, 2017). Par exemple, le potentiel addictif de la morphine est attribué à son incapacité à induire le recrutement de la β-Arrestine et donc l'endocytose et désensibilisation. La morphine, le fentanyl et la méthadone sont considérés comme de puissants agonistes de μ, même si l'efficacité de la morphine est beaucoup plus faible que celle du fentanyl. La codéine et l'oxycodone sont quant à eux considérés comme des agonistes modérés (Duttaroy et Yoburn, 1995). De nombreux agonistes opioïdes peuvent produire de l'HIO, tels que le fentanyl, le rémifentanil, la morphine, la codéine ou le DAMGO (Célérier *et al.*, 2000 ; Aguado *et al.*, 2015 ; Johnson *et al.*, 2014 ; Drdla *et al.*, 2009). Il est suggéré que les agonistes de μ avec peu d'efficacité intrinsèque comme la morphine, sont capables de renforcer le phénomène de tolérance car ils induisent la désensibilisation du récepteur, mais peu d'internalisation. Cela retarde donc la déphosphorylation de μ et sa resensibilisation, qui est nécessaire au renforcement de son efficacité observée avec d'autres agonistes (Schulz et *al.*, 2004). D'autres études suggèrent l'implication de la β-arrestine 2 dans les effets secondaires induits par la morphine. En effet, la morphine améliore l'analgésie et réduit les phénomènes de dépression respiratoire et autres effets secondaires gastro-intestinaux chez des souris KO (*Knock Out*) pour la β-arrestine 2 (Bohn et *al.*, 1999 ; Maguma, Dewey et Akbarali, 2012 ; Raehal, Walker et Bohn, 2005). Les agonistes sélectifs du récepteur δ, tel que le SNC-80, limitent la tolérance et la dépendance mais peuvent provoqués des convulsions causées par le recrutement de la β-arrestine 2. La sélectivité de chacun des ligands pour son récepteur semble donc être en faveur de l'apparition d'effets secondaires délétères.

4.2. Pharmacologie des récepteurs opioïdes

La voie de signalisation classique des récepteurs opioïdes implique la protéine G_i. Cependant, des études soulignent une interaction physique entre μ et G_s, qui s'intensifie en cas de traitement chronique à la morphine (Chakrabarti *et al.*, 2005). Parmi les effecteurs des récepteurs opioïdes, on retrouve l'AC, les canaux calciques voltage dépendants de type N, les canaux GIRK (modulés par les sous-unités Gβγ) et les MAPK, qui sont impliquées dans de nombreux processus cellulaires tels que la croissance, la différenciation, la migration et la mort cellulaire. Tous ces phénomènes sont notamment impliqués dans la plasticité synaptique (Sweatt, 2004). Or, la plasticité synaptique est le support des mécanismes adaptatifs observés en cas de tolérance aux opioïdes, ce qui suggère un lien mécanistique étroit entre ces différentes observations (Eitan *et al.*, 2003).

Comme sont capables de le faire les récepteurs à la somatostatine ou à la sérotonine, les récepteurs aux opioïdes ont la faculté d'interagir de manière croisée avec d'autres RCPGs, notamment par l'intermédiaire de la PLCβ. Par exemple, l'activation de δ peut entraîner une libération de calcium intracellulaire si l'activation d'un récepteur muscarinique a lieu simultanément (Connor et Henderson, 1996 ; Chen *et al.*, 2000). Ce mécanisme est réalisé grâce à la capacité du RCPG à réactiver la PLCβ, stimulée au préalable par une protéine G_q. Comme la plupart des RCPGs, l'activation des récepteurs aux opioïdes entraîne une phosphorylation des domaines intracellulaires, responsables des phénomènes de désensibilisation. La phosphorylation du récepteur dépend de la nature de son agoniste ainsi que du modèle cellulaire. Par exemple, la morphine n'induit pas la désensibilisation de μ dans des cellules HEK293, mais entraîne son internalisation dans des cultures primaires de neurones striataux de rat (Haberstock-Debic *et al.*, 2005). D'autre part, l'agoniste total DAMGO, induit une phosphorylation du récepteur plus importante que la morphine, qui est un agoniste partiel (Marie *et al.*, 2006). Les récepteurs opioïdes sont également capables d'hétérodimérisation, un phénomène responsable d'une diversité dans les signaux intracellulaires, en apportant des réponses fonctionnelles différentes de celles provoquées par l'activation d'un monomère. L'association des récepteurs δ et κ entraîne une diminution de l'affinité des agonistes spécifiques, mais augmente celle des agonistes non sélectifs, car elle est à l'origine d'un nouveau site de liaison impliquant ainsi une nouvelle pharmacologie (Jordan et Devi, 1999). Parmi la multitude de combinaisons hétérodimériques possibles et connues, le couple μ-δ est le plus étudié. D'autre part, des hétérodimères μ-NOP ont été détectés grâce à la technique de BRET (*Bioluminescence Resonance Energy Transfer*), et une étude montre que l'activation de NOP entraîne une désensibilisation croisée de μ (Wang *et al.*, 2005). Des études de FRET (*Förster Resonance Energy Transfer*) et de co-immunoprécipitation ont démontré la capacité des récepteurs opioïdes à se dimériser avec d'autres RCPGs tels que les récepteurs CCR5 (*C-C chemokine receptor type 5*), SST2A (*Somatostatin receptor subtype 2A*), NK1 (*Neurokinin 1 receptor*), CB1 (*Cannabinoid receptor 1*), ou encore NPFF2R (Roumy *et al.*, 2007). Un agoniste des récepteurs NPFFR -le 1DMeNPFF (D-Tyr1-*N*-Me-Phe3)-NPFF)-

augmente le transfert de fluorescence d'environ 10 à 15% en FRET entre NPFF2R et μ, tout en diminuant de moitié l'internalisation de μ (Roumy *et al.*, 2007). En modifiant la diffusion latérale de μ dans la membrane cellulaire, il promeut l'association de μ et NPFF2R. Au niveau de la moelle épinière, le 1DMeNPFF bloque les autorécepteurs δ, ce qui augmente le courant potassique induit par la libération de Met-enképhaline (Mauborgne *et al.*, 2001). Dans des neurones isolés du noyau du raphe périventriculaire et dorsal qui co-expriment les récepteurs NPFF et la nociceptine, des analogues du NPFF inhibent la diminution induite par la nociceptine de la conductance des canaux calciques de type N (Roumy *et al.*, 1999, 2003). Dans l'ensemble, ces résultats renforcent l'importance des peptides RF-amides dans la régulation des phénomènes impliquant les opioïdes et leurs récepteurs.

La complexité des phénomènes mis en jeu par les systèmes opioïdes oriente la recherche vers le développement de ligands à multiples cibles d'actions plutôt que vers la caractérisation d'agonistes très sélectifs, dans le traitement de la douleur par exemple. Les effets secondaires sont surtout observés avec la morphine, sélective du récepteur μ. Les agonistes sélectifs de κ et δ n'entraînent pas de constipation, ni de dépression respiratoire ou addiction, mais induisent de la diurèse, sédation ou encore dysphorie. La co-administration de la morphine et d'un antagoniste de δ tel que le naltrindole provoque une analgésie plus soutenue, et réduit la tolérance et la dépendance. Les agonistes du récepteur κ induisent plus d'effets secondaires que les agonistes non sélectifs κ/μ. De manière générale, les douleurs sont traitées par la morphine. La méthylnaltrexone est un analogue de la naltrexone, un antagoniste bien connu des récepteurs opioïdes. Incapable de passer la BHE, elle peut bloquer le développement de la tolérance à la morphine et l'hyperalgésie secondaire à son injection chronique (Corder *et al.*, 2017). Elle est par exemple utilisée en clinique pour bloquer la constipation chez des patients souffrant de douleurs chroniques (Portenoy et *al.*, 2008).

Malgré quelques controverses évoquées dans la littérature, la tolérance à la morphine est très souvent réduite de manière significative chez les KO NOP et N/OFQ (Ueda *et al.*, 1997 ; Chung *et al.*, 2006). En ce qui concerne l'addiction, plusieurs tests intégrés sont réalisables pour la mesurer chez l'animal. C'est le cas de la préférence de place conditionnée (CPP) pour les rongeurs, qui consiste à évaluer les effets appétitifs ou aversifs d'une drogue donnée, comme la cocaïne, l'alcool ou la méthamphétamine. Il a été démontré que le système N/OFQ/NOP bloque par exemple l'auto-administration d'alcool (Ciccocioppo *et al.*, 2004). Enfin, certaines fonctions motrices sont également affectées par la nociceptine et son récepteur. Le récepteur NOP et la nociceptine sont des molécules découvertes récemment, et leurs mécanismes d'action sont moins connus que ceux des récepteurs opioïdes classiques. Il existe ainsi plusieurs études contradictoires sur la fonction de ce système. D'une part, il a été montré que N/OFQ bloquait l'activité antinociceptive de μ, δ et κ. De l'autre, N/OFQ ne diminue pas de lui-même le seuil nociceptif, mais bloque l'analgésie induite par le stress lors d'injections icv.. Il n'aurait donc pas d'activité nociceptive, mais plutôt anti-opioïde (Mogil *et al.*, 1996a,b).

4.3. Rôle des opioïdes endogènes dans les circuits de la douleur

Le système opioïde joue un rôle aux niveaux périphérique, spinal et supraspinal. En périphérie, les messages douloureux stimulent les nocicepteurs Aδ et C. Ces fibres font synapses avec des neurones de projection se situant dans la corne dorsale de la moelle épinière, qui envoie à leur tour l'information vers les aires supraspinales. De plus, en périphérie, les opioïdes peuvent être libérés par des cellules immunitaires lors d'une inflammation. Localement, ces derniers vont inhiber la transmission de la douleur (Rittner *et al.*, 2008). Dans la corne dorsale de la moelle épinière, le nocicepteur va stimuler les récepteurs aux opioïdes présents au niveau présynaptique, ce qui engendre une inhibition de la libération de neurotransmetteurs. A partir de la corne dorsale de la moelle épinière, le signal nerveux poursuit son chemin jusqu'au thalamus et aux aires limbiques, où il sera relayé aux cortex somatosensoriels S1 et S2, dans lequel se fera l'encodage et la traduction de la discrimination spatiale et temporelle du stimulus désagréable. L'activation des aires limbiques est connue pour traduire les conséquences affectives et émotionnelles de la douleur. Une activation soutenue de ces zones cérébrales peut entraîner des symptômes psycho-physiologiques plus ou moins graves comme de l'anxiété liée à la douleur, la dépression ou encore des troubles cognitifs. Les récepteurs opioïdes sont très fortement exprimés dans les zones liées aux émotions et à l'ensemble des processus mentaux qui se rapportent à la pensée, telles que l'amygdale, le noyau accumbens et les cortex mediopréfrontal et cingulé antérieur. En effet, il est connu que certains facteurs émotionnels et cognitifs peuvent moduler la perception de la douleur (Bushnell *et al.*, 2013).

Les opioïdes endogènes et leurs récepteurs modulent l'activité des neurones noradrénergiques au sein du locus coeruleus, qui inhibe la transmission synaptique au niveau de la moelle épinière. A ce niveau, les opioïdes bloquent l'influx douloureux par une inhibition directe des cellules favorisant la transmission nociceptive (Nadal *et al.*, 2013). Les opioïdes sont également impliqués dans le contrôle des systèmes de récompense, émotionnels, cognitifs et locomoteur, la régulation de l'appétit, de la température corporelle, et des fonctions endocrines, cardiovasculaires, respiratoires ou encore gastro-intestinales (Bodnar, 2017). Ce système joue un rôle majeur dans l'évolution des organismes, pour lesquels il met en place des adaptations à long terme évitant la répétition des stimuli néfastes.

Enfin, à travers la « *Brain opioid theory of social attachment* », en 1978 Jaak Panksepp souligne l'implication des opioïdes endogènes dans le comportement social des individus (Pellissier *et al.*, 2017 ; Panksepp *et al.*, 1978). Il existe des similitudes entre l'attachement social et l'addiction aux drogues. De plus, des études montrent qu'un isolement social induit une détresse sociale et une recherche de contact dont le tonus opioïde insuffisant en serait responsable. Le retour à des contacts humains permet de pallier cet aspect négatif par le déclenchement de la libération d'opioïdes et notamment d'endorphines. En revanche, la morphine à faible dose utilisée de manière chronique chez des anciens malades et donc anciens grands consommateurs, peut entraîner un déficit d'interactions sociales, voire le développement

de troubles autistiques (McDonald *et al.*, 2013 ; Johnson *et al.*, 2014). De ces études est né un modèle de balance entre les récepteurs opioïdes et les systèmes participant à l'évitement social, dans lequel une suractivation ou un déficit du système opioïde entraîneraient un retrait ou une indifférence sociale.

5. Les adaptations à long-terme causées par les opioïdes

La morphine est utilisée pour soulager les douleurs par ses propriétés analgésiques. Or, des injections répétées provoquent un effet totalement opposé à celui pour lequel on l'utilise, appelé effet iatrogène. Au fur et à mesure des injections d'opioïdes, le seuil nociceptif basal de l'individu va diminuer progressivement, notamment *via* la montée en puissance d'un système anti-opioïde, qui va venir rétablir un équilibre homéostatique. D'autre part, avec la répétition des injections dans le temps, la dose initiale de morphine ne va plus avoir le même effet analgésique que lors de la première administration. En effet, l'obtention d'un effet similaire nécessitera l'augmentation de la dose d'opioïdes administrée, par rapport au premier jour d'injection. C'est ce que l'on appelle la tolérance. Ces deux effets secondaires liés à l'administration chronique d'opioïdes sont des phénomènes complexes à étudier car ils sont sous l'influence du sexe, de l'espèce, du fond génétique, et dépendent de la dose d'opioïdes utilisée. Par exemple chez le rat, l'hyperalgésie induite par la morphine et la tolérance analgésique sont plus prononcées chez les femelles que chez les mâles (Holtman and Wala, 2005, 2007 ; Hopkins *et al.*, 2004), alors que ces phénomènes sont comparables entre les deux sexes chez des souris C57Bl/6J (Juni *et al.*, 2010).

5.1. L'hyperalgésie induite par les opioïdes

Plusieurs hypothèses ont été mises en avant pour tenter de décrypter ce phénomène neuroadaptatif, comme la désensibilisation et la régulation négative des récepteurs opioïdes (Kieffer et Evans, 2002). De nombreuses études fondamentales et cliniques montrent que les opioïdes activent divers systèmes anti-opioïdes qui produisent des effets opposés et à long-terme menant à l'HIO, et à la réduction de l'analgésie (tolérance) (Rothman, 1992 ; Celerier *et al.*, 2000 ; Simonnet et Rivat, 2003). Cela illustre la théorie d'un équilibre homéostatique entre système opioïde et anti-opioïde. En effet, supposons l'existence en conditions basales de deux mécanismes antinomiques, l'un anti-nociceptif (pro-opioïde), et l'autre pro-nociceptif (anti-opioïde). Afin d'assurer le maintien d'un équilibre, un système pro-nociceptif va émerger suite aux injections répétées d'opioïdes, ce qui provoquera l'hyperalgésie secondaire observée. En retour, lors de l'arrêt de la stimulation des récepteurs opioïdes par ces injections répétées, le système pro-opioïde va à son tour être stimulé, afin de rétablir un nouvel équilibre, appelé allostasie (Figure 7 ; Célérier *et al.*, 2001). Ce nouvel équilibre serait à l'origine de la sensibilisation latente à la douleur, illustrée par l'apparition d'une hyperalgésie suite à l'administration

d'un antagoniste des récepteurs opioïdes chez des animaux ayant reçu un traitement chronique à la morphine dans le passé. Les mécanismes moléculaires qui sous-tendent l'HIO sont encore mal connus. Ce phénomène est également associé à la sensibilisation des voies pro-nociceptives en réponse à un traitement par les opioïdes.

Plusieurs neuromodulateurs ont été décrits comme ayant des propriétés anti-opioïdes. Des familles de peptides, notamment les RF-amides, participent à l'équilibre homéostatique entre systèmes opioïdes et anti-opioïdes. C'est aussi le cas du NMDA ou encore de la cholecystokinine, certaines cytokines et chimiokines ainsi que leurs récepteurs, l'ATP, le BDNF (*Brain-derived neurotrophic factor*), ou encore les sphingolipides (Figure 8 ; Roeckel *et al.*, 2016). Par ailleurs, des arguments sont en faveur d'une responsabilité du récepteur μ lui-même dans les effets secondaires consécutifs à l'injection chronique de morphine.

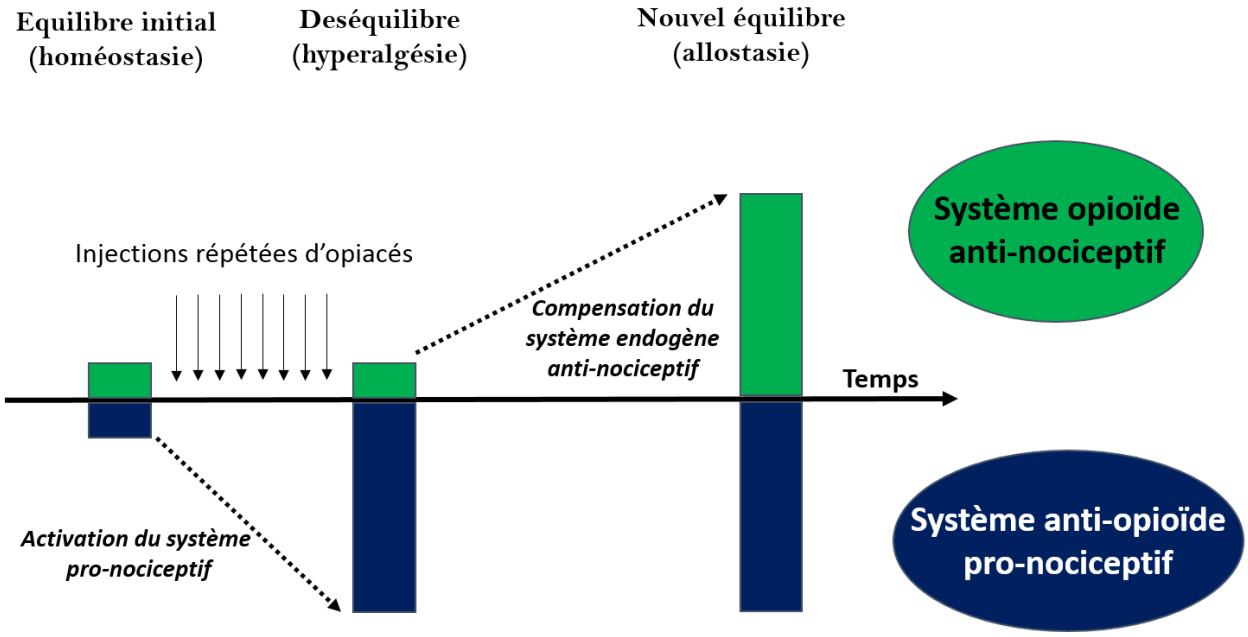

Figure 7 : Modèle simplifié de la balance homéostatique et neuroadaptative mise en jeu dans la douleur. *Avant la première exposition aux opioïdes, un équilibre initial (homéostasie) entre une faible expression du système pro- et anti-nociceptif permet d'obtenir un seuil nociceptif basal. Des injections répétées d'opioïdes entraînent une activation du système anti-opioïde pro-nociceptif et donc un déséquilibre de la balance en faveur d'une hyperalgésie. Après l'arrêt du traitement chronique d'opioïdes, une compensation par le système anti-nociceptif pro-opioïde endogène est observée, et un nouvel état d'équilibre (allostasie) permet le retour au seuil nociceptif basal. D'après (Célérier et al., 2001).*

5.1.1. Rôle du récepteur μ dans les effets secondaires liés à l'administration chronique de morphine

Les effets paradoxaux induits par l'administration d'opioïdes s'expliqueraient en partie par l'existence d'isoformes du récepteur μ. Le récepteur μ est codé par le gène OPRM1 chez l'Homme, et *Oprm1* chez la souris. L'épissage alternatif de ses transcrits permet de générer des variants du récepteur à 7TM, 6TM ou encore 1TM. Notamment, l'isoforme à 6TM est dépourvu de certains résidus dans la région N-terminale correspondant à la queue extracellulaire et au premier domaine transmembranaire. Contrairement au récepteur à 7TM, qui inhibe les neurones en activant la protéine G_i, le variant à 6TM induit des effets excitateurs *via* la protéine G_s (Gris et *al.*, 2010). De plus, on observe une diminution de l'hyperalgésie induite par la morphine chez des souris déficientes pour le variant à 6TM par rapport aux souris sauvages, suggérant que le couplage à G_s est probablement impliqué dans l'HIO (Convertino *et al.*, 2015 ; Oladosu *et al.*, 2015).

La désensibilisation d'un récepteur se caractérise par la diminution de la réponse cellulaire suite à sa suractivation. Elle est généralement liée à une perte de l'expression membranaire du dit récepteur, suite à un déséquilibre entre sa dégradation et son recyclage ou sa néosynthèse. Elle est homologue si elle est induite par un ligand endogène du récepteur concerné. En revanche, les ligands d'un autre récepteur, qui mettent en jeu une voie de signalisation intracellulaire dans l'environnement proche du RCPG d'intérêt peuvent aussi provoquer ce phénomène, et on parle alors de désensibilisation hétérologue.

Parmi les phénomènes de désensibilisation connus du récepteur μ, la désensibilisation hétérologue par les récepteurs aux chimiokines (CxCRs) ont été bien décrits (Mélik-Parsadaniantz *et al.*, 2015). De plus, des études ont révélé l'existence d'une interaction fonctionnelle entre les récepteurs μ et CxCR4 dans la moelle épinière, dans laquelle l'administration de CXCL12 diminue l'analgésie morphinique (Rivat *et al.*, 2014). Les récepteurs aux chimiokines activent la PKC, qui phosphoryle le domaine intracytoplasmique de μ (Williams *et al.*, 2013). La phosphorylation de μ entraîne un découplage du récepteur à la protéine G_i, permettant le recrutement des GRKs et des arrestines, conduisant à l'internalisation du récepteur et par la suite à la tolérance analgésique (Williams *et al.,* 2013). Des études mettent en exergue le rôle du récepteur δ et des α2-adrénorécepteurs dans les phénomènes d'interaction fonctionnelle avec le récepteur μ (Ong and Cahill, 2014 ; Fujita *et al.*, 2015 ; Milne *et al.*, 2008 ; Chabot-Dore *et al.*, 2015).

Le couplage du récepteur μ aux protéines G hétérotrimériques est un champ d'investigation très riche. En effet, différents couplages sont susceptibles de se produire en fonction de la dose de morphine utilisée ou des conditions physiopathologiques. Les propriétés analgésiques consécutives à l'activation du récepteurs μ s'expliquent par son couplage à la protéine G_i dans les neurones, qui conduit à une hyperpolarisation et ainsi à une inhibition de la nociception. En revanche, une ligation des nerfs spinaux L5-L6, responsable de douleurs neuropathiques, entraîne un changement de couplage vers la protéine

G_s. Cette transition est atténuée par l'administration d'une très faible concentration de naltrexone, un antagoniste des récepteurs aux opioïdes (Largent-Milnes *et al.*, 2008). De plus, l'injection de PTX (qui inhibe l'activation de la protéine G_i), entraîne l'augmentation du couplage entre G_s et μ. L'hyperalgésie induite par la morphine chronique est corrélée à une augmentation du couplage μ-G_s, qui est bloquée par l'administration d'une très faible dose de naloxone (Tsai *et al.*, 2009). En revanche, l'hyperalgésie induite par une très faible dose de morphine dans la substance grise périaqueducale de la souris préfère le couplage à G_i plutôt que G_s (Bianchi *et al.,* 2009). Par ailleurs, une technique utilisant des oligodeoxynucléotides antisens $G\alpha_{i1-3}$ et $G\alpha_{o1}$ nous montre l'importance de ces sous-unités dans le paradigme d'HIO précédemment décrit (Bianchi *et al.*, 2011).

5.1.2. L'hyperalgésie induite par les opioïdes dans un contexte neuronal

Au niveau neuronal, plusieurs mécanismes impliquant différents niveaux anatomiques du passage de l'influx nerveux ont été avancés pour expliquer l'hyperalgésie induite par les opioïdes. Parmi les hypothèses proposées, on note :

i) La sensibilisation des neurones primaires afférents
ii) La sensibilisation des neurones de second ordre face aux neurotransmetteurs excitateurs
iii) Une adaptation des voies descendantes menant à une augmentation de l'expression des modulateurs pro-nociceptifs et une libération de glutamate par les afférences primaires (Chu *et al.*, 2008).

L'hypersensibilisation des neurones se traduit par une potentialisation à long-terme (LTP) des synapses. La potentialisation à long terme désigne le renforcement d'une synapse, *via* une augmentation de la transmission de signaux nerveux entre les deux neurones qui la composent, et par sa stimulation à haute fréquence. La LTP a déjà été observée entre des fibres C et des neurones des couches superficielles de la corne dorsale de la moelle épinière (Liu et Sandkuhler, 1997). L'étroite relation entre LTP et hyperalgésie a été mise en évidence par l'utilisation de substances capables de bloquer la LTP, telles que la kétamine ou la minocycline, qui sont capables de prévenir également l'hyperalgésie (Klein *et al.*, 2007 ; Zhong *et al.*, 2010). De plus, le sevrage consécutif à une infusion de rémifentanil conduit à la potentialisation des synapses entre les fibres C et les neurones de la couche superficielle de la moelle épinière (*opioid-induced withdrawal LTP*, Drdla *et al.*, 2009). Or, les trois récepteurs opioïdes sont exprimés sur les fibres C primaires afférentes et sur les neurones de la couche superficielle de la moelle épinière (Besse *et al.*, 1990). Par ailleurs, la LTP et l'HIO partagent des voies de signalisation communes. Le BDNF (*Brain-Derived Neurotrophin Factor*), libéré par les cellules gliales, qui joue un rôle dans le développement de l'HIO (Ferrini *et al.*, 2013) est impliquée dans la maintenance de la LTP (Zhou *et al.*,

2010). Les seconds messagers CaMKII (Ca^{2+}/*calmoduline-dependent protein kinase II*), PKC, PKA et PLC sont tous impliqués à la fois dans l'hyperalgésie ainsi que dans la potentialisation à long-terme (Chen et Huang, 1991 ; Yang *et al.*, 2004 ; Drdla *et al.*, 2009). Les antagonistes des récepteurs NMDA sont capables d'empêcher ces deux phénomènes (Benrath *et al.*, 2005 ; Haugan *et al.*, 2008 ; Frankiewicz *et al.*, 1996 ; Li *et al.*, 2001). Les cellules gliales et les cytokines pro-inflammatoires sont des médiateurs de la LTP et de l'HIO. L'IL-1β (*Interleukin 1β)* et le TNF-α (*Tumor necrosis factor α*) stimulent la fréquence et l'amplitude des potentiels post-synaptiques excitateurs spontanés (EPSCs) qui conduisent à une excitation des neurones de la moelle épinière (Zhang *et al.*, 2011). Ces cytokines agissent surtout sur les cellules gliales pour induire la libération d'autres médiateurs qui sont capables de déclencher la LTP et l'HIO (Gruber-Schoffnegger *et al.*, 2013 ; Kronschläger *et al.*, 2016). Par ailleurs, une hyperalgésie rapide et courte consécutive à l'administration de carragénine induit un phénomène de sensibilisation à long terme à la douleur dans les nocicepteurs primaires, engendrant une augmentation durable des réponses aux molécules inflammatoires (Aley *et al.*, 2000).

La facilitation de la signalisation pro-nociceptive dans des modèles de douleurs persistantes, ainsi que l'hyperalgésie à long terme et la tolérance analgésique induites par les opioïdes, impliquent également les voies descendantes venant de la PAG et de la medulla rostral ventro-medial (RVM) (Donovan-Rodriguez *et al.*, 2006 ; Svensson *et al.*, 2006 ; Dogrul *et al.*, 2009 ; Vanderah *et al.*, 2001). L'hyperalgésie induite par les opioïdes concerne, comme les phénomènes de douleurs chroniques, le système glutamatergique, et notamment les récepteurs NMDA, localisés au niveau pré-synaptique sur les terminaisons centrales des neurones primaires afférents et au niveau post-synaptique sur les neurones de la corne dorsale de la moelle épinière (Antal *et al.*, 2008). Dans un contexte d'HIO, la morphine chronique entraîne l'augmentation de l'expression de la sous-unité NMDAR1 dans le cerveau. Sa co-administration chronique avec un antagoniste des récepteurs NMDA induit une diminution de la transcription de la β-arrestine-2 dans le cerveau de la souris (Ohnesorge *et al.*, 2013). De plus, l'ARNm de la β-arrestine-2 voit sa transcription augmenter en cas de tolérance analgésique dans la PAG, le cortex et le striatum, ce qui serait une des causes de la désensibilisation du récepteur μ et de la tolérance analgésique (Bohn *et al.*, 2000 ; Hurle, 2001 ; Jiang *et al.*, 2006 ; Williams *et al.*, 2013). La régulation de la β-arrestine-2 par les récepteurs NMDA serait donc responsable des phénomènes d'hyperalgésie secondaire et de tolérance. Une autre hypothèse énonce que l'administration chronique d'opioïdes induit une translocation de la PKC à la membrane plasmique, ce qui entraînerait une activation des récepteurs NMDA pré-synaptiques par l'élimination du magnésium. Il en résulterait ainsi une libération plus soutenue de glutamate (Zhao *et al.*, 2012b). De plus, après une administration soutenue de morphine, une diminution des transporteurs de glutamate GLAST (*Glutamate Aspartate Transporter*) et GLT1 (*Glutamate transporter 1*) est observée (Mao *et al.*, 2002). En conséquence, une augmentation du taux de glutamate dans la fente synaptique et une sur-stimulation de la synapse pourrait conduire à une

hyperalgésie. L'implication des récepteurs NMDA dans ces effets secondaires liés à l'administration chronique de morphine ne se limite pas au système nerveux central. En particulier, une augmentation de l'expression de EAAT3 (*glutamate transportor excitatory amino acid transporter-3*) et de la sous-unité 2B du récepteur NMDA (GluN2B) a lieu dans les DRG (Gong *et al.*, 2016).

Parmi les autres systèmes que l'on peut citer comme impliqués dans les mécanismes d'HIO, des études ont démontré le rôle du transporteur KCC2 (*K^+-Cl^- cotransporter isoform 2*) et de l'homéostasie chlorique, dans laquelle cette dernière est critique pour le développement de l'hyperalgésie dans différents modèles de douleur au niveau de la corne dorsale de la moelle épinière (Zeilhofer *et al.*, 2012). Par ailleurs, le récepteur TRPV1 à la capsaïcine (*transient receptor potential vanilloid 1*), un canal activé par la chaleur mais également des ligands chimiques, et présent sur les terminaisons centrales et périphériques des neurones sensoriels primaires joue un rôle dans la transduction des signaux thermiques et douloureux, ainsi que dans le développement de la tolérance et de l'hyperalgésie (Caterina *et al.*, 2000 ; Julius, 2013 : Zhou *et al.*, 2010 ; Chen *et al.*, 2008 ; Vardanyan *et al.*, 2009).

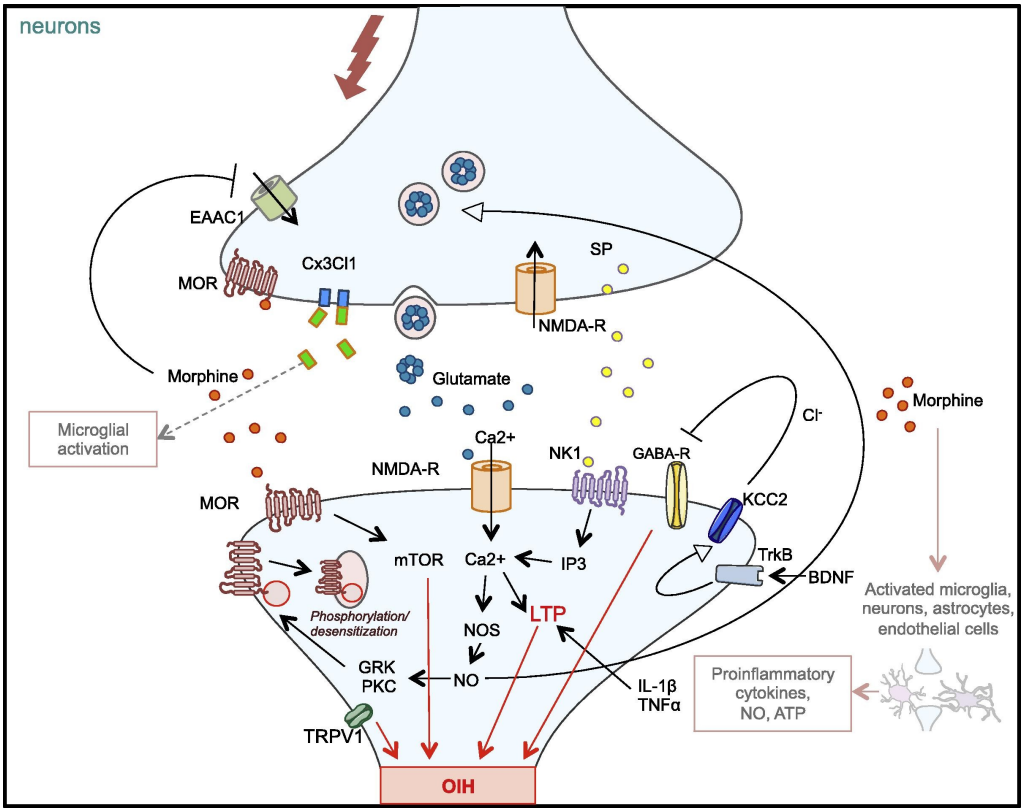

Figure 8 : Voies de signalisation neuronales impliquées dans l'hyperalgésie induite par les opioïdes*. La liaison de la morphine sur le récepteur μ entraîne l'activation de la signalisation mTOR (mammalian target of rapamycin), responsable d'une excitation neuronale et de l'HIO. La morphine peut aussi bloquer le transporteur du glutamate EEAC1 (excitatory amino acid carrier 1) impliqué dans la recapture de l'acide aminé, entraînant une augmentation de la concentration synaptique du glutamate. A travers l'activation de son récepteur NMDA et de la potentialisation à long terme (LTP) qu'il entraîne, il participe au développement de l'HIO. D'autres facteurs tels que TrkB (Tropomyosin receptor kinase B, le récepteur au BDNF) diminuent l'activation de KCC2 (K^+-Cl^- cotransporter isoform 2), modulant l'homéostasie du chlore et inhibant GABA-R pour produire de l'HIO. L'activation des cellules gliales et des neurones par la morphine contribue à l'HIO en produisant des cytokines telles que IL1β (Interleukin 1β) ou TNF-α (Tumor necrosis factor α), impliquées dans la LTP. Ces multiples mécanismes participent à l'établissement et au maintien de l'HIO. D'après (Roeckel et al., 2016).*

De nombreux acteurs de premiers et seconds rôles semblent ainsi impliqués dans le développement des adaptations à long-terme décrites précédemment. Or, il n'est pas aisé de faire le lien entre tous ces protagonistes. Nous allons maintenant nous concentrer particulièrement sur la place des peptides RF-amides et de leurs récepteurs dans ce réseau complexe des modulateurs des effets des opioïdes.

5.2. Les systèmes RF-amides dans les effets secondaires liés à l'administration d'opioïdes

Une des parties de la revue que nous avons écrite au laboratoire traite de l'implication des systèmes RF-amides dans la nociception et ses interactions avec le système opioïde. Je détaillerai particulièrement ici les liens de ce dernier avec les systèmes RFRPs/NPFF1R et NPFF/NPFF2R. Depuis sa découverte, les effets anti-nociceptifs du NPFF ont été démontrés dans différentes études (Yang *et al.*, 1985, Oberling *et al.*, 1993 ; Devillers *et al.*, 1995). Cependant, la découverte de l'existence de deux récepteurs couplés aux protéines G comme cibles du NPFF a complexifié la compréhension mécanistique de ses effets (Bonini *et al.*, 2000). Ces deux récepteurs, baptisés NPFF1R et NPFF2R sont capables de liés le RFRP-3 et le NPFF avec une affinité de l'ordre du nanomolaire, bien que ces deux ligands aient des affinités préférentielles respectives pour NPFF1R et NPFF2R (Hinuma *et al.*, 2000 ; Elshourbagy *et al.*, 2000). Ces premières découvertes ont permis l'émergence de réponses révélant les raisons pour lesquelles le NPFF est capable d'exercer des actions opposées, notamment dans le domaine de la nociception. D'ailleurs, leur expression est régulée d'une façon différente chez des souris génétiquement déficientes en récepteurs opioïdes (Gouardères *et al.*, 2004). Alors qu'il existe une augmentation de l'expression de NPFF2R dans certaines régions bien spécifiques du cerveau, le récepteur NPFF1R est quant à lui sous-exprimé au sein d'autres régions, ce qui suggère qu'ils ont un impact différent selon la fonction régulée par les opioïdes. En outre, le rôle distinct de chacun des deux récepteurs est soutenu par leurs localisations différentes (Gouardères *et al.*, 2002 ; Gouardères *et al.*, 2004).

C'est pourquoi de nombreuses études ont utilisé des agonistes sélectifs d'un des deux sous-types de récepteurs pour tenter de mieux discriminer les fonctions de ces deux protagonistes. D'une part, le RFRP-3, qui présente une sélectivité supérieure à 300 fois pour le NPFF1R par rapport au NPFF2R (en test de liaison avec le ligand radioactif [^{3}H]-FFRF, Elhabazi *et al.*, 2013) est utilisé pour étudier le rôle de NPFF1R. D'autre part, le dNPA (D.Asn-Pro-(*N*-Me)-Ala-Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2 ; Roussin *et al.*, 2005) et le 1DMeNPFF, qui sont des dérivés du NPFF ou de ses précurseurs, sont plutôt utilisés pour cibler préférentiellement le récepteur NPFF2R. En effet, des agonistes endogènes dérivés des précurseurs pro-$NPFF_A$ ou pro-$NPFF_B$, qui présentent des affinités légèrement sélectives pour NPFF1R ou NPFF2R, engendrent des activités pharmacologiques différentes (Quelven *et al.*, 2005). Alors que le RFRP-3 semble impliqué dans les phénomènes d'hypothermie et d'amplification de l'analgésie morphinique, les peptides associés au NPFF entraînent une augmentation de la température corporelle ainsi qu'un effet anti-opioïde lorsqu'ils sont injectés directement au niveau du système nerveux central (Quelven *et al.*, 2005 ; Moulédous *et al.*, 2000). A l'aide des mêmes outils, une étude a mis en avant l'implication du récepteur NPFF2R dans la modulation des effets centraux de l'endomorphine-1, alors que les deux sous-types de récepteurs intensifient l'antinociception induit par l'endomorphine-2 (Wang *et al.*, 2014). Des différences au niveau du couplage intracellulaire de ces deux RCPGs ont également été

soulignées. Le NPFF1R est plutôt couplé aux protéines $G\alpha_{i3}$ et $G\alpha_s$, tandis que NPFF2R induit le recrutement des protéines $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$ et $G\alpha_s$ (Gouardères *et al.*, 2007).

En 2006, l'équipe de Frédéric Simonin identifie le premier antagoniste doté d'une haute affinité (nanomolaire) pour les deux sous-types de récepteurs, et présentant une affinité micromolaire pour les trois autres récepteurs à peptides RF-amides (Simonin *et al.*, 2006). Baptisé RF9, ce composé bloque l'hyperalgésie secondaire consécutive à l'administration de morphine et de fentanyl et réduit la réponse nociceptive induite par différents peptides RF-amides dans différents modèles de douleur chez la souris (Simonin *et al.*, 2006 ; Fang *et al.*, 2011 ; Elhabazi *et al.*, 2012 ; Elhabazi *et al.*, 2013). Néanmoins, le RF9 n'est pas sélectif d'un des deux sous-types de récepteurs NPFF et présente des propriétés agonistes sur le récepteur Kiss1R à fortes concentrations. En effet, de nombreux articles montrent qu'il est capable de stimuler Kiss1R *in vitro* et *in vivo*, et de provoquer notamment la libération de LH, ce qui est problématique pour l'étude du système RFRPs/NPFF1R dans le domaine de la reproduction (Min *et al.*, 2015 ; Kim *et al.*, 2015). C'est pourquoi la découverte d'un antagoniste sélectif et dépourvu de toute activité sur Kiss1R est primordiale pour analyser la contribution des RFRPs dans la régulation de l'axe HHG et ses interactions avec la kisspeptine et son récepteur (Quillet *et al.*, 2016 ; Kriegsfeld *et al.*, 2006 ; Johnson *et al.*, 2007 ; Clarke *et al.*, 2008 ; Kadokawa *et al.*, 2009 ; Sari *et al.*, 2009 ; Pineda *et al.*, 2010). Des antagonistes sélectifs ont été développés depuis ce premier composé, mais tous conservent une affinité inférieure au micromolaire sur le récepteur NPFF2R, ce qui est insuffisant. Par exemple, des antagonistes synthétisés par l'entreprise Acadia Pharmaceuticals, dont l'un est très sélectif pour NPFF1R mais toujours submicromolaire sur NPFF2R, et dont le second est moins de 20 fois sélectif, ont mené les scientifiques à conclure sur le rôle pro-nociceptif du récepteur NPFF1R dans différents modèles de douleur *in vivo* (Lameh *et al.*, 2010). De plus, en dépit d'un effet pronociceptif du récepteur NPFF2R au niveau cellulaire, d'autres composés d'Acadia Pharmaceuticals caractérisés comme agonistes totaux ou partiels présentent des propriétés pro-opioïdes *in vivo* (Lameh *et al.*, 2010 ; Gaubert *et al.*, 2009). Plus récemment, le composé GJ14, construit à partir d'un squelette ^{D}Arg-^{D}Phg, dont la structure est étroitement liée au BIBP 3226, un antagoniste sélectif du récepteur du NPY (Aiglsforter *et al.*, 2000 ; Mollereau *et al.*, 2002) a été décrit comme un antagoniste des récepteurs NPFFR (sélectivité ≈ 10 fois). Ce dernier est dénué d'effet sur le récepteur Kiss1R, comme en témoigne son absence d'effet sur la circulation de LH lorsqu'il est injecté au niveau central, contrairement au RF9 (Kim *et al.*, 2015). En revanche, son insuffisante sélectivité en fait un mauvais candidat pour discriminer les rôles respectifs de NPFF1R et NPFF2R chez l'animal, notamment dans des fonctions physiologiques impliquant ces deux récepteurs. En l'occurrence, il ne pourrait être utilisé dans le champ des effets secondaires liés à l'administration chronique d'opioïdes (Ayachi *et al.*, 2014 ; Quillet *et al.*, 2016). Il est donc nécessaire de mettre en évidence des ligands sélectifs de chacun des récepteurs NPFF, utilisable *in vivo*, pour nous aider à comprendre les mécanismes régulés par l'un, l'autre ou les deux récepteurs. En effet, cet outil est

nécessaire à l'étude de ces systèmes, et leur découverte reste un défi, car irrésolu depuis le début de la recherche de ligands sélectifs des récepteurs NPFFR.

6. Le développement de ligands sélectifs pour les récepteurs aux peptides RF-amides

Le développement de ligands antagonistes est une des stratégies utilisées pour l'étude du rôle d'un récepteur dans une fonction donnée. Une autre démarche, complémentaire, consiste à bloquer le système que l'on veut étudier de manière génétique. Cependant, l'un des inconvénients de cette technique, est que la délétion d'un gène dans l'embryon peut amener le génome à compenser cette perte par diverses adaptations, ou même ne pas être viable. Cela peut entraîner l'apparition d'un système compensatoire comme par exemple la surexpression d'autres gènes impliqués dans la même fonction, ou alors l'inverse, si notre gène d'intérêt est impliqué dans l'expression d'autres gènes. Pour pallier ce problème, il est possible de procéder à un KO inductible du gène, qui est déclenché à l'âge adulte de l'animal par l'expérimentateur, ce qui permet d'éviter le développement des adaptations consécutives à la perte d'un gène. Le blocage pharmacologique par un antagoniste est une des façons de contourner ces aléas. C'est pourquoi cela représente un enjeu majeur pour la communauté scientifique.

La caractérisation de ligands de synthèse requiert l'étroite collaboration entre chimistes et biologistes. Une série d'échanges entre les chimistes, qui fabriquent les molécules selon des données théoriques et les biologistes qui les testent dans des modèles *in vitro* et *in vivo* est fondamentale.

6.1. Stratégies pharmacochimiques de la découverte de nouveaux ligands de synthèse

Une des principales stratégies dans le domaine de la pharmacochimie est la conception rationnelle d'une chimiothèque à partir du ligand endogène du récepteur considéré (Figure 9). Afin de déterminer quels sont les acides aminés importants dans la liaison et/ou l'activité du ligand, les chimistes procèdent généralement à une délétion successive de chaque acide aminé, ou à un alanine-scan (Ala-scan) : une substitution pas à pas de chaque acide aminé par une alanine, qui est utilisé dans cette technique pour son caractère non-encombrant et chimiquement inerte. C'est une façon de faire disparaitre un acide aminé sans modifier la structure et la conformation des autres amino-acides composant le peptide. Cela permet également de déterminer la séquence minimale nécessaire à la liaison du ligand sur son récepteur d'intérêt. En effet, les petits peptides sont métabolisés en des acides aminés facilement dégradables par l'organisme, leur conférant une faible toxicité. En revanche, les peptides passent généralement très mal les différentes barrières physiologiques, comme la barrière hémato-encéphalique ou la paroi digestive. Ils sont très sensibles à l'hydrolyse enzymatique, et un haut poids moléculaire

réduit leur chance de passer la barrière gastro-intestinale, ce qui est incompatible avec une administration par voie orale (Lipinski *et al.*, 2001). Certaines études suggèrent l'existence d'un transport actif des petits peptides au niveau de la BHE, car ils exercent des effets supposés centraux malgré leur injection périphérique, et leur faible probabilité de traversée de la BHE (Simonin *et al.*, 2006). La structure d'un peptide, garnie d'une ossature centrale plus ou moins longue et de chaînes latérales positionnées de l'un ou l'autre côté de la structure centrale rend la molécule plus ou moins flexible. Les chimistes peuvent jouer sur cette notion de flexibilité pour améliorer la sélectivité des peptides. En effet, une flexibilité trop marquée est susceptible de favoriser la liaison du ligand avec d'autres récepteurs, d'où un défaut de sélectivité et un risque d'effets hors cibles (Giannis et Kolter, 1993). Plusieurs manœuvres sont possibles afin de contourner les différents inconvénients cités précédemment. Selon la position de sa chaîne latérale par rapport au carbone de référence, il existe deux énantiomères de chaque acide aminé : L ou D. Les deux énantiomères sont donc latéralisés de deux façons différentes, et sont dit chiraux, c'est-à-dire que l'un constitue l'image miroir de l'autre. Ils sont asymétriques, à l'instar d'une main gauche et d'une main droite par exemple. Les acides aminés sont les briques de construction du vivant. Ils sont présents majoritairement sous une forme L, qu'on appelle forme naturelle. L'inversion de l'acide aminé L en son énantiomère D (non-naturel), permet de réduire leur reconnaissance par les enzymes qui hydrolysent les peptides. Par ailleurs, une rigidification au niveau des chaînes latérales ou du squelette peptidique peut pallier la non-sélectivité du peptide en diminuant sa flexibilité. Les chimistes peuvent optimiser les dérivés peptidiques grâce à des décorations en N ou C-terminal. Parmi les autres facteurs influençant les liaisons ligand-récepteur, on retrouve l'orientation conformationnelle des chaînes latérales. Les acides aminés possèdent différents angles de rotation (2 pour la phénylalanine et 4 pour l'arginine par exemple), et il est possible de réduire le nombre de degrés de liberté octroyé par la chaîne latérale de l'acide aminé afin de jouer sur ces interactions.

Figure 9 : Démarche rationnelle du développement de ligands peptidiques : du peptide endogène à la caractérisation d'un dérivé actif chez l'animal.

Diverses liaisons sont présentes au sein d'un peptide entre les différents atomes qui constituent chaque acide aminé. Les liaisons hydrogène (liaison H) sont des forces qui attirent un atome d'hydrogène et un autre atome électronégatif, tel que l'oxygène, l'azote ou le fluor. Ce sont des liaisons

intermédiaires, plus faibles que les liaisons covalentes, qui peuvent être modifiées en fonction de la température pour changer la nature de la molécule (comme pour l'eau par exemple). Elles constituent les interactions nécessaires à la formation des hélices α ou des feuillets β. Les liaisons covalentes sont des liaisons plus fortes que les liaisons H car elles mettent en jeu un partage de paires d'électrons entre deux atomes. Les liaisons hydrophobes sont permises grâce au regroupement de molécules non polaires (c'est-à-dire non chargées, caractéristiques de bonnes candidates au passage de la BHE). Au contraire, une liaison ionique (ou électrovalente) a lieu entre deux atomes qui ont une très grande différence d'électronégativité (e.g. le NaCl formé par les ions Na^+ et Cl^-).

Après une série de synthèses, les molécules sont criblées par les biologistes sur des cellules recombinantes surexprimant le récepteur d'intérêt afin de déterminer leur affinité, et leur sélectivité vis-à-vis d'autres récepteurs. A partir des molécules les plus intéressantes, les chimistes en déduisent les décorations et/ou les conformations amenant une affinité et une sélectivité significatives. Plusieurs jeux d'aller-retours selon cette logique sont alors entrepris pour aboutir à la sélection du ou des meilleur(s) dérivés, qui seront ensuite testés dans des essais fonctionnels afin de déterminer leur caractère agoniste ou antagoniste. Le composé le plus prometteur, et dont la biodisponibilité, la solubilité et la demi-vie sont les meilleures est alors choisi pour être mis à l'essai chez l'animal. Notons que certaines entreprises pharmaceutiques proposent le criblage de la molécule d'intérêt sur un nombre exhaustif de récepteurs humains, et que la révélation d'une inhibition du récepteur canal hERG entraîne l'arrêt de la procédure pour ladite molécule, à cause d'un risque d'arythmie cardiaque trop important. La recherche de la sélectivité d'une molécule est un enjeu crucial pour éviter au maximum les risques d'effets secondaires dus à des interactions sur des récepteurs étrangers à sa cible principale.

6.2. La caractérisation de ligands pour les récepteurs aux peptides RF-amides

L'arginine et la phénylalanine présentes à l'extrémité COOH des peptides RF-amides sont cruciales dans leur activité. Cette caractéristique se retrouve chez un autre peptide qui stimule l'appétit : le NPY (FY-NH_2). Impliqué également dans cette fonction, l'α-MSH contient une arginine et une phénylalanine consécutive au milieu de sa séquence, ainsi qu'une fonction amide en C-terminale. Notons que l'homologie de séquence entre les récepteurs NPFFR et le récepteur de NPY est de 40%, et de 59% entre les deux sous-types NPFF1R et NPFF2R (Hinuma *et al.*, 2000 ; Bonini *et al.*, 2000).

Des études précédentes de SAR soulignent l'importance de l'introduction de modifications au niveau de l'extrémité N-terminale (Gealageas et al., 2012) ou C-terminale des dipeptides Arg-Phe-NH_2 pour le développement de ligands sélectifs des récepteurs NPFF. La substitution des résidus arginine ou phénylalanine a conduit notre équipe à la caractérisation du RF313. Dans ce composé, l'arginine est remplacée par une ornithine portant une fraction pipéridine sur sa chaîne latérale (Schneider *et al.*,

2015). Un projet auquel j'ai contribué a permis de le décrire comme un antagoniste non sélectif des récepteurs NPFF1R et NPFF2R, qui bloque les effets du RFRP-3 et de l'hyperalgésie induite par les opioïdes chez les rongeurs (Elhabazi *et al.*, 2017).

L'histoire des ligands des récepteurs NPFFR commence avec la caractérisation du dansyl-PQRF-NH_2 qui présente une affinité de 6,1 nM dans des homogénats de moelle épinière de rat (Payza *et al.*, 1993). Plus de dix ans plus tard, un dipeptide flanqué d'un cycle adamantane à son extrémité N-terminale, nommé RF9, fut identifié dans notre laboratoire. Celui-ci présente une affinité de l'ordre du nanomolaire sur les deux sous-types de récepteurs NPFFR (Simonin *et al.*, 2006). Il agit comme un antagoniste des récepteurs NPFFR en bloquant l'hyperalgésie induite par le fentanyl et la morphine chez les rongeurs, ainsi que la tolérance. C'est un outil qui fut par la suite très utilisé dans des études portant sur la nociception ou la reproduction, mais la mise en exergue de son côté agoniste sur le récepteur Kiss1R l'a discrédité du point de vue des biologistes spécialistes de l'étude de la régulation de la reproduction.

En ce qui concerne le récepteur Kiss1R, la recherche de ligands sélectifs s'est également basée sur la modification du ligand endogène, la kisspeptine. Réduite à son pentapeptide C-terminal et modifiée par rapport au ligand natif, cette dernière conserve une affinité subnanomolaire, mais reste agoniste total du récepteur Kiss1R (Tomita *et al.*, 2008). Les délétions successives effectuées du côté N-terminal du pentapeptide (à partir de la phénylalanine) entraînent une chute significative de son affinité. D'un point de vue général, l'insertion d'un tryptophane améliore la stabilité face aux protéases, et la rigidification permet d'augmenter la demi-vie. Un autre antagoniste, baptisé P234 est lui aussi issu de la troncature du ligand endogène, et a montré son efficacité *in vivo* sur la libération de la LH et de la GnRH. Des hétérocycles ont aussi été caractérisés pour ce récepteur, grâce à des criblages réalisés par Takeda Pharmaceuticals de dérivés 2-acylamino-4,6-diphénylpyridine. Comme pour les ligands NPFFR, on note la présence d'un cation sous la forme d'une guanidine ou d'une amine protonable à pH physiologique, ce qui est un élément crucial pour la conservation d'une haute affinité.

6.2.1. L'importance de l'extrémité N-terminale des peptides RF-amides

Des premières études de SAR ont été réalisées avec les différents neuropeptides RF-amides sur leurs récepteurs endogènes (Findeisen *et al.*, 2011). Les résidus en position 5.27 et 6.59 du récepteur sont importants pour la reconnaissance du ligand par le récepteur, suggérant la formation d'une poche de liaison acide et chargée négativement dans les deux sous-types de récepteurs. Le résidu en position 7.35 jouerait quant à lui un rôle dans la sélectivité fonctionnelle (Findeisen *et al.*, 2012). Par la suite, ces travaux ont été complétés par notre équipe grâce à l'exploration de la SAR des différents neuropeptides sur tous les récepteurs à peptides RF-amides (Figure 10 ; Rouméas *et al.*, 2015). Dans ce dernier travail, tous les peptides synthétisés ont été benzoylés à leur extrémité N-terminale. En effet, il est connu que les

peptides benzoylés présentent une très bonne affinité sur les récepteurs NPFFR ou Kiss1R (Bihel *et al.*, 2015 ; Gealageas *et al.*, 2012 ; Tomita *et al.*, 2007, 2006 ; Simonin *et al.*, 2006).

Le RFRP-3 et le NPFF partagent la signature carboxy-terminale PQRF-NH_2, mais différent par leur extrémité N-terminale. Cette différence confère à RFRP-3 une sélectivité pour NPFF1R, alors que le NPFF est un meilleur agoniste sur NPFF2R que sur NPFF1R. Une étude a été réalisée en ce qui concerne l'impact des délétions N-terminales systématiques du NPFF et du RFRP-3 sur les propriétés pharmacologiques des peptides sur NPFF1R et NPFF2R (Roumeas *et al.*, 2015). La délétion de la leucine en position 4 entraîne une perte complète de la sélectivité pour NPFF1R. Le RFRP-3 est un agoniste complet des deux récepteurs mais est plus puissant sur NPFF1R. La délétion de la proline en position 2 abolit cette sélectivité pour NPFF1R. Les délétions N-terminales jusqu'à la proline en position 4 n'altèrent pas l'efficacité maximale des dérivés de RFRP-3 sur les deux récepteurs. La perte de la leucine en position 4 entraîne une forte diminution de l'affinité et de l'activité sur les deux récepteurs. Même s'ils présentent encore une affinité submicromolaire pour les deux sous-types de récepteurs, les tri- et dipeptides sont des agonistes partiels à très fortes concentration. A l'inverse du RFRP-3, le NPFF présente des affinités nanomolaires pour les deux récepteurs NPFFR et son affinité est conservée jusqu'au peptide tronqué Bz-PQRFa. En revanche, le NPFF présente une sélectivité fonctionnelle pour NPFF2R par rapport à NPFF1R, et ce jusqu'à la délétion de la phénylalanine en position 3. Les peptides plus petits sont agonistes partiels à fortes concentrations (Figure 10).

En bref, le raccourcissement progressif du côté N-terminal du RFRP-3 et du NPFF altère leur activité agoniste sur leur récepteur endogène. De plus, la sélectivité fonctionnelle de RFRP-3 pour NPFF1R et de NPFF sur NPFF2R est principalement conservée avec des peptides de taille normale. L'extrémité N-terminale du RFRP-3 et du NPFF est donc cruciale pour avoir une activité agoniste sur les récepteurs NPFF, tout comme celle du 26RFa pour le récepteur QrFPR (Roumeas *et al.*, 2015).

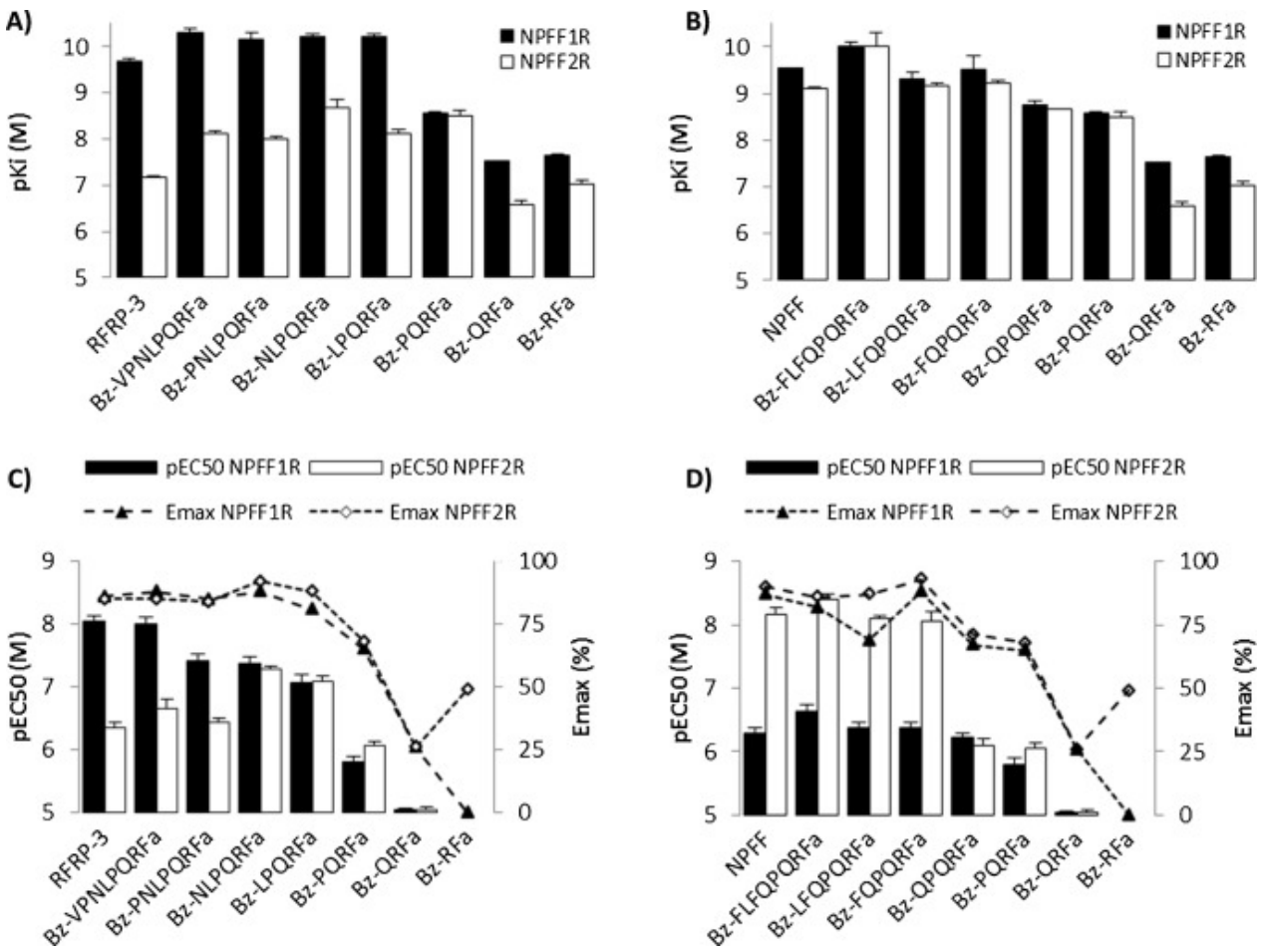

Figure 10 : Impact des délétions N-terminales systématiques du NPFF et du RFRP-3 sur les propriétés pharmacologiques des peptides. *Les panels A et B illustrent les affinités de RFRP-3 (A) et NPFF (B) et de leurs dérivés tronqués au niveau N-terminal pour les récepteurs NPFF1R et NPFF2R (cellules recombinantes CHO-NPFF1R ou CHO-NPFF2R, [^{3}H]-FFRF-NH$_2$). Les panels C et D représentent les profils d'activité du RFRP-3 (C) et du NPFF (D) et de leurs dérivés tronqués sur NPFF1R et NPFF2R. D'après (Roumeas et al., 2015).*

6.2.2. L'importance de l'extrémité C-terminale des peptides RF-amides

L'amide C-terminale a un rôle fondamental sur l'affinité et l'activité des peptides RF-amides, tout comme l'arginine et la phénylalanine. Par exemple, des analogues plus courts que l'arginine en position 7 du NPFF entraînent une diminution de la réponse maximale du récepteur NPFF1R. Au niveau de la phénylalanine en position 8, il existe une corrélation entre la réponse maximale du récepteur NPFF1R et la longueur de la liaison entre la colonne vertébrale du peptide et son groupe phényle. Le remplacement de la phénylalanine en position 8 par un tryptophane améliore la sélectivité pour NPFF2R (Findeisen *et al.*, 2011).

Pour résumer, les ligands des récepteurs NPFF ont besoin d'un groupement lipophile aromatique ou non-aromatique pour renforcer leur interaction avec le récepteur. Les transformations L/D sont susceptibles d'avoir un impact sur l'affinité, la stabilité métabolique et l'efficacité *in vivo*. L'arginine est un acide aminé très basique (pK_A = 13.2). Au pH physiologique (7.4), il est donc fortement ionisé. Or, plus la molécule est chargée, et plus il lui est difficile de passer à travers la BHE. Mais comme une activité centrale est détectée *in vivo* malgré une injection systémique, des transporteurs actifs sont supposés entrer en jeu. Un deuxième groupe phényle en position ortho du résidu benzoyle améliore l'affinité et la sélectivité pour les récepteurs NPFFR. Notons que l'effet de l'inversion L en D est dépendant de la nature de la décoration ajoutée en N-terminal. Si l'on remplace la Phe par un Trp, les dérivés présentent une plus faible activité. En revanche, les composés dérivés de la séquence Arg-Trp-NH_2 ont une bonne affinité pour le récepteur Kiss1R. Pour construire une chimiothèque complète, il faut donc considérer les différentes combinaisons possibles mêlant le choix de la décoration N-terminale (e.g. adamantane, phenyle), l'orientation L ou D des chaînes latérales, et la conservation de l'extrémité NH_2 présente au niveau de l'extrémité carboxylée.

L'univers de la synthèse chimique ne se cantonne pas aux dipeptides. Les hétérocycles, qui sont des cycles constitués d'atomes d'au moins deux éléments différents, représentent aussi de bons candidats dans la course à la recherche de composés sélectifs. Deux séries d'hétérocycles ont été décrites dans la littérature pour nos cibles d'intérêt. D'une part, les quinolinyle-guanidines, caractérisés comme antagonistes NPFF1R mais inexplorés sur NPFF2R (Kawakami *et al.*, 2003). D'autre part, les aminoguanidines hydrazones, qui sont la base d'un projet auquel j'ai contribué et sur lequel nous reviendrons ultérieurement, présentent un caractère antagoniste et légèrement sélectif pour le récepteur NPFF2R par rapport au récepteur NPFF1R. D'autre part, sachant que la kisspeptine, le PrRP et le 26RFa se lient non seulement à leurs récepteurs endogènes, mais aussi aux récepteurs NPFF1R et NPFF2R, il est difficile de les utiliser pour analyser le rôle spécifique de ces ligands et de leurs récepteurs.

OBJECTIFS

Les objectifs principaux de ma thèse ont été de caractériser de nouveaux outils spécifiques des récepteurs à peptides RF-amides, en l'occurrence des récepteurs NPFF1R, NPFF2R et Kiss1R. Grâce à l'un de ces composés, nous avons été en mesure de caractériser le rôle du système RFRP-3/NPFF1R dans la modulation de la nociception.

Nous avons mis en lumière un composé très affin et hautement sélectif pour le récepteur NPFF1R qui nous a permis de caractériser ce composé *in vivo* dans des modèles de douleur et de reproduction pour i) en confirmer son pouvoir antagoniste chez l'animal, et ii) déterminer le rôle spécifique du système RFRP-3/NPFF1R chez la souris en ce qui concerne la nociception. Nous avons également comparé l'effet du blocage pharmacologique avec notre antagoniste et celui du blocage génétique grâce à nos souris KO NPFF1R. De plus, nous nous sommes intéressés à la transcription et la distribution des ARNm du récepteur NPFF1R et de son ligand dans la moelle épinière et les ganglions rachidiens dorsaux de la souris. Notre outil sélectif a été caractérisé chez le hamster Syrien mâle, chez lequel son effet antagoniste a été confirmé, et grâce auquel nous avons pu montrer l'importance du RFRP-3 dans la modulation de son axe HHG. Cette partie sera présentée sous forme d'article. J'ai également contribué à une publication sur la caractérisation du RF313, un antagoniste des récepteurs NPFF, capable de prévenir les effets du RFRP-3 et de l'hyperalgésie induite par les opioïdes chez les rongeurs. Ma contribution se retrouve également dans la caractérisation d'un dérivé aminoguanidyle hydrazone sélectif des récepteurs NPFFR, qui a mené à la soumission d'un article. Au sujet du récepteur NPFF2R, des criblages successifs ont permis d'identifier des composés hautement sélectifs par rapport au récepteur NPFF1R, mais cette-fois constitué de 3 acides aminés.

Dans une autre partie, nous nous sommes intéressés au système RFRP-3/NPFF1R chez le hamster Syrien. D'une part, nous avons étudier la modulation de l'expression du récepteur NPFF1R chez cet animal, selon le sexe et la photopériode. De l'autre, grâce à l'identification de notre composé sélectif NPFF1R, nous avons tenté de répondre aux questions concernant le rôle déterminant du système RFRP-3/NPFF1R dans la photoréactivation de l'axe reproducteur du hamster Syrien mâle.

Enfin, à l'égard du récepteur Kiss1R, nous nous somme basés sur les publications d'une équipe japonaise, portant sur la synthèse, l'étude systématique et l'évaluation d'antagonistes (Kobayashi *et al.*, 2010a, 2010b). Nous avons ainsi réalisé une caractérisation *in vitro* de deux composés antagonistes sélectifs du récepteur Kiss1R.

PARTIE 1 : CARACTERISATION DE LIGANDS SELECTIFS DES RECEPTEURS NPFF1R ET NPFF2R

Cette partie s'intéresse au développement de ligands spécifiques des récepteurs NPFF1R. Elle s'articule autour de trois axes majeurs :

i) La caractérisation d'un ligand très affin et sélectif du récepteur NPFF1R, et la détermination du rôle du système RFRP-3/NPFF1R chez la souris dans la modulation de la nociception et chez le hamster concernant l'axe HHG.

ii) La caractérisation de deux ligands antagonistes des récepteurs NPFF : le 22e et le RF313.

iii) La caractérisation de ligands sélectifs pour le récepteur NPFF2R

1. Matériels et méthodes non cités dans l'article

1.1. Préparations d'homogénats de tissus nerveux et expérience de compétition

Les préparations d'homogénats de bulbes olfactifs ont été réalisées selon le protocole décrit dans l'étude de Nathalie Gherardi et Jean-Marie Zajac de 1997 (Gherardi et Zajac, 1997). Brièvement, les bulbes olfactifs de souris et les hypothalamus de hamsters ont été rapidement disséqués, pesés et homogénéisés dans 12 volumes (1g/12mL) de tampon froid Tris-HCl 50mM pH 7.4 à l'aide d'un Ultraturrax. L'homogénat ainsi obtenu est centrifugé à 100000 x g durant 30 minutes à 4°C. Le culot est à son tour homogénéisé dans 12 volumes de tampon avec l'Ultraturrax et centrifugé comme précédemment. Le culot final est à son tour resuspendu dans 1mL de Tampon + Sucrose 0.32 M, puis homogénéisé à l'aide d'un potter environ 10 fois, puis dosé et stocké à -80°C. Les tests de liaison sont réalisés avec le radioligand [^{125}I]-1DMeNPFF et de 25 µg à 100 µg de tissus selon l'expérience. Les échantillons sont incubés pendant 1h à 25°C sous agitation et filtrés sur un Brandel Harvester à travers des filtres GF/B. Les filtres sont ensuite incubés dans des fioles de comptage pendant 1h à l'obscurité avec 3 mL de liquide scintillant. La radioactivité est ensuite quantifiée dans chaque échantillon à l'aide d'un compteur à scintillation (Topcount, Perkin Elmer).

2. Résultats

2.1. Le RF3286, un nouvel et puissant antagoniste du récepteur NPFF1R : caractérisation du système RFRP-3/NPFF1R chez la souris et le hamster

Ce travail s'inscrit dans la continuité des explorations déjà effectuées entre notre laboratoire et le laboratoire des chimistes de l'équipe de Frédéric Bihel de Strasbourg, dont les efforts fournis durant ces dix dernières années n'ont pas faiblis dans la recherche de ligands sélectifs des récepteurs NPFF. Pour cela, nous avons poursuivi la réflexion sur des composés dérivés de l'extrémité N-terminale Arg-Phe-NH_2 des peptides RF-amides. En 2012, nos deux laboratoires caractérisaient des molécules issues de l'exploration chimique de la partie N-terminale du dipeptide amidé Arg-Phe-NH_2 (Gealageas *et al.*, 2012). Notamment, l'introduction d'un acide cinnamique (groupement aryle) pour les composés 37 et 38 de cette publication montrent que l'introduction d'un espaceur entre la plateforme aromatique et la séquence dipeptidique (cpd 37 : ^{D}Arg/^{L}Phe, 0,8 nM pour NPFF1R et 117 nM pour NPFF2R ; cpd 38 : ^{D}Arg/^{D}Phe, 84 nM pour NPFF1R et 1620 nM pour NPFF2R) améliore la sélectivité pour NPFF1R par rapport à NPFF2R, et la ^{D}Arg en joue un rôle crucial. Bien que possédant un facteur de sélectivité supérieur à 100 fois, l'affinité du composé 37 sur NPFF2R est encore trop importante pour qu'il puisse être considéré comme un candidat idéal. En revanche, malgré son affinité micromolaire sur NPFF2R, le composé 38 présente une mauvaise sélectivité (seulement 20 fois).

Nous avons donc pris la décision de démarrer notre projet en modifiant ces deux composés, que l'on nommera RF480 (cpd 37) et RF487 (cpd 38), par l'ajout d'un deuxième groupement phényle. Ainsi, ces deux composés et leurs dérivés diphényles (respectivement RF479 et RF3296) ont été évalués en tests de liaison sur membranes de cellules recombinantes CHO surexprimant soit le récepteur NPFF1R, soit le récepteur NPFF2R humains. Comme reporté dans l'article qui suit, le RF3296 a un K_i sur NPFF1R de 7,2 ± 1 nM et sur NPFF2R de 727 ± 130 nM, soit une sélectivité de 101 fois, alors que le RF479 ne présente qu'une sélectivité de 48 fois. L'ajout d'un cycle aromatique sur un dipeptide composé d'une ^{D}Arg améliore significativement la sélectivité, à la fois en augmentant l'affinité sur NPFF1R et en la diminuant sur NPFF2R. Nous avons ainsi réalisé une nouvelle série de dérivés à partir du RF3296 en conservant la ^{D}Arg et en substituant la phénylalanine par un bromophényle ou un autre acide aminé en conformation L ou D, en l'occurrence le tryptophane, l'alanine et la leucine. De ces nouvelles synthèses, nous avons pu déduire que la bromophénylalanine et le tryptophane amélioraient significativement l'affinité pour NPFF2R, baissant donc la sélectivité NPFF1R vs NPFF2R. Notons tout de même une amélioration de plus de 200 fois avec la ^{L}Phe(2Br) pour le RF3283. L'alanine en conformation L diminue fortement la capacité de liaison du dipeptide à NPFF2R, mais la perte d'affinité concerne également dans une moindre mesure NPFF1R. Alors que la ^{D}Leu entraîne une diminution de l'affinité sur les deux récepteurs, la ^{L}Leu donne lieu à la plus grande sélectivité (>400 fois). De cette première série de dérivés, d'autres composés ont été

synthétisés. Les acides aminés utilisés dans notre première série sont tous des acides aminés dont la chaîne latérale est hydrophobe. Etant donné que le composé le plus convaincant de la première série était en conformation L, nous avons décidé de baser la deuxième série de synthèse sur des dérivés de ^{D}Arg-^{L}XX. Plusieurs sous-séries de dipeptides ont été générées selon la nature du ^{L}XX :

i) XX : Glycine (Gly), Valine (Val), Ile (Isoleucine), Cha (Cyclohexylalanine) et Nle (Norleucine).

ii) XX : Tyrosine (Tyr), Phenylalanine (Phe)

iii) XX : Glutamine (Gln), Asparagine (Asp)

La ^{D}Arg qui constitue ces dérivés permet une flexibilité dans la nature de l'acide aminé choisi en seconde position, car elle confère une très bonne affinité pour NPFF1R dans la plupart des cas, par rapport à la ^{L}Arg. Cette étude nous a permis de mettre en évidence deux composés très affins sur NPFF1R et très sélectifs vis-à-vis de NPFF2R : le RF3286 et le RF3287. Dans la suite de notre étude *in vitro*, nous avons caractérisé le RF3286 et le RF3287, et poursuivi la caractérisation *in vitro* et *in vivo* du RF3286, chez la souris et le hamster. Nous avons été ainsi en mesure d'identifier un nouvel antagoniste hautement sélectif du récepteur NPFF1R, et de caractériser le rôle de RFRP-3/NPFF1R dans la modulation de la nociception chez la souris et de l'axe HHG chez le hamster. Ce projet a mené à la rédaction d'un manuscrit présenté dans les pages suivantes, qui sera soumis dans les semaines à venir.

RF3286, a new potent and highly selective NPFF1 receptor antagonist to decipher the role of RFRP-3/NPFF1R system in nociception and reproduction

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Abbreviations:

AUC, area under the curve; BBB, brain-blood barrier ; CHO, Chinese hamster ovary; DMR, dynamic mass redistribution assay ; DOP, delta opioid receptor; GOWS, global opiates withdrawal syndrom ; HEK, human epithelial kidney; icv., intracerebroventricular ; KOP, kappa opioid receptor; LH, luteinizing hormone ; MOP, mu opioid receptor; NOP, nociceptin receptor; NPFF, neuropeptide FF; NPFFR, neuropeptide FF receptor; OIH, opioid-induced hyperalgesia; PrRP, Prolactin releasing peptide ; QRFP, pyroglutamylated RFamide peptide ; RFRP-3, RFamide related peptide 3; sc., subcutaneous.

ABSTRACT

RF-amide-relatide peptide-3 (RFRP-3) and Neuropeptide FF (NPFF), belongs to the family of so-called RF-amide peptides. In mammals, they are involved in the modulation of several function including metabolism, reproduction and nociception. They target two different G protein coupled receptor subtypes called Neuropeptide FF1 receptor (NPFF1R alias GPR148 or GnIH receptor) and Neuropeptide FF2 receptor (NPFF2R or GPR74), respectively. However the study of their function *in vivo* is severely limited by the lack of highly selective antagonists. In this report, we describe the identification of small compounds that display nanomolar affinity for NPFF1R, micromolar affinity for NPFF2R and very low or no affinity for other RF-amide and opioid receptors. We furher show that one of them RF3286 displays potent antagonist activity toward NPFF1R both *in vitro* and *in vivo* in two different species, mouse and hamster. Pharmacological blockade of NPFF1R in mouse models of opioid-induced hyperalgesia and analgesic tolerance revealed that NPFF1R and its endogenous ligand RFRP-3 are critically involved in neuroadataption associated with acute and chronic opioid administration. These results were further confirmed in NPFF1R knockout animals. Morover, we observed that NPFF1R mRNA is expressed in the dorsal horn of the spinal cord indicating that its modulatory role on the action of opioids is mediated, at list partly, by spinal mechanisms. Altogether, our data allowed to identify the NPFF1R/RFRP-3 system as a *bona fide* anti-opioid system and futher suggest that antagonists of this receptor might represent interesting therapeutic tools to improve adverse side effects associated with chronic opioid administration.

Keywords:

NPFF receptors – RF-amide peptides - Opioid systems - NPFF receptors - RF-amide peptides - opioid analgesia - opioid-induced hyperalgesia – analgesic tolerance - physical dependence - LH release

Introduction

RF-amide-relatide peptide-3 (RFRP-3) and Neuropeptide FF (NPFF) belong to the family of so-called RF-amide peptides, which display a typical Arg-Phe-NH_2 C-terminal sequence. They are involved in a variety of complex and highly regulated physiological functions including metabolism, reproduction and nociception (Quillet et al., 2016). In the begining of 2000, several groups identified two G protein coupled receptor subtypes for Neuropeptide FF that were called NPFF1 and NPFF2 receptors (NPFF1R and NPFF2R, respectively; (Bonini et al., 2000; Elshourbagy et al., 2000; Hinuma et al., 2000). RFRP-3 was further proposed to be the cognate ligand for NPFF1R, while NPFF was shown to display preferential binding affinities for NPFF2R (Elshourbagy et al., 2000; Hinuma et al., 2000). Although several lines of evidence suggest that both receptors and their endogenous ligands play a role in the modulation of nociception and opioid analgesia (Ayachi and Simonin, 2014), their respective involvement in these functions is still unclear. This is particularly due to the absence of highly selective antagonists for these two receptor subtypes that could be used *in vivo* to investigate the different functions modulated by these two systems.

In 2006, the RF9 dipeptide was introduced as the first antagonist endowed with a high affinity for both NPFF receptors while interacting in the micromolar concentration range with the three other RF-amide receptors (Simonin et al., 2006). Numerous experiments proved its efficacy in opposing morphine- or fentanyl-induced hyperalgesia and in reducing the nociceptive response of mice to RF-amide peptides in various *in vivo* pain models (Elhabazi et al., 2013; Elhabazi et al., 2012; Fang et al., 2011; Simonin et al., 2006). Nevertheless, RF9 exhibited no NPFF1/NPFF2 receptor selectivity and turned also to be a Kiss1R agonist, albeit with moderate affinity and efficacy. Indeed, a series of recent reports dedicated to the examination of the respective roles of RFRPs and kisspeptin in reproduction pointed to its ability to stimulate Kiss1 receptors, both *in vitro* and *in vivo* (Kim et al., 2015; Min et al.,

2015; Quillet et al., 2016). Thus, the discovery of a highly selective NPFF1R antagonist is mandatory to examine under safe conditions the contribution of the RFRP system in the regulation of the hypothalamic-pituitary-gonadal axis and its relationship with the kisspeptin system (Clarke et al., 2008; Johnson et al., 2007; Kadokawa et al., 2009; Kriegsfeld et al., 2006; Pineda et al., 2010a; Quillet et al., 2016; Sari et al., 2009). Since the initial discovery of RF9, several teams have reported peptidic and non peptidic compounds that target NPFF receptors but none of them displayed high selectivity and antagonist activity at NPFF1R or NPFF2R (Bihel et al., 2015; Gaubert et al., 2009; Journigan et al., 2014; Kim et al., 2015; Lameh et al., 2010), which would enable to study unambiguously the role these two receptor subtypes in the modulation of different functions. Moreover, in most cases the selectivity and activity toward the other RF-amide receptors have not been investigated. The main goal of this study, was therefore to identified and characterize a highly selective antagonist of NPFF1R in order to better understand the different function in which NPFF1R/RFRP-3 system is involved.

Previous SAR studies led us to consider the importance of introduction of modifications at the N-terminus or at the carboxy-amidated parts of Arg-Phe-NH_2 dipeptides to investigate the selectivity and antagonistic nature at NPFF1 and NPFF2 receptors (Gealageas et al., 2012). Substitutions of either Arg or Phe residues, conducted us to recently characterize RF313, replacing arginine with a non-natural ornithine derivative bearing a piperidine moiety on its side chain (Bihel et al., 2015) but this compound displayed similar affinity at both NPFF1 and NPFF2 receptors (Elhabazi et al., 2017). Based on these previous observations, we describe here the design of a novel series of compounds and the systematic comparison of their binding affinity properties at the five RF-amide receptors and at the four opioid receptors (MOP, DOP, KOP and NOP). We identified a novel compound, RF3286, that displays a nanomolar affinity and a high selectivity toward human and mouse NPFF1

receptors, with a NPFF1/NPFF2 receptor selectivity around 500 fold and almost no affinity for the other RF-amide receptors as well as opioid receptors. We further showed that RF3286 clearly behaves as a selective antagonist at the NPFF1 receptor *in vivo* in two different species (mouse and hamster). Moreover, RF3286 completely blocked the long-lasting hyperalgesia induced by acute fentanyl or chronic morphine injections and partially prevented the development of morphine tolerance. These results were further confirmed in NPFF1R knockout animals. Altogether, our data show that NPFF1 receptor is critically involved in neuroadaptative responses to acute and chronic opiate treatments in mice, and the secretion of LH in male Syrian hamsters. Altogether, our results clearly indicate that RF3286 represent a very promising tool to examine the role of NPFF1R receptor and its endogenous ligand in the fields of reproduction and pain.

Results

In this study we sought to identify small, highly selective compounds for NPFF1R in order to study the function of this receptor *in vivo*. To this goal, we started from two Arg-Phe-NH_2 dipeptide derivatives that we previously described (cp37 and cp38 in Gealageas et al, 2012). In these compounds (respectively named RF480 and RF487 in this study), the introduction of an aromatic platform at the N-terminus of the dipeptidic sequence (D-Arg/L-Phe for RF480 and D-Arg/D-Phe for RF487, see Figure 1) increased the selectivity for NPFF1R versus NPFF2R (Gealageas et al., 2012) and Table 1). We first replaced the phenyl ring of these two compounds by a diphenyl functional group and evaluate the affinity of the two novel compounds (RF479 and RF3296 in table 1) for recombinant human NPFF1 or NPFF2 receptors. Both compounds displayed nanomolar affinity for NPFF1R and a 50 to 100 fold lower affinity for NPFF2R. By comparison, in the same experiments RF9 displayed no selectivity and GJ14, a previously reported selective NPFF1R compound (Kim et al., 2015), showed only a 4 fold lower affinity for NPFF2R (Table 1). In order to further improved the selectivity of these compounds, we further synthesized 18 other dipeptides in which Phe was replaced by different L- and D-amino acids and identified 2 compounds, RF3286 and RF3287, which displayed high affinity for NPFF1R (4.7 ± 0.7 nM and 11.2 ± 1.3 nM, respectively) and a selectivity factor for hNPFF1 versus hNPFF2 of 471 and 150, respectively (Table 1, Figure 2). We further confirmed the high affinity and high selectivity of RF3296, RF3286 and RF3287 on recombinant mouse NPFF1 and NPFF2 receptors (Table 2, Figure 2). We then evaluated the selectivity of these compounds toward the other RF-amide receptors, PrRPR, Kiss1R and QRFPR as well as the opioid receptors, MOPr, DOPr, KOPr and NOPr. As shown in Table 3, they displayed almost no affinity for the three RF-amide receptors (Ki > 5-50μM). RF3296 displayed a slightly better affinity for MOPr, KOPr and NOPr (Ki > 1μM)

while RF3286 and RF3287 displayed lower affinity for the four opioid receptors (Ki > 5-50 μM).

Based on these results we selected RF3286 and RF3287 for further evaluation in different functional assays with HEK293 recombinant cells stably expressing the human NPFF1 receptor. We first evaluated whether these compounds displayed agonist activity by measuring their capacity to decrease the forskolin-stimulated cAMP level in those cells. As shown in Figure 3A, the NPFF1R endogenous agonist RFRP-3 efficiently decreased cAMP level with an EC_{50} around 50 nM while RF3286 and RF3287 had no effect up to 10 μM. We further showed that both compounds induced a rightward shift of the dose-response curve of RFRP-3 in a potent manner, displaying a K_b of 16 ± 2nM (pA_2=7.8, RF3286 Fig. 3B) and 20 ± 5 nM (pA_2=7.7, RF3287 Fig. 3C). For both compounds the slope of the shild plot was close to 1 indicating that they are both competitive antagonists. We then studied the activity of RF3286 on hNPFF1R HEK293 cells in a label-free dynamic mass redistribution assay (DMR). As shown in Figure 3D the agonist RFRP-3 was able to generate a dose-dependent response with and EC_{50} around 30 nM. RF3286 induced no signal in this assay up to 10 μM (Figure 3E) and the activity of RFRP3 was clearly attenuated in presence of 1μM RF3286 with a rightward shift in the dose-response curve of 53 fold (Figure 3F), Finally, we also confirmed the antagonist activity of RF3286 in a GTPγS assay with membranes from CHO cells stably expressing hNPFF1R and found a K_b value of 32 nM (pA_2=7.5, Fig. S1). We also investigated the activity of RF3286 in HEK-293 cells expressing human NPFF2 receptor. As expected from binding experiment this compound displayed neither agonist nor antagonist activity on those cells up to 10 μM (Fig. S2). To complete the selectivity profile of these two compounds, we tested them in functional calcium assays with recombinant CHO cells expressing Kiss1, PrRP or QRFP receptors. As expected from binding experiments, the two compounds up to 10μM did not show any agonist or antagonist activity at these 3 receptors,

except RF3286 that displayed a slight agonist activity on Kiss1R with an IC_{50} around 10 µM (see Fig. S2). Altogether our data show that we have identified two highly potent and highly selective NPFF1R antagonists. As RF3286 displayed a better selectivity than RF3287 toward human and mouse NPFF2R and a very good selectivity (≥ 1000) toward all the other receptors, we then decided to further characterize this compound in *in vivo* experiments.

We first evaluated the pharmacokinetic parameters of RF3286 in mouse plasma. When injected subcutaneously in mice (5 mg/kg, sc.), we observed a peak concentration 10 min. after the administration with a half-life of around 30 minutes (Fig. 4). We then evaluated the capacity of RF3286 to antagonize RFRP-3-induced hyperalgesia in mice following icv. administration. As shown in Figure 5A and 5B, when administered alone, RF3286 (10 nmol, icv.) had no effect while RFRP3 (10 nmol, icv.) induced a strong decrease of the nociceptive threshold of the animals (AUC = -43 ± 113 a.u. for saline vs -499 ± 98 a.u. for RFRP-3; $p < 0.01$, one way ANOVA followed by Bonferroni post hoc test). When co-administered with RFRP-3 at the same dose, RF3286 completely prevented the decrease of the basal nociceptive threshold (AUC = -499 ± 98 a.u. for RFRP-3 vs -87 ± 81 a.u. for RFRP-3 + RF3286; $p < 0.01$, one way ANOVA followed by Bonferroni post hoc test, Fig. 5A and 5B), confirming that this compound display antagonist activity at NPFF1R *in vivo*. We next studied the effect of RF3286 on the anti-opioid analgesia induced by the endogenous NPFF2R agonist NPFF in mice. When administered icv., morphine (1.5 nmol) caused analgesia (AUC = 286 ± 48 a.u. for morphine vs -7 ± 18 a.u. for saline; $p < 0.001$, one way ANOVA followed by Bonferroni post hoc test, Fig. 5C and 5D) that was significantly inhibited by NPFF (AUC = 31 ± 37 a.u. for morphine + NPFF vs 286 ± 48 a.u. for morphine; $p < 0.001$, one way ANOVA followed by Bonferroni post hoc test, Fig 5C and 5D). RF9, the mixed NPFF1/2R antagonist, efficiently reversed the effect of NPFF on morphine analgesia while RF3286 did not (AUC = 32 ± 28 a.u. for morphine + NPFF + RF3286 (ns) and 189 ± 16 a.u. for morphine + NPFF +

RF9 vs 31 ± 37 a.u. for morphine + NPFF ; $p < 0.05$, one way ANOVA followed by Bonferroni post hoc test). These data clearly show that RF3286 can selectively antagonize RFRP-3 action *in vivo* and clearly suggest that NPFF action on morphine analgesia is mediated by NPFF2R and not NPFF1R.

We then studied whether RF3286 can cross the blood- brain-barrier to block the effect of RFRP-3 in mice. As shown in Figure 5E and 5F, subcutaneous administration of RF3286 (5 mg/kg) efficiently prevented hyperalgesia induced by the central administration of 10 nmol of RFRP-3 (AUC = -205 ± 58 a.u. for RFRP-3 icv vs 90 ± 27 a.u. for RF3286 sc + RFRP-3 icv ; $p<0.001$, one way ANOVA followed by Bonferroni post hoc test) indicating that this compound can cross the blood- brain-barrier to block central action of RFRP3.

We next investigated the consequences of NPFF1 receptor blockade with RF3286 on acute fentanyl-induced analgesia and secondary hyperalgesia. As shown previously (Elhabazi et al., 2017), when injected subcutaneously in mice, fentanyl (4 x 60 µg/kg) induced a short lasting analgesia (AUC = 658 ± 91 a.u. for fentanyl vs AUC = 40 ± 56 a.u. for saline, Fig. 6A) that was followed on D2 and D3 by a significant decrease of the nociceptive threshold (AUC = -0.61 ± 1.11 a.u. for saline vs -5.80 ± 1.04 a.u. for fentanyl ; $p < 0.01$, one way ANOVA followed by Bonferonni post hoc test) of the animals indicating that they displayed pain hypersensitivity. When administered alone at a high dose (30 mg/kg, sc.), RF3286 had no effect on the nociceptive threshold of animals. When co-administered at low doses (from 0.005 mg/kg to 0.05 mg/kg) with fentanyl, RF3286 did not alter the primary fentanyl-induced analgesia (respective AUC = 810 ± 142; 702 ± 127; 797 ± 49; 806 ± 108 a.u. for 0.005; 0.02 and 0.05 mg/kg, Fig. 6B) but very efficiently prevented the secondary hyperalgesia induced by fentanyl with an ED_{50} = 0.01mg/kg (Fig. 6A and 6B). We further examined the consequences of NPFF1R blockade on the development of hyperalgesia and analgesic tolerance following chronic morphine administration. As RF3286 displayed a shorter half life

than morphine (30 min versus 6 hours) we decided to administered RF3286 three times per day at a relatively high dose (1 mg/kg, sc.) as compared to the ED_{50} value that we determined in our previous experiment, while morphine was administered only once a day. We performed daily injections of morphine (10 mg/kg, sc.) for 7 consecutive days and the thermal nociceptive threshold of the animals was measured every day before morphine administration. Such a daily morphine treatment led to a progressive decrease of the nociceptive threshold (Fig. 6C and 6D), indicative for the development of a robust hyperalgesic state in mice (AUC = -7 ± 2 a.u. for saline vs -23 ± 2 a.u. for morphine ; $p < 0.001$, one way ANOVA followed by Bonferroni post hoc test. While RF3286 had no effect when administered alone, it completely blocked the development of hyperalgesia induced by morphine (AUC = -22.75 ± 1.62 a.u. for morphine vs -7.93 ± 5.21 a.u. for morphine + RF3286; $p < 0.001$, one way ANOVA followed by Bonferroni post hoc test, Fig. 6C and 6D). We then examined the development of analgesic tolerance in those mice and considered the impact of RF3286 (Fig.6C, 6E-F). To this end, morphine (5 mg/kg, sc.) analgesic effect was measured in time-course experiments at day 1 and at day 7 (Fig. 6C, 6E-F), on mice daily pre-treated with either morphine or RF3286. As expected, at day 0, subcutaneous injection of morphine induced an increase of the tail withdrawal latency, reflecting acute analgesia that was not modified by administration of RF3286 (AUC = 11.79 ± 0.91 a.u. for morphine vs -0.70 ± 0.96 a.u. for RF3286 alone ; $p < 0.001$, one way ANOVA followed by Bonferroni post hoc test, Fig. 6C and 6E). After 8 days of treatment, the analgesic effect of morphine alone was strongly reduced (AUC = 12 ± 1 a.u. for morphine at D1 vs -3 ± 1 a.u. for morphine at D8 ; $p < 0.001$, one way ANOVA followed by Bonferroni post hoc test, Fig. 6C and 6F) indicating that tolerance did develop in these animals, while in mice co-administered with RF3286 and morphine tolerance was reduced (AUC = 7.54 ± 0.86 a.u for morphine + RF3286 at D8 vs -3.38 ± 1.45 a.u. for morphine at D8 ; $p < 0.001$, one way ANOVA followed by Bonferroni post hoc test, Fig. 6C and 6F). In a

second step, we performed the same experiment with female mice and obtained the same results (Fig. S3). Altogether our results show that NPFF1 receptor is critically involved in the development of opioid-induced hyperalgesia and analgesic tolerance following chronic opioid administration.

We then assessed the effect of NPFF1R receptor pharmacological blockade on the withdrawal syndrome induced by chronique morphine treatment in mice. Animals were treated with escalating doses of morphine (10 to 50 mg/kg, sc.) and co-administered with saline or increasing doses of RF3286 (1 to 5 mg/kg) for 5 days. 2 hours after the last administration the withdrawal syndrome was precipitated with naltrexone (1 mg/kg, sc.). Several behavioral and autonomic signs of withdrawal were scored including jumps, paw tremors, teeth chattering, diarrhea and weight loss (Fig. S4) and a global opiate withdrawal score (GOWS; (Papaleo and Contarino, 2006) was calculated for each group (Fig. 6G). In morphine-dependent mice, naltrexone increased significantly the apparition of the different signs of withdrawal compared to non-dependent mice while co-administration of escalating doses of RF3286 had no impact on these different signs of withdrawal (Fig. S4, Fig. 6G). This result suggests that NPFF1 receptor is not critically involved in the development of the naltrexone-precipitated physical signs of withdrawal induced by chronic morphine treatment.

In order to confirm the involvement of NPFF1R in neuroadaptive changes associated with chronic morphine administration, we generated knock out (KO) mice for NPFF1R gene (Fig S5) and studied their phenotype upon chronic morphine treatment. NPFF1R KO mice behaved normally and displayed no gross abnormality as observed in a full range of behavioral tests, metabolic evaluation and clinical observations (Fig. S6, S7 and S8). The basal nociceptive threshold of wild-type and knockout animals was not significantly different (Fig. 7). As shown in Figure 7, acute morphine administration induced similar analgesia in both genotypes (AUC = 29 ± 2 a.u. for WT vs 36 ± 2 a.u. for KO, ns, figure 7B) while

morphine-induced hyperalgesia and analgesic tolerance were significantly attenuated in NPFF1R KO compared to wild-type animals (AUC = -25 ± 3 a.u for WT vs -15 ± 3 for KO ; $p<0.05$, one_way ANOVA followed by Bonferroni post hoc test, Fig. 7D; AUC = -2 ± 3 a.u. for WT at D8 vs 15 ± 2 a.u. for KO NPFF1R at D8 ; $p<0.001$, one-way ANOVA followed by Bonferroni post hoc test, Fig. 7C). We then examined the effect of this deletion on naltrexone-precipitated withdrawal syndrome and observed no difference between wild-type and NPFF1R KO animals (Fig. 7E). These data are consistent with those obtained with RF3286 and clearly demonstrate the involvement of NPFF1R in nociceptive adaptations associated with chronic opioid treatment but not in the neuroadaptive modifications associated with behavioral and autonomic signs of morphine withdrawal.

In order to further investigate by which mechanism RFRP-3 and its receptor can modulate nociception and opioid analgesia, we decided to investigate by fluorescent in situ hybridization the presence of RFRP-3 and NPFF1R transcripts in the spinal cord and DRG in wild-type and NPFF1R KO mice. In the dorsal horn of the spinal cord, only NPFF1R receptor mRNA was detected in few cells while no signal was detected in NPFF1R KO mice (Fig. 8A and 8B). As expected, we also observed MOP transcript on the same sections but in a larger number of cells. No co-localization was observed between NPFF1R and MOR transcripts. In DRG of wild-type and NPFF1R KO animals, we observed no labeling with NPFF1R and RFRP-3 probes while MOP transcript was easily detected (not shown). Our data show for the first time that NPFF1R transcript is expressed in the dorsal horn of the spinal cord at the same level than MOP positive cells. Conversely, we did not detect RFRP-3 transcript in spinal cord and DRG suggesting that this ligand could be released by descending pain controls.

As RFRP-3/NPFF1R system has been involved in the modulation of reproduction of seasonal mammals (Henningsen et al., 2016), we further investigated the effect of RF3286 in male Syrian hamster. Several studies have shown that RFRP-3 is able to provoke LH release

in male Syrian hamster, contrary to other species or female Syrian hamster (Ancel et al., 2012; Simonneaux et al., 2013). We first evaluated the consequences of the administration of RF3286 alone on LH release. As shown in Figure 9 and conversely to RF9 that increased LH in blood (Elhabazi et al., 2017), RF3286 (10 nmol, icv.) did not induced LH release but rather reduced the basal level of LH that was observed in vehicule treated animals. This result clearly indicates that RF3286 at the dose used did not displayed any Kiss1R agonist activity and further suggests that endogenous tonic activation of NPFF1R is responsible for the basal level of LH that is observed in male Syrian hamster. As expected, acute central injection of RFRP-3 (10 nmol, icv.) stimulated the blood release of LH (Fig. 9) and RF3286 fully block this effect. Altogether these data highlight the involvement RFRP-3/NPFF1R in the control of LH blood levels in male Syrian hamster and further confirm that RF3286 is a highly selective NPFF1R receptor antagonist *in vivo* that will be useful to study the functions that are modulated by the RFRP-3/NPFF1R system in different species.

4. Discussion

We describe here the discovery of RF3286, the first small, high affinity antagonist for NPFF1R that displays a high selectivity *in vitro* and *in vivo* toward the other RFamide receptor as well as opioid receptor. Moreover, we show that upon peripheral administration this compound can cross the brain blood barrier to efficiently block central action of RFRP-3. Several studies have previously described small peptidic or heterocyclic molecules that display good affinity for and antagonist activity at NPFF receptors (for review see (Quillet et al., 2016). However, none of these compounds displayed a level of selectivity toward NPFF1R or NPPF2R that is good enough ($\geq$ 100) to allow to efficiently discriminate between both receptor subtypes *in vivo*. We showed here that RF3286 displays a selectivity of around 500 fold for human and mouse NPFF1R versus NPFF2R. Moreover, we showed that in mice

RF3286 efficiently reversed RFRP-3 induced hyperalgesia while it had no effect on NPFF-induced reversion of morphine analgesia. These results clearly demonstrate that RF3286 can efficiently block NPFF1R both in vitro and in vivo without blocking NPFF2R and is therefore a very useful pharmacological tool to delineate the role played by NPFF1R in the modulation of different functions, particularly those in which NPFF1 and NPFF2 receptor subtypes have been involved like the modulation of nociception and opioid analgesia.

Another issue to consider when developing a novel NPFF1R antagonist is the selectivity toward the other RF-amide receptors and particularly kisspeptin receptor. Indeed, previous studies aiming at understanding the role of NPFF1R in the modulation of reproduction have used the non selective NPFF1/2R antagonist RF9 (Simonin et al., 2006) in *in vivo* experiments (Caraty et al., 2012; Pineda et al., 2010b; Rizwan et al., 2012). In these studies, RF9 was shown to stimulate LH release suggesting that NPFF receptors and most likely NPFF1R subtype, which is expressed in GnRH neurons, tonically inhibits LH release and that blocking this tonic inhibition leads to an increase of LH. This conclusion was later questioned by several studies reporting agonist activity of RF9 at kisspeptin receptor (Kim et al., 2015; Min et al., 2015). Although this activity was observed at micromolar concentrations, while RF9 displays affinity at NPFF receptors in the 10 nmolar range, Min and collaborators clearly demonstrated that *in vivo* RF9 stimulation of LH release was mainly due to its agonist activity at kisspeptin receptor activity (Min et al., 2015). In this study we therefore examined the affinity of RF3286 at PrRP, QRFP and kisspeptin receptors and observed no or very low (> 50 μM) affinity for the three receptors. We further examined its activity at these receptors and detected no activity at PrRP and QRFP receptor while we observed a slight agonist activity at kisspeptin receptor at 10 μM. In order to address this issue *in vivo* we evaluated the activity of RF3286 in hamsters in condition in which RF9 was previously shown to strongly stimulate LH release (Elhabazi et al., 2017). As shown in Figure 5 we observed no

stimulatory effect of RF3286 indicating it did not display agonist activity at kisspeptin receptor in these conditions. Moreover, we showed that it efficiently block the activity of RFRP-3 on LH release showing that RF3286 is a useful pharmacological tool to study the involvement of NPFF1R in the modulation of reproduction without interfering with kisspeptin receptor function.

In this study we used RF3286 to study the role of NPFF1R/RFRP-3 in the modulation of nociception and opioid analgesia. Previous studies suggested that both NPFF1R/RFRP-3 and NPFF2R/NPFF systems are involved in the modulation of these functions but the absence of selective antagonists for these two receptor subtypes did not allow to clearly delineate their respective contribution (see (Ayachi and Simonin, 2014). For example RFRP-3, was reported to increase morphine analgesia upon icv. administration (Quelven et al., 2005) while experiments performed with NPFF1R antagonists that show some selectivity for NPFF1R versus NPFF2R (Lameh et al., 2010) rather suggested that NPFF1R/RFRP-3 system display pro-nociceptive action. In agreement with our previous observations (Elhabazi et al., 2013), we show in this study that icv. administration of RFRP-3 in mice induced a strong decrease of the nociceptive threshold, which was fully blocked by RF3286 indicating that this effect is mediated by NPFF1R. We further confirmed the pronociceptive action of NPFF1R/RFRP-3 system in experiments in which hyperalgesia was induced by actute (fentanyl) or chronic (morphine) opioid administration. In both experiments, NPFF1R receptor blockade with RF3286 fully prevented the development of opioid-induced hyperalgesia. Moreover, the development of morphine-induced analgesic tolerance was significantly attenuated by RF3286 but not fully blocked. These results strongly suggest that (i) NPFF1R/RFRP-3 system is a pronociceptive system, which activation is induced by opioids and is reponsible for the development of pain hypersensitivity associated with opioid administration; and (ii) opioid analgesic tolerance is at least partly due to the activation of NPFF1R/RFRP-3 system. These

conclusions were further supported by experiments performed in NPFF1R knockout mice showing an attenuation of morphine-induced hyperalgesia and analgesic tolerance both in males and females. It is notworthy that the consequence of NPFF1R genetic blockade on morphine-induced hyperalgesia appeared less drastic than pharmacological blockade of this receptor. This could be due to the fact that in this study we used constitutive KO animals in which the absence of NPFF1R since early developmental stages could have been compensated by other neuropetide/receptor systems like NPFF/NPFF2R and PrRP/PrRPR systems, which have already been shown to display anti-opioid properties (Laurent et al., 2005; Simonin et al., 2006).

Finally, we showed that the severity of behavioral and somatic signs of naloxone-precipitated withdrawal syndrome that develop after chronic morphine administration in mice was not affected neither by pharmacological nor by genetic blockade of NPFF1R. This result suggest that anti-opioid action of RFRP-3/NPFF1R is specific to nociceptive pathways. As we have shown previously that RF9 the mixed NPFF1R/NPFF2R antagonist efficiently attenuated opioid withdrawal syndrome (Elhabazi et al., 2013), it is tempting to speculate that this effect could be due specifically to NPFF/NPFF2R system. This hypothesis is in agreement with a previous study reporting that in rats NPFF significantly potentiates the overall morphine withdrawal syndromes (Tan et al., 1999).

In conclusion, we describe in this study the design of a highly selective NPFF1R antagonist that allowed us to identify the RFRP-3/NPFF1R system as an important player in the neuroadaptations that occurs upon exogenous opioid administration particularly in the nociceptive pathways. These results were further confirmed with NPFF1R KO animals. We also provide the first evidence that NPFF1R mRNA is expressed in the dorsal horn of the spinal cord in a cell population that is distinct from MOP-positive cells suggesting that the modulatory role of this receptor on opioid action could take place at this level. Overall, our

results point to selective NPFF1R antagonists as potential therapeutic tools to improve the effect of opiates for the treatment of chronic pain. In the future, the development of a highly selective antagonist for NPFF2R will be mandatory to better understand the respective role of RFRP-3/NPFF1R and NPFF/NPFF2R in the modulation of nociception and opioid analgesia.

Materials and methods

Materials

Human RF-amide peptides were from Polypeptide (Strasbourg, France; Kp-10, NPFF, PrRP-20), Peptanova (Sandhausen, Germany ; 43RFa) and Tebu-Bio (Le Perray-en-Yvelines, France ; 26RFa and RFRP-3). Mouse RFRP-3 was from Genecust (Luxembourg). Orphanin FQ (nociceptin), fentanyl citrate, naloxone hydrochloride, forskolin, 3-isobutyl-1-methylxanthine (IBMX) and probenecid were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Morphine hydrochloride was from Francopia (Paris, France) and Fluo-4 acetoxymethyl ester was from Molecular Probes (Invitrogen, Cergy Pontoise, France). D-luciferin was purchased from Synchem (Felsberg-Altenburg, Germany). N-adamantanecarbonyl-Arg-Phe-NH_2 trifluoroacetate (**RF9**) was synthesized as reported (Simonin et al., 2006).

[^{3}H]-PrRP-20 (150 Ci/mmol) and [^{35}S]Guanosine 5'-*O*-[g-thio] triphosphate ([^{35}S]GTPgS; 1250 Ci/mmol) were from Hartmann Analytic (Braunschweig, Germany). [^{125}I]-1DMe-NPFF (2200 Ci/mmol), [^{125}I]-Kp-10 (2200 Ci/mmol), [^{125}I]-QRFP43 (2200 Ci/mmol), [Leucyl-^{3}H]-nociceptin (123 Ci/mmol) and [^{3}H]-Diprenorphine (53 Ci/mmol) were purchased from Perkin Elmer Life and Analytical Sciences (Courtaboeuf, France).

Methods

General solid-phase peptide synthesis procedures

Fmoc-protected natural amino acids were purchased from commercial suppliers. All chemicals and solvents were used without further purification. All peptides were synthesized by manual solid-phase synthesis by using a Fmoc strategy with a Rink-amide resin SS (Advanced ChemTech, 0.70 mmol/g). The side-chain protective groups included t-butyl (tBu) for tyrosine, t-butoxycarbonyl (Boc) for lysine and tryptophane, Trityl (Trt) for histidine,

asparagine and glutamine, and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for arginine. Fmoc deprotection was performed by using a solution of 2% piperidine and 2% DBU in DMF. With natural amino acids, coupling reactions were performed by the addition of N,N-diisopropylethylamine (DIEA, 6 equiv), Fmoc-protected amino acid or carboxylic acid (3 equiv), and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU, 2.9 equiv) in DMF, and the mixture was agitated for 1 h. All coupling reactions were monitored by Kaiser test. Resin cleavage was performed by using a TFA/thioanisol/triisopropylsilane (92:5:3) solution. The mixture was filtered, and the peptide solution was concentrated in vacuo, and precipitated by adding cold diethyl ether. The mixture was centrifuged, and the resulting crude peptide was purified by flash chromatography (Simply connect C18 column (50g, 40 – 60 µm) from AIT; Phase A: 0.05% TFA in water; B : MeOH; eluent : phase B : from 10 to 100% in 30 min). Final compounds were recovered with purity > 95% by HPLC.

Pharmacokinetic study of RF3286 in mice

5mg/kg of RF3286 was injected (sc.) in C57Bl/6n mice and blood was drawn by intracardiac puncture at different time points (n =3 per point), from 5 minutes to 2 hours. Blood samples were then placed into EDTA-coated tubes and centrifuged at 12,000g (4°C), during 10 minutes and the supernatant were collected and immediatly placed at -80°C. For the detection of RF3286, 40µL of each plasma sample was mixed with 100 µL of acetonitrile to precipitate and extract the compound. Samples were stirred for 3 minutes, sonicated for 3 minutes and then centrifuged at 15,000g (4°C) during 5 minutes to sediment the proteins. Surpernatants were transfered in a microplate before LC-MS/MS analysis.

Receptor cDNA constructs, cell expression and membrane preparations

Human NPFF1R, NPFF2R and Kiss1R cDNAs were subcloned into the pCDNA3.1 expression vector (Invitrogen, Cergy Pontoise, France) and transfected into CHO cells before

selection for stable expression, as reported (Elhabazi et al., 2013). CHO cells expressing human PrRPR and QFRPR were a gift from M. Parmentier (IRIBHM, Brussells, Belgium).

cDNAs encoding human NPFF1R, NPFF2R or each of the four opioid receptors (mu, MOR; delta, DOR; kappa, KOR; Orphanin FQ/Nociceptin, OFQR) were further transfected into HEK293 cells stably expressing the cAMP GloSensor^{TM} before G418 selection for stable receptor expression. Membranes from CHO cells expressing RF-amide receptors were prepared as reported (Elhabazi et al., 2013). Membranes from HEK293 cells with transient or stable expression of human mu, delta or kappa opioid receptors were obtained as previously described (Becker et al., 1999; Simonin et al., 2006). All these membrane preparations were stored at –80°C as aliquots (1 mg prot./mL) until use in radioligand binding experiments.

Radioligand binding assays

RF-amide receptors

Competition experiments were conducted essentially as described (Elhabazi et al., 2013). Briefly, 5 to 10 µg of membranes from cells expressing the different receptors were incubated at 25°C in 96-well plates with the appropriate radioligand and increasing concentrations of unlabelled peptides or test compounds under binding equilibrium conditions. [^{125}I]-1DMe-NPFF (0.015nM) was used to label NPFF1 and NPFF2 receptors. [^{3}H]-PrRP-20 (0.3 nM), [^{125}I]-Kp-10 (0.02 nM) and [^{125}I]-43RFa (0.03 nM) were used to label PrRP, Kiss1 and QRFP receptors, respectively. Dilution and binding buffers for [^{125}I]-1DMe-NPFF, [^{3}H]-PrRP-20, [^{125}I]-Kp-10, and [^{125}I]-43RFa were composed of Hepes pH 7.4 50mM, $MgCl_2$ 1mM, $CaCl_2$ 1mM, BSA 0.10% ; Hepes pH 7.4 50mM, $MgCl_2$ 5mM, $CaCl_2$1mM, BSA 0.50% ; Tris-HCl pH 7.4 50mM, $MgCl_2$10mM, EDTA 1mM, BSA 0.50% and Tris-HCl pH 7.4 50mM, Bacitracine 100µg/mL, Saponine 10µg/mL, BSA 0.50%, respectively . Washing buffers for [^{125}I]-1DMe-NPFF, [^{3}H]-PrRP-20, [^{125}I]-Kp-10, and [^{125}I]-43RFa were composed of Hepes pH 7.4 25mM, $MgCl_2$ 1mM and $CaCl_2$ 1mM ; Hepes pH 7.4 25mM,

$MgCl_2$ 5mM, $CaCl_2$ 2mM and NaCl 0.5M ; Tris-HCl pH 7.4 50mM and Tris-HCl pH 7.4 50mM, NaCl 0.5M, respectively. Membrane-bound radioactivity was separated from free radioligand by rapid filtration through GF/B filtermats presoaked in 0.5% PEI for [^{125}I]-1DMe-NPFF ; 0.1% PEI for [^{3}H]-PrRP-20 and [^{125}I]-Kp-10 ; and Tris-HCl pH 7.4 50mM, BSA 0.50% and PEI 0.10% - diluted in washing buffer - using a cell harvester (Perkin Elmer Life and Analytical Sciences, Courtaboeuf, France). Filter-bound radioactivity was quantified using a TopCount scintillation counter (Perkin Elmer).

Opioid receptors

Opioid receptor-rich membranes were incubated at 25°C with [^{3}H]-Diprenorphine (0.6 nM; MOR, DOR and KOR) or [^{3}H]-OFQ/Nociceptin (0.15 nM; OFQR) and increasing concentrations of compounds to be tested. Dilution and binding buffers for [^{3}H]-Diprenorphine and [^{3}H]-OFQ/Nociceptin were respectively composed of Tris-HCl pH 7.4 50mM, EDTA 1mM and Tris-HCl pH 7.4 50mM, BSA 0.10%. Washing buffer (Tris-HCl pH 7.4 50mM) and saturation buffer (PEI 1% diluted in washing buffer) are common for both radioligands. Non specific binding was defined in the presence of 10 µM naloxone (MOR, DOR and KOR) or OFQ/Nociceptin (OFQR). Membrane-bound radioactivity was determined by scintillation counting as described above.

[^{35}S]GTPgS binding

Stimulation by endogenous RF-amide peptides of [^{35}S]GTPgS binding to membranes from CHO cells expressing NPFF1R and its inhibition by test compounds was examined as previously reported (Simonin et al., 2006) .

cAMP accumulation

Variations in the overall cAMP content of HEK cells expressing NPFF1 and NPFF2 receptors were monitored using the GloSensor^{TM} cAMP assay according to manufacturer

recommendations (Promega, Madison WI, USA) with a few modifications. HEK cells were suspended (10^6 cells per mL) in physiological Hepes buffer (10 mM HEPES, 0.4 mM NaH_2PO_4, 137.5 mM NaCl, 1.25 mM $MgCl_2$, 1.25 mM $CaCl_2$, 6 mM KCl, 10 mM glucose and 1 mg/mL bovine serum albumin, pH 7.4) supplemented with 1 mM D-Luciferin. After equilibration for 2 h at 25°C, cells were recorded in real time for their luminescence levels in 96-well plates using a FlexStation^R 3 (Molecular Devices, Sunnyvale CA, USA). The agonist/antagonist nature of test compounds was determined according to two distinct experimental paradigms. In the agonist mode, compounds were injected at various concentrations 15 min before forskolin (0.4 µM final concentration) and readings were pursued for 90 min. In the antagonist mode, compounds were allowed to pre-incubate with cells for 15 min before the addition of prototypical agonists for NPFF1R and NPFF2R (RFRP-3, NPFF). Experiments were conducted at 25°C in the presence of 0.1 mM IBMX to prevent the degradation of cAMP by phospodiesterases. According to their preferential coupling to $G_{i/o}$ proteins, the stimulation of NPFF1 and NPFF2 receptors by agonists was monitored as a dose-dependent reduction in steady-state luminescence levels, reflecting the inhibition of forskolin-induced cAMP accumulation.

Calcium mobilization

CHO cells stably expressing G_q-coupled receptors (PrRPR, Kiss1R and QFRPR) were loaded with 2.5 µM Fluo-4 AM in the presence of 2.5 mM probenecid, as described (Elhabazi et al., 2013). Compounds were serial diluted in physiological Hepes buffer and antagonists were pre-incubated with cells for 10 min before agonist challenge. Agonist-evoked increases in intracellular calcium were recorded over time at 37 °C through fluorescence emission at 520 nm using a FlexStation 3 microplate reader. Peak response amplitudes were normalized to basal and maximal (cells permeabilized with 20 µM digitonin) fluorescence levels.

Label-Free dynamic mass redistribution assay

HEK293 cells expressing NPFF1 receptor were seeded at 10,000 per well in Perkin Elmer 6057408 plate pre-coated during 45 minutes with 15µL of collagen 60µg/mL and containing 10µL of DMEM medium. After 24h of culture in a cell culture incubator at 37°C with 5% CO_2, cells were washed 4 times in PBS with Robot Biomek FXP and plate were incubated 2h in Enspire reader to equilibriate the temperature. Agonists and antagonists were dissolved in Hepes/BSA buffer and deposited in Greiner 784201 plate. After a baseline of 6 minutes, cells were incubated 15 minutes with antagonist and then 90 minutes with agonist, while DMR measurements were recorded.

Animals

Male or female C57BL/6N mice (25-30g weight; Janvier Labs, Le Genest St Isle, 53941 Saint Berthevin Cedex, France) were housed in groups of five per cage and kept under a 12h/12 h light/dark cycle at 21 ± 1°C with *ad libitum* access to food and water. Experiments were performed during the light-on phase of the cycle. Mice were habituated to the testing room and equipment and handled for 1-2 weeks before starting behavioural experiments. Control and treated group assignment as well as pain responses measurements were performed in a blinded manner. Every animal was used only once. At the end of experiments, animals were euthanized with pentobarbital sodium (120 mg/kg, i.p.) or CO_2 (fill rate of 20% of the chamber volume per minute). Adult male Syrian hamsters were bred in-house and maintained under a 14h/10h light/dark cycle at 22 ± 2°C with free access to food and water. All experiments were carried out in strict accordance with the European guidelines for the care of laboratory animals (European Communities Council Directive 2010/63/EU) and approved by the local ethical committee. All efforts were made to minimize animal discomfort and to reduce the number of animals used.

Drug administration

All drugs were dissolved in physiological saline (0.9%) and administered subcutaneously (sc.; 10mL/kg mice bodyweight), or intracerebroventricular as previously described (Haley and McCormick, 1957). RF3286 (10 nmol), morphine (1.5 nmol), RFRP-3 (10 nmol), NPFF (10 nmol) were administered (alone or in combination; 5 μL total volume) in the left lateral ventricle using a modified Hamilton syringe. Proper injection site was verified in pilot experiments through administration and localization of methylene blue dye. Male hamsters were prepared for i.c.v. injections as reported (Ancel et al., 2012). A stainless steel 30-gauge cannula was placed in the lateral ventricle and animals were allowed to recover from surgery for one week, prior to further i.c.v. injections.

Measurement of nociception in mice

The nociceptive threshold of naive and treated mice was assessed in the tail immersion test (Elhabazi et al., 2012; Simonin et al., 1998) by measuring the latency time to withdraw their tail from a thermostated (47 ± 0.5 °C) water bath. In the absence of any nociceptive reaction, a cut-off of 25 sec was set to prevent tissue damage.

Fentanyl-induced analgesia and hyperalgesia

Experiments were designed according to a protocol enabling the visualization of an early analgesic effect of opiates and of a delayed hyperalgesic state lasting for several days (Celerier et al., 2000; Elhabazi et al., 2012). The impact of the dose of RF3286 on the short-term analgesia to four consecutive injections of fentanyl (4 x 60µg/kg, sc., 15 minutes interval) was evaluated using the tail immerson test. RF3286 (0.005, 0.02 and 0.05 mg/kg) was administered subcutaneously 20 minutes before fentanyl and nociceptive threshold were measured every 1h after the last fentanyl injection, during 3h. The impact of RF3286 on long-term changes in nociceptive sensitivity induced by fentanyl injection on day 1 was evaluated using the tail immerson test once daily over a 6-days period, until recovery of the pre-drug baseline value.

Morphine-induced hyperalgesia and analgesic tolerance

On day 1, RF3286 (1mg/kg) or saline were administered (sc.) 20 minutes before morphine (5mg/kg, sc.) or saline. Nociceptive response of the animals was measured using the tail immersion test, at 30 min-interval, over a 0-180 min period following morphine or saline injections. Over the next 5 days, morphine (10 mg/kg, sc.) was administered once a day (9 am) and saline or RF3286 (1 mg/kg) were administered 3 times a day (9 am, 12 am and 6 pm). During this period, the nociceptive threshold of the animals was measured every day, 60 min before morphine injection. On day 7, we repeated the same procedure than on day 1.

Morphine dependence and withdrawal

A procedure of intermittent and escalating doses of morphine was used to induce morphine physical dependence. Mice were injected subcutaneously twice a day during 5 days with morphine (9 am and 5 pm). On the first day, mice received doses of 10 mg/kg at 9 am and 20 mg/kg at 5 pm. On the second day, mice received doses of 20 mg/kg at 9 am and 30 mg/kg at 5 pm. On the third day, mice received doses of 30 mg/kg at 9 am and 40 mg/kg at 5 pm. On the fourth day, mice received doses of 40 mg/kg at 9 am and 50 mg/kg at 5 pm. Finally, on the fifth day, mice received a unique dose of 50 mg/kg at 9 am and 2 h later the withdrawal syndrome was precipitated with naltrexone 5 mg/kg (sc.). Behavioral and Somatic signs of withdrawal were evaluated immediately after naloxone injection during a period of 30 min, as described (Maldonado et al., 1996). Body weight was measured before naltrexone administration and at the end of the observation period. To investigate the effect of RF3286 on morphine withdrawal syndrome, escalating doses of compound were injected 3 times a day and after the first injection of morphine, during 5 days as following : 1mg/kg at 9 and 12 am, and 2 mg/kg at 5 pm on the fist day ; 2 mg/kg at 9 and 12 am, and 3 mg/kg at 5 pm on the second day; 3 mg/kg at 9 and 12 am, and 4 mg/kg at 5 pm on the third day; 4 mg/kg at 9 and 12 am, and 5 mg/kg at 5 pm on the fourth day; 5 mg/kg at 9 am on the fifth day.

Generation of RFamide receptor knock-out (KO) mice

KO mice for NPFF1R receptor were engineered at Mouse Clinical Institute at the University of Strasbourg by homologous recombination between a targeted vector and mouse embryonic stem cells. The targeting vector contained NPFF1R exon 4 flanked by LoxP sequences, and a Neomycin resistance cassette flanked by FRT sequences. Electroporation was used to introduce this targeting vector in embryonic stem cells. Then, cells were selected for their resistance to Neomycin. PCR and Southern blot experiments were used to select clones that undergo homologous recombination. A positive clone was implanted in Balb/cN mouse blastocyst and in a pseudo-pregnant mouse. Chimeric mice were crossed with C57Bl/6N mice constitutively expressing Flip Recombinase, an enzyme that delete fragment between FRT sequence. Animals generated will present floxed exon 4 NPFF1R and deleted Neomycin resistance cassette. Finally, these mice were crossed with C57Bl/6N mice constitutively expressed Cre recombinase (CMV-Cre mice), that delete fragment between LoxP sequences allowing the generation of NPFF1R exon 4 knockout mice.

Genotyping protocol : Ear fragments from WT and KO mice were incubated with protease K in a reaction medium containing 200 µL of lysis buffer (1M Tris-HCl pH 8.5 ; 0.5 M EDTA ; SDS 10% ; 5M NaCl) and 4 µL proteinase K (20 mg/mL Fermentas), overnight at 55°C in rotation in an oven. Then, PCR reactions were performed using specific primers for exon 4 (Lf : TGCTGCTGCTCATCGACTAC ; Lr : AGAAGGCCAGCCAGTGTG) in a reaction mixture containing : reaction buffer, 4 x10 mM NTP, 50 mM $MgCl_2$, Go Taq polymerase Promega, 10 mM of each primer, genomic DNA. PCR reactions were realised in 28 cycles : 1 x 94°C for 3 min ; 28 x (94°C for 30 sec ; 68°C for 30 sec ; 72 °C for 1 min) ; 1 x 4°C, ∞. Amplified DNA fragments were analyzed on an agarose gel (1.5%) after staining with Ethidium Bromide (0.5 µg/mL) and UV visualization.

In situ hybridization

Lumbar spinal cord and dorsal root ganglia of WT and KO NPFF1R mice were removed and directly incorporated in OCT (optimum cutting temperature) medium and frozen in dry ice. Tissues were cryosectionned (14 µM ; -20°C) and collected on Superfrost Plus glass slides (Fisher, Pittsburg, PA, USA). Commercially available RNAscope Multiplex fluorescent v2 kits and probes were purchased directly from Advanced Cell Diagnostics (Hayward, CA, USA). In situ hybridization probes were designed against mouse NPFF1R exon 4 (bp 432-1294 ; NM_001177511.1 ; C1), mouse MOPr (Mm-Oprm1 ; C2), and mouse RFRP-3 (bp 2-557 ; NM_021892.1 ; C3). Different fluorophores were assigned to the C1, C2, C3 channels depending on the TSA plus fluorophores (Perkin Elmer) selected diluted at the optimized concentration (C1 : TSA Plus Cyanine 3, 1/1500 ; C2 : TSA Plus Cyanine 5, 1/3000 ; C3 : TSA Plus Fluoresceine, 1/1500). In situ hybridization was performed according to the manufacturer's recommendation with slight optimization. Briefly, a post-fixation step (freshly made pre-chilled 4% paraformaldehyde at 4°C during 1h) was followed by 3 wash with PBS (3*1 min, RT) and dehydration with ethanol bath (50%-70%-100%-100%, 5 min each, RT). After slides pretreatment with Protease Pretreat 4 (Advanced Cell Diagnostics, Hayward, CA, USA) at RT for 40 min for spinal cord samples and 20 min for DRG samples, hybridization was performed at 40°C for 2h in a HybEZ oven (Advanced Cell Diagnostics, Hayward, CA, USA). Following wash and amplification steps, DAPI was used to label nuclei and Prolong Gold antifade reagent was used to mount coverslips. Images were acquired using a confocal laser scanning microscope (Leica TSC SPE objective 20X) and analyzed with the ImageJ software.

Measurement of the effect of drugs on LH secretion in the male Syrian hamster

I.c.v. injections of drugs, alone or in combination, were performed (2µL/animal; flow rate 0.15µL/h) in the morning under light anaesthesia with isoflurane vapour. 30 min after injection, hamsters were deeply anesthetized with CO_2 vapor and the blood was taken by intracardial puncture for subsequent LH dosage using a validated radioimmunoassay (Tena-Sempere et al., 1993).

Data and statistical analyses

Binding and functional data were analyzed using Prism 5.02 (GraphPad Software, San Diego, CA, USA). Occupancy curves were fit to a four-parameter logistic equation to derive top and bottom plateau values, the drug concentration producing 50% of the maximal effect (EC_{50} value) or inhibiting 50% of specific binding or actual response (IC_{50} value) and the midpoint Hill slope. When the slope was not significantly different from unity, it was constrained as such. IC_{50} values of drugs derived from binding competition experiments were converted into equilibrium dissociation constants (K_i values) using the Cheng and Prusoff relationship. Antagonist-induced rightward shifts of agonist dose-response curves were globally fitted to the Schild equation to derive the pA_2 value as well as Hill and Schild slopes. If not significantly deviating from 1, the slope parameters were constrained to 1 enabling the assimilation of the pA_2 value for a competitive antagonist to pK_B, the negative log of its equilibrium dissociation constant for the receptor.

Data for nociceptive tests are expressed as mean values ± S.E.M. for 6 to 15 animals depending on the group. Analgesia and hyperalgesia were quantified as the area under the curve (AUC) calculated by the trapezoidal method (Celerier et al., 2000). Data were analyzed using one-way or two-way analysis of variance (ANOVA). Post-hoc analyses were performed with Bonferonni's post-hoc test. The level of significance was set at $P < 0.05$. All statistical analyses were carried out using the StatView software.

LH levels are mean values ± S.E.M. from 7 hamsters. Statistical analyses were performed using Statistica (StatSoft Inc., Tulsa, OK). Data were analyzed by *t* test or one-way ANOVA, followed by *post hoc* analysis: Tukey's honestly significant difference test, as appropriate. Statistical significance was set at $p < 0.05$.

Disclosures

The authors declare no conflict of interest.

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Table 1: binding affinity constants at human NPFF1 and NPFF2 receptors of the different compounds designed and characterized in this study

Cpd	R	Xaa	NPFF1R	NPFF2R	Selectivity
			Binding affinity constant (K_i, nM)		K_i R2 / K_i R1
RF480	H	^{L}Phe	7.2 ± 2.8	175 ± 45	24
RF487	H	^{D}Phe	73 ± 15	2,150 ± 154	29
RF479	Ph	^{L}Phe	4.3 ± 0.3	205 ± 55	48
RF3296	Ph	^{D}Phe	7.2 ± 1.0	727 ± 130	101
RF3283	Ph	^{L}Phe(2Br)	0.73 ± 0.03	3.3 ± 0.9	5
RF3295	Ph	^{D}Phe(2Br)	10 ± 1	84 ± 11	8
RF3297	Ph	^{D}Trp	6.3 ± 0.6	267 ± 88	42
RF3289	Ph	^{L}Trp	21 ± 3	153 ± 187	7
RF3288	Ph	^{L}Tyr	32 ± 8	512 ± 80	16
RF3284	Ph	^{L}hPh	6.6 ± 1.0	557 ± 220	84
RF3294	Ph	^{D}Ala	16 ± 10	557 ± 122	35
RF3285	Ph	^{L}Ala	79 ± 5	6,035 ± 3,735	76
RF3299	Ph	^{D}Leu	523 ± 672	3,045 ± 1963	6
RF3286	Ph	^{L}Leu	4.7 ± 0.7	2,213 ± 163	471
RF3287	Ph	Gly	11.2 ± 1.3	1,685 ± 257	150
RF3290	Ph	^{L}Val	68 ± 42	4,775 ± 1,562	70
RF3292	Ph	^{L}Ile	39 ± 9	1,492 ± 338	38
RF3300	Ph	^{L}Cha	591 ± 664	1,113 ± 674	2
RF3298	Ph	^{L}Nle	18 ± 6	805 ± 296	45
RF3291	Ph	^{L}Gln	58 ± 15	1,742 ± 380	30
RF3293	Ph	^{L}Asn	132 ± 46	4,838 ± 1,125	37
RF9			14	18	1.3

GJ14	58 ± 2	255 ± 5	4
RFRP-3	0.01	0.9	90
NPFF	0.06	0.02	0.3

Binding affinity constants (Ki) are from competition experiments performed with [^{125}I]-1DMeNPFF according to material and methods. Ki values are means ± SEM for of least 2 independent experiments performed in duplicate

Table 2 : Binding affinity constants of RF3296, RF3286 and RF3287 for mouse NPFF1 and NPFF2 receptors

Compound	mNPFF1R	mNPFF2R	Selectivity
	Binding affinity constant (K_i, nM)		K_i R2 / K_i R1
RF3296	22 ± 2	1,450 ± 150	166
RF3286	8 ± 1	4,300 ± 100	538
RF3287	45 ± 6	2,750 ± 50	61
GJ14	72	821	11
RFRP-3	0.18	nd	
NPFF	nd	1.1	

Data were obtained from at least two independent experiments performed in duplicates. Ki values are mean ± S.E.M. Nd: not determined

Table 3. binding affinity constants of RF3296, RF3286 and RF3287 at the five human RF-amide receptors and the four human opioid receptors

Compound	RF-amide receptors			Opioid receptors			
	PrRPR	Kiss1R	QRFPR	MOPr	DOPr	KOPr	NOPr
	Binding affinity constants (K_i, nM)			Binding affinity constants (K_i, nM)			
RFRP-3	ND	ND	ND	ND	ND	ND	ND
NPFF	ND	ND	ND	ND	ND	ND	ND
PrRP-20	2.1 ± 0.4[a]	ND	ND	ND	ND	ND	ND
Kp-10	ND	0.062±0.009[a]	ND	ND	ND	ND	ND
26RFa	ND	ND	2.04 ± 0.58[a]	ND	ND	ND	ND
DAMGO	ND	ND	ND	14.6 ± 3.8[a]	ND	ND	ND
DPDPE	ND	ND	ND	ND	57.1 ± 18.8[a]	ND	ND
Naloxone	ND	ND	ND	ND	ND	8.4 ± 4.3[a]	ND
Nociceptin	ND	ND	ND	ND	ND	ND	0.0013±0.0002[a]
RF3296	> 50,000	> 50,000	> 5,000	> 1,000	> 5,000	>1,000	> 1,000
RF3286	> 50,000	> 50,000	> 50,000	> 5,000	> 50,000	> 10,000	> 5,000
RF3287	> 50,000	> 50,000	> 50,000	> 5,000	> 50,000	> 20,000	> 50,000
GJ14	ND	>5,000	ND	>10,000	>30,000	2,685 ± 1,745	>30,000

Data are means ± S.E.M. of at least 2 independent experiments performed in duplicate. [a] from Drieu la Rochelle *et al.*, in press

Figure legends

Figure 1. General formula of the compounds that were synthesized and characterized in this study

Figure 2. Competition binding experiments with RF3296, RF3286 and RF3287 on human and mouse NPFF1R and NPFF2R receptors.

Competition experiments were performed with [^{125}I]-1DMeNPFF as radiotracer and increasing concentrations of RF3296, RF3286 and RF3287 on membrane preparations from recombinant CHO-cells expressing human NPFF1R (**A**), human NPFF2R (**B**), mouse NPFF1R (**C**) or mouse NPFF2R (**D**). Experiments on each receptor were performed at least 3 times in duplicate. A representative experiment is shown for each receptor.

Figure 3. In vitro functional characterization of RF3286 and RF3287

A: Inhibition of forskolin-induced cAMP accumulation in HEK293-Glo2hNPFF1R cells induced by RFRP-3 or RF3286 and RF3287 alone.

B, C: Inhibition of forskolin-induced cAMP accumulation in HEK293-Glo2hNPFF1R cells induced by RFRP-3 alone or in increasing concentration of RF3286 (**B**) or RF3287 (**C**). Shild plots are shown in inset.

D-G: Experiments of dynamic mass redistribution (DMR) induced by a dose effect of RFRP-3 alone (**D**), RF3286 alone (**E**) RFRP-3 with RF3286 1µM (**F,G**) in HEK293-hNPFF1R cells. Representative data of at least two experiments performed in duplicate are shown here.

Figure 4. Evaluation of pharmacokinetic properties of RF3286.

RF3286 (5 m/kg, sc.) was administered to mice and blood samples were obtained from 3 mice for each time point. Plasmatic concentration of RF3286 was by measured in each sample by LC-MS/MS (see Methods). Data are means ± S.E.M.

Figure 5. In vivo selectivity of RF3286.

A: Intracerebroventricular injections of RFRP-3 (10 nmol) and RF3286 (10 nmol) alone or in combination were performed in mice, and nociceptive threshold of the animals was measured every 15 minutes post-injection using the tail immersion test.

B: Comparison of AUC between the different groups over the total time-course. Data are expressed as mean ± SEM, n=6-8. *$p < 0.05$; **$p < 0.01$ by one way ANOVA test followed by a Bonferroni post hoc test.

C: Intracerebroventricular injection of morphine (1.5 nmol) and blockade of the morphine analgesia by the co-administration of NPFF (10 nmol). Effect of RF9 (10 nmol, icv.) and RF3286 (10 nmol, icv.) on the prevention of morphine analgesia by NPFF.

D: Comparison of AUC between the different groups over the total time-course. Data are expressed as mean ± SEM, n=6-8. *$p < 0.05$; **$p < 0.01$; $p < 0.001$ by one way ANOVA test followed by a Bonferroni post hoc test.

E: Intracerebroventricular injections of RFRP-3 (10 nmol), alone or in combination with RF3286 (5 mg/kg, sc.), were performed in mice and nociceptive threshold of the animals was measured at different time intervals during 3h using the tail immersion.

F: Comparison of AUC between the different groups over the total time-course. Data are expressed as mean ± SEM, n = 8. ***$p < 0.001$; **$p < 0.01$ by two-way ANOVA (A) or one way ANOVA (B) test followed by a Bonferroni post hoc test.

Figure 6. Effect of RF3286 on acute and chronic administration of opiates.

A: Effect of RF3286 on analgesic and hyperalgesic responses of mice to fentanyl. On day 1, different doses of RF3286 (0.005 to 0.05 mg/kg, sc.) were administered to mice 20 minutes before fentanyl injections (4 x 60 µg/kg; 15 min interval; sc.). Nociceptive response of the animals was measured using the tail immersion test every 30 min. after fentanyl injection until return to baseline and then once daily from D2 to D7.

B: AUC from D2 to D4 (without analgesic measure) were calculated for each group. Data are expressed as mean ± SEM, n=8-14. *$p < 0.05$ by one-way ANOVA followed by a Bonferroni post hoc test.

C: Effect of RF3286 on morphine-induced analgesia, hyperalgesia and morphine tolerance in male mice. On the first day, RF3286 (1 mg/kg) or saline were injected 20 min before morphine (5 mg/kg) and tail withdrawal latencies were measured, at 1 h interval, over a 4 h period after morphine injection. From D2 to D7, mice received daily injections of RF3286 (3 x 1 mg/kg) and morphine (10 mg/kg) alone or in combination. Basal nociceptive values were measured every day before treatment. On day 8, mice received either saline or RF3286 20 min prior to saline or morphine (5 mg/kg) injections, and nociceptive responses were measured as on day 1.

D,E,F,G: Comparison between tested groups of AUC values over the 0-4 h time course, for secondary hyperalgesia (**D**), first day analgesia (**D**) tolerance (**F**) and global opiates withdrawal score (GOWS, **G**). Data are expressed as mean ± SEM, n = 7-8. *$p < 0.05$, ***$p < 0.001$ by one-way ANOVA followed by a Bonferroni post hoc test.

Figure 7. Effect of NPFF1R genetic deletion on morphine-induced analgesia, hyperalgesia and morphine tolerance in WT and KO NPFF1R mice.

A: On the first day, Morphine (5 mg/kg) was injected and tail withdrawal latencies were measured, at 1 h interval, over a 4 h period after morphine injection. From D2 to D7, mice received daily injections of morphine (10 mg/kg). Basal nociceptive threshold of the animals was measured every day before treatment. On day 8, mice received morphine (5 mg/kg) injection, and nociceptive responses were measured as on day 1. ***$p < 0.001$, *$p < 0.05$ by two-way ANOVA followed by a Bonferroni post hoc test.

B,C,D,E: Comparison between tested groups of AUC values for analgesia (**B**), secondary hyperalgesia (**C**), tolerance (**D**) and GOWS (**E**). Data are expressed as mean ± SEM, n = 7-8. *$p < 0.05$ by t-test; ***$p < 0.001$ by one-way ANOVA followed by a Bonferroni post hoc test.

Figure 8. In situ hybridization for MOP, NPFF1R and RFRP-3 transcripts in spinal cord of WT and KO NPFF1R mice.

Fluorescent in situ hybridization of MOP (cyan), NPFF1R (red) and RFRP-3 (green) in spinal cord of WT (**A, C**) and KO NPFF1R (**B, D**) mice. Cell nuclei are visualized in blue using DAPI staining. High magnification images show target expression of MOP and NPFF1R but not RFRP-3 in spinal cord of WT (**A**). In spinal cord of NPFF1R KO (**B**), MOP expression is maintained while NPFF1R transcrip is absent.

Figure 9. Effects of central injection of RFRP-3 and RF3286 on LH secretion in the male Syrian hamster.

RFRP-3 and RF3286 (10 nmol) were injected i.c.v., alone or in combination, in a 2 µL final volume. 30 min later, the blood was sampled and assayed for LH content (ng/mL plasma) as described under Materials and methods. Data are expressed as mean ± SEM for 6-8 animals. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA followed by Fisher's test.

Figure 1

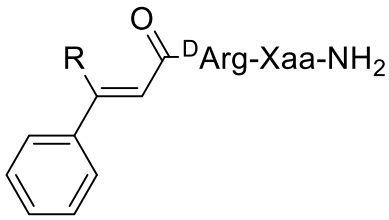


Figure 2

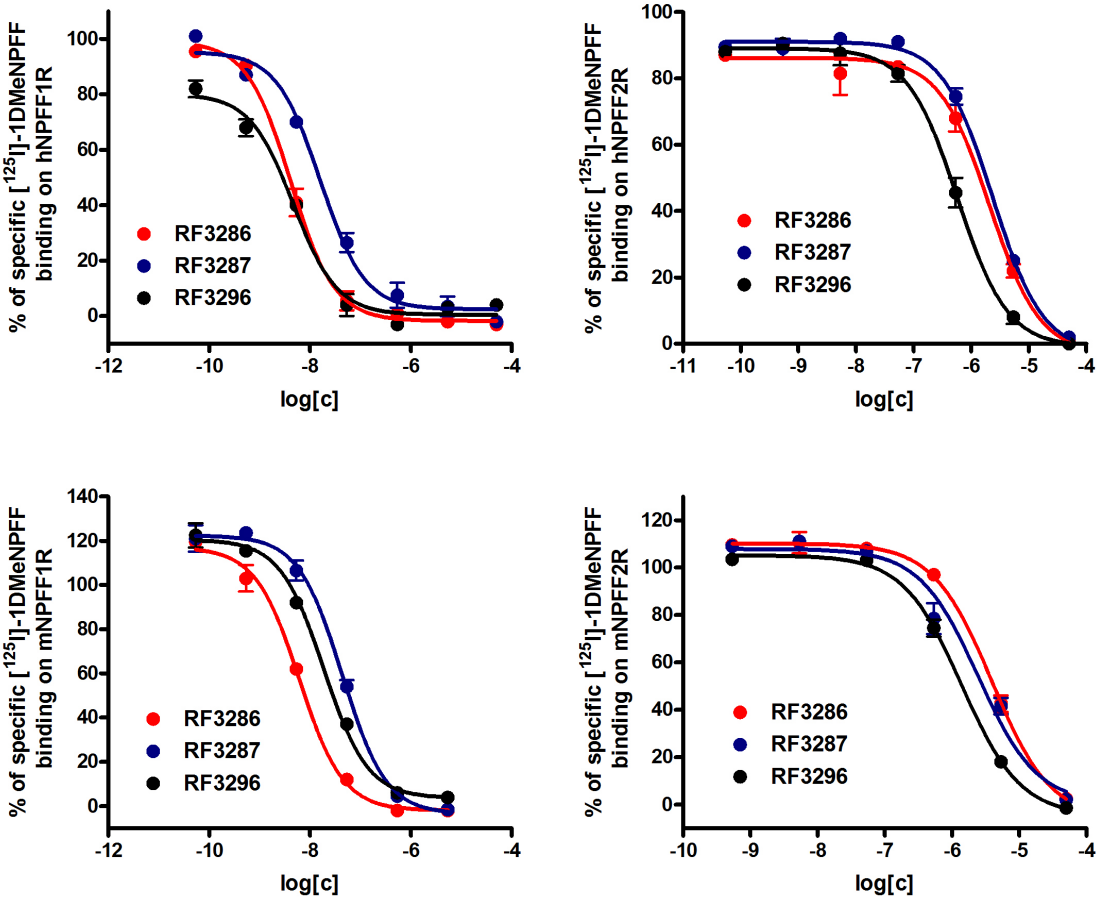

% of specific [125I]-1DMeNPFF binding on hNPFF1R
RF3286
RF3287
RF3296
log[c]
% of specific [125I]-1DMeNPFF binding on hNPFF2R
RF3286
RF3287
RF3296
log[c]
% of specific [125I]-1DMeNPFF binding on mNPFF1R
RF3286
RF3287
RF3296
log[c]
% of specific [125I]-1DMeNPFF binding on mNPFF2R
RF3286
RF3287
RF3296
log[c]

Figure 3

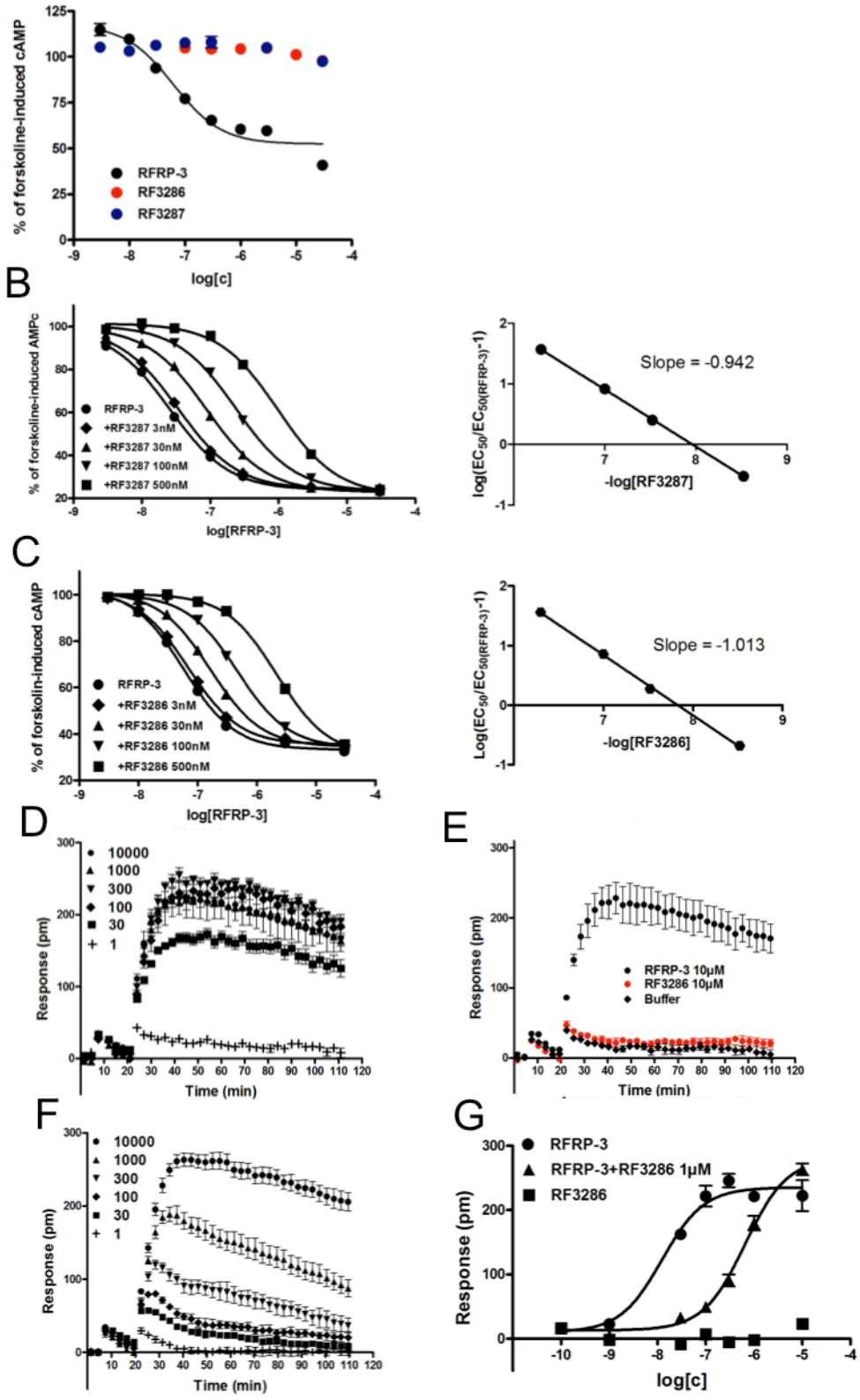

% of forskoline-induced cAMP
RFRP-3
RF3286
RF3287
log[c]
B
% of forskoline-induced AMPc
RFRP-3
+RF3287 3nM
+RF3287 30nM
+RF3287 100nM
+RF3287 500nM
log[RFRP-3]
log(EC50/EC50(RFRP-3)-1)
Slope = -0.942
-log[RF3287]
C
% of forskolin-induced cAMP
RFRP-3
+RF3286 3nM
+RF3286 30nM
+RF3286 100nM
+RF3286 500nM
log[RFRP-3]
Log(EC50/EC50(RFRP-3)-1)
Slope = -1.013
-log[RF3286]
D
Response (pm)
10000
1000
300
100
30
1
Time (min)
E
Response (pm)
RFRP-3 10μM
RF3286 10μM
Buffer
Time (min)
F
Response (pm)
10000
1000
300
100
30
1
Time (min)
G
Response (pm)
RFRP-3
RFRP-3+RF3286 1μM
RF3286
log[c]

Figure 4

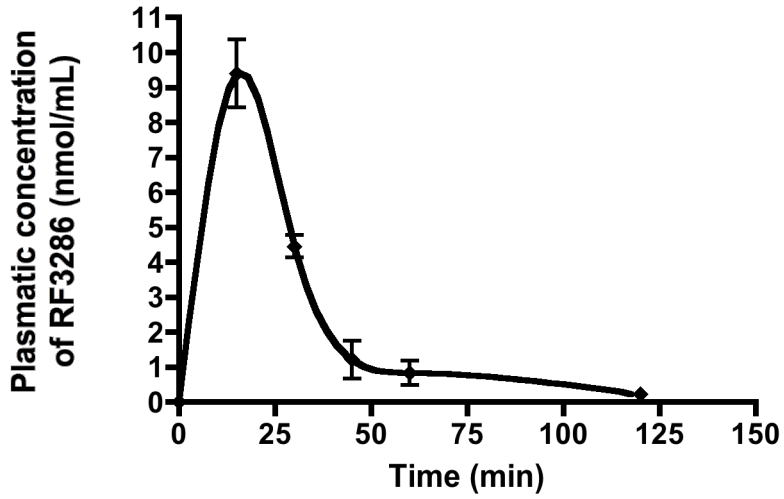

Plasmatic concentration of RF3286 (nmol/mL)
11
10
9
8
7
6
5
4
3
2
1
0
0
25
50
75
100
125
150
Time (min)

Figure 5

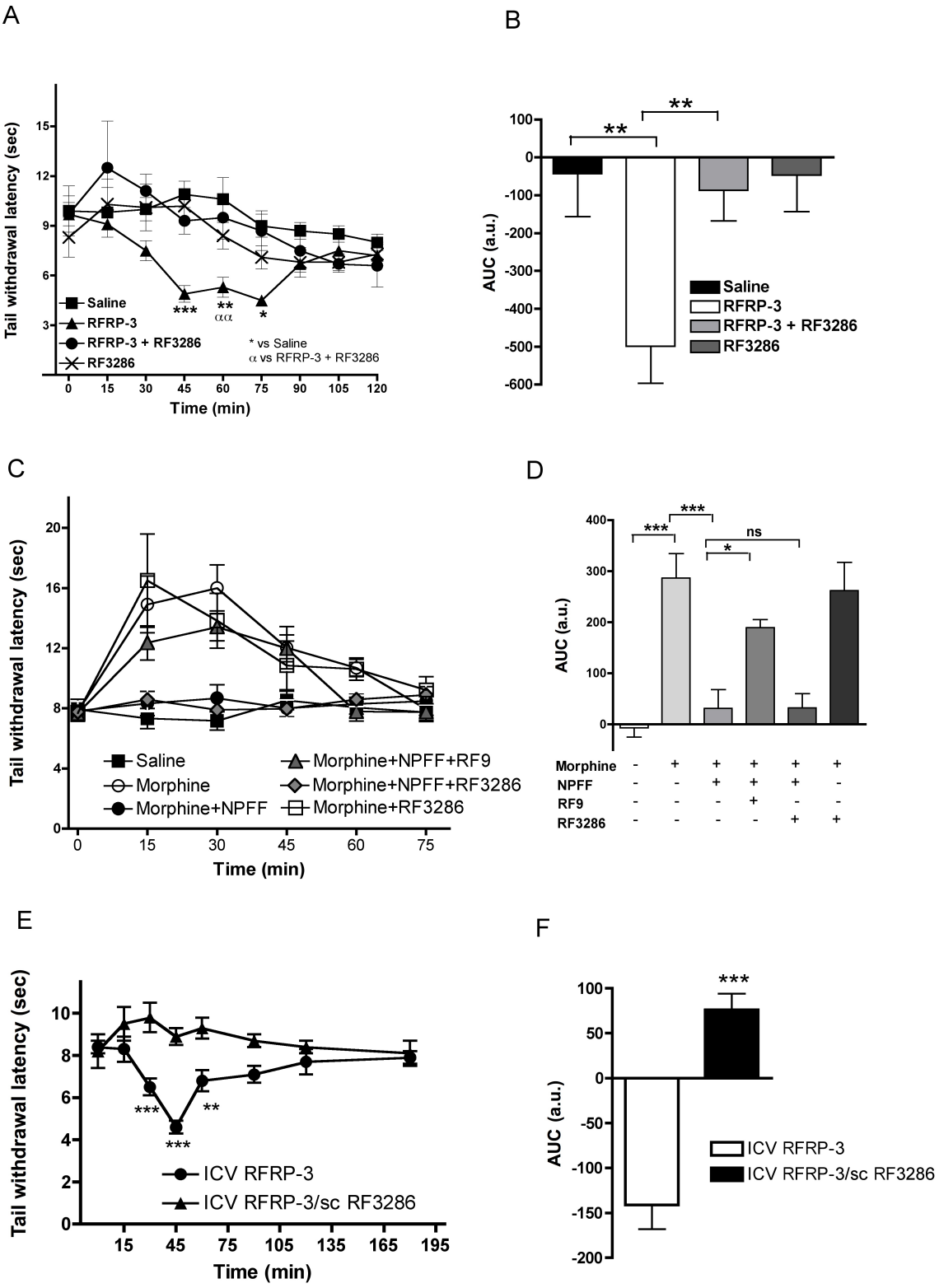

A
Tail withdrawal latency (sec)
Time (min)
Saline
RFRP-3
RFRP-3 + RF3286
RF3286
* vs Saline
α vs RFRP-3 + RF3286
B
AUC (a.u.)
Saline
RFRP-3
RFRP-3 + RF3286
RF3286
C
Tail withdrawal latency (sec)
Time (min)
Saline
Morphine
Morphine+NPFF
Morphine+NPFF+RF9
Morphine+NPFF+RF3286
Morphine+RF3286
D
AUC (a.u.)
Morphine
NPFF
RF9
RF3286
ns
E
Tail withdrawal latency (sec)
Time (min)
ICV RFRP-3
ICV RFRP-3/sc RF3286
F
AUC (a.u.)
ICV RFRP-3
ICV RFRP-3/sc RF3286

Figure 6

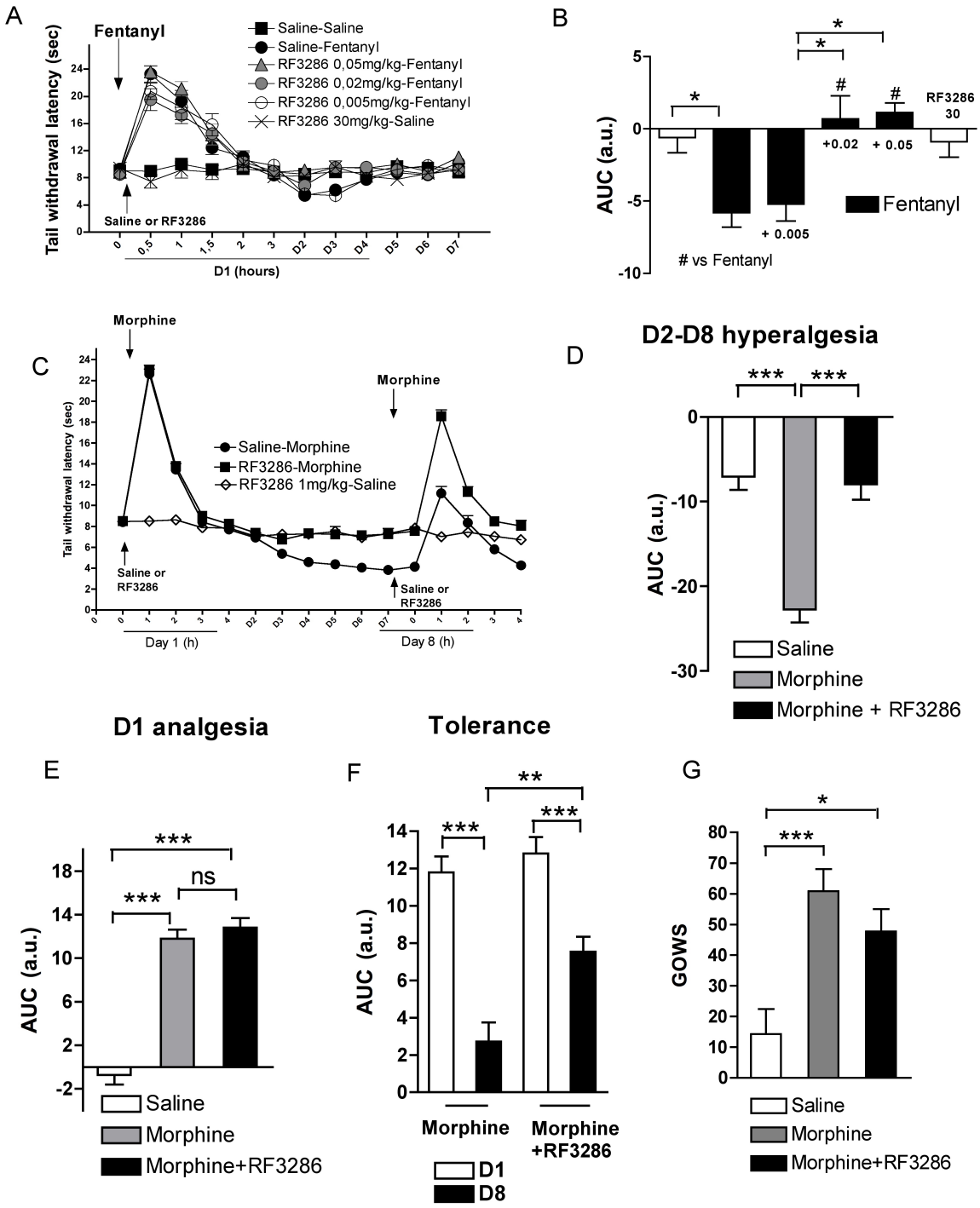

A
Fentanyl
Saline-Saline
Saline-Fentanyl
RF3286 0,05mg/kg-Fentanyl
RF3286 0,02mg/kg-Fentanyl
RF3286 0,005mg/kg-Fentanyl
RF3286 30mg/kg-Saline
Tail withdrawal latency (sec)
Saline or RF3286
D1 (hours)
B
AUC (a.u.)
RF3286 30
+0.02
+ 0.05
+ 0.005
Fentanyl
vs Fentanyl
C
Morphine
Morphine
Saline-Morphine
RF3286-Morphine
RF3286 1mg/kg-Saline
Tail withdrawal latency (sec)
Saline or RF3286
Saline or RF3286
Day 1 (h)
Day 8 (h)
D
D2-D8 hyperalgesia
AUC (a.u.)
Saline
Morphine
Morphine + RF3286
D1 analgesia
E
AUC (a.u.)
ns
Saline
Morphine
Morphine+RF3286
Tolerance
F
AUC (a.u.)
Morphine
Morphine +RF3286
D1
D8
G
GOWS
Saline
Morphine
Morphine+RF3286

Figure 7

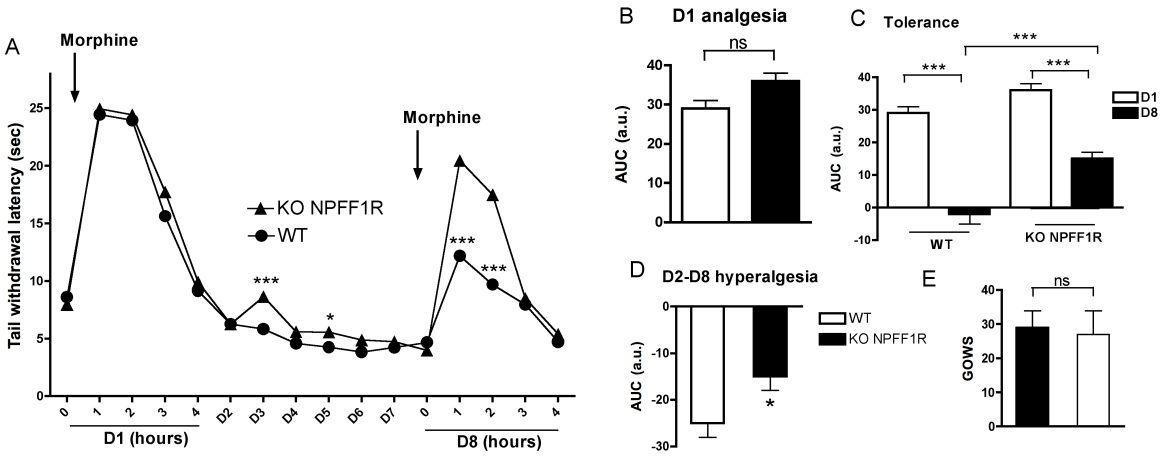
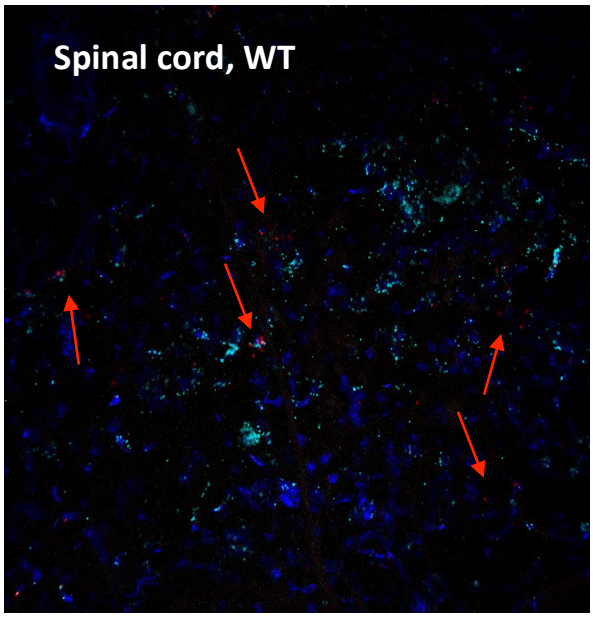

A
Morphine
Morphine
Tail withdrawal latency (sec)
KO NPFF1R
WT
***
*
***
***
D1 (hours)
D8 (hours)
B
D1 analgesia
ns
AUC (a.u.)
C
Tolerance
***
***
***
D1
D8
AUC (a.u.)
WT
KO NPFF1R
D
D2-D8 hyperalgesia
WT
KO NPFF1R
AUC (a.u.)
*
E
ns
GOWS

Figure 8

A



B

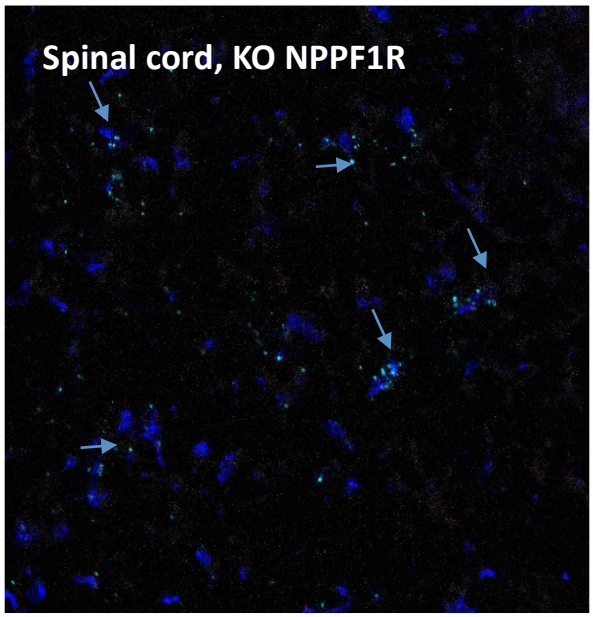
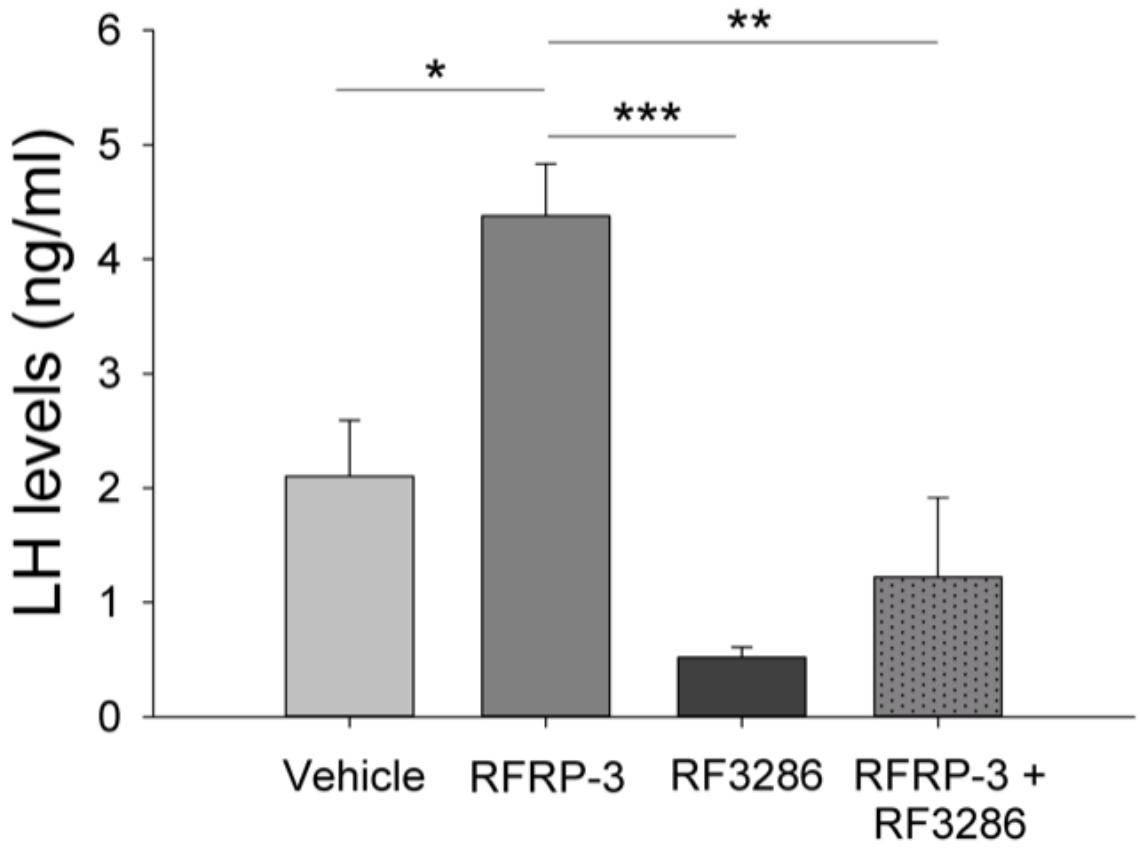


Figure 9



SUPPLEMENTARY INFORMATION

Figure S1. Antagonist activity of RF3286 on NPFF1R in a [^{35}S]-GTPgS assay. An increasing concentration of RF3286 rightward shifted the dose-response curve of RFRP-3 (left panel). Shild plot for RF3286 (right panel).

Figure S2. Functional *in vitro* characterization of RF3286 and RF3287 on hNPFF2R (cAMP assay), hKiss1, hPrRP and hQRFP receptors (Ca^{2+} mobilization assay). Neither agonist nor antagonist activity were observed for both compounds up to 10 µM on the four receptors, except a slight agonist activity of RF3286 Kiss1R at 10µM. A representative experiment from at least two independent experiments performed in duplicate is shown for each receptor.

Figure S3. Effect of RF3286 on morphine-induced analgesia, hyperalgesia and morphine tolerance in female mice.

A: On the first day, RF3286 (1 mg/kg) or saline were injected 20 min before morphine (5 mg/kg) and tail withdrawal latencies were measured, at 1 h interval, over a 4 h period after morphine injection. From D2 to D7, mice received daily injections of RF3286 (3 x 1 mg/kg) and morphine (10 mg/kg) alone or in combination. Basal nociceptive values were measured every day before treatment. On day 8, mice received either saline or RF3286 20 min prior to saline or morphine (5 mg/kg) injections, and nociceptive responses were measured as on day 1.

B,C,D: Comparison between tested groups of AUC values over the 0-4 h time course, for first day analgesia (**B**), secondary hyperalgesia (**C**) and tolerance (**D**). For AUC values, data are expressed as mean ± SEM, n=7-9. ***p<0.001 ; **p<0.01 by one-way ANOVA followed by Bonferroni post hoc test.

Figure S4. Behavioral and somatic signs of naloxone-precipitated morphine withdrawal signs in male mice. Comparison of morphine withdrawal signs in male mice chronically treated with saline, increasing doses of morphine and increasing doses of morphine and RF3286 as described in Material and Methods

Figure S5. Strategy for the generation and genotyping of NPFF1R-KO mice. Conditional NPFF1R-KO vector contains exon 4 flanked by LoxP sequences (black triangles), and FRT sequences (gray triangles). Conditionnal NPFF1R-KO mice were crossed with Cre-CMV mice to generate NPFF1R-KO mice. PCR primers are represented by black arrows. Neomycin resistance cassette deletion using FRT sequences is not shown. Results of genotyping PCR of 3 littermate mice (#1-3), using genomic DNA and 8 NPFF1R-KO mice (#4-11), using genomic DNA. WT gene was revealed at 309bp whereas KO NPFF1R gene was revealed at 393 bp.

Figure S6. General behavioral phenotyping of NPFF1R KO mice. No significant difference were observed between WT and NPFF1R KO mice concerning all the studied parameters.

Figure S7. Metabolic phenotyping of WT and NPFF1R KO mice. No difference were observed between WT and KO NPFF1R mice concerning energy expenditure, activity and food and water consumption.

Figure S8. Comparison of body composition, blood chemistry and blood hematology between KO NPFF1R and WT mice. Among all the evaluated parameters only small differences were observed for potassium and total bilirubin concentrations.

Figure S1

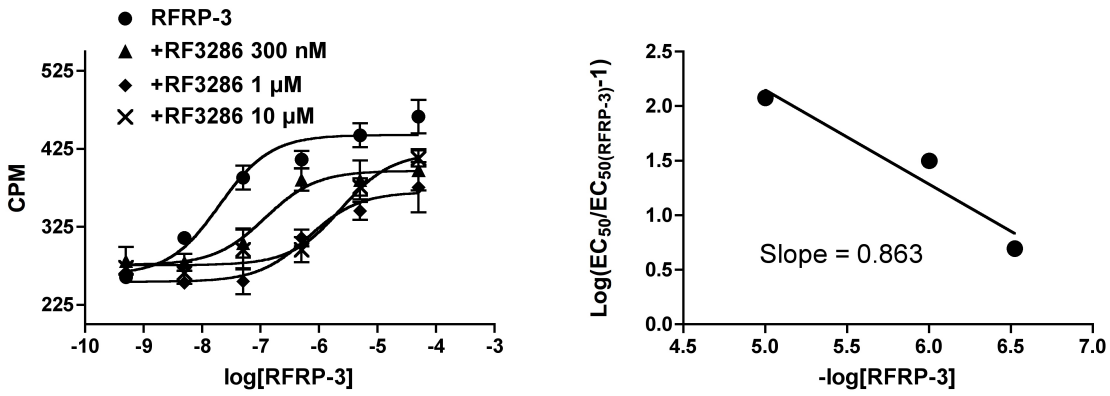

RFRP-3
+RF3286 300 nM
+RF3286 1 µM
+RF3286 10 µM
CPM
525
425
325
225
-10
-9
-8
-7
-6
-5
-4
-3
log[RFRP-3]
Log(EC50/EC50(RFRP-3)-1)
2.5
2.0
1.5
1.0
0.5
0.0
Slope = 0.863
4.5
5.0
5.5
6.0
6.5
7.0
-log[RFRP-3]

Figure S2

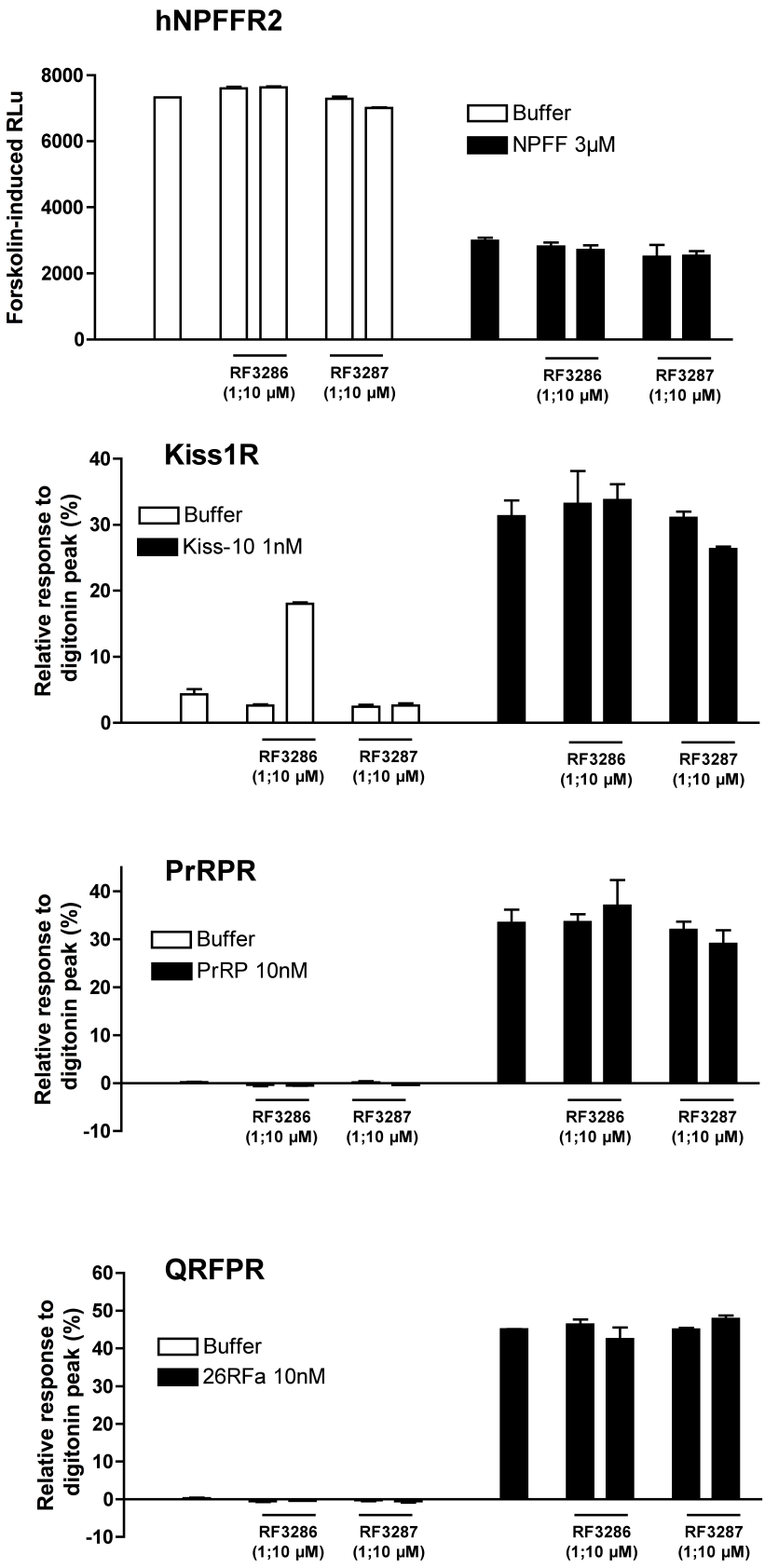

hNPFFR2
Forskolin-induced RLu
8000
6000
4000
2000
0
Buffer
NPFF 3µM
RF3286 (1;10 µM)
RF3287 (1;10 µM)
RF3286 (1;10 µM)
RF3287 (1;10 µM)
Kiss1R
Relative response to digitonin peak (%)
40
30
20
10
0
Buffer
Kiss-10 1nM
RF3286 (1;10 µM)
RF3287 (1;10 µM)
RF3286 (1;10 µM)
RF3287 (1;10 µM)
PrRPR
Relative response to digitonin peak (%)
40
30
20
10
0
-10
Buffer
PrRP 10nM
RF3286 (1;10 µM)
RF3287 (1;10 µM)
RF3286 (1;10 µM)
RF3287 (1;10 µM)
QRFPR
Relative response to digitonin peak (%)
60
50
40
30
20
10
0
-10
Buffer
26RFa 10nM
RF3286 (1;10 µM)
RF3287 (1;10 µM)
RF3286 (1;10 µM)
RF3287 (1;10 µM)

Figure S3

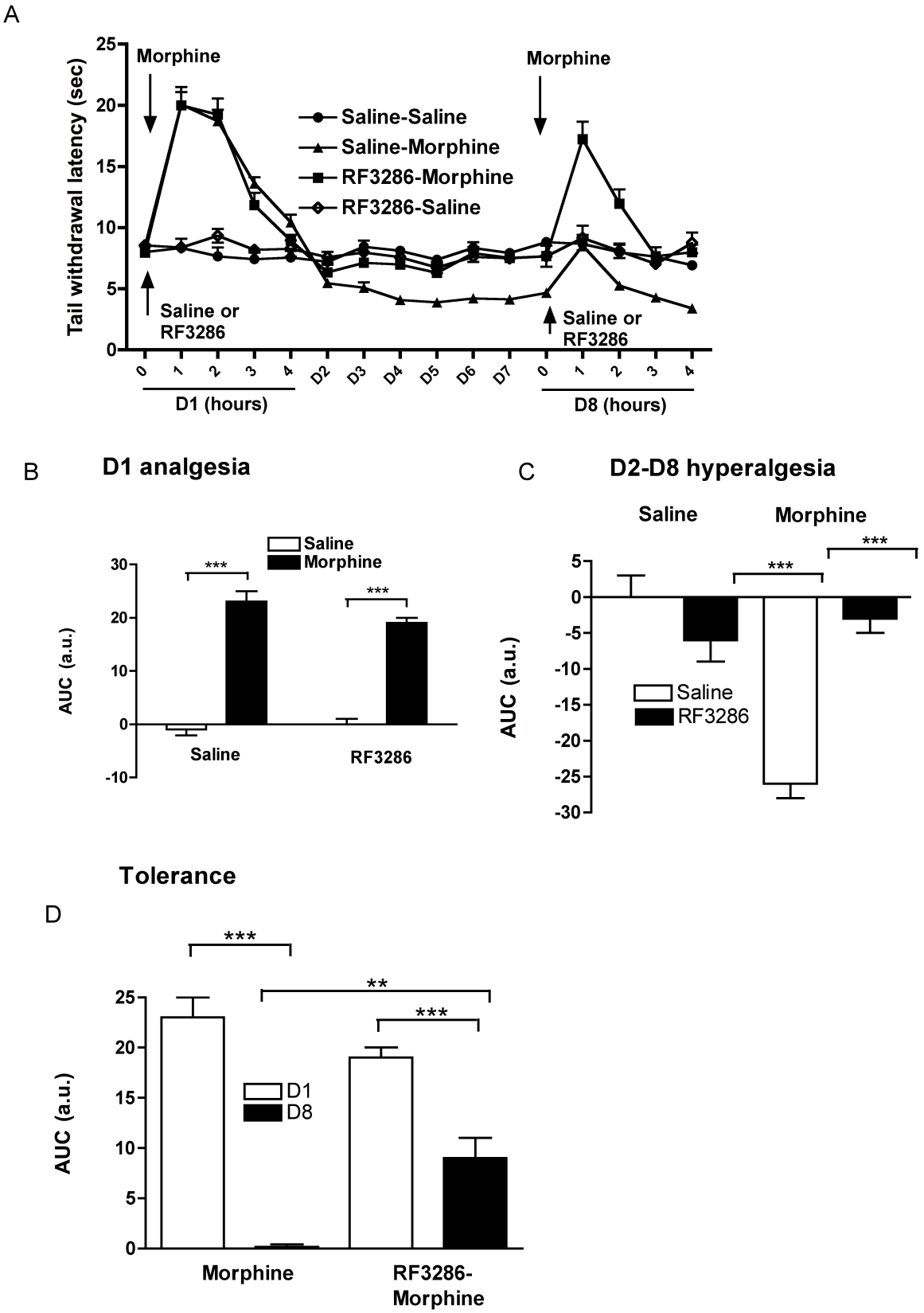

A
Tail withdrawal latency (sec)
Morphine
Morphine
Saline-Saline
Saline-Morphine
RF3286-Morphine
RF3286-Saline
Saline or RF3286
Saline or RF3286
D1 (hours)
D8 (hours)
B
D1 analgesia
Saline
Morphine
AUC (a.u.)
Saline
RF3286
C
D2-D8 hyperalgesia
Saline
Morphine
AUC (a.u.)
Saline
RF3286
D
Tolerance
AUC (a.u.)
D1
D8
Morphine
RF3286-Morphine

Figure S4

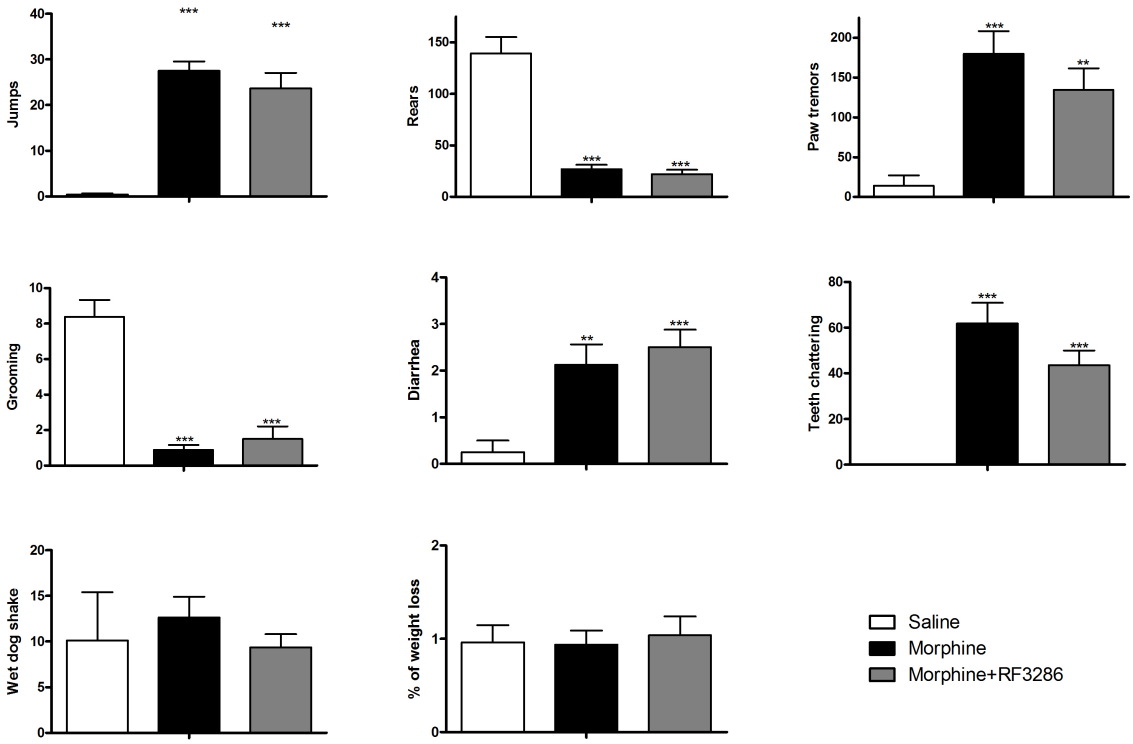

Jumps
Rears
Paw tremors
Grooming
Diarrhea
Teeth chattering
Wet dog shake
% of weight loss
Saline
Morphine
Morphine+RF3286

Figure S5

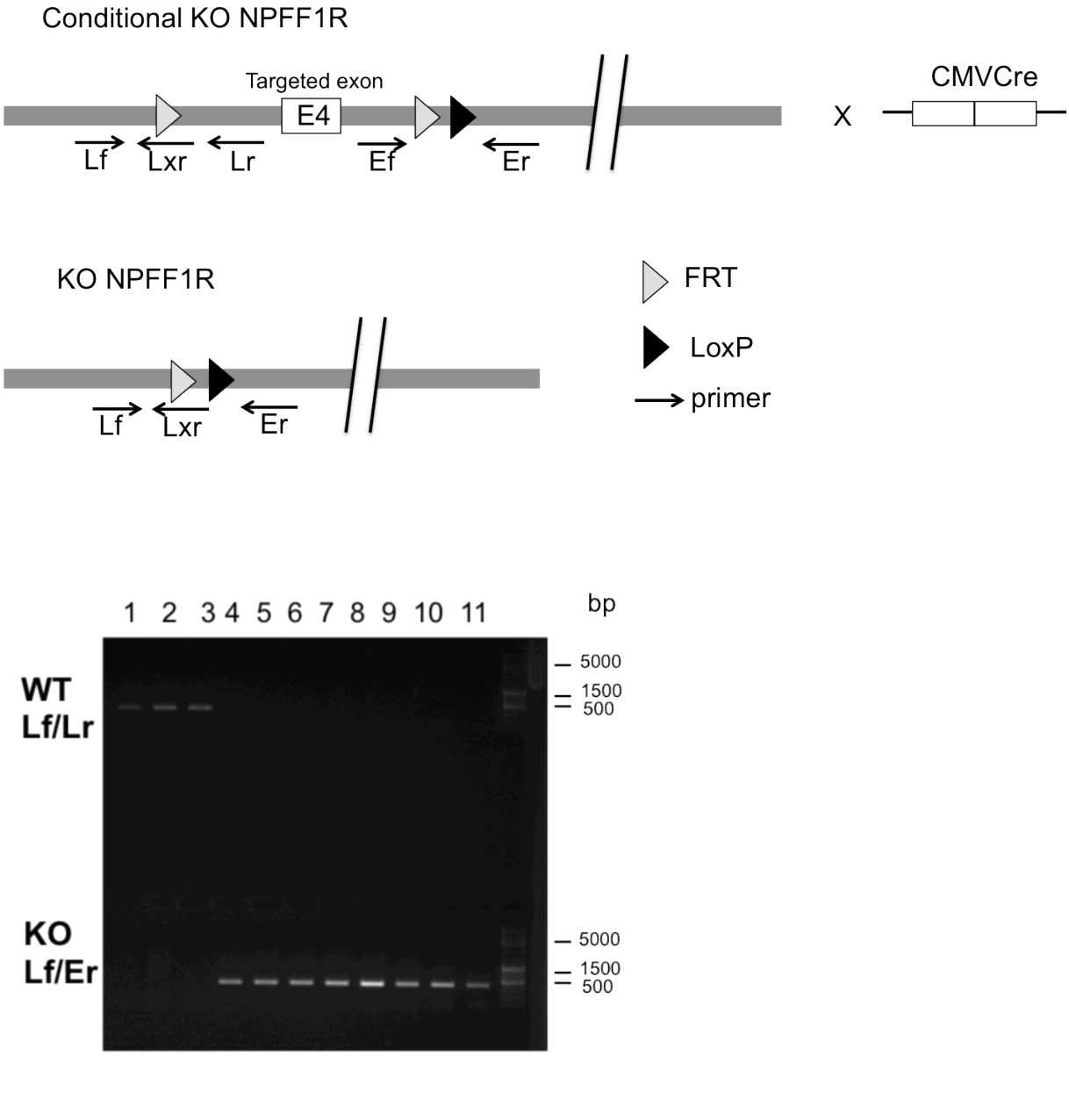
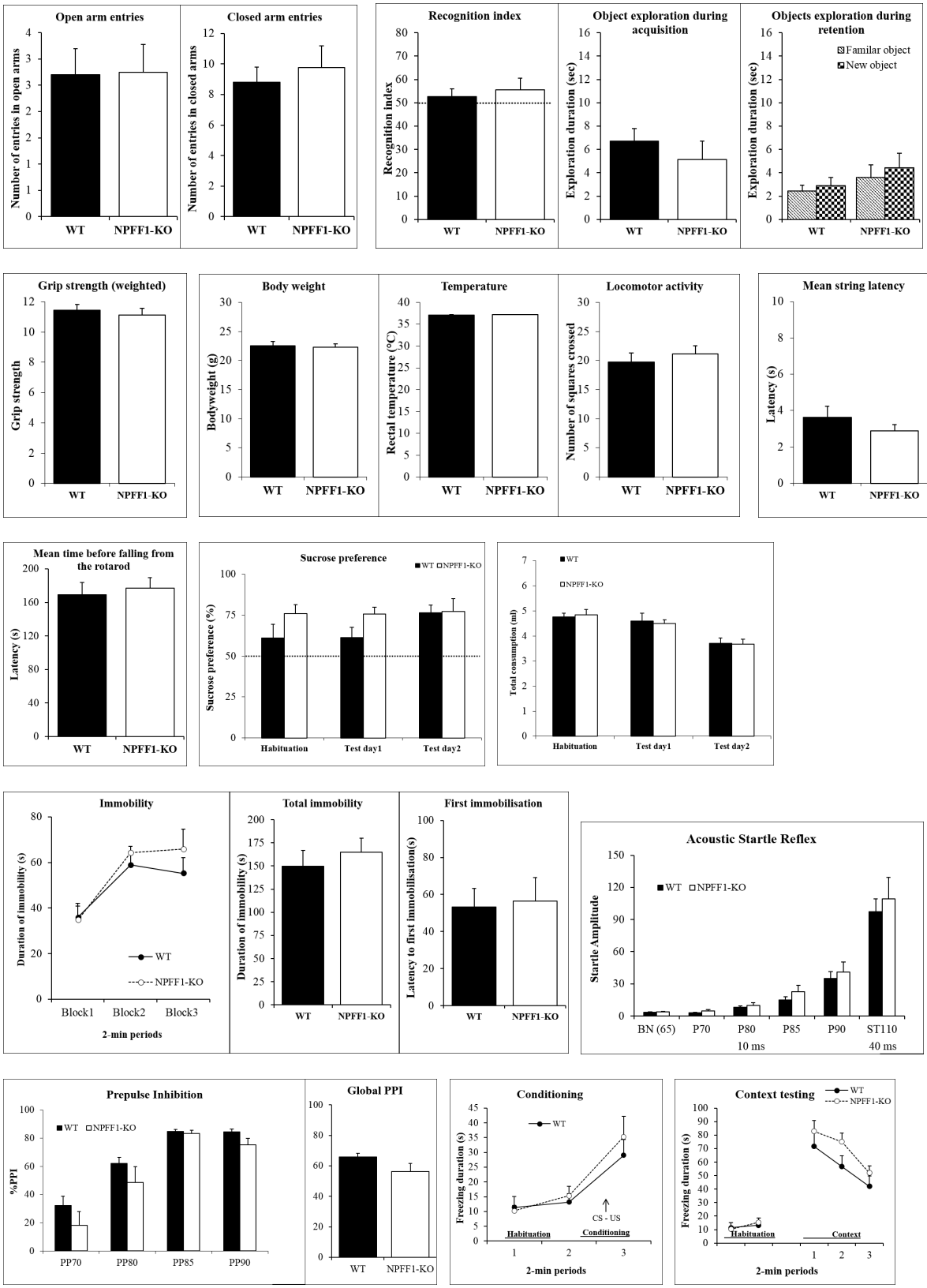
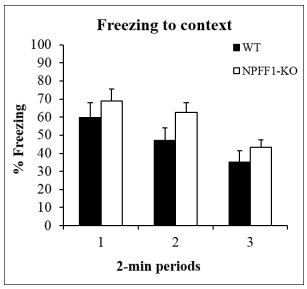
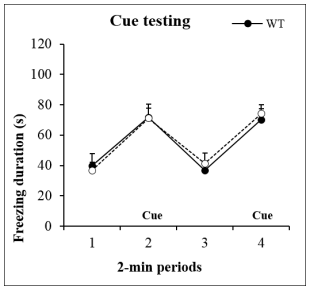
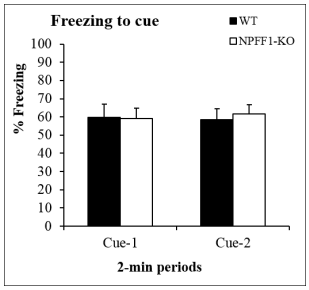

Conditional KO NPFF1R
Targeted exon
E4
Lf
Lxr
Lr
Ef
Er
X
CMVCre
KO NPFF1R
Lf
Lxr
Er
FRT
LoxP
primer
1 2 3 4 5 6 7 8 9 10 11
bp
WT
Lf/Lr
5000
1500
500
KO
Lf/Er
5000
1500
500

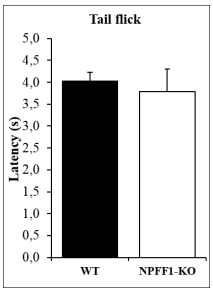
Figure S6

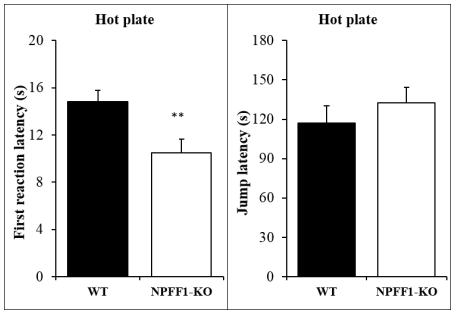

Open arm entries
Number of entries in open arms
WT
NPFF1-KO
Closed arm entries
Number of entries in closed arms
Recognition index
Object exploration during acquisition
Exploration duration (sec)
Objects exploration during retention
Familar object
New object
Grip strength (weighted)
Grip strength
Body weight
Bodyweight (g)
Temperature
Rectal temperature (°C)
Locomotor activity
Number of squares crossed
Mean string latency
Latency (s)
Mean time before falling from the rotarod
Sucrose preference
Sucrose preference (%)
Habituation
Test day1
Test day2
Total consumption (ml)
Immobility
Duration of immobility (s)
Block1
Block2
Block3
2-min periods
Total immobility
First immobilisation
Latency to first immobilisation(s)
Acoustic Startle Reflex
Startle Amplitude
BN (65)
P70
P80
P85
P90
ST110
10 ms
40 ms
Prepulse Inhibition
%PPI
PP70
PP80
PP85
PP90
Global PPI
Conditioning
Freezing duration (s)
CS - US
Context testing
Context

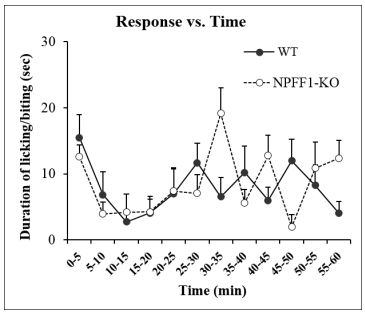

Freezing to context
WT
NPFF1-KO
% Freezing
2-min periods


Cue testing
WT
Freezing duration (s)
Cue
Cue
2-min periods


Freezing to cue
WT
NPFF1-KO
% Freezing
Cue-1
Cue-2
2-min periods


Tail flick
Latency (s)
WT
NPFF1-KO


Hot plate
First reaction latency (s)
**
WT
NPFF1-KO
Hot plate
Jump latency (s)
WT
NPFF1-KO


Response vs. Time
WT
NPFF1-KO
Duration of licking/biting (sec)
Time (min)

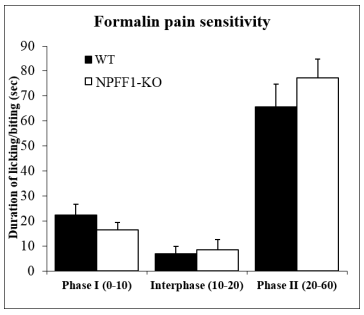
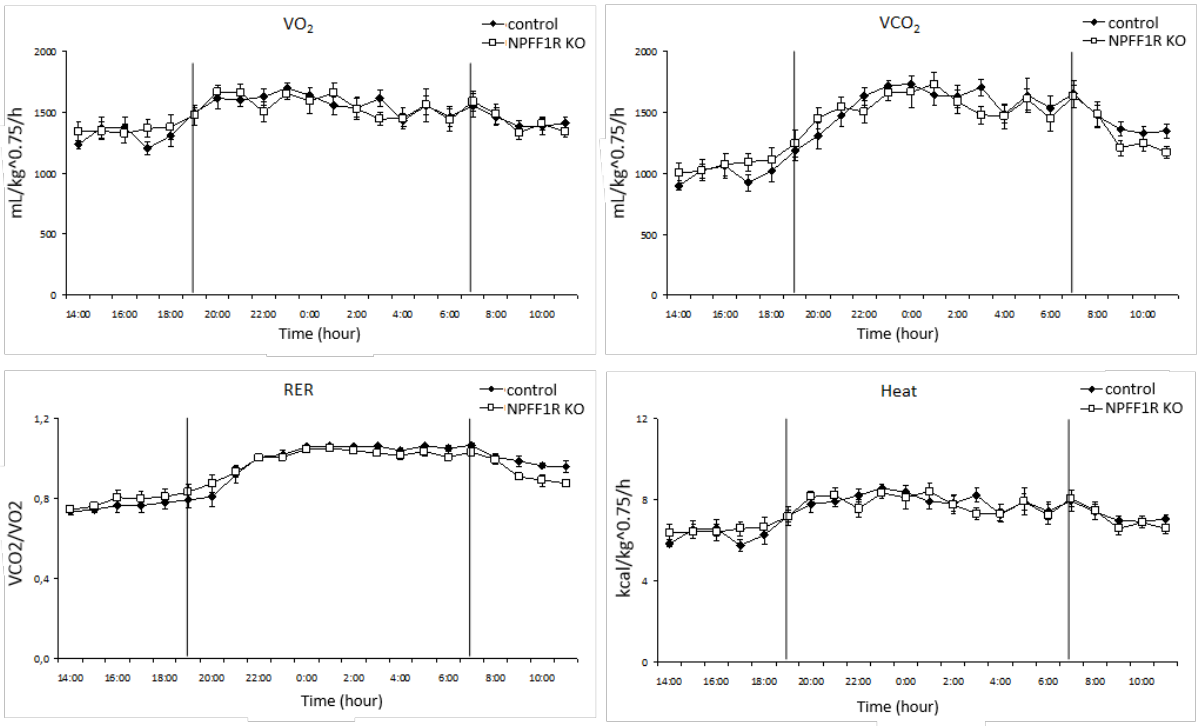
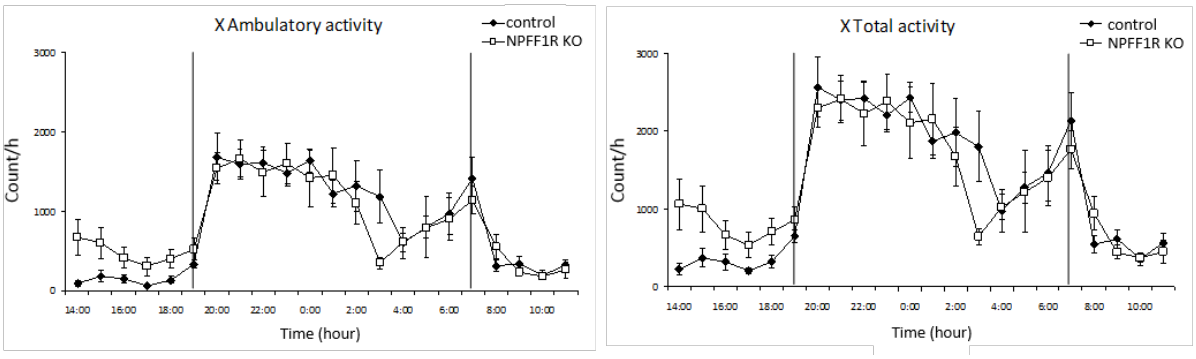

Formalin pain sensitivity
WT
NPFF1-KO
Duration of licking/biting (sec)
Phase I (0-10)
Interphase (10-20)
Phase II (20-60)

Figure S7

Energy expenditure



Activity



Food and water consumption

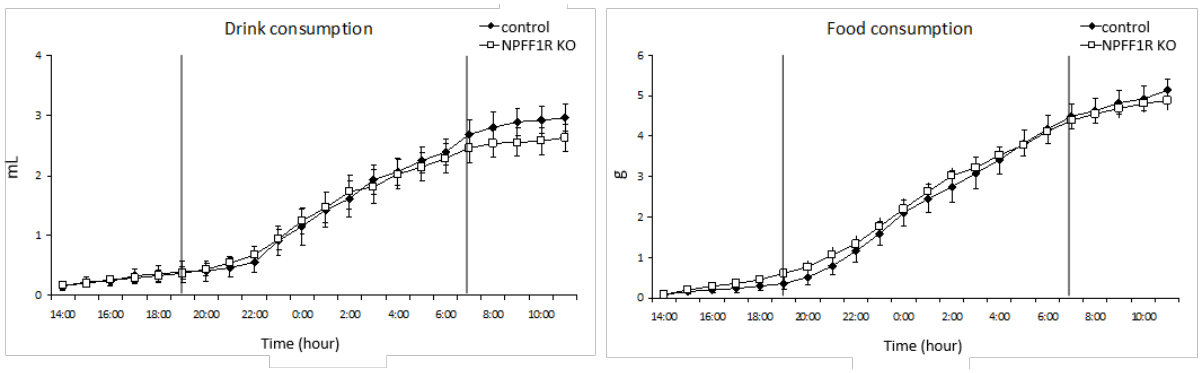
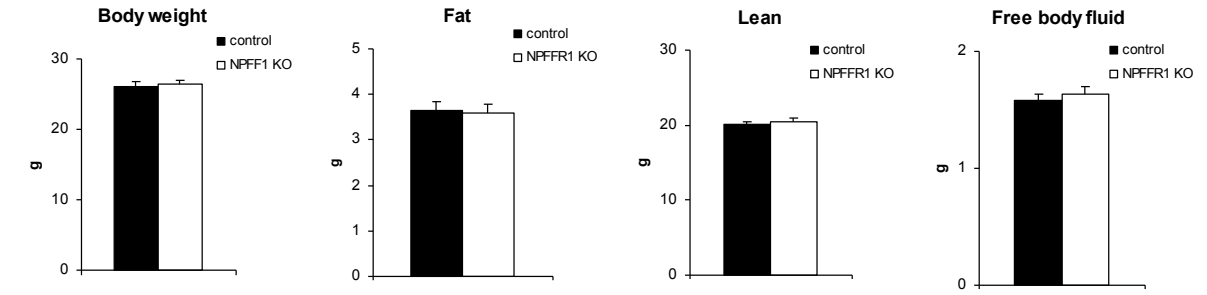
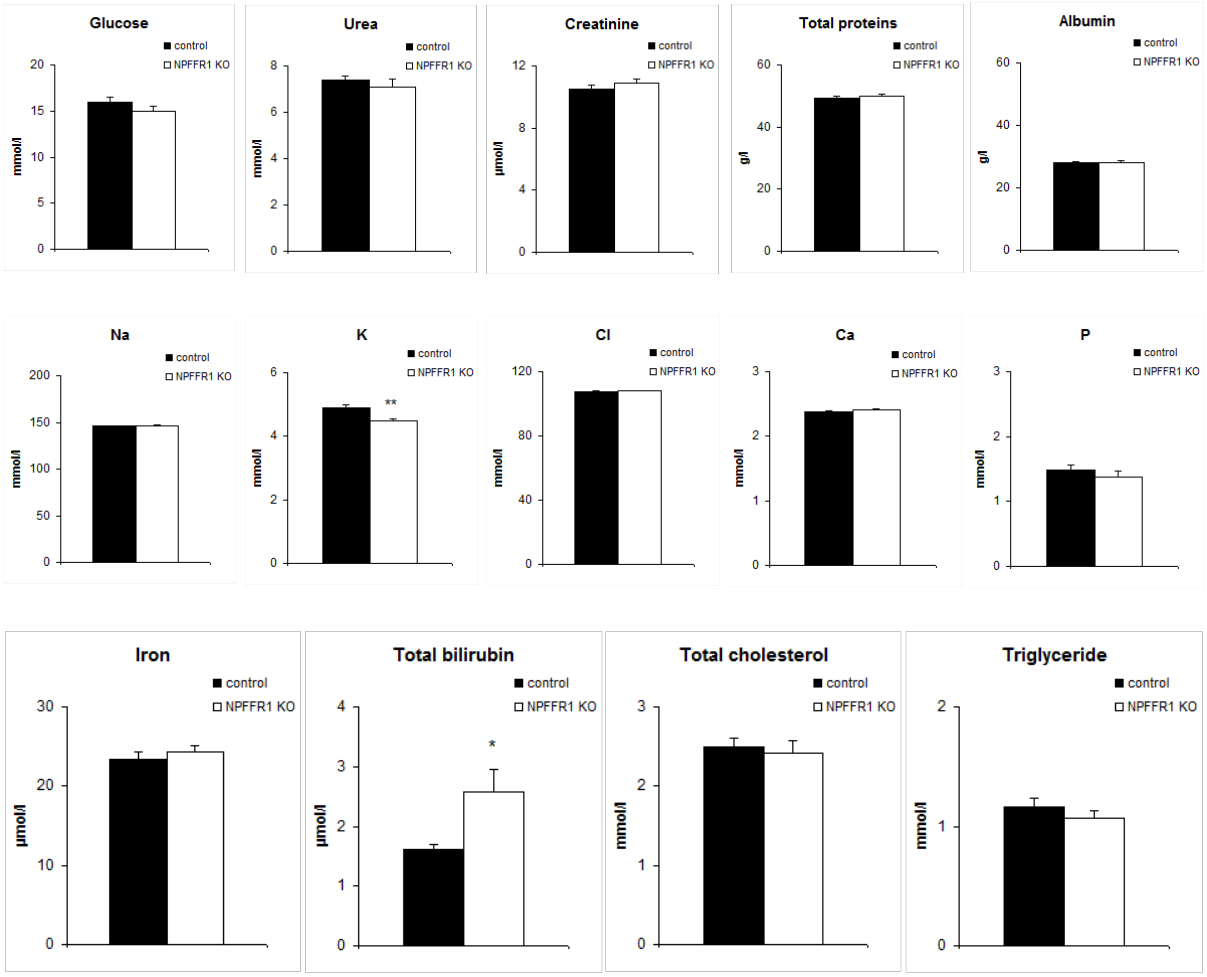


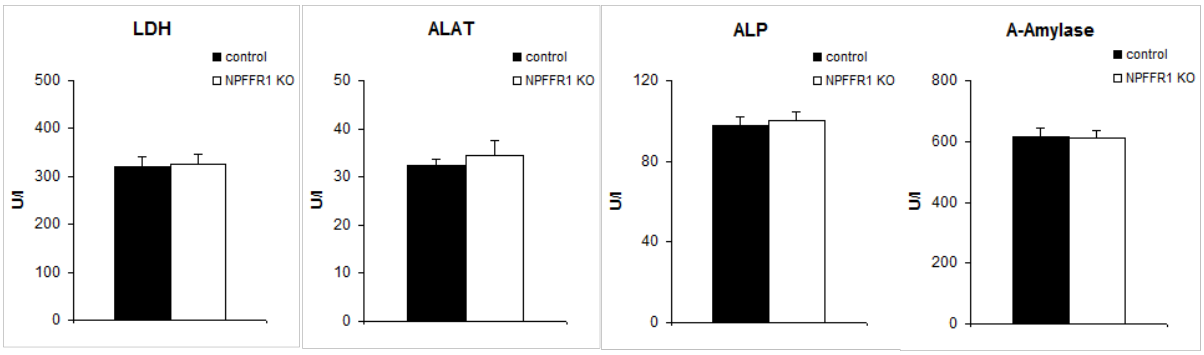
Figure S8

Body composition

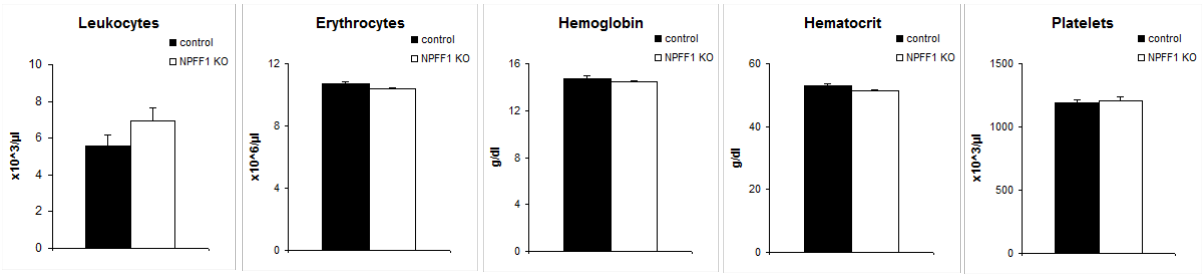


Blood chemistry





Blood hematology



2.2. Caractérisation de dérivés aminoguanidines hydrazones en tant que ligands des récepteurs NPFFR

Une étude à laquelle j'ai collaboré s'intéresse à l'effet de dérivés aminoguanidines hydrazones (AGH) sur l'hyperalgésie induite par les opioïdes chez les rongeurs. L'article qui en découle, intitulé « *Aminoguanidine hydrazone derivatives as non-peptide NPFF receptors ligands reverse opioid-induced hyperalgesia* » (Hammoud *et al.*, 2018) est soumis pour publication (Annexe 1). Cet article s'appuie sur des données précédemment obtenues dans la littérature mettant en avant le rôle critique du cation guanidinium présent dans la structure des peptides contenant une N-acyle-Arg. Cette découverte constitue l'une des principales sources de recherche concernant les ligands non-peptidiques des récepteurs NPFFR. Dans notre projet, deux séries de dérivés ont été identifiées :

i) La série I, constituée de dérivés hétéro-aryle guanidines
ii) La série II, constituée de dérivés (hétéro)-aryle aminoguanidine hydrazones

La série II s'intéresse à des dérivés décrits par Acadia Pharmaceuticals. Parmi eux, les composés AC-1045 et AC-2616 ont été sélectionnés comme composés de référence internes. Les objectifs de cette étude sont :

i) La modification de la structure des AGH par l'introduction de diversités chimiques telles que des substitutions, de nouvelles interactions, de l'homologie et de l'isostérie (caractéristique de deux molécules ayant une forme ou un volume semblables).
ii) La construction d'un nouvel échafaudage qui pourrait remplacer et mimer le chimiotype présent dans la structure des AGH, qui est probablement nécessaire pour la liaison aux récepteurs NPFFR (liaisons hydrogènes, interactions électrostatiques)
iii) La caractérisation pharmacologique *in vitro* des composés d'intérêt
iv) La caractérisation *in vivo* des composés d'intérêt dans l'hyperalgésie induite par les opioïdes chez le rat et la souris.

L'équipe de chimistes à l'origine de cette étude a synthétisé des dérivés de la seconde série d'AGH en ajoutant des liaisons C-N ou N-N qui relient 2 atomes de la partie aminoguanidine. Les interactions des liaisons H ont été remplacées par un noyau capable de mimer le chimiotype selon une conformation spécifique. Brièvement, l'introduction de chlores en position méta et para du noyau phényle entraîne une perte de la sélectivité entre les deux récepteurs NPFFR. Ma contribution à ce projet a consisté en l'évaluation de la sélectivité de ces composés envers les autres récepteurs à peptides RF-amides. Mis à part le composé 1p (AC-3099) dont le K_i est compris entre 0,5 et 5 µM sur QRFPR, les autres ont tous un K_i au moins supérieur à 5 µM sur tous les autres récepteurs aux peptides RF-amides testés. J'ai ensuite

poursuivi la caractérisation *in vitro* par des tests fonctionnels sur les deux sous-types de récepteurs NPFFR. A 50 µM, tous les composés ont un effet non spécifique sur les cellules HEK-Glo. A 1 µM et à 10 µM, ils n'ont aucun effet agoniste sur les deux récepteurs. En revanche, ils bloquent tous complètement l'effet de l'agoniste endogène RFRP-3 sur NPFF1R à 10 µM. De plus, à la concentration de 1 µM les composés 16a, 16b, 22c et 22e ont un effet similaire, ce qui montre qu'ils sont plus puissants que le 1n (AC-1045), 1p (AC-3099) et 1r (AC-216). En revanche, aucun effet, ni agoniste ni antagoniste n'est observé sur les HEK-Glo exprimant le récepteur NPFF2R, mis à part un effet agoniste du 1r à 10 µM. Nous avons donc décidé de poursuivre notre étude avec les composés 1n et 22e, dans des tests d'hyperalgésie induite par le fentanyl. Chez le rat, une injection de fentanyl induit de manière aigue une analgésie durant 4h, et une hyperalgésie se développe les jours suivant. Une injection au préalable du composé 1n (0,5mg/kg, sc.) n'a pas d'effet sur l'analgésie, mais atténue de manière significative l'hyperalgésie induite par le fentanyl. De plus, le composé 22e a été testé dans la même modalité chez la souris (5mg/kg, sc.), où il bloque cet effet secondaire sans en atténuer la fonction principale, comme observé chez le rat. Au vue des données fonctionnelles *in vivo*, ces résultats sont interprétés comme la conséquence du blocage des récepteurs NPFF1R, bien que leurs profils pharmacologiques ne soit pas clair, étant donné que l'on n'observe aucune activité fonctionnelle sur les cellules HEK-Glo NPFF2R malgré une liaison de l'ordre du nanomolaire sur les CHO-NPFF2R.

2.3. Caractérisation du RF313

La caractérisation du RF313 est un projet auquel j'ai contribué et qui a mené à une publication (Elhabazi *et al.*, 2017, annexe 2). Le RF313 est un dérivé du RF9, un antagoniste des récepteurs NPFFR précédemment synthétisé et décrit par notre laboratoire, en collaboration avec le laboratoire de Jean-Jacques Bourguignon (faculté de pharmacie de Strasbourg), et Guy Simonnet (Université de Bordeaux). En effet, la fonction amidée en C-terminale a été éliminée, le résidu arginine a été remplacé par une ornithine portant une pipéridine au niveau de sa chaîne latérale, et l'adamantane a été substitué par un biphényle. Pour résumer, le RF313 est un composé qui avait été identifié en 2015 (composé 12e ; Bihel *et al.*, 2015). Cette première étude décrit une nouvelle classe de dérivés d'ornithine utilisés en tant que bioisostères de l'arginine, dont fait partie le composé 12e, un antagoniste peptidomimétique des récepteurs NPFFR et actif par voie orale. Son injection en périphérie bloque l'hyperalgésie induite par les opioïdes à faible dose, sans effet secondaire apparent. De plus, il potentialise l'analgésie induite par les opioïdes. Dans notre article récent (Elhabazi *et al.*, 2017), nous avons entrepris de caractériser de manière plus approfondie ce composé, dans des tests *in vitro*, et chez la souris. Pour résumer, le RF313 est un antagoniste du récepteur NPFF1R présentant une légère sélectivité par rapport au NPFF2R (≈3 fois) et une bonne sélectivité par rapport aux autres récepteurs à peptides RF-amides (Ki > 20µM pour PrRPR et QRFPR ; Ki > 100µM pour Kiss1R). J'ai participé à cette publication en montrant qu'il n'avait

aucun effet propre dans des tests de mesure de l'activité des protéines G, ni d'inhibition de l'AC, mais qu'il était capable d'inhiber l'effet de l'agoniste endogène RFRP-3 sur des cellules recombinantes exprimant le récepteur NPFF1R. En dépit de sa faible sélectivité pour NPFF1R par rapport à NPFF2R, notre composé n'a ni effet agoniste, ni effet antagoniste dans ces mêmes tests fonctionnels sur le récepteur NPFF2R. Comme chez le rat, le RF313 prévient l'hyperalgésie induite par le RFRP-3 chez la souris, ce qui montre que c'est un antagoniste du récepteur NPFF1R *in vivo*. De plus, il empêche le développement de l'hyperalgésie secondaire induite par le fentanyl à la fois lorsqu'il est injecté dans la circulation systémique, mais aussi par voie orale. Sa co-administration avec la morphine potentialise l'analgésie aigüe, et réduit l'hyperalgésie à long-terme. Enfin, à la différence du RF9, le RF313 antagonise la stimulation de la sécrétion de LH par le RFRP-3 chez le hamster. En conclusion, le RF313 est un bon outil pharmacologique par rapport au RF9 car il n'a pas d'activité sur le récepteur Kiss1R, mais ne présente pas une assez bonne sélectivité par rapport à NPFF2R pour être considéré comme un outil permettant d'aborder les rôles respectifs des récepteurs NPFF1R et NPFF2R *in vivo*.

2.4. Caractérisation de ligands sélectifs du récepteur NPFF2R

Afin d'éliminer toute ambiguïté sur les rôles propres des deux sous-types de récepteurs NPFF, il est primordial de développer et caractériser un antagoniste sélectif du récepteur NPFF2R. C'est pourquoi une partie de ma thèse a consisté, toujours en collaboration avec les chimistes de l'équipe de Frédéric Bihel, en la recherche d'une molécule ayant ces qualités, selon la même logique que celle mise en œuvre pour la découverte du ligand spécifique NPFF1R.

A défaut d'antagoniste spécifique du récepteur NPFF2R, les équipes intéressées par le rôle du NPFF2R ont longtemps utilisé des agonistes sélectifs, comme le dNPA (D.Asn-Pro-(N-Me)Ala-Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2), analogue du NPFF synthétisé pour la première fois par Roussin et ses collaborateurs en 2005 (Roussin *et al.*, 2005). Le dNPA a un K_i de 0,027 nM sur NPFF2R et est plus de 100 fois sélectif par rapport à NPFF1R. Il est aussi puissant en termes d'inhibition de l'accumulation d'AMPc que le NPA-NPFF. Des effets anti-opioïdes sont observés lorsque ce composé est administré au niveau central où il prévient l'analgésie morphinique (Roussin et *al.*, 2005). De plus, injecté dans le troisième ventricule, le dNPA a des propriétés hyperthermiques, contrairement au RFRP-3 qui fait baisser la température corporelle (Moulédous *et al.*, 2010). Les études utilisant ce composé mettent en évidence des interactions fonctionnelles entre les récepteurs NPFF2R et μ (Gouardères *et al.*, 2007 ; Kersanté *et al.*, 2011). Par ailleurs, en observant la stimulation des courants potassiques voltage dépendant dans une lignée cellulaire issue de ganglions rachidiens dorsaux par le dNPA, le récepteur NPFF2R fut décrit comme un important modulateur des courants potassiques (Mollereau et *al.*, 2011). Néanmoins, le dNPA présente des limites. En dépit de sa haute sélectivité pour NPFF2R, il reste toute de

même d'une très haute affinité sur le récepteur NPFF1R, ce qui n'en exclut pas sa liaison aux doses utilisées *in vivo*. Ainsi, il est nécessaire de développer des outils plus performants pour l'étude du récepteur NPFF2R, qui confirmeraient que les effets observés à l'aide du dNPA résultent en effet de l'activation de ce dernier. Depuis une dizaine d'années de travail de recherche sur des ligands sélectifs, notre laboratoire s'est sans cesse heurté à des difficultés, notamment au regard du récepteur NPFF2R.

En collaboration avec les chimistes, l'équipe a caractérisé précédemment une molécule dotée pour la première fois d'une sélectivité de dix fois pour le récepteur NPFF2R par rapport au NPFF1R (composé 12d, Bihel et *al.*, 2015). Le motif BnPip (Benzylpipéridine) présent sur sa chaîne latérale a été identifié comme déterminant pour la bonne sélectivité pour le récepteur NPFF2R. C'est pourquoi nous avons entrepris de synthétiser une première série de dipeptides R-BnPip-Xaa-NH_2. Après avoir effectué un premier criblage sur cellules hétérologues CHO-hNPFF1R et CHO-hNPFF2R, nous avons identifié des composés très sélectifs pour NPFF2R. En revanche, une perte de sélectivité est apparue à la suite de nouvelles déterminations de l'affinité de ces mêmes composés, issus d'un nouveau lot. C'est ainsi que les chimistes ont remarqué une contamination des premiers échantillons avec des tripeptides possédant deux amino-acides BnPip. La resynthèse de ces tripeptides a permis de confirmer que ces derniers étaient bien sélectifs du récepteur NPFF2R, *a contrario* des dipeptides constituant la base de notre étude. Nous avons donc décidé premièrement d'explorer le rôle de l'environnement C-terminal de ces tripeptides en jouant sur le remplacement du troisième acide aminé (Tableau 1).

De ce premier criblage, nous avons mis en évidence trois molécules présentant une sélectivité supérieure à 100 fois pour le second sous-type de récepteur NPFF (Tableau 1). Le meilleur de ces trois dérivés disposant d'une affinité subnanomolaire sur NPFF2R et d'une sélectivité de plus de 300 fois par rapport à NPFF1R est constitué d'une Valine en C-terminal (RF3353). Nous avons donc décidé de conserver cette Valine et d'explorer la partie N-terminale du tripeptide Bz-Xaa-BnPip-Val-NH_2, par l'introduction d'acides aminés naturels ou non naturels. La structure de ces derniers est présentée dans la figure 11.

Bz-BnPip-BnPip-**Xaa-**NH_2

Composés	Xaa	K_i NPFF1R (nM)	K_i NPFF2R (nM)	Sélectivité R1/R2
RF3352	Gly	294	10	29
RF3348	Ala	276	9	31
RF3353	Val	275 ± 40	0,8 ± 0,3	344
RF3343	Ile	269	2	135
RF3350	Leu	152	2	76
RF3344	Nle	228	5	46
RF3347	cHex	95	11	9
RF3345	Phe	253	1	253
RF3346	Hphe	174	5	35
RF3351	Tyr	31	0,9	34
RF3349	Trp	44	2	22

***Tableau 1** : **Exploration de l'acide aminé C-terminal du tripeptide Bz-BnPip-BnPip-Xaa-NH_2.** K_i obtenus par compétition des composés avec une dose fixe de [^{125}I]-1DMeNPFF, sur CHO-hNPFF1R et CHO-hNPFF2R. La présence d'une SEM signale que l'expérience a été réalisée au moins 4 fois en duplicat. (Gly : Glycine ; Ala : Alanine ; Val : Valine ; Ile = Isoleucine ; Leu = Leucine ; Nle = Norleucine ; cHex : cyclohexyle ; Phe : Phénylalanine ; Hphe : homophénylalanine ; Tyr : Tyrosine ; Trp : tryptophane).*

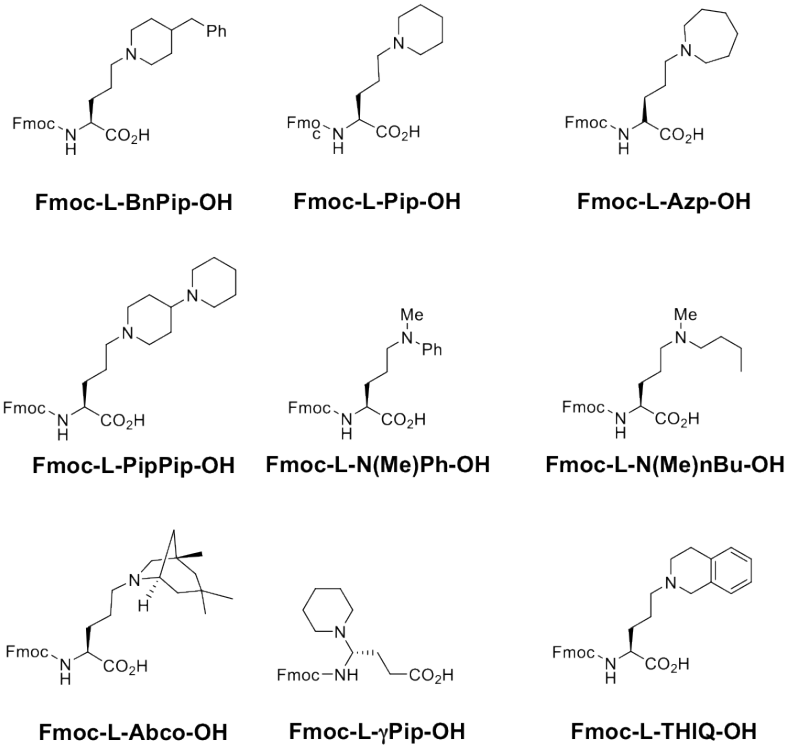


***Figure 11 : Description des acides aminés non naturels utilisés pour l'exploration N-terminale du tripeptide Bn-Xaa-BnPip-Val-NH_2.** Fmoc est un groupement protecteur utilisé pour la synthèse des dérivés sur résine. L représente l'énantiomère naturel de la molécule. (BnPip : Benzopipéridine ; Pip : Pipéridine ; Azp : Azepam ; PipPip : Piperidino-piperidine ; N(Me)Ph-OH : N(méthyle)phényle ; N(Me)nBu-OH : N(méthyle)nButyl ; Abco : azabicyclo-octane ; γPip : γ Pipéridine ; THIQ : Tétrahydroisoquinolin*

Bz-**Xaa-**BnPip-Val-NH_2

Composés	Xaa	Ki NPFF1R (nM)	Ki NPFF2R (nM)	Sélectivité R1/R2
RF3358	Ala	1518	10	152
RF3362	Val	530	12	44
RF3363	Ile	386	87	4
RF3359	Leu	82	73	1
RF3356	Phe	78	16	5
RF3360	Tyr	58	35	2
RF3361	Trp	97	12	8
RF3357	Gln	17	> 500	0,03
RF3378	Arg	233	7	33
RF3383	Lys	636	12	53
RF3382	Orn	846	16	53
RF3353	BnPip	275 ± 40	0,8 ± 0,3	344
RF3368	Pip	233	43	5
RF3372	Azp	11	11	1
RF3370	PipPip	401 ± 401	18 ± 10	22
RF3375	THIQ	8	14	1
RF3371	N(Me)Ph	1483	90	16
RF3374	N(Me)nBu	445	27	16
RF3373	Abco	498	1	498
RF3369	γ-Pip	2889	118	24

Tableau 2 : Exploration de l'acide aminé N-terminal du tripeptide Bz-Xaa-BnPip-Val-NH_2. *K_i obtenus par compétition des composés avec une dose fixe de [^{125}I]-1DMeNPFF, sur CHO-hNPFF1R et CHO-hNPFF2R. La présence d'une SEM signale que l'expérience a été réalisée au moins 4 fois en duplicat.*

Aucun des dérivés synthétisés n'atteint une aussi bonne affinité sur NPFF2R que le RF3353, ni une meilleure sélectivité, à part le RF3373 (Tableau 2). Nous avons donc entrepris de poursuivre notre étude en conservant la Valine en position C-terminale du tripeptide, et d'ainsi modifier la partie N-terminale du composé R-BnPip-BnPip-Val-NH_2. Comme présenté dans le tableau 3, le remplacement du benzoyle par un hydrogène (H), un acétyle (Ac) ou un pyroglutamate (Pg) améliore considérablement la

sélectivité pour le récepteur NPFF2R, notamment en diminuant l'affinité pour le récepteur NPFF1R (Tableau 3).

R-BnPip-BnPip-Val-NH_2

Composés	R	Ki NPFF1R (nM)	Ki NPFF2R (nM)	Sélectivité R1/R2
RF3353	Bz	275 ± 40	0,8 ± 0,3	344
RF3364	H	2580 ± 285	0,2 ± 0,04	12900
RF3367	Ac	1040 ± 257	0,4 ± 0,2	2600
RF3381	Pg	2535	0,3	8450

Tableau 3 : Exploration N-terminale du tripeptide R-BnPip-BnPip-Val-NH_2. *K_i obtenus par compétition des composés avec une dose fixe de [^{125}I]-1DMeNPFF, sur CHO-hNPFF1R et CHO-hNPFF2R. La présence d'une SEM signale que l'expérience a été réalisée au moins 4 fois en duplicat.*

L'activité des meilleurs de ces composés a tout d'abord été testée dans des cellules HEK-Glo-hNPFF2R. Dans cet essai, le RF3364, le RF3367 et le RF3381 sont tous les trois des agonistes partiels, avec des EC_{50} respectives de 11 nM, 16 nM et 6 nM (EC_{50} = 5 nM pour le NPFF). Par rapport au NPFF (Emax à 100%), les efficacités maximales des trois composés sont respectivement de 63 %, 45 % et 45 % (Figure 12).

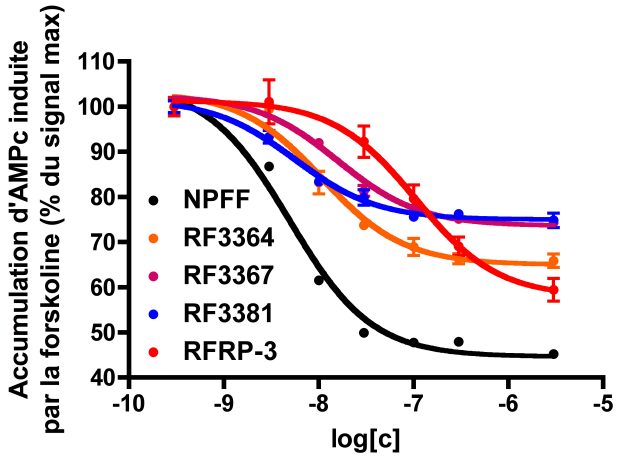


Figure 12 : Activité agoniste du RF3364, RF3367 et RF3381 sur des cellules HEK-Glo-hNPFF2R. *L'activation de NPFF2R stimule la protéine Gi, visualisée par une baisse de l'accumulation d'AMPc au sein de la cellule. Expérience représentative de 2 expériences réalisées en duplicat.*

En conclusion, nous avons identifié au cours de ce travail, 3 composés hautement sélectifs pour le récepteur NPFF2R. Ces molécules semblent toutefois être des agonistes partiels. C'est pourquoi nous avons choisi de ne pas poursuivre la caractérisation *in vivo* de ces composés. Par la suite, il s'agira de poursuivre la synthèse de dérivés qui garderont leur sélectivité mais auront une activité antagoniste plus marquée.

3. Discussion

3.1. Le RF3286, un nouvel outil puissant et sélectif du récepteur NPFF1R

Le RF3286, notre antagoniste sélectif du récepteur NPFF1R nous a permis d'ajouter un argument à la discrimination des rôles propres de NPFF1R et NPFF2R dans la nociception. Dans la première expérience des effets d'une administration chronique de morphine, nous avons co-administré une dose de RF3286 pour une dose de morphine. Or, le blocage de l'hyperalgésie secondaire, total au début de l'apparition du phénomène tendait à être atténué au fur et à mesure des jours, pour ne devenir que partiel (Figure 13).

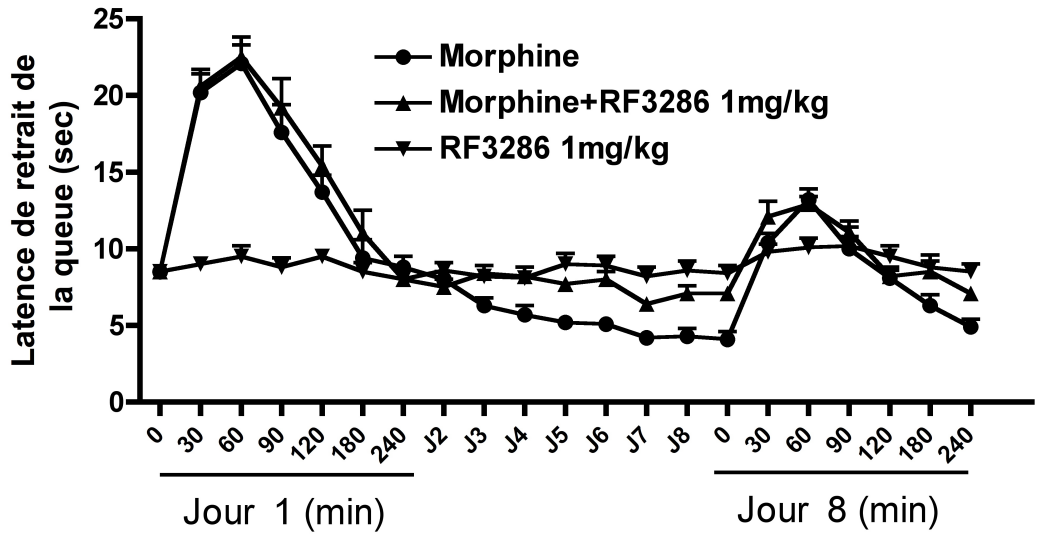


Figure 13 : Effet du RF3286 injecté une seule fois sur l'analgésie, l'hyperalgésie secondaire et la tolérance liées à l'administration chronique de morphine. *Le RF3286 bloque totalement l'hyperalgésie secondaire jusqu'au jour 4, mais son effet tend à s'atténuer par la suite. Au jour 8, la tolérance à l'analgésie morphinique n'est pas modifiée chez le groupe qui a reçu du RF3286.*

La faible demi-vie que nous avons mesurée pour le RF3286 par rapport à celle de la morphine, qui est de 2 à 6 heures, semblerait être responsable de la diminution de cet effet. C'est pourquoi nous avons

décidé d'administrer le composé 3 fois par jour au lieu d'1 fois pour la morphine. En effet, avec 3 doses réparties dans la journée, l'effet du RF3286 sur l'hyperalgésie secondaire à l'administration de morphine reste total tout au long de l'expérience.

Contrairement au RF3286, le RF9 potentialise et prolonge l'effet analgésique produit par une injection aigue de fentanyl ou de morphine, et prévient presque totalement la tolérance à la morphine. L'absence de sélectivité du RF9 entre les deux sous-types de récepteurs nous permet d'émettre l'hypothèse que d'une part, le récepteur NPFF1R serait impliqué dans l'hyperalgésie induite par les opioïdes, et que d'autre part, le NPFF2R aurait également une part de responsabilité dans le phénomène de tolérance (Elhabazi *et al.*, 2012). De plus, le RF9 réduit significativement les signes du syndrome de sevrage morphinique (Elhabazi *et al.*, 2012). Comme le RF3286 n'a aucun effet sur ces derniers, et que les animaux KO NPFF1R ne présentent pas de différence par rapport aux animaux sauvages concernant le syndrome de sevrage à la morphine, nous pouvons nous permettre de conclure que le système impliqué dans le syndrome de sevrage serait plutôt NPFF/NPFF2R. Dans le futur, il sera intéressant d'évaluer l'effet d'un antagoniste du récepteur NPFF2R sur le syndrome de sevrage morphinique pour confirmer ces hypothèses.

Nous avons démontré clairement le passage de la barrière hémato-encéphalique de notre antagoniste *in vivo*, *via* le blocage de l'hyperalgésie induite par le RFRP-3 central par une injection périphérique de RF3286. Les données obtenues au laboratoire par spectrométrie de masse ne mesurent pourtant qu'une très faible proportion de RF3286 dans le cerveau lorsque l'on injecte 5 mg de RF3286 en périphérie (tableau 4). La diffusion d'un composé de la périphérie au cerveau est calculée par le log[cerveau]/[plasma], et plus ce rapport est proche de 0, plus la concentration cérébrale est proche de la dose de composé plasmatique, illustrant ainsi une bonne distribution. Une valeur de -2,6 soutient ici un très faible passage, de l'ordre de 0,3%.

log [cerveau]/[plasma]		
-2.6	±	0.6

Tableau 4 : Evaluation du passage de la barrière hémato-encéphalique du RF3286.

Bien que nous ayons démontré *in vivo* le passage de la BHE par le RF3286, sa faible diffusion nous a conduit à émettre l'hypothèse d'une action de notre antagoniste en périphérie. C'est pourquoi il nous est apparu intéressant de tester les effets périphériques du RFRP-3, en assumant que lorsqu'il est injecté

s.c., il est incapable de passer la BHE, à l'instar de nombreux peptides. Ainsi, nous en déduisons que les effets observés avec ce mode d'administration ne seront pas centraux. Pour cela, nous avons injecté 5 mg/kg de RFRP-3 chez des animaux sauvages en périphérie (s.c.). Trente minutes après l'injection, nous avons observé une diminution du seuil nociceptif des animaux, illustrant une hyperalgésie (AUC = -6 ± 18 u.a. pour le groupe saline vs -76 ± 14 u.a. pour le groupe RFRP-3 ; $p < 0,01$, *one way* ANOVA suivi d'un test de Bonferroni, figure 14). En revanche, l'injection préalable de RF3286 (5 mg/kg ; s.c.) n'a aucun effet sur l'hyperalgésie induite par le RFRP-3 dans ces conditions (AUC = -76 ± 14 u.a. pour le groupe RFRP-3 vs -71 ± 8 u.a. pour le groupe RFRP-3 + RF3286, ns). En conclusion, le RFRP-3 est capable d'induire de l'hyperalgésie en périphérie, mais son effet n'est pas bloqué par le RF3286, suggérant une action sur des récepteurs distincts du NPFF1R.

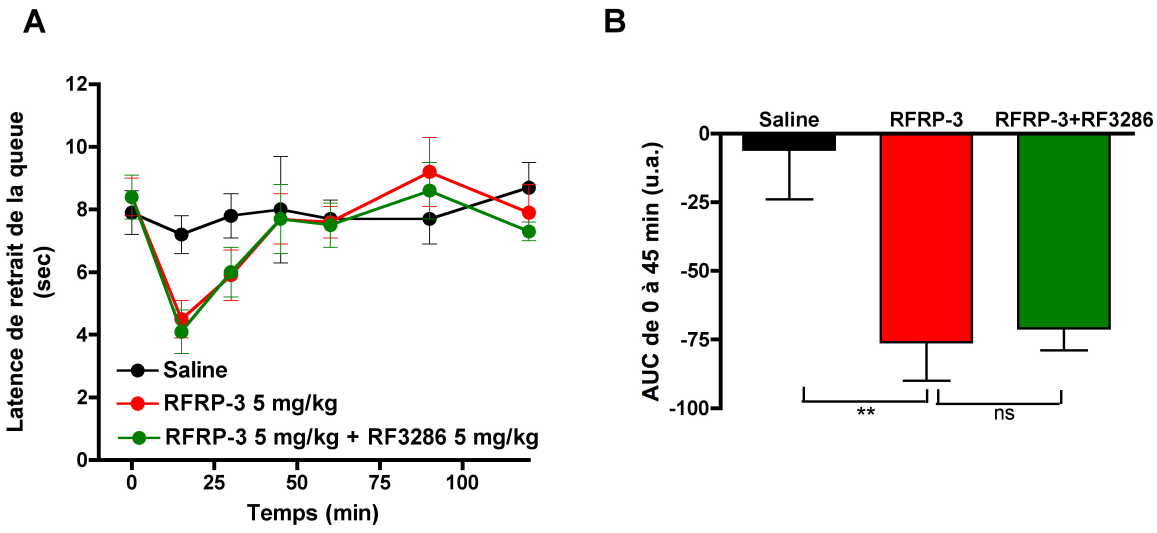


Figure 14 : Effet du RFRP-3 injecté en périphérie (s.c.) sur le seuil nociceptif des animaux. *(A) Au temps 0, une injection de 5 mg/kg de RFRP-3 (s.c.) est réalisée chez les souris et la latence de retrait de la queue est mesurée toutes les 15 minutes pendant 2 heures. Dans le groupe concerné, le RF3286 est administré 20 minutes avant l'injection de l'agoniste. (B) AUC (Aire sous la courbe) des différents groupes de 0 à 45 minutes. Le RFRP-3 induit une hyperalgésie, qui n'est pas modifiée par la co-administration de RF3286 (N=6-7).*

Afin de confirmer que le NPFF1R n'est pas impliqué dans l'effet hyperalgésique induit par le RFRP-3 en périphérie, nous avons testé l'impact de son administration sous-cutanée chez des souris déficientes pour le récepteur NPFF1R. Comme le montre la figure 15, le RFRP-3 (5 mg/kg ; s.c.) provoque une hyperalgésie chez les souris WT et chez les souris KO NPFF1R (*** $p < 0,001$ entre T0 et T15 chez les souris WT ; ** $p < 0,01$ entre T0 et T15 chez les souris KO NPFF1R, *one way* ANOVA suivi d'un test de Bonferroni). Bien que légèrement atténuée chez les souris KO NPFF1R, l'hyperalgésie

observée n'est pas significativement différente de celle observée chez les souris WT (AUC = -154 ± 58 u.a. pour les souris WT vs -99 ± 44 u.a. pour les souris KO NPFF1R, ns, *t-test*).

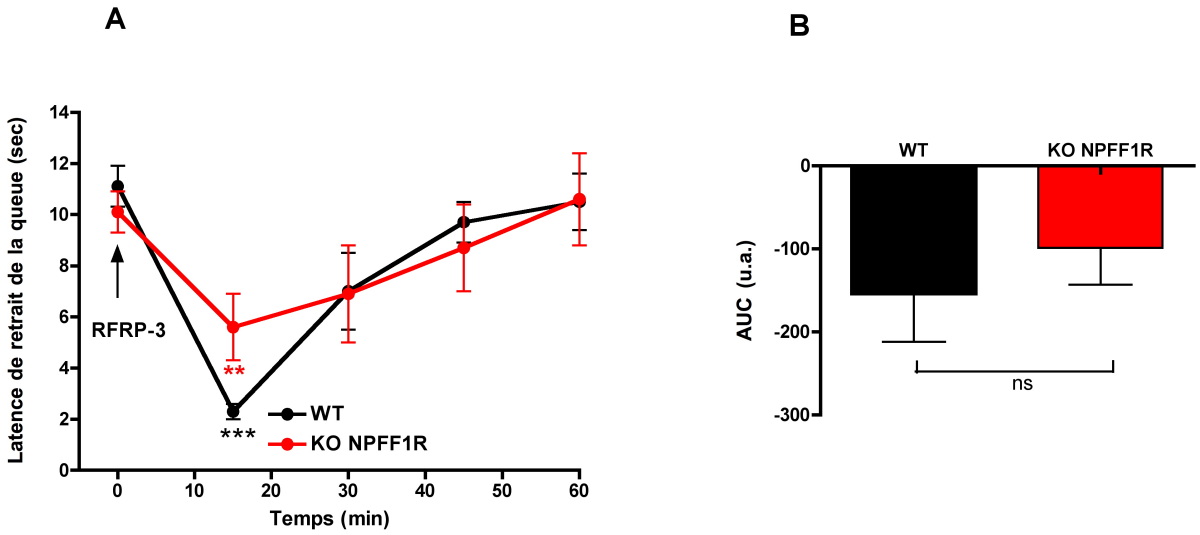


Figure 15 : Comparaison de l'effet de l'injection du RFRP-3 en périphérie chez les souris WT et KO NPFF1R. *(A) Au temps 0, une injection de 5 mg/kg de RFRP-3 en s.c. est réalisée chez les souris WT et KO NPFF1R et la latence de retrait de la queue est mesurée toutes les 15 minutes pendant 1 heure. (B) AUC des différents groupes pendant la totalité de l'expérience. Aucune différence significative n'est observée entre les 2 groupes de souris (N=7).*

En conclusion, le RFRP-3 provoque une hyperalgésie comparable chez des souris WT et KO NPFF1R, suggérant que cet effet est indépendant du récepteur NPFF1R. Cette observation confirme ainsi les résultats obtenus par l'absence d'effet de notre antagoniste sur l'hyperalgésie induite par le RFRP-3 en périphérie.

Considérant l'expression du récepteur NPFF2R dans les DRGs et la bonne activité du RFRP-3 sur NPFF2R, nous avons émis l'hypothèse que l'hyperalgésie induite par le RFRP-3 en périphérie serait relayée par ce dernier. C'est pourquoi, nous avons entrepris de tester l'effet du RFRP-3 (s.c.) chez des souris KO NPFF2R. Comme le montre la figure 16, nous observons une hyperalgésie 15 minutes après l'injection de RFRP-3 (5 mg/kg ; s.c.), chez les animaux sauvages et KO NPFF2R (*** $p < 0,001$ entre T0 et T15 chez les souris WT ; *** $p < 0,001$ entre T0 et T15 chez les souris KO NPFF1R, *one way* ANOVA suivi d'un post hoc test de Bonferroni). Les AUC calculées pour la totalité de l'expérience indiquent qu'il n'y a pas de différence entre l'hyperalgésie observée chez les souris WT et les souris KO NPFF2R.

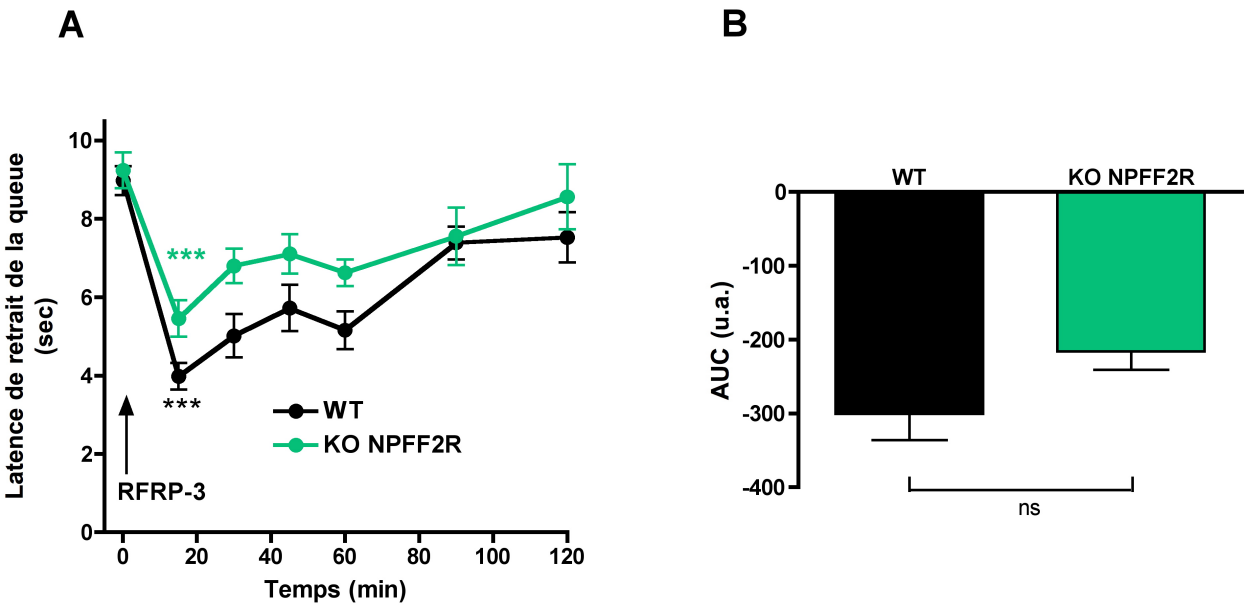


Figure 16 : Comparaison de l'effet de l'injection du RFRP-3 en périphérie chez les souris WT et KO NPFF2R. *(A) Au temps 0, une injection de 5 mg/kg de RFRP-3 en s.c. est réalisée chez les souris WT et KO NPFF2R et la latence de retrait de la queue est mesurée toutes les 15 minutes pendant 2 heures. (B) AUC des différents groupes pendant la totalité de l'expérience. Aucune différence significative n'est observée entre les 2 groupes de souris (N=9).*

L'effet hyperalgésique du RFRP-3 administré par voie périphérique ne semble donc pas être tributaire des deux sous-types de récepteurs NPFFR. Il existe plusieurs arguments dans la littérature démontrant l'existence de récepteurs –autres que NPFF1R et NPFF2R- présents au niveau des ganglions dorsaux de la moelle épinière, capables d'être activés par des peptides RF-amides tels que le NPFF ou le NPAF (Dong *et al.,* 2001 ; Han *et al.,* 2002). Ces récepteurs, isolés par l'équipe de Young et ses collaborateurs en 1986 (Young *et al.*, 1986), et caractérisés pour leurs caractéristiques oncogéniques, sont appelés *Mas receptors*, ou encore Mrgs (*Mas-related genes*). Il a été montré que le RFRP-3 activait l'un de ces récepteurs –le MrgC11- avec une EC_{50} de 113 nM (Han *et al.,* 2002). D'après nos résultats et ces données précédemment citées, nous pourrions avancer l'hypothèse d'un effet du RFRP-3 en périphérie, au niveau des neurones sensoriels de la corne dorsale de la moelle épinière, ce dernier activant des récepteurs Mrgs pour induire une hyperalgésie.

3.2. Expériences complémentaires pour la caractérisation des souris KO NPFF1R et KO NPFF2R

Des études ayant recours aux radiotraceurs [^{125}I]YVP et [^{125}I]EYF pour caractériser respectivement les récepteurs NPFF1R et NPFF2R ont démontré que le bulbe olfactif était une région du cerveau très

enrichie en NPFF2R. En revanche, aucun site de liaison du récepteur NPFF1R n'y a été détecté (Gouardères *et al.*, 2004a,b). Afin de caractériser les souris KO NPFF2R, nous avons donc décidé de prélever les bulbes olfactifs des souris WT et des KO NPFF2R, afin d'étudier la liaison du [^{125}I]-1DMeNPFF. Comme le montre la figure 17, nous observons que le signal spécifique du [^{125}I]-1DMeNPFF est totalement aboli chez les souris KO NPFF2R, en comparaison aux souris WT, soulignant l'absence de ces récepteurs chez les souris génétiquement modifiées. Ces résultats ont été publiés dans l'article concernant le rôle du NPFF dans le métabolisme, dont je suis co-auteure (Waqas *et al.*, 2017, Annexe 3). Par ailleurs, nous avons également publié récemment un résumé de cet article dans la revue médecine et science (Quillet et Simonin, 2018, Annexe 4).

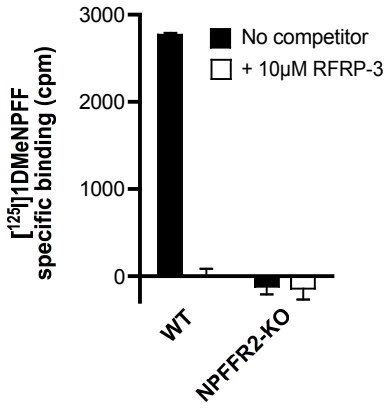


Figure 17 : Liaison du [^{125}I]-1DMeNPFF sur des bulbes olfactifs de souris WT et KO NPFF2R, en présence ou non de 10µM de RFRP-3*. D'après (Waqas et al, 2017).*

Contrairement au récepteur NPFF2R, le récepteur NPFF1R est très peu exprimé dans le cerveau de la souris, et sa détection reste un défi. Malgré tout, des sites de liaison du récepteur NPFF1R ont été identifiés surtout au niveau du septum latéral et de différentes parties de l'hypothalamus chez les souris C57Bl/6 (Gouardères *et al.*, 2004a,b). En revanche, contrairement à l'hypothalamus, aucun site de liaison du récepteur NPFF2R n'a été détecté dans le septum latéral des souris, ce qui en fait un tissu idéal pour des tests de liaison visant à caractériser la présence spécifique du récepteur NPFF1R dans nos souris KO et WT NPFF1R. En dépit de cet avantage, le récepteur NPFF1R semble bien trop peu exprimé pour être détectable à l'aide de cette technique. C'est pourquoi nous avons eu recours à la technique d'hybridation *in situ* pour pallier cet inconvénient dans l'article précédemment intégré.

3.2. Vers la caractérisation d'un antagoniste sélectif du récepteur NPFF2R

L'étonnante sélectivité observée de nos tripeptides pour le récepteur NPFF2R par rapport au NPFF1R est attribuée au résidu BnPip. En effet, les deux résidus BnPip modifient la nature chimique du dipeptide C-terminal originel, en rendant les composés très basiques, par rapport au NPFF. Ces

différences chimiques au sein du peptide pourraient engendrer des différences dans la liaison au récepteur NPFF2R, expliquant ainsi la sélectivité.

L'étude concernant les rôles respectifs des récepteurs NPFF1R et NPFF2R dans la modulation de la nociception et de l'analgésie morphinique ne pourra être complétée qu'à l'aide d'un antagoniste sélectif de NPFF2R. Bien que nos expériences nous aient conduits à l'identification de composés très sélectifs du récepteur NPFF2R, il ne s'agit pas d'antagonistes. Nos résultats nous indiquant que la sélectivité était améliorée de manière drastique par le remplacement du résidu benzoyle en N-terminal, nous testerons dans le futur des composés de la série Bz-Xaa-BnPip-Xaa-NH_2, afin d'identifier un ou plusieurs antagonistes du récepteur NPFF2R. Le cas échéant, nous en modifierons le benzoyle en N-terminal en espérant leur faire gagner de la sélectivité sans affecter leur nature antagoniste. De plus, il serait intéressant de confirmer dans d'autres tests fonctionnels, comme le GTPγS, la nature agoniste des composés cités précédemment et de les tester *in vivo*, en commençant par son effet sur l'analgésie induite par une injection icv. de morphine chez la souris.

PARTIE II : CARACTERISATION DU SYSTEME RFRP-3/NPFF1R CHEZ LE HAMSTER

1. Introduction

La saisonnalité chez certains mammifères est une stratégie adaptative qui permet à la descendance de naître au moment opportun, lorsque les conditions environnementales sont les plus favorables. Dans ces conditions, ces animaux sont fertiles seulement durant une partie de l'année. Leur système reproducteur peut se mettre en veille grâce aux modifications neuronales, hormonales et sexuelles, dépendantes des signaux réceptionnés à partir de l'environnement extérieur. Ces animaux sont donc très sensibles à la photopériode (la durée du jour), ou à leur rythme circannuel, c'est-à-dire leur horloge interne. Il existe deux catégories d'animaux saisonniers. D'une part, les animaux qui se reproduisent lorsque les jours sont longs (e.g. les hamsters), et d'autre part, ceux qui deviennent sexuellement actifs uniquement lorsque les jours sont courts (e.g. les moutons). Cette disparité s'explique par la durée de la gestation. En effet, le printemps étant le moment opportun pour mettre bas, grâce aux retours des sources énergétiques par exemple, les animaux dont le temps de gestation est long ont intérêt à se reproduire quelques mois avant (lorsque les jours sont plus courts, en automne ou en hiver), contrairement aux animaux dont le temps de gestation est court, qui peuvent se permettre de se reproduire durant cette période. De manière très surprenante, deux signaux exactement opposés (i.e. la durée de jour : soit courte, soit longue) conduisent à un état sexuellement identique chez ces deux types d'animaux saisonniers. La mélatonine a un rôle primordial dans cet effet. Sécrétée par la glande pinéale durant la nuit, sa concentration augmente en fonction de la durée de la nuit. C'est pourquoi on la trouve plus élevée durant les photopériodes courtes que longues. Grâce à la traduction de la photopériode et de ses variations par un signal mélatoninergique, le corps et en particulier le système nerveux central se tiennent au courant des changements environnementaux et sont en mesure de mettre en place certaines modifications afin de s'adapter. Plusieurs molécules voient leur taux d'expression varier lors de ces changements. Parmi les molécules impliquées dans la photoactivation de l'axe de reproduction, on retrouve la mélatonine, les hormones thyroïdiennes hypothalamiques (TH) et les RF-amides. La kisspeptine est très connue pour un être un puissant stimulateur de la libération de GnRH, varie selon la photopériode, et a longtemps été considérée comme l'acteur principal du passage d'un phénotype sexuellement inactif à un phénotype sexuellement actif. Or, depuis quelques années les RFRPs semblent aussi y jouer un rôle critique. Une implication du système RFRP-3/NPFF1R dans les adaptations photopériodiques du hamster Syrien a été démontrée chez nos collaborateurs. Alors que le RFRP-3 est considéré comme un inhibiteur de l'axe gonadotrope chez différents mammifères étudiés comme la souris, le rat, le mouton ou le hamster Syrien femelle (Clarke *et al.*, 2008 ; Kriegsfeld *et al.*, 2006 ; Ducret

et al., 2009 ; Wu *et al.*, 2009 ; Johnson *et al.*, 2007 ; Anderson *et al.*, 2009 ; Kadokawa *et al.*, 2009 ; Sari *et al.*, 2009 ; Pineda *et al.*, 2010), il est étonnant de constater que le RFRP-3 administré au niveau central provoque une augmentation dose-dépendante des niveaux plasmatiques de LH et de FSH, ainsi que du taux de testostérone circulant du hamster Syrien mâle (Ancel *et al.*, 2012). La cible centrale du RFRP-3 est confirmée par l'absence d'effet du RFRP-3 injecté en périphérie chez le hamster, et sur des cellules d'hypophyse en culture (Ancel *et al.*, 2012). De plus, une administration icv. de RFRP-3 entraîne l'activation de deux sous-populations de neurones de l'hypothalamus : les neurones à GnRH présents dans la POA, et les neurones à kisspeptines du noyau arqué (ARC). Par ailleurs, le RFRP-3 et son récepteur semblent être inhibés lorsque la photopériode est courte (chez des hamsters sexuellement inactifs), et cela de manière dépendante de la mélatonine (Revel *et al.*, 2008). Bien que plusieurs études soulignent une régulation différente et opposée de plusieurs molécules chez des animaux sexuellement actifs versus des animaux quiescents, les acteurs impliqués dans la transition entre les deux états reproducteurs des animaux saisonniers ne sont pas encore connus. Le système RFRPs/NPFF1R pourrait faire partie des intermédiaires permettant la traduction du signal nocturne de mélatonine au niveau des régions participant au contrôle central de la reproduction.

En effet, une administration centrale et chronique de RFRP-3 permet de réactiver l'axe reproducteur des hamsters syriens mâles en dépit de conditions photo-inhibitrices (Ancel *et al.*, 2012). Cet effet est visualisé par une augmentation significative de la taille des testicules et du taux de testostérone circulant, comparables à ceux des animaux maintenus en photopériode longue (LP). De plus, une augmentation de l'expression de kiss1 est observée dans le noyau arqué de l'hypothalamus des animaux ayant reçu une infusion chronique de RFRP-3 (Ancel *et al.*, 2012). La régulation différente de l'axe reproducteur chez les hamsters Syriens mâles et femelles est expliquée dans plusieurs études par l'impact des œstrogènes (Kriegsfeld *et al.*, 2006 ; Gibson *et al.*, 2008 ; Molnar *et al.*, 2011). En revanche, chez le rat, l'effet du RFRP-3 est le même quel que soit le sexe de l'animal (Pineda *et al.*, 2010), et l'expression de son ARNm n'est pas affectée par la concentration circulante d'œstrogènes (Quennell *et al.*, 2010). Par ailleurs, les RFRPs ont aussi un effet stimulateur de l'axe reproducteur chez le saumon (Amano *et al.*, 2006).

L'implication du RFRP-3 dans la transition entre les deux photopériodes du hamster Syrien serait également cohérente avec l'augmentation de l'expression de RFRP-3 observée chez les hamsters sexuellement actifs (Revel *et al.*, 2008). De plus, des études montrent que les niveaux d'ARNm de NPFF1R au sein de l'hypothalamus du hamster Syrien sont diminués en photopériodes courtes, chez les deux sexes, même si ces taux sont plus élevés chez la femelle que chez le mâle quelle que soit la photopériode (Henningsen *et al.*, 2016). A ce jour, aucune étude n'a confirmé ces résultats au niveau de l'expression de la protéine. C'est pourquoi nous avons décidé de tenter d'illustrer les résultats obtenus par l'équipe de Valérie Simonneaux avec laquelle nous collaborons, au niveau protéique, par la

détermination du nombre de récepteurs (Bmax) en test de liaison sur des hypothalamus de hamsters Syriens mâles et femelles, soit en photopériode courte, soit en photopériode longue.

De plus, les arguments cités ci-dessus suggèrent une implication du système RFRP-3/NPFF1R dans les changements physiologiques impliqués dans la saisonnalité du hamster. A partir de toutes ces observations, nous avons émis l'hypothèse que le système RFRP-3/NPFF1R serait un acteur clé dans le passage d'un état sexuellement inactif à un état sexuellement actif. Pour la tester, nous avons étudié l'effet de notre antagoniste sélectif du récepteur NPFF1R (le RF3286), sur la photoréactivation de l'axe reproducteur du hamster Syrien mâle.

2. Matériels et méthodes

2.1. Préparations d'homogénats de tissus nerveux et expérience de compétition

Les préparations d'homogénats d'hypothalamus de hamsters sont réalisées comme celles des bulbes olfactifs précédemment décrites.

2.2. Infusion chronique d'aCSF ou de RF3286 chez le hamster

Les infusions chroniques des hamsters ont été réalisées selon les méthodes décrites par nos collaborateurs (Revel *et al.*, 2012 ; Ancel *et al.*, 2012). Les animaux ont été anesthésiés et une canule (Alzet Brain infusion Kit, Durect, Cupertino, CA) a été implantée dans le ventricule droit (coordonnées stéréotaxiques : +0.8 mm antérieur au Bregma, +2.0 mm par rapport à la ligne médiane et 23.0 mm par rapport à la surface piale). Une minipompe osmotique (modèle 2006; débit : 0.15 µl/h.; durée : 6 semaines; Durect, Cupertino, CA) est placée sous la peau du dos, et connectée au ventricule *via* un tube médical en polyvinylchloride sous-cutané. Les pompes sont remplies avec du liquide céphalo rachidien artificiel (aCSF) avec ou sans RF3286 (1,25 nmol et 0,15 µL administré toutes les heures). La canule est fixée dans le crâne avec deux petites vis en acier inoxydable, et le site entier implanté est recouvert de ciment dentaire. Après suture du scalp, les animaux sont placés seuls dans une cage jusqu'au moment de l'euthanasie.

2.3. Hybridation *in situ*

Les animaux sont anesthésiés au dioxyde de carbone et tués par décapitation. Les cerveaux sont prélevés et placés à -80°C jusqu'à leur utilisation. Des tranches d'ARC et de DMH (16 µM) sont sectionnées à l'aide d'un cryostat (Leica, Leica Microsystems, Rueil-Malmaison, France) à -20°C, puis

montées sur des lames SuperFrost*Plus (Menzel-Gläser, Braunschweig, Allemagne) et placées à -80°C jusqu'à l'ISH. Les caractéristiques des sondes utilisées sont, pour la kisspeptine : 266pb, Rattus Norvegicus, 90-359, NM_181692 ; et pour RFRP : 440pb, Phodopus Sungorus, 87-529, JF727837 ; et sont marquées à la digoxigénine. En bref, les sections sont fixées dans du paraformaldéhyde (PFA) 4%, acétylée dans du tampon triethanolamine et déshydratées dans des bains d'éthanol de concentrations croissantes. Les sondes sont dénaturées et diluées dans le tampon d'hybridation (200 ng/mL), avant d'être appliquées sur les tissus, qui seront incubés pendant 40 h à 60°C. Six rinçages sont ensuite réalisés à 72°C. Les sondes marquées à la digoxigénine sont détectées grâce à des anticorps antidigoxigénine marqué à la phosphatase alcaline (Roche). L'activité de la phosphatase alcaline est détectée avec du phosphate de bromochloroindolyle et du nitrobleu de tétrazolium en présence de 5% d'alcool polyvinylique (Sigma). Finalement, les lames sont montées et la densité de cellules *Kiss1* dans l'ARC est évaluée grâce à un microscope Leica DMRB (Leica Microsystems) D'après (Ancel *et al.*, 2012).

3. Résultats

3.1. Détermination de Bmax chez le hamster selon les conditions sexuelles et photopériodiques

Le Bmax, ou quantité maximale de récepteurs que l'on peut trouver à la surface d'une membrane par exemple, se détermine en effectuant une courbe de saturation réalisée à partir de concentrations croissantes d 'un ligand radioactif. Or, dans ces conditions, la consommation de radioligand est très élevée. Pour contourner cet aspect délétère, nous avons décidé d'utiliser la méthode d'Antonio DeBlasi décrite en 1989 dans le journal TIPS (DeBlasi *et al.*, 1989), qui consiste en la compétition entre une concentration fixe de radioligand, et des concentrations croissantes du même ligand non marqué. Dans ces conditions le ligand marqué et le ligand froid se lie au même récepteur avec exactement la même affinité. La courbe de compétition obtenue nous permet de déterminer trois données importantes :

i) La valeur du plateau maximal, qui correspond à la liaison totale du radioligand, en l'absence de compétiteur froid.

ii) La valeur du plateau minimal, correspondant à la liaison non-spécifique du radioligand (c'est-à-dire en présence du ligand froid en excès).

iii) L'IC_{50} : la concentration de ligand non marqué qui déplace la moitié de la liaison spécifique du radioligand.

Selon l'équation de Cheng et Prussoff (Cheng and Prussoff, 1973) :

$$\boldsymbol{IC50 = Kc(1 + L/Kh)}$$

Avec Kc et Kh, les constantes de dissociation des ligands froid (Kc) et chaud (Kh), et L, la concentration de radioligand utilisée.

Si les deux composés ont la même affinité sur le récepteur (Kd), alors Kc = Kh = Kd, et on peut alors écrire :

$$\boldsymbol{IC50 = Kd(1 + L/Kd)}$$

$$\boldsymbol{IC50 = Kd + L}$$

$$\boldsymbol{Kd = IC50 - L}$$

Selon la loi d'action des masses :

$$\boldsymbol{Bmax = Bo(Kd + L)/L}$$

$$\boldsymbol{Bmax = Bo(IC50 - L + L)/L}$$

$$\boldsymbol{Bmax = Bo.IC50/L}$$

Grâce à cette méthode, le nombre de récepteur peut donc être déterminé à partir de l'amplitude de la réponse (Bo), de l'IC_{50} du ligand non marqué, et de la concentration du ligand chaud.

Cette expérience a été appliquée à des homogénats hypothalamiques de hamsters mâles et femelles sexuellement actifs ou inactifs, par compétition entre le [^{125}I]-1DMeNPFF et le I-1DMeNPFF, possédant un isotope de l'iode non radioactif. Les résultats obtenus sont visualisés sur la figure 18. La quantité la plus élevée de récepteurs se retrouve chez les mâles et les femelles en photopériode courte (SP, Bmax = 94 ± 12 et 71 ± 9 fmol/mg pour les mâles SP et les femelles SP, respectivement). La photopériode longue (LP) entraîne quant à elle une diminution -bien que non significative- du Bmax pour les deux sexes (Bmax = 59 ± 3 vs 36 ± 9 fmol/mg pour les mâles LP et les femelles LP, respectivement). Enfin, chez les femelles LP, le nombre de récepteurs est significativement plus bas que chez les mâles SP (Bmax = 94 ± 12 et 36 ± 9 fmol/mg pour les mâles SP et les femelles LP respectivement ; **p < 0,01 *one way ANOVA* suivi d'un post hoc test de Bonferroni). Ces résultats sont en totale contradiction avec ceux obtenus pour l'étude des ARNm de NPFF1R par nos collaborateurs, chez lesquels les transcrits de RFRPs sont plus nombreux en LP, et chez les femelles (Henningsen *et al.*, 2016).

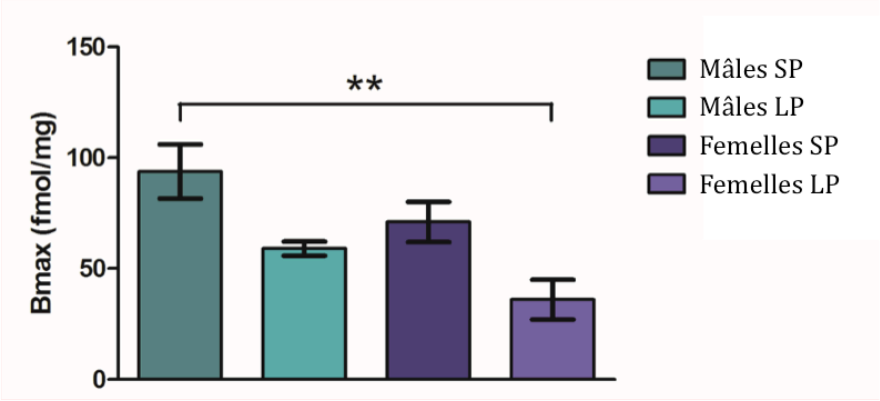


Figure 18 : Evaluation du nombre de récepteurs NPFFR selon le sexe et la photopériode du hamster*. Ces résultats sont la moyenne ± SEM d'expériences de compétition réalisées entre le [^{125}I]-1DMeNPFF et le I-1DMeNPFF (N = 5). SP : Short Photoperiod, LP : Long Photoperiod.*

3.2. Implication du système RFRP-3 dans la transition SP/LP chez le hamster syrien mâle.

Dans cette partie du travail, nous avons utilisé l'antagoniste du récepteur NPFF1R –le RF3286- afin de déterminer si le système RFRP-3/NPFF1R était un déclencheur de la transition saisonnière du hamster Syrien mâle. Le schéma de l'expérience réalisée est présenté dans la figure 19. Quatre groupes d'animaux ont été constitués pour cette expérience. Aux termes d'un maintien de 11 semaines en SP, deux groupes d'animaux ont reçu une administration chronique de aCSF ou RF3286 et ont été placés en LP pendant 6 semaines. Deux autres groupes de hamsters sont restés pendant la totalité de l'expérience en LP et ont également reçu soit une infusion de aCSF, soit de RF3286. A la fin de l'expérience, les animaux ont été euthanasiés et différents paramètres ont été mesurés.

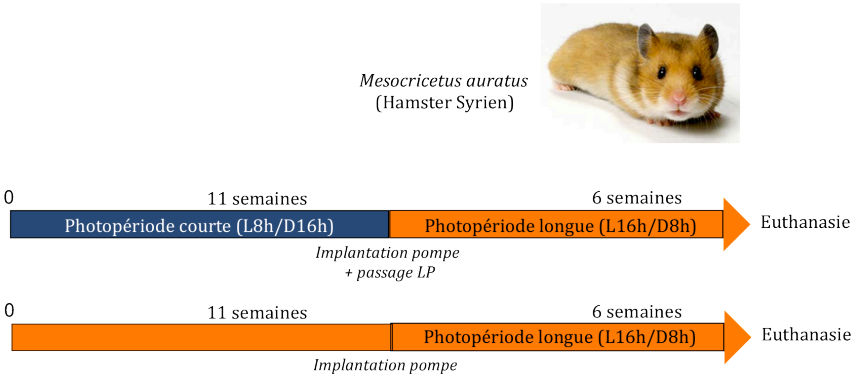


Figure 19 : Schéma de l'expérience d'évaluation de l'effet du RF3286 sur la photoréactivation du hamster Syrien mâle. *Après 11 semaines de photopériode courte (SP), des groupes d'animaux sont implantés avec une pompe contenant du RF3286 ou de l'aCSF, et placés 6 semaines en photopériode longue (LP). Deux autres groupes de hamsters recevant également soit de l'aCSF, soit du RF3286 sont maintenus en photopériode longue durant la totalité de l'expérience (N=8 dans chaque groupe).*

Parmi les différents paramètres qui attestent du passage à l'état sexuellement actif, nous nous intéressons au poids des animaux, ceux de leurs testicules et des vésicules séminales. Nous quantifions également le nombre de neurones exprimant la kisspeptine dans le noyau arqué de l'hypothalamus et des neurones à RFRP dans le DMH. Comme nous l'observons sur la figure 20, chez les animaux SP/LP (aCSF), le poids corporel et celui des testicules atteint ceux des animaux LP/LP, soit respectivement environ 120 g et 3500 mg. Alors que les vésicules séminales des hamsters LP/LP pèsent environ 1500mg, celles des hamsters SP/LP sont significativement plus légères (environ 1250 mg, a-b $p<0,05$ *one way ANOVA* suivi d'un post hoc test de Tukey). Notre antagoniste ne modifie aucun de ces trois paramètres physiologiques en comparaison aux animaux aCSF dans des conditions SP/LP et LP/LP (Figure 20). D'autre part, il provoque une augmentation significative de l'expression de la kisspeptine dans le noyau arqué, en comparaison aux animaux SP/LP, aCSF (Figure 21, 100000 u.a. pour les animaux SP/LP aCSF vs 150000 u.a. pour les animaux SP/LP RF3286, $p < 0,01$ *one way ANOVA* suivi d'un post hoc test de Tukey). Or, comme nous l'avons mentionné précédemment, l'administration chronique de l'agoniste endogène du récepteur NPFF1R –le RFRP-3- augmente lui aussi ce paramètre. Nous pouvons expliquer cet effet paradoxal par une intervention des kisspeptines. Il n'est en effet pas impossible d'imaginer un effet compensatoire du système kisspeptines/Kiss1R en réponse à une inhibition du système RFRP-3/NPFF1R dans ces conditions. Enfin, aucune différence concernant le nombre de corps cellulaires exprimant RFRP n'est à noter entre les groupes SP/LP, aCSF et SP/LP, RF3286 (Figure 21), et notre antagoniste n'empêche pas le passage à l'état sexuellement actif des hamsters. Dans l'ensemble, ces résultats suggèrent que le système RFRP-3/NPFF1R n'est pas responsable de la réactivation de l'axe reproducteur et que cette réactivation provoquée par l'infusion centrale de RFRP-3 (Ancel *et al.*, 2012) serait forcée par un système non représentatif de la physiologie.

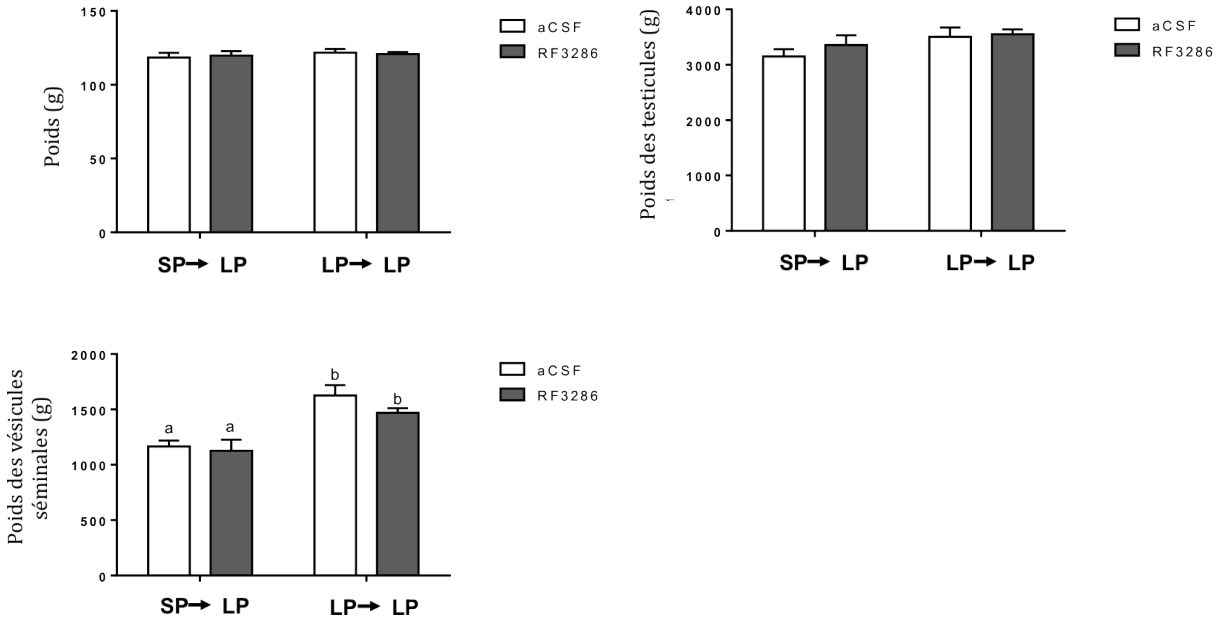


Figure 20 : Mesures des indices physiologiques d'un retour à l'état sexuellement actif des hamsters. *Après 6 semaines de traitement, les poids global et testiculaire des animaux SP/LP ont atteint ceux des animaux LP/LP. La photoréactivation de l'axe hypothalamo-hypophyso-gonadotropique des hamsters SP/LP n'est pas complète car le poids de leurs vésicules séminales n'a pas encore atteint celui des animaux LP/LP. Le RF3286 n'a pas d'effet sur ces signes de la réactivation de l'axe reproducteur. a et b représentent des valeurs significativement différentes. (SP : Short Photoperiod/LP : Long Photoperiod).*

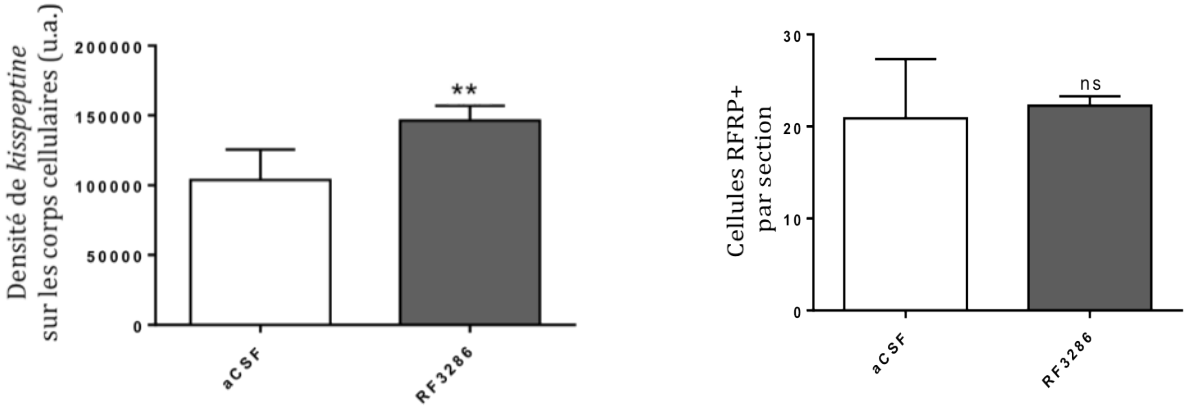


Figure 21 : Comparaison du nombre de transcrits de kisspeptine dans l'ARC (à gauche) et rfrp dans le DMH (à droite) chez des animaux SP/LP. *L'infusion chronique de RF3286 provoque une augmentation de la transcription de la kisspeptine dans l'ARC. Il n'a aucun effet sur le taux de transcription de rfrp dans le DMH. RF3286 n'empêche pas le retour à l'état sexuellement actif des hamsters.*

Durant la totalité de l'expérience, la prise alimentaire des animaux a été mesurée. Comme le montre la figure 22, une diminution de la prise alimentaire d'environ 7 à 4 g/jour est observée après l'opération chez tous les animaux opérés, sauf chez les hamsters LP/LP qui ont reçu du RF3286 (**p < 0,01 ; *p < 0,05 *one way ANOVA* suivi d'un post hoc test de Tukey).

Durant les 7 jours qui suivent l'opération, ce paramètre est significativement plus élevé que chez les autres groupes. Ces résultats peuvent être interprétés comme un blocage de l'effet hyperalgésique induit par le RFRP-3 endogène après l'opération, ce qui faciliterait l'alimentation des animaux. D'autre part, chez le hamster, le RFRP-3 est également connu pour avoir un effet sur les modifications du métabolisme énergétique, en intervenant sur la prise alimentaire et le contrôle des motivations sexuelles (Schneider *et al.*, 2017).

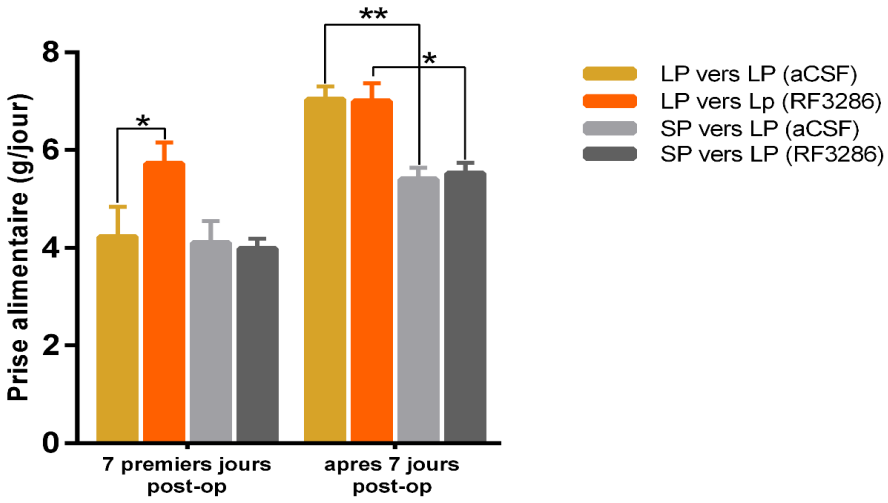


Figure 22 : Comparaison de la prise alimentaire des hamsters entre les 4 groupes de l'étude, et effet de l'infusion chronique de RF3286 sur la photoréactivation de l'axe reproducteur. *Durant les 7 premiers jours qui suivent l'opération, les animaux LP/LP ayant reçu du RF3286 mangent significativement plus que les autres. Les 7 jours suivant, les animaux LP/LP et SP/SP augmentent leur apport alimentaire, mais il existe toujours une différence significative entre les LP/LP et les SD/LP, indiquant que le retour en photopériode longue n'est pas encore complet. Le RF3286 n'a pas d'effet chez les animaux SP/LP, par rapport au véhicule.*

Dans l'ensemble, l'infusion chronique de RF3286 n'a pas d'effet sur la photoréactivation de l'axe reproducteur chez le hamster Syrien mâle. En effet, il n'est pas capable de bloquer les signes physiologiques d'un état sexuellement actif, tels que l'augmentation du poids global, de celui des testicules et des vésicules séminales. De plus, il provoque une augmentation de la transcription de la kisspeptine dans le noyau arqué de l'hypothalamus chez les animaux SP/LP par rapport aux animaux

contrôles SP/LP. Ce résultat est également un indice d'un état sexuellement actif. Ces expériences semblent ainsi nous indiquer que le système RFRP-3/NPFF1R n'a pas de rôle en amont dans la réactivation de l'axe reproducteur du hamster Syrien mâle.

4. Discussion

4.1. Quantification des récepteurs NPFFR sur les hypothalamus de hamsters

Les Bmax obtenus dans nos conditions sont en désaccord avec l'hypothèse d'une augmentation de transcrits du NPFF1R en période sexuellement active. Nous pouvons tenter d'expliquer ces résultats par la méthode et les tissus choisis dans notre expérience. En effet, le radioligand que nous utilisons ne présente pas de forte sélectivité entre les deux sous-types de récepteurs NPFFR. Si l'hypothalamus de hamster contient à la fois les deux récepteurs, alors cette méthode est non applicable, car elle a pour prérequis qu'il n'existe qu'une seule classe de récepteurs sur laquelle le radioligand puisse se fixer. En effet, la présence de plusieurs récepteurs cibles rend l'équation plus complexe, surtout si ces récepteurs sont liés avec une affinité très différente, et que leur proportion varie dans les différentes conditions que l'on a étudiées. Contrairement aux autres rongeurs, pour lequel la présence du récepteur NPFF2R a été largement explorée au niveau du système nerveux central, aucune donnée n'est reportée dans la littérature en ce qui concerne le hamster (Gouardères *et al.*, 2004a,b ; Bonini *et al.*, 2000).

4.2. Le RF3286 ne bloque pas la photoréactivation de l'axe reproducteur des hamsters

Chez les animaux SP/LP, aCSF, nous nous attendions à observer un retour à l'état sexuellement actif, dont les paramètres physiologiques seraient semblables à ceux des animaux LP/LP. En revanche, bien que le poids corporel et celui des testicules soient semblables chez les animaux LP/LP et SP/LP, celui des vésicules séminales est plus faible chez les derniers. La croissance des vésicules séminales fait suite à l'augmentation de la concentration de testostérone, elle-même provoquée par l'accroissement du poids des testicules. Six semaines de maintien en photopériode longue ne semblent donc pas suffire pour arriver au terme du processus de réactivation de l'axe reproducteur.

Les résultats obtenus avec l'infusion chronique de RF3286 suggèrent que le RFRP-3 n'est pas physiologiquement impliqué dans la photoréactivation de l'axe reproducteur du hamster Syrien mâle. Cependant, nous n'excluons pas que la dose de RF3286 utilisée soit peut-être insuffisante pour entraîner un effet physiologique, ou que notre composé se soit dégradé au fil des 6 semaines d'expérience. D'autre part, il pourrait exister une compensation mise en place par le système kisspeptine/Kiss1R en conséquence de l'inhibition du récepteur NPFF1R, les deux systèmes ayant un rôle commun dans le phénomène étudié ici. En outre, il n'est pas exclu que la prévention de l'inhibition de l'axe reproducteur

soit due à un effet des phéromones des animaux LP/LP et SP/LP (aCSF) placés dans la même pièce. Une séparation des différents groupes serait donc à prévoir dans ce type d'expérience. Par ailleurs, il existe des données très récentes révélant que la diminution de l'expression de la déiodinase 3 (Dio3) serait l'évènement le plus précoce dans la photoréactivation de l'axe de reproduction (Milesi *et al.*, 2017). Ces observations étayent nos résultats, en suggérant que le RFRP-3 ne serait pas l'élément déclencheur de ce phénomène. La chronologie précise et les changements séquentiels d'expressions protéiques ne sont pas encore connus, bien qu'une étude récente montre que la sécrétion de FSH soit augmentée plusieurs semaines avant un changement détectable des expressions de la kisspeptine et du RFRP-3 (Milesi *et al.*, 2017). Plutôt que de participer au déclenchement de la photoréactivation, le RFRP-3 serait une de ses conséquences, et permettrait de maintenir ou de participer à l'état sexuellement actif du hamster.

PARTIE III : DEVELOPPPEMENT DE LIGANDS SELECTIFS DU RECEPTEUR KISS1R

1. Introduction

Il existe à ce jour 4 dérivés de la kisspeptine décrits comme antagonistes, dont le plus puissant est le peptide 234, ou p234 (Ac-^{D}Ala-Asn-Trp-Asn-Gly-Phe-Gly-^{D}Trp-Arg-Phe-NH_2, Roseweir *et al.*, 2009). Celui-ci est capable de réduire l'effet de la kiss-10 dans différents modèles cellulaires et *in vivo*, comme par exemple la stimulation de l'inositol phosphate ou encore la libération de LH chez le rat, la souris ou la brebis. En revanche, ces effets ne sont jamais totalement bloqués, et un effet résiduel de la kiss-10 reste observable malgré l'application d'une forte concentration de p234 (Roseweir *et al.*, 2009). Par ailleurs, une étude très récente a démontré l'inefficacité de cet antagoniste sur le blocage de la mobilisation calcique intracellulaire provoquée par la kiss-10 sur des cellules recombinantes exprimant le récepteur Kiss1R, ainsi que sur la libération de LH induit par la kisspeptine chez le chien (Albers-Wolthers *et al.*, 2017). Enfin, aucune donnée concernant sa sélectivité vis-à-vis d'autres cibles n'a été reportée. Le p234 ne représente donc pas l'antagoniste idéal pour étudier les effets du système kisspeptine/Kiss1R *in vitro* et *in vivo*. En marge de ces peptides, des petites molécules non peptidiques ont été également développées dans une démarche de recherche d'antagonistes du récepteur Kiss1R.

C'est ainsi que notre étude prend appui sur les travaux de l'équipe d'Atsuo Baba, qui décrivent pour la première fois des hétérocycles comme antagonistes du récepteur Kiss1R. Des composés dérivés de 2-acylamino-4,6-diphenyle-pyridine ont été criblés au sein de leur laboratoire. Une étude de SAR a été effectuée grâce à plusieurs modifications de la structure précédemment citée, et en particulier au niveau des cycles 4-phenyle et 6-phényle et du groupe 2-acylamino (Figure 23, Kobayashi *et al.*, 2010a). Une première investigation concernant la chaîne latérale d'amines a permis d'identifier l'importance du groupement β-alanine amidé. Ensuite, une optimisation du cycle 6-phényle a révélé que le groupe hydroxyle en position 2' était essentiel pour l'activité, et que l'introduction d'un accepteur d'électrons en position 4' l'améliorait. Enfin, le groupement 2-furoyle fut le plus approprié des groupements 2-acyle. La combinaison de ces différentes modifications chimiques a permis l'identification de composés dotés d'une affinité nanomolaire sur Kiss1R, et jusqu'à 1000 fois plus actifs que le composé « hit » avec lequel leurs travaux ont débuté (Kobayashi *et al.*, 2010a). De surcroît, il est pourvu d'une activité antagoniste dans un test de mobilisation calcique. Un deuxième article du même groupe porte sur l'amélioration de ces composés, notamment au regard de leur stabilité métabolique et de leur faculté à traverser la barrière hémato-encéphalique (Kobayashi *et al.*, 2010b). Pour ce faire, les chercheurs ont réalisé un travail de conversion de l'amine primaire de la chaîne latérale en amine secondaire ou tertiaire, et

identifié un composé doté d'un groupe pipérazine. Ce dernier possède une très haute affinité sur Kiss1R et est capable d'inhiber la mobilisation de calcium intracellulaire induite par la kisspeptine avec une IC_{50} proche de 1 µM. *In vivo*, son administration par voie intraveineuse provoque la diminution du tonus de LH chez le rat castré (Kobayashi *et al.*, 2010b).

Dans notre travail, nous avons voulu caractériser plus en détail deux composés décrits par Kobayashi et collaborateurs. Les composés 9b (Kobayashi *et al.*, 2010a, figure 23), ainsi qu'un dérivé fluoré (composé 1 dans Kobayashi *et al.*, 2010b, figure 24), qui présentait une bonne affinité sur Kiss1R. Nous appellerons respectivement ces dérivés RF1042 et RF1537 dans nos expériences. Nous avons réalisés des tests de liaison afin d'identifier leur affinité et sélectivité sur les autres récepteurs RF-amides, ainsi que des tests d'activité fonctionnelle sur le récepteur Kiss1R.

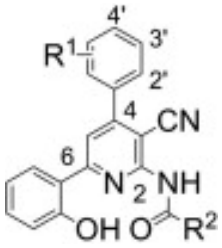


***Figure 23 : Structure du composé 9b de Kobayashi et al., 2010a**. R^1 : 3'-$NHCO(CH_2)_2NH_2$; R^2 : 2-Thienyl. Composé RF1042 dans notre étude.*

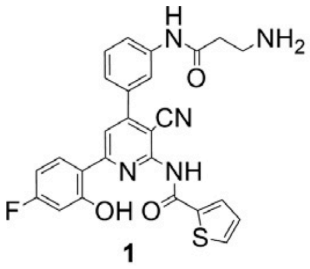


***Figure 24 : Structure du composé 1 de Kobayashi et al., 2010b**. Composé RF1537 dans notre étude.*

2. Résultats

En premier lieu, les constantes d'affinité des deux hétérocycles RF1042 et RF1537 ont été évaluées sur les cinq récepteurs à peptides RF-amides humains et les quatre récepteurs opioïdes humains. Les résultats sont reportés dans le tableau 5. Nos résultats montrent que le RF1537 présente une sélectivité supérieure à 100 fois entre Kiss1R et les autres récepteurs testés, à l'exception de QRFPR pour lequel la sélectivité ne serait que de 20 fois. En outre, il possède une meilleure affinité sur Kiss1R et une sélectivité améliorée vis-à-vis de tous les autres récepteurs testés, par rapport au RF1042.

	RF1042	RF1537
Kiss1R	55 ± 18	24 ± 11
NPFF1R	3100 ± 1200	> 5000
NPFF2R	> 5000	> 5000
PrRPR	> 50000	> 50000
QRFPR	> 500	> 500
MOP	> 5000	> 5000
DOP	> 10000	> 50000
KOP	> 5000	> 5000
NOP	> 20000	> 30000

Tableau 5 : Constantes d'affinité (Ki, nM) des antagonistes du récepteur Kiss1R sur les cinq récepteurs à peptides RF-amides et les quatre récepteurs opioïdes humains*. Expériences de compétition réalisées en présence de radioligands sélectifs de chacun des récepteurs. Les résultats reportés ci-dessus sont la moyenne ± SD d'au moins 2 expériences différentes réalisées en duplicat.*

J'ai ensuite évalué l'activité *in vitro* du RF1042 et du RF1537 dans un test de mobilisation calcique sur cellules CHO exprimant le récepteur Kiss1R humain. Le RF1537 ne présente aucune activité agoniste dans ce test jusqu'à 50µM. Le RF1042 est doté d'une légère activité agoniste à 50 µM, représentant 1/5ème de l'effet agoniste de la kisspeptine à 1 nM (Figure 25). Testés dans un mode antagoniste, ces deux composés décalent vers la droite la courbe effet-dose de la kisspeptine, de manière dose-dépendante (Figure 25), et présentent des K_B respectifs de 1050 ± 14 nM et 133 ± 6 nM. Le RF1042 et le RF1537 sont donc des antagonistes du récepteur Kiss1R *in vitro*.

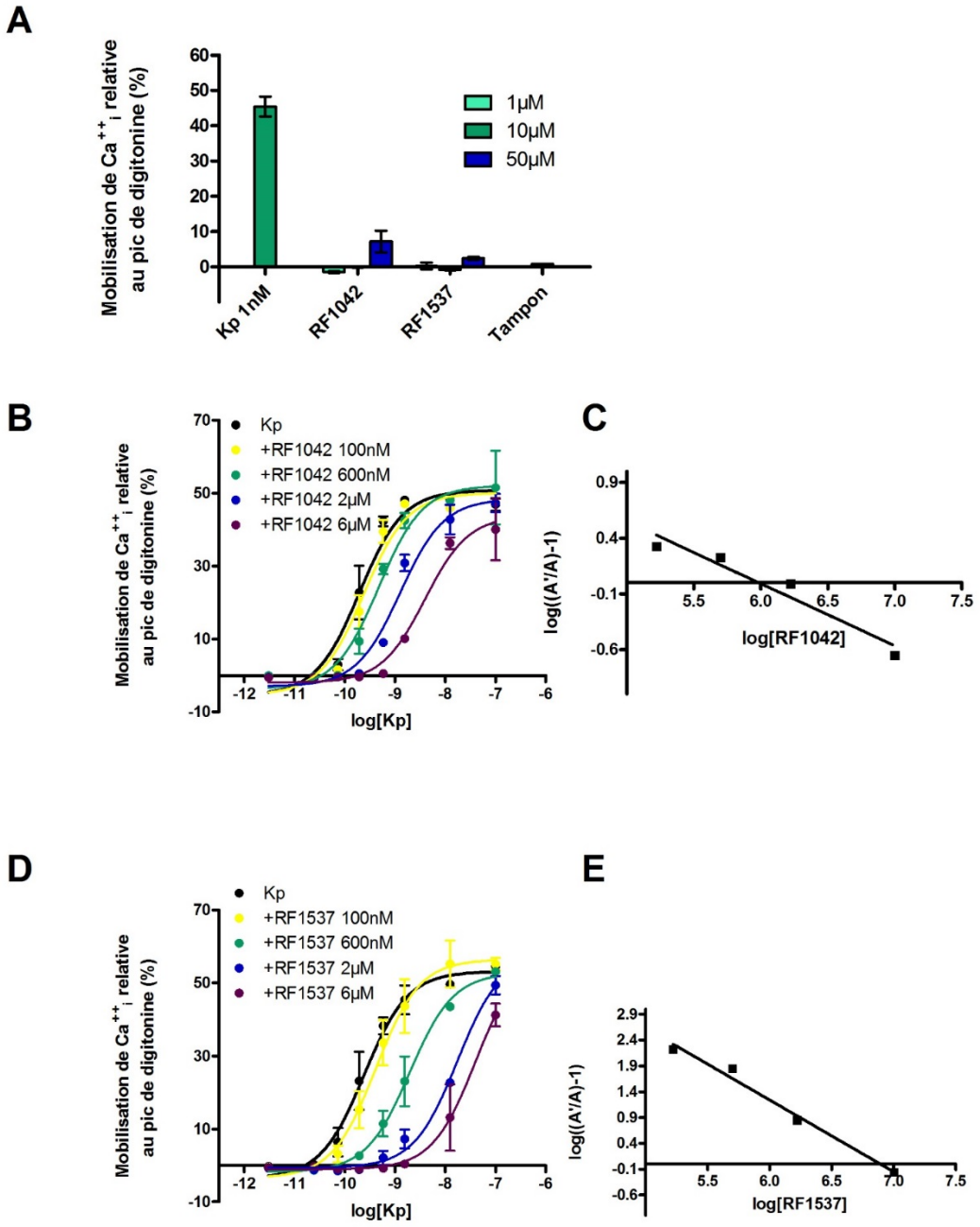


Figure 25 : Activité du RF1042 et du RF1537 sur le récepteur Kiss1R. *Sur des cellules CHO surexprimant le récepteur Kiss1R, la kisspeptine (Kp) induit la libération du calcium à partir des stocks intracellulaires, via son activation de Kiss1R couplé à la protéine G_q. (A) Seuls, le RF1537 et le RF1042 n'ont pas d'effet jusqu'à 50µM. Le RF1042 (B) et le RF1537 (D) déplacent la courbe effet-dose de la kisspeptine de manière dose-dépendante, illustrant son pouvoir antagoniste. Détermination du pA_2 du RF1042 (C) et du RF1537 (E). Les expériences représentatives de manipulations réalisées au moins 2 fois en duplicat sont montrées ci-dessus.*

3. Discussion

Dans l'ensemble, nos résultats indiquent que nous avons identifié deux antagonistes sélectifs du récepteur Kiss1R, dont le RF1537 est le plus puissant et le plus sélectif. Bien que sa sélectivité vis-à-vis du récepteur QRFPR mériterait d'être améliorée, ce composé pourrait déjà être utilisé *in vivo* pour étudier le rôle du récepteur Kiss1R dans la reproduction mais aussi dans la nociception. En effet, des études récentes rapportent son implication dans cette dernière fonction. Alors qu'une étude de notre laboratoire suggérait un rôle central hyperalgésique de la kisspeptine passant par les récepteurs NPFFR (car bloqué par le RF9, Elhabazi *et al.*, 2013), ses effets spinaux et périphériques semblent impliquer le récepteur Kiss1R. Par ces voies d'administration, la kisspeptine provoque une hyperalgésie accompagnée de changements biochimiques associés à des mécanismes de sensibilisation nociceptive centrale et périphérique (Spampinato *et al.*, 2011). Dans ces expériences, la kisspeptine diminue le seuil basal nociceptif des animaux et favorise les comportements en réponse à la douleur chez la souris. Des ARNm et protéines de Kiss1R sont retrouvés dans des fibres sensorielles périphériques et les DRGs (Dun *et al.*, 2003 ; Mi *et al.*, 2009). De plus, une injection intraplantaire du peptide 234 provoque une analgésie robuste dans un modèle de douleur inflammatoire induite par la formaline. Cette réponse comportementale est associée à une augmentation de la phosphorylation du canal TRPV-1, un évènement majeur intervenant dans la sensibilisation nociceptive périphérique (Hucho *et al.*, 2007 ; Stucky *et al.*, 2009 ; Studer *et al.*, 2010). D'autre part, dans la seconde phase du test de douleur inflammatoire induite par la formaline, l'hyperalgésie et l'analgésie induites respectivement par la kisspeptine et l'antagoniste de son récepteur reflètent le développement de la sensibilisation nociceptive centrale des cornes dorsales de la moelle épinière (Tominaga *et al.*, 2001). Le récepteur NPFF2R a été écarté en tant que cible potentielle de la kisspeptine dans ce cas, car on sait que le NPFF a des propriétés analgésiques par voie intrathécale. L'implication du récepteur Kiss1R dans les circuits de la douleur justifie donc les besoins d'un outil très sélectif de Kiss1R, et notamment vis-à-vis de NPFF1R et NPFF2R, les deux récepteurs à peptides RF-amides liant la kisspeptine, et dont on connaît le rôle dans la nociception.

En perspective, il s'agirait de tester par voies périphérique et intrathécale, l'effet du RF1537 sur l'hyperalgésie induite par la kisspeptine, elle même périphérique ou intrathécale. D'une part, cela nous indiquerait si i) le RF1537 se comporte comme un antagoniste *in vivo* et ii) si cette hyperalgésie est due à l'activation des récepteurs NPFFR, comme il l'a été évoqué par le passé, ou si le récepteur Kiss1R a un rôle distinct dans la modulation de la nociception.

Composés / Récepteurs	22e	RF313	RF3286	RF3364	RF1537
NPFF1R	57 ± 1,3	172 ± 13	4,7 ± 0,7	2580 ± 285	> 5000
NPFF2R	84 ± 13	560 ± 160	2213 ± 163	0,2 ± 0,04	> 5000
Kiss1R	> 20	> 100000	> 50000	nd	24 ± 11
PrRPR	± 5	> 20000	> 50000	nd	> 50000
QRFPR	± 5	> 20000	> 50000	nd	> 500
MOP	nd	1020 ± 50[a]	> 5000	nd	> 5000
DOP	nd	> 50000	> 50000	nd	> 50000
KOP	nd	> 1000	> 10000	nd	> 5000
NOP	nd	> 1000	> 5000	nd	> 30000

Tableau 6 : Valeurs de la constante d'affinité (Ki, nM) des différents composés caractérisés au cours des différentes études pour les récepteurs RF-amides et opioïdes. *nd : non déterminé ; [a] : Bihel et al., 2015.*

Composés / Récepteurs	22e	RF313	RF3286	RF3364	RF1537
NPFF1R	**Antagoniste** 100% d'inhibition RFRP-3 (50 nM) à 1µM	**Antagoniste** Kb = 977 ± 145 nM	**Antagoniste** Kb = 16 ± 2 nM	nd	nd
NPFF2R	nm	nm	nm	**Agoniste** EC_{50} = 11 nM Emax = 63%	nd
Kiss1R	nm	nm	50% de l'effet de Kiss-10 (1 nM) à 10 µM	nd	**Antagoniste** Kb = 133 ± 6 nM

Tableau 7 : Activité des différents composés caractérisés au cours des différentes études pour les récepteurs NPFF1R, NPFF2R et Kiss1R. *nd : non déterminé ; nm : non mesurable.*

CONCLUSION

Les cinq peptides RF-amides et leurs récepteurs représentent des systèmes complexes à étudier en raison de l'absence d'outils pharmacologiques sélectifs disponibles. Il était jusqu'à présent difficile de dissocier les rôles respectifs de NPFF1R et NPFF2R à l'aide des antagonistes existant en raison de leur affinité similaire sur ces deux sous-types de récepteurs. Néanmoins, les effets observés dans la modulation de la nociception ont souvent été attribués à NPFF2R, car de plus nombreuses études reportent son implication dans ce domaine.

Nos travaux nous ont permis de mettre à jour le premier antagoniste hautement sélectif du récepteur NPFF1R, à la fois vis-à-vis du récepteur NPFF2R, des autres récepteurs à peptides RF-amides, et des récepteurs opioïdes. Grâce à cet outil et les souris génétiquement déficientes en NPFF1R, nous avons pu mettre en évidence l'implication du système RFRP-3/NPFF1R dans les phénomènes d'hyperalgésie induite par les opioïdes et de tolérance analgésique à la morphine. L'allostasie consécutive à une injection chronique de morphine résulterait en une suractivation de plusieurs systèmes, qui semblent travailler en collaboration, et nécessaires pour l'obtention d'un effet pronociceptif. Cependant, il est intéressant de noter que le blocage d'un des récepteurs à peptides RF-amides ne résulte pas en une simple atténuation de l'effet, mais mène à sa disparition totale. Ces observations suggèrent qu'il n'existe pas de redondance dans la fonction des différents acteurs participant à cet effet. Dans le cas de l'hyperalgésie induite par les opioïdes, il suffit qu'un seul de ces systèmes soit défaillant pour conduire à la perte totale de la fonction, et il n'existe pas de phénomène compensatoire.

Concernant le rôle du système RFRPs/NPFF1R dans la reproduction, il existe un lien étroit entre balance énergétique et système reproducteur. La reproduction nécessite une dépense énergétique importante, et un déséquilibre négatif de cette balance peut entraîner une suppression de la reproduction. Des études suggèrent que les neurones RFRPs sont le chaînon manquant entre ces deux systèmes, en jouant un rôle par dans le contrôle de la reproduction en cas de conditions métaboliques stringentes (Clarke *et al.*, 2012). Dans le futur, l'antagoniste que nous avons identifié devrait se révéler d'une grande utilité pour étudier le rôle du système RFRP-3/NPFF1R dans la reproduction et son éventuelle connexion avec le métabolisme.

Au cours de ce travail, nous avons également identifié des ligands hautement sélectifs pour le récepteur NPFF2R. Cependant, ces ligands présentent une activité agoniste partielle qui n'est pas compatible avec l'utilisation de ces composés *in vivo* en tant qu'antagoniste sélectif. Ils constituent

cependant une base solide qui devrait permettre à l'avenir l'identification d'un antagoniste hautement sélectif du récepteur NPFF2R utilisable *in vivo*.

Enfin, nous avons également caractérisé un antagoniste sélectif du récepteur Kiss1R, le RF1537. Bien que ce composé pourrait déjà être utilisé *in vivo* pour étudier les différentes fonctions du système kisspeptine/Kiss1R, il serait certainement préférable d'améliorer au préalable sa sélectivité vis-à-vis du récepteur QRFPR. En effet, concernant la modulation de la nociception, des résultats obtenus au laboratoire indiquent un rôle de ce récepteur et de son ligand en tant que système pronociceptif.

Dans l'ensemble, ces travaux ont contribué aux objectifs de l'équipe consistant à identifier des antagonistes sélectifs des 5 récepteurs à peptides RF-amides, dont les caractéristiques *in vitro* sont récapitulées dans les tableaux 6 et 7. Bien que des progrès restent encore à faire concernant les composés ciblant les récepteurs NPFF2R et Kiss1R, l'antagoniste sélectif du récepteur NPFF1R que j'ai identifié et caractérisé devrait s'avérer être un outil pharmacologique utile à la communauté scientifique pour étudier les différentes fonctions dans lequel ce récepteur est impliqué.

ANNEXE I

Aminoguanidine hydrazone derivatives as non-peptide NPFF receptor ligands reverse opioid induced hyperalgesia

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Aminoguanidine hydrazone derivatives as non-peptide NPFF receptor ligands reverse opioid induced hyperalgesia

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KEYWORDS: GPCR, neuropeptide FF, GPR74, aminoguanidine hydrazone, Opioid-induced hyperalgesia.

ABSTRACT

Neuropeptide FF receptors (NPFF1R and NPFF2R) and their endogenous ligand Neuropeptide FF have been shown previously to display anti-opioid properties and to play a critical role in the adverse effects associated with chronic administrations of opiates including the development of opioid-induced hyperalgesia and analgesic tolerance. In this work, we sought to identify novel NPFF receptors ligands by focusing our interest on a series of heterocycles as rigidified non-peptide NPFF receptor ligands, starting from already described aminoguanidine hydrazones (AGH's). Binding experiments and functional assays highlighted AGH **1n** and its rigidified analog 2-amino-dihydropyrimidine **22e** for in vivo experiments. As earlier shown with the prototypical dipeptide antagonist RF9, both **1n** and **22e** reduced significantly the long lasting fentanyl-induced hyperalgesia in rodents. Altogether these data indicate that AGH rigidification maintains nanomolar affinities for both NPFF receptors, while improving antagonist character towards NPFF1R.

INTRODUCTION

Neuropeptide FF (NPFF) is an endogenous octapeptide member of a family of neuropeptides that display a conserved Arg-Phe-NH_2 at their C-terminal end and have therefore been named RF-amide peptides. It binds to specific G-protein-coupled-receptors (GPCRs) that exist as two different sub-types, NPFF1R (GPR147) and NPFF2R (GPR74).[1-4] These two receptors have been characterized in mammals including man. They display different cellular and tissue distributions in the central nervous system and at the periphery. [3, 5] NPFF2R are mostly co-localized with μ-opioid receptors, in particular in the spinal cord, whereas NPFF1R are mainly present in the brain. The physiological roles of these systems are still poorly understood. However they have been involved in several physiological processes, such as insulin release, food intake, [6-8] memory, [9- 11] blood pressure [12] and electrolyte balance.[13] More interestingly, several lines of evidence support the involvement of NPFF receptors in the modulation of nociception and opioid-induced analgesia. [14]

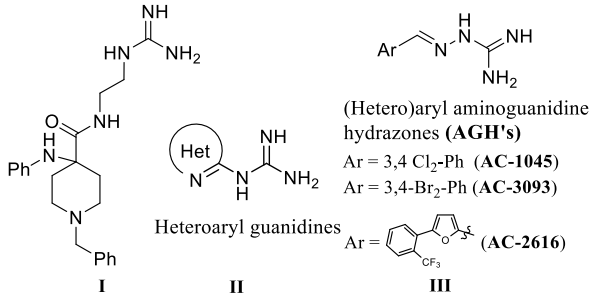
Long term opioid treatments are associated with several adverse effects, including the development of analgesic tolerance,[15] which leads to the need of increasing doses of the opioid analgesic to be used for relieving pain.[16-17] It has been proposed that this phenomenon may involve a balance between opioid and so-called anti-opioid systems able to modulate nociception. More specifically, stimulation of mu-opioid receptors triggers activation of anti-opioid systems such as NPFF,[14, 18] that in turn produce hyperalgesia [19-21] thus diminishing the net analgesic effect of the opioid analgesic.

A better understanding of the respective physiological roles played by both NPFF1R and NPFF2R in the modulation of nociception is limited by the lack of pharmacological tools, including agonists (metabolically stable NPFF mimetics) and antagonists acting in a selective manner on one of the two NPFF receptor subtypes. In our previous work, we focused our efforts on the prototypical signature of the C-terminal part of NPFF (Arg-Phe-NH_2) and

reported the first non-selective NPFF receptor antagonist (RF9, N-adamantane-carbonyl L-Arg L-Phe $CONH_2$). [22, 24] We further showed that RF9 did not show any intrinsic analgesic or hyperalgesic effect but efficiently blocks hyperalgesia induced by repeated administration of opiates such as heroin and morphine as well as analgesic tolerance. [22, 24]

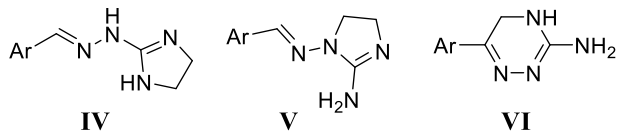
The very few SAR analyses published so far in the literature support the critical role played by the guanidinium cation present in the structure of N-acyl arginine-containing dipeptides recently described by us, [25- 26] or in the few reported nonpeptide structures (Fig. 1, guanidine-piperidines I, heteroaryl guanidines II and aminoguanidine hydrazones III). [27, 29]

Figure 1. Guanidine-containing nonpeptide NPFF-R ligands



AGHs were initially reported by Acadia pharmaceuticals, but only very few derivatives were described in terms of binding and functional activities. [23, 29-30] As AGHs are versatile pharmacological tools, compatible with in vivo oral administration (ex: Guanabenz, an antihypertensive drug), we describe in this paper an in-depth SAR analysis of the AGH moiety, as well as a scaffold hopping strategy leading to promising heterocyclic structures with in vivo efficacy against opioid-induced hyperalgesia (Fig. 2).

Figure 2. Scaffold hopping strategy along the structure of AGH chemotype.

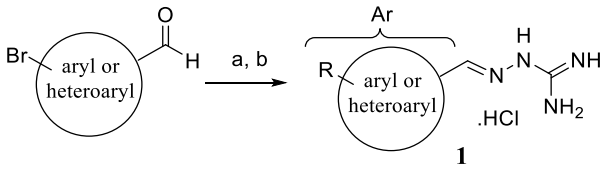


CHEMISTRY

The different aminoguanidine arylhydrazones **1** could be easily obtained by reacting the corresponding aryl or heteroaryl carboxaldehyde with amino guanidine hydrochloride in refluxing ethanol or microwaves, when the carboxaldehyde was less reactive (yields ranging from 75 to 90%).[31- 32] Most of aldehydes were commercially available (cpds **1a-e, 1g-p**), or could be prepared by a Suzuki-Miyaura reaction under microwaves **(cpds 1f, 1q-s)**, as depicted in Scheme 1.

All the synthesized aryl and heteroaryl aminoguanidine hydrazones are listed in Tables 1-2.

Scheme 1. Synthesis of AGH's **1**

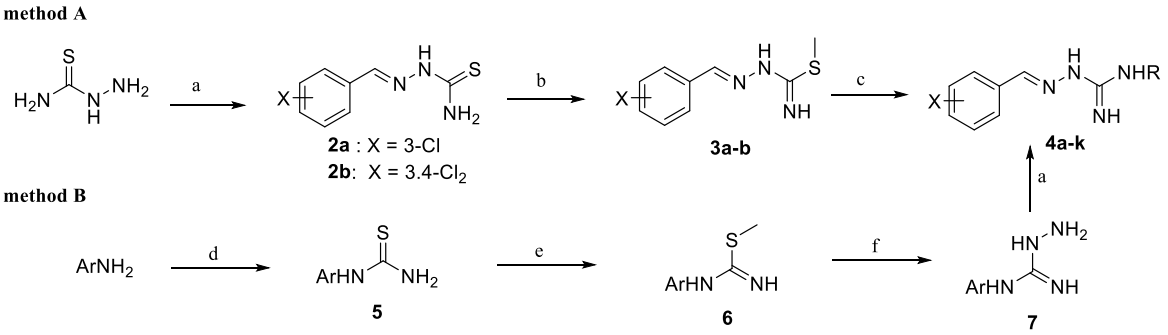


1

Reagents and conditions: (a) $ArB(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, DME/H_2O, MW, 150°C, 89-97%, 10min; (b)) Amino guanidine, HCl, EtOH, reflux, 5h, 75-90%.

The guanidine fragment constitutes a second anchor point allowing further introduction of various substituents at either the terminal (ω), or hydrazine (α) nitrogen. The substitution on ω position was performed as indicated in Scheme 2. Chemical diversity (NHR) could be introduced either at the final step (method A), or at the beginning (method B). Both are yielding similar cumulative yields. However, with fairly active nucleophiles (anilines), the method B involving sodium thiocyanate was found more efficient because of its strong electrophilic character.

Scheme 2. Substitution on ω position of the aminoguanidines hydrazones

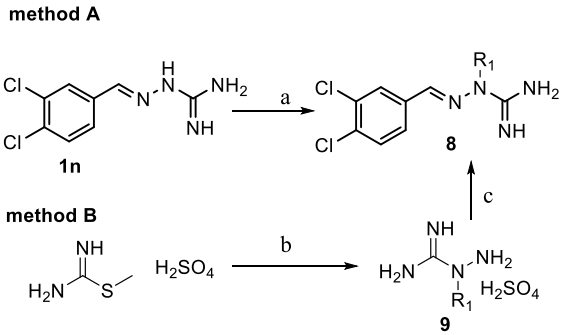


Reagents and conditions: (a) ArCHO, EtOH, reflux, 5h; (b) Iodomethane, DMF, rt, 4h; (c) NH_2R, EtOH, reflux, 5h; or NH_2R, EtOH, MW, 120°C, 15min; (d) Sodium thiocyanate, EtOH, reflux, 30 min. (e) Iodomethane, DMF, rt; (f) Hydrazine monohydrate, MeOH, reflux, 3h.

The thiosemicarbazone **2,** obtained by refluxing the title carboxaldehyde with thiosemicarbazide, was quantitatively methylated by means of iodomethane in DMF. The resulting S-methyl intermediate **3** could be reacted with various primary and secondary amines (method A).[33-34] In the method B, the N-phenyl thiourea intermediate **5** was further methylated. The resulting S-methyl thiourea derivative **6** was reacted with hydrazine and the final N-aryl aminoguanidine hydrazone **4f** could be prepared in satisfactory yield.[35-36]

Direct substitution of the highly nucleophilic aminoguanidine hydrazone was performed in various solvents, bases and halides (method A, Scheme 3). The reaction of **1n** with benzyl bromide in DMF was efficient and regioselective, leading to the N-α-benzyl derivative **8** as the single isomer. However alkylation with highly reactive methyl iodide afforded a mixture of mono and di-methyl derivatives, whereas no reaction occurred with the less electrophilic phenethyl bromide, even in DMF solution. An alternative method (method B) could be generalized to all derivatives. S-methyl thiourea was reacted with N-alkylhydrazine in ethanol leading in a non-ambiguous manner to the N-alkyl aminoguanidine intermediate **9**. All the N-substituted aminoguanidine hydrazones are summarized in Table 3.

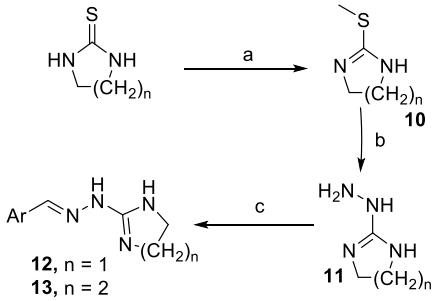
Scheme 3. Substitution at the non-terminal nitrogen (AGH'S **8)**



Reagents and conditions: (a) BnBr, DMF or MeOH, rt, 48h; (b) NH_2-NHR_1, EtOH, reflux; (c) 3,4-dichlorobenzaldehyde, EtOH, reflux.

The guanidine moiety was also introduced in various cyclic systems, as depicted in Table 4. Cyclic thioureas were first S-methylated, and the resulting intermediates **10** were reacted with hydrazine affording the 2-hydrazino cyclic amidines **11** as the key intermediate (Scheme 4).

Scheme 4. Access to the cyclic aminoguanidine derivatives **12** and **13.**

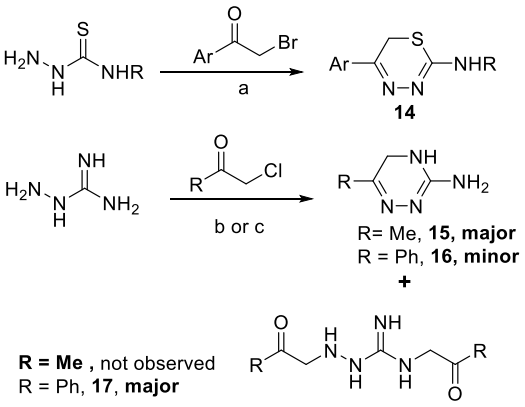


Reagents and conditions: (a) MeI, MeOH, reflux, 1h; (b) Hydrazine monohydrate, EtOH, reflux, 6h; (c) ArCHO, EtOH, reflux, 5h.

Other AGH cyclic derivatives bridging the imine carbon to the terminal guanidine nitrogen were also synthesized. The 5-aryl-2-aminothiadiazines **14 a-c** were prepared in one step by reacting thiosemicarbazide with a substituted phenacyl bromide in methanol.[37] In a similar way the different dihydrotriazines **15** could be obtained by replacing thiosemicarbazide by aminoguanidine in cyclocondensation reaction. As shown in Scheme 5, the 6-methyl dihydrotriazine **15** could be recovered in good yield, [38] whereas the reaction performed with

phenacyl chloride yielded a mixture of dihydrotriazine **16** as the minor product along with the di-adduct **17**. As the expected compound **16** could not be isolated from the reaction mixture, a second approach was carried out with success.

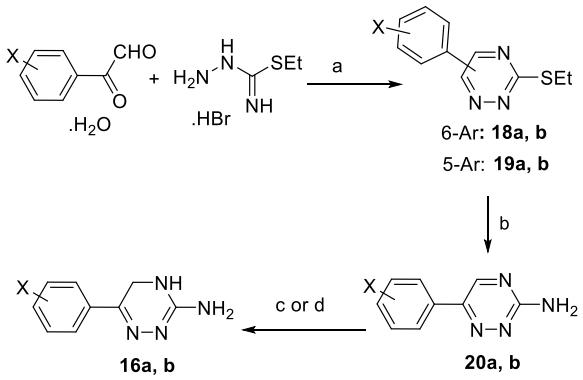
Scheme 5. Rapid access to 5-aryl-2-aminothiadiazines **14** and 6-methyl-3-aminodihydrotriazine **15** .



Reagents and conditions: (a) MeOH, reflux, 30min, 48-86%; (b) H_2O, 25°C, 48h, 93%; (c) H_2O, 25°C; or EtOH, reflux.

The key step was a careful reduction of the corresponding 2-amino-1,2,4-triazines **20a** and **20b** into the corresponding dihydrotriazines **16a-b** by means of triethylsilane in TFA, [39] avoiding removal of critical chlorine atoms from the phenyl ring observed when using catalytic hydrogenation. The 2-amino-1,2,4-triazines **18** were prepared by cyclocondensation of different aryl glyoxals with ethyl hydrazinocarbimidothioate leading to a mixture of two regioisomers **18** (major) and **19** (minor, <10%), respectively.[40] They were easily recovered by silica gel column chromatography (Scheme 6).

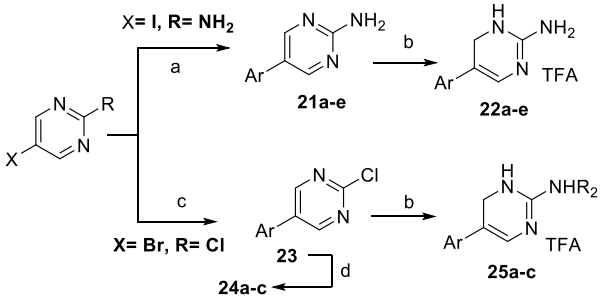
Scheme 6. Access to 6-aryl-3- amino-dihydrotriazines **16a, b**



Reagents and conditions: (a) EtOH, reflux, 30 min; (b) 7N NH_3 in MeOH, reflux, 15h; (c) H_2/Pd, 60psi, 15h; (d) Et_3SiH, TFA, 15h, rt.

In a similar manner reduction of 5-aryl-2-aminopyrimidines **21a-e,** obtained by reacting the commercially available 2-amino-5-iodopyrimidine with different arylboronic acids, [41] afforded the corresponding dihydropyrimidines **22a-e** (Scheme 7).

Scheme 7. Access to 5-aryl-2-amino-dihydropyrimidines **22a-e** and **25a-c**.



Reagents and conditions: (a) $ArB(OH)_2$, $Pd(OAc)_2$, S-Phos, K_2CO_3, MeCN, H_2O, 95°C, 15h; (b) Et_3SiH, TFA, rt, 1h; (c) $ArB(OH)_2$, $Pd(OAc)_2$, KF, MeOH, µw, 120°C; (d) NH_2R_2, THF, µw, 100°C, 12min

The use of umpolung principle allowed rapid access to 2-N-substituted 2-amino pyrimidine derivatives **24**, as illustrated in Scheme 7. The 5-aryl-2-chloropyrimidine **23** was submitted to amination using microwaves. The resulting derivatives **24a-c** were reduced as described

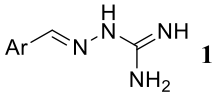
earlier, giving the expected dihydropyrimidines **25a-c** in satisfactory yields. [39] Cyclic Compounds **14**, **16**, **22** and **25** are listed in Table 5.

RESULTS AND DISCUSSION

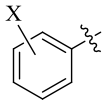
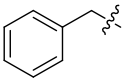
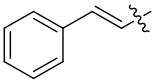
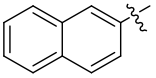
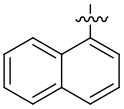
Binding experiments were performed first by using [^{3}H]-FFRF-NH_2 as radioligand on membranes from CHO cells stably expressing SF-hNPFFR1 or SF-hNPFFR2.[42] The percentages of inhibition of FFRF-NH_2 binding by the different compounds at 0.5 and 5 µM are shown in Tables 1-4. Ki values were determined for the most potent compounds.

Altogether our data allowed the following comments: i) The unsubstituted AGH **1a** presented a good affinity for NPFF1R (Ki= 76 nM), associated with a satisfactory selectivity towards NPFF2R (Ki > 3 µM). ii) Surprisingly substitution in para position by chlorine (**1d**), or methoxy (**1j**) afforded compounds with similar affinities towards NPFF1R (Ki ± 20 nM). iii) Introduction of a second chlorine atom in position meta (**1n**), or ortho (**1l**) gave potent ligands but with different selectivity profiles. Compound **1n** displayed a good affinity of NPFF1R (Ki = 12 nM) and was relatively selective toward NPFF2R (twentyfold), whereas **1l** is not selective. iv) Replacing the m-Cl substituent in **1n (AC-1045)** by a CF_3 group dramatically reversed selectivity profile. Finally, within this chemical sub-series the AGH **1p (AC-3099**) displayed the best affinity for NPFF2R (Ki = 4 nM) and a ten fold lower affinity for NPFF1R.

Table 1. AGH derivatives and their binding affinities for NPFF1R & NPFF2R.



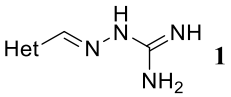
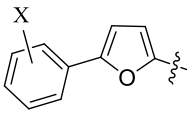
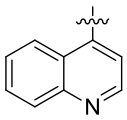
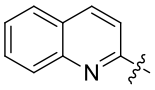
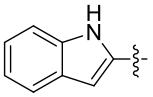
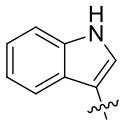
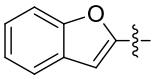
1

			NPFF1-R			NPFF2-R		
			% inhibition at		Ki [a] (nM)	% inhibition at		Ki [a] (nM)
n°	Ar	X	5µM	0.5µM		5µM	0.5µM	
1a		H	62	34	76	28	10	3,600
1b		2-Cl	55	25	nd[b]	13	1	nd
1c		3-Cl	56	17	nd	24	0	nd
1d		4-Cl	98	69	20	60	18	490
1e		2-Ph	70	22	na[c]	35	17	na
1f		3-Ph	9	0	nd	30	0	nd
1g		4-Ph	58	11	nd	23	5	nd
1h		2-OMe	48	34	nd	8	0	nd
1i		3-OMe	59	33	nd	20	0	nd
1j		4-OMe	77	65	25	28	14	2,500
1k		4-CF_3	100	57	341	89	37	66
1l		2,4-Cl_2	66	22	47	48	14	13
1m		2,6-Cl_2	77	16	86	28	5	7,800
1n (AC-1045)		3,4-Cl_2	90	40	**12**	92	50	270
1o		3,5-Cl_2	88	46	83	83	32	219
1p (AC-3099)		3-CF_3,4-Cl	100	71	40	100	71	**4**
1u		-	26	8	nd[b]	11	0	nd
1v		-	38	19	nd	32	16	nd
1w		-	90	36	156	63	19	nd
1x		-	99	36	115	42	13	345

[3]H]-FFRF-NH_2 was used as radioligand for competition assays with both hNPFF1 & 2 receptors; [a] Values are means of two experiments performed in duplicates; [b] nd : not determined. [c] na: not active, Ki > 5 µM

Table 2 Heteroaryl AGH derivatives and their binding affinities for NPFF1R & NPFF2R

.

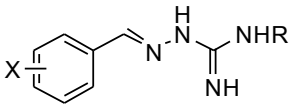
	 **1**		**NPFF1-R**			**NPFF2-R**		
			% inhibition at		Ki (nM)[a]	% inhibition at		Ki (nM)[a]
n°	Het		5µM	0.5µM		5µM	0.5µM	
		X						
1q		H	83	14	185	32	0	1,510
1r (AC-2616)		2-CF_3	100	93	**7**	92	52	**4**
1s		2-NH_2	74	7	na	26	0	na
1t		3,4-Cl_2	98	26	83	54	0	2,350
1y			87	21	nd	38	13	nd
1z			32	0	nd	5	0	nd
1aa			47	0	nd	68	31	nd
1ab			98	47	84	46	10	nd
1ac			32	22	nd	30	0	nd

[^{3}H]-FFRF-NH_2 was used as radioligand for competition assays with both hNPFF1 & 2 receptors; [a]Values are means of two experiments performed in duplicates; [b] nd: not determined.

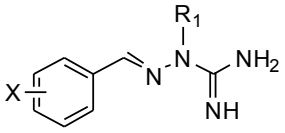
As firstly introduced by Acadia researchers, the 5-arylfuran-2-yl aminoguanidine hydrazones **1q** and **1r** (table 2) appeared as another promising chemical sub-series. However, no binding data were available for SAR analysis. [29] The specific role played by the furan ring remains unclear. Again, aromatic substitution led to compounds with significantly increased affinities. In particular the 2-CF_3 derivative **1r (AC-2616)** showed a strong affinity towards both

NPFFR subtypes. The other compounds listed in Table 1 are homologues (**1n**, **1v**), naphtalen (**1w**, **1x**), and heterocyclic derivatives (**1y-1ac**), to be compared with **1a**. They were found poorly active, except the 3-indolyl compound **1ab**, which presented a binding profile similar to that of **1a** (Ki = 84 nM, NPFF1R).

Table 3: N-substituted aminoguanidine hydrazones and their affinities for NPFF1R and NPFF2R



4



8

					NPFF1-R			**NPFF2-R**		
					% inhibition at			% inhibition at		
n°	X	R (or R_1)	Method	Yield (%)	5μM	0.5μM	Ki (nM)	5μM	0.5μM	Ki (nM)
4a	3-Cl	Me	A	69	27	28	nd[a]	64	25	nd
4b	3-Cl	Ph	B	79	42	28	nd	26	12	nd
4c	3-Cl	Bn	A	91	52	18	nd	34	12	nd
4d	3-Cl	$(CH_2)_2Ph$	A	70	50	11	nd	49	8	nd
4e	3,4-Cl_2	Me	A	92	78	27	nd	81	47	nd
4f	3,4-Cl_2	Ph	B	57	48	13	nd	27	0	nd
4g	3,4-Cl_2	Bn	A/B	71/59	70	26	nd	45	13	nd
4h	3,4-Cl_2	$(CH_2)_2Ph$	A	72	76	14	nd	56	3	nd
4i	3,4-Cl_2	$(CH_2)_3Ph$	A	58	78	13	nd	30	0	nd
4j	3,4-Cl_2	N-benzylpiperidinyl	A	75	82	21	nd	59	11	nd
4k	3,4-Cl_2	N-benzylpiperazino	A	60	57	27	nd	32	5	nd
8a	3,4-Cl_2	Me	A	80	41	8	nd	16	5	nd
8b	3,4-Cl_2	Bn	B	92	51	14	nd	23	3	nd

Further substitutions either at the terminal nitrogen (cpds **4**, R ≠ H), or non-terminal guanidine nitrogen (cpds **8**, R_1 ≠ H), led to inactive compounds. It may highlight some critical interactions involving the whole aminoguanidine fragment present in AGH compounds **1**, **4** and **8**. This hypothesis was further supported by inactivity of imidazoline **12** and tetrahydropyrimidine **13** (table 4). The last mode of rigidification, (type V, Chart 2) is new and particularly efficient, as illustrated by data in Table 5.

Table 4. Affinities of cyclic AGH'S **12-13**

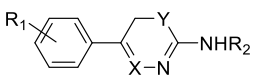
	NPFF1-R			NPFF2-R		
	% inhibition at			% inhibition at		
n°	5µM	0.5µM	Ki (nM)	5µM	0.5µM	Ki (nM)
12	34	12	nd[a]	18	0	nd
13	41	19	nd	39	11	nd

[^{3}H]-FFRF-NH_2 was used as radioligand for competition assays with both hNPFF1 & 2 receptors; [a] nd: not determined.

The resulting dihydrotriazines **16** showed potent affinities towards NPFF receptors. The unsubstituted derivative **16a** showed a Ki of less than 20 nM on NPFF1R with a good selectivity profile, whereas introduction of two chlorine atoms in meta and para position (cpd **16b**), restored a good affinity for NPFF2R besides a similar affinity for NPFF1R (Ki = 29 nM and 44 nM on NPFF1R and NPFF2R, respectively). Other series in Table 5 could be considered as two different types of bioisosteres of compounds **16**. The first series (cpds **14**, X= N, Y= S) was found inactive, whereas the second series (cpds **22**, X= C, Y= NH) was again more promising with highly potent representatives.

The unsubstituted carbaisostere (dihydropyrimidine **22a**) showed a moderate affinity for both receptor subtypes with Ki in the 100 nM range. Among the different substitutions introduced onto the phenyl ring of **22a**, beneficial chlorine effects were again particularly interesting. Chlorination in para position (cpd **22c**) led to a potent (Ki = 1.3nM) and selective (2 orders of magnitude) ligand of NPFF1-R. Introduction of a second chlorine atom in meta position did not modify the affinity towards NPFF1-R, but restored a good affinity for NPFF2R (**22e**, Ki = 0.91 nM and 37 nM for NPFF1R and NPFF2R, respectively). Finally, as observed in the AGH series, substitution at the exocyclic nitrogen led to compounds with strongly decrease (cpd **25a**), or loss of affinity (cpds **25b, c**).

Table 5. 6-aryl dihydro-3-aminotriazines and their isosteres



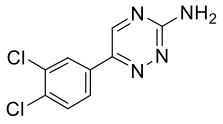
					NPFF1-R			NPFF2-R		
					% inhibition at		Ki (nM)[a]	% inhibition at		Ki (nM)[a]
n°	X	Y	R_1	R_2	5µM	0.5µM		5µM	0.5µM	
14a	N	S	H	H	12	0	na[b]	0	0	na
14b	N	S	3-Cl	H	0	0	na	0	0	na
14c	N	S	3,4-Cl_2	H	21	0	na	15	0	na
14d	N	S	H	Ph	21	16	na	2	0	na
14e	N	S	H	Bn	20	0	na	14	14	na
16a	N	NH	H	H	72	30	16	18	0	na
16b	N	NH	3,4-Cl_2	H	64	54	29	70	52	48
22a	CH	NH	H	H	78	58	150	47	24	69
22b	CH	NH	3-Cl	H	74	33	74	21	6	2,740
22c	CH	NH	4-Cl	H	84	28	**1.3**	41	5	260
22d	CH	NH	4-OMe	H	93	45	21	48	6	210
22e	CH	NH	3,4-Cl_2	H	100	85	**1**	93	65	37
25a	CH	NH	3,4-Cl_2	Bn	65	28	60	32	20	430
25b	CH	NH	3,4-Cl_2	n-Pr	27	30	na	36	33	na
25c	CH	NH	3,4-Cl_2	$(CH_2)_4$-Ph	95	21	1563	56	16	358

[^{3}H]-FFRF-NH_2 was used as radioligand for competition assays with both hNPFF1 & 2 receptors; [a] Values are means of at least two experiments performed in duplicates; [b]na: not active, Ki > 5 µM

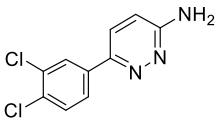
Some other structural analogues resembling the structure of active azines **16, 22** were synthesized (for preparation see SI) and evaluated for their affinity toward NPFF1R and NPFF2R (Figure 3). All of them showed low or no affinity towards both NPFF receptor subtypes. This inactivity may result from electronic considerations emphasizing i) the lack of NH group within the ring system (aromatic azines **20b** and **26**). ii) the presence of an NH

group within a strongly basic cyclic guanidine (cpd **27**). iii) some critical steric hindrance (cpd **28**).

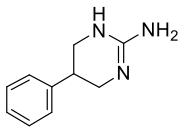
Figure 3. Structurally analogs of compound **20, 22** and their binding affinity for NPFF1R and NPFF2R



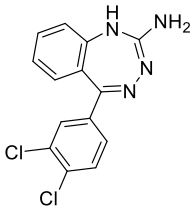
20b
0.5 □M / □ 1□M



26
>>1 □M / >> 1□M



27 [40]
>>1 □M / >> 1□M



28
□1 □M/ □ 1□M

The compounds earlier synthesized by Acadia researchers (AC-series) and our most promising hits from new chemical series (**16a-b**, **22c** and **22e**) were selected for further pharmacological evaluation. In a first step, affinities for both NPFF1R and NPFF2R were evaluated by using [^{125}I]-1DMe-NPFF as radioligand and compared to the values obtained with [^{3}H]-FFRF-NH_2 used for primary screening. Ki values for these compounds are summarized in Table 6.

When comparing Ki values obtained from both binding assays using ^{3}H-FFRFamide or [^{125}I]-1DMe-NPFF, we found similar results in terms of affinity and selectivity for **1p (AC-3099), 1r** (**AC-2616)** and **16b**. Surprisingly, **1n** (**AC-1045)**, **16a**, **22c** and **22e** displayed significantly less affinity (about one order of magnitude less) on NPFF1-R, when using [^{125}I]-1DMe-NPFF as radioligand. It is interesting to note that our binding data obtained with [^{125} I]-1DMe-NPFF for 3,4-dichloro AGH **1n (AC-1045)** on both NPFF1-R and NPFF2-R (214 nM and 73 nM,

respectively) are very similar to those reported for the 3,4-dibromo AGH derivative **AC-3093** with the same radioligand (pki = 7.0 and 6.9).[30]

Table 6. Binding data for the most relevant NPFF-R ligands

		[^{3}H]-FFRF-NH_2, Ki, nM[a]		[^{125}I]-1DMe-NPFF, Ki, nM[a]	
N°	Code AC [29]	**NPFF1-R** (SEM)	**NPFF2-R** (SEM)	**NPFF1-R** (SEM)	**NPFF2-R** (SEM)
1n	**1045**	12 (2)	270 (10)	214 (54)	73 (10)
1p	**3099**	40 (4)	4 (0.5)	83 (34)	13 (1.3)
1r	**2616**	7 (1)	4 (1)	10 (2)	25 (4.6)
16a	-	16 (3)	> 1µM	116 (6)	2,800 (554)
16b	-	29 (6)	44 (14)	48 (14)	143 (34)
22c	-	1.3 (0.4)	260 (50)	50.5 (7)	202 (23.5)
22e	-	0.9 (0.1)	37 (8)	57 (1.3)	84 (13)

[^{3}H]-FFRF-NH_2 was used as radioligand for competition assays with both hNPFF1 & 2 receptors; [a] Values are means of at least two experiments performed in duplicates.

Overall, these results show that previously reported compounds **1n** (**AC-1045)**, **1p** (**AC-3099)**, and **1r** (**AC-2616)** as well as compounds **16b**, **22c** and **22e** display a good affinity for both NPFF receptors subtypes. Interestingly, compound **16a** displayed a good affinity for NPFF1R but low for NFF2R with a selectivity factor between 25 and 60 depending on the radioligand that was used in competition experiments. When comparing selectivity profiles of **16a** and **16b**, introduction of chlorines in meta and para position of the phenyl ring led to a non-selective ligand, as earlier observed in the AGH series (compare **1a** and **16b**).

We further evaluated the selectivity of these compounds toward the other RF-amide neuropeptide receptors PrRPR (alias GPR 10), Kiss1R (alias GPR54) and QRFPR (alias GPR103) as illustrated in Table 7.

Table 7. Affinity of the most relevant NPFFR ligands for PrRP, Kiss1 and QRFP receptors.

N°	Code[29]	Ki PrRPR (μM)[a]	Ki Kiss-1R (μM)[a]	Ki QRFPR (μM)[a]
1n	**AC-1045**	± 5	> 20	> 5
1r	**AC-2616**	> 20	> 20	± 5
1p	**AC-3099**	> 5	> 5	0.5 > Ki > 5
16a		> 5	> 5	> 5
16b		> 5	> 5	> 5
22c		> 20	> 20	> 5
22e		± 5	> 20	± 5

[^{3}H]-PrRP-20, [^{125}I]-Kp-10 and [^{125}I]-43RFa were used as radioligand for competition assays with PrRP, Kiss1 and QRFP receptors (respectively);
[a]Values are results of at least two experiments performed in duplicates.

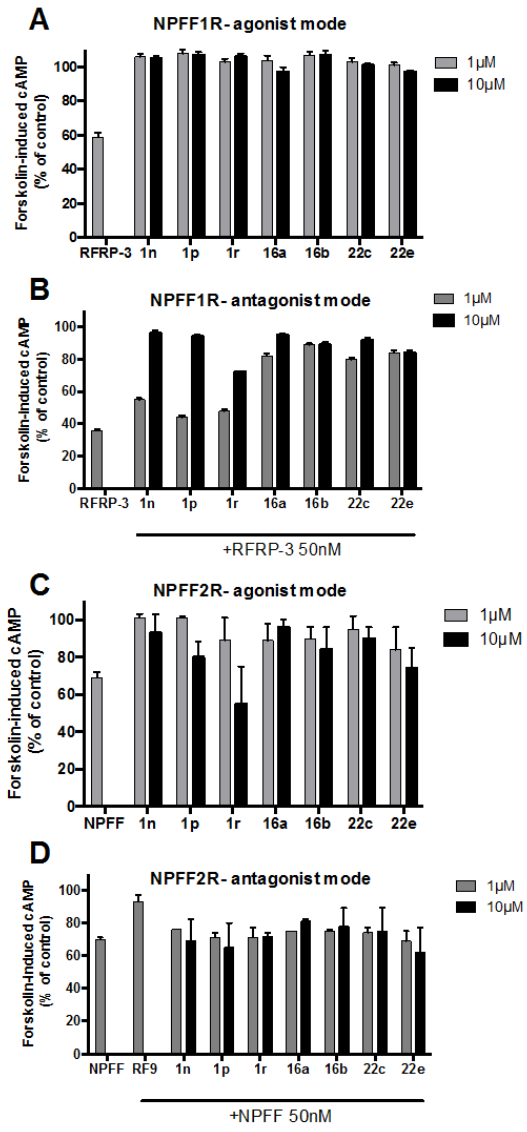
Except **1p** (**AC-3099)** that displayed an affinity between 0,5μM and 5μM on QRFPR, all other compounds had Ki values that were over 5 μM (or even over 20 μM for some compounds on PrRPR and Kiss1R) for the three RF-amide receptors. Altogether these data indicate that the selected compounds display a good selectivity for NPFF receptors.

Examination of in vitro functional activity of the selected compounds

We then examined the agonist or antagonist activity of the seven selected compounds at both NPFF1R and NPFF2R with HEK293-hNPFF1R/NPFF2R GloSensor^{Tm} stable cell lines. We first observed that all the selected compounds displayed a non-specific effect on cAMP accumulation in HEK-Glo cells lacking recombinant hNPFF1R or hNPFF2R, when used at concentration exceeding 10 μM (data not shown). We therefore tested these seven compounds at 1 and 10 μM on both NPFF receptor subtypes. As shown in Figure 4A, RFRP-3, the endogenous agonist of NPFF1R, inhibited forskolin-stimulated production of cAMP in hNPFF1R expressing cells while none of the seven evaluated compounds displayed agonist activity in this assay. When tested in combination with RFRP-3 (antagonist mode; Fig. 1B), 10 μM of all the compounds completely blocked the RFRP-3 inhibition of forskolin-stimulated production of cAMP. Moreover, 1 μM of compounds **16a**, **16b**, **22c** and **22e** displayed a similar effect indicating that they are more potent than compounds **1n** (**AC-1045)**,

1p (**AC-3099)** and **1r** (**AC-2616)**. These data clearly demonstrate that the seven selected compounds display antagonist activity at hNPFF1R *in vitro*. When tested on cells expressing NPFF2R in both agonist (Fig. 4C) and antagonist mode (Fig. 4D), except compounds **1r** that showed a significant agonist activity at 10 µM, all the others compounds displayed neither agonist nor antagonist activity on this receptor up to 10 µM. These results contrast with previous data on **1n** and **1p** (compound **1** and **9)** [29], reporting that these two compounds were full NPFF2R agonists with no activity (agonist or antagonist) or partial agonist activity at NPFF1R, respectively. Although we cannot exclude that in our assay **1n** and **1p** could display agonist activity at NPFF2R at concentrations higher than 10 µM, we clearly show that these two compounds display antagonist activity at NPFF1R. This discrepancy could rely on the R-SAT assay that was originally used to characterize these compounds as compared to more classical radioligand binding and cAMP assays we used in this study. In conclusion, the different compounds we characterized here, all bind to both NPFF receptor subtypes, all display antagonist activity at NPFF1R while we detected significant agonist activity at NPFF2R only for compound **1r (AC 2616)**.

Figure 4. Evaluation of the activity of seven selected compounds on NPFF1 and NPFF2 receptors. Forskolin-induced cAMP accumulation was recorded in the presence of the seven selected compounds on NPFF1R and NPFF2R stably expressed in HEK-Glo recombinant cells, in the agonist (A and C, respectively) and antagonist (B and D, respectively) modes. RFRP-3 (50 nM) inhibited ≈40% of the forskolin -stimulated cAMP production in HEK-NPFF1R-Glo cells while NPFF (50 nM) inhibited ≈30% of the forskolin-stimulated cAMP production in HEK-NPFF2R-Glo cells. Antagonist activity of the seven compounds was evaluated in presence of the 50 nM RFRP-3 or NPFF as indicated in B and D. Error bars represent the mean ± SEM of data from at least two experiments performed in duplicate.

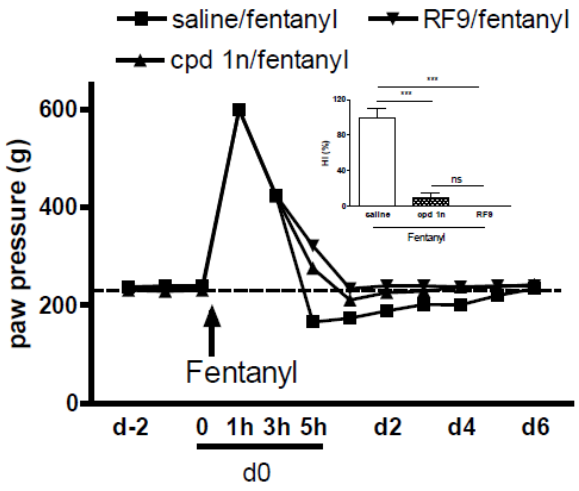


*Cpd **1n** and cpd **22e** prevent long lasting hyperalgesia induced by fentanyl in rodents*

In a previous work, we have shown that the pharmacological blockade of the two NPFF receptors (NPFF1R and NPFF2R) by RF9 prevents the development of opioid-induced hyperalgesia in rodents.[22, 24] In this study, we therefore investigated the activity of two representative compounds of the non-peptide antagonists of the NPFF receptors, compounds **1n** (**AC-1045)** and **22e**, in a model of hyperalgesia induced by acute administration of fentanyl in rats and mice, respectively.[24, 17] RF9 was also tested in these experiments as a positive control. As shown in Figure 5, fentanyl (4x80 µg/kg, sc.) promoted a short lasting analgesic response in rats (modulation of paw pressure), which reached a maximum at 1 h after the last injection of fentanyl and returned to basal level within 5 h. On the next days following fentanyl injection, rats exhibited a delayed hyperalgesic response that lasted for four days.

As expected, RF9 (0.5 mg/kg) almost blocked fentanyl-induced hyperalgesia (HI: 100 ± 10 for fentanyl vs 0.5 ± 0.1 for RF9, $F(2, 25) = 55$; $p < 0.001$, one way ANOVA followed by Fisher's PLSD test $p < 0.01$). Similarly, pretreatment with compound **1n** (0.5 mg/kg, sc.) before fentanyl had no effect on short lasting fentanyl analgesia but significantly attenuated the long lasting fentanyl-induced hyperalgesia (HI: 100 ± 10 for fentanyl vs 9.5 ± 5 for compound **1n**, $F(2, 25) = 55$; $p < 0.001$, one way ANOVA followed by Fisher's PLSD test $p < 0.001$) .

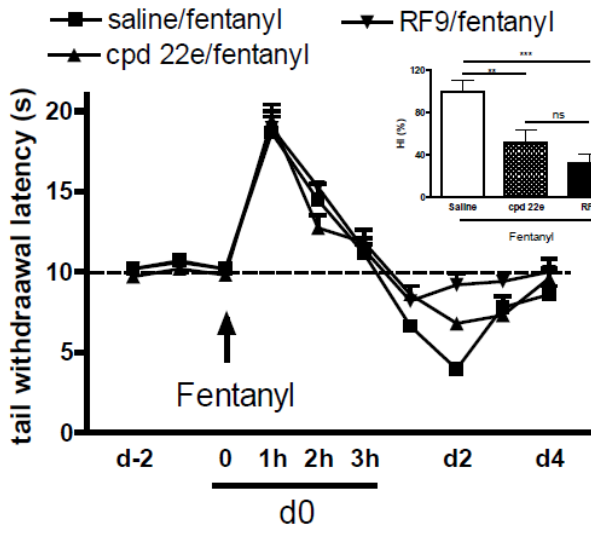
Figure 5: Effect of compound **1n** on analgesic and hyperalgesic responses of rats to fentanyl. On day 0, a single dose (0.5 mg/kg, sc.) of cpd **1n** or **RF9** was administrated 20 min before four consecutive fentanyl injections (80 µg/kg, sc ; 15 min interval). Nociceptive threshold of the animals was measured using the paw pressure test once daily before experiment start (d-2, d-1, d0) to verify baseline stability, at 1h, 3h and 5h after the last fentanyl injection to monitor analgesia amplitude (d0), and once daily (d1 to d6) to follow the development of hyperalgesia. Hyperalgesia indexes (HI) were calculated as described in the Data and statistical analyses section and presented as histograms. Data are expressed as mean ± S.E.M, *n* = 8-10. ***: $p < 0.001$ by Fisher's test as compared with the fentanyl-pretreated saline group. ^{ns} : $p > 0.05$, by Fisher's test as compared with the fentanyl-pretreated R9 group



We next examined the effect of compound **22e** on fentanyl-induced analgesia and hyperalgesia in mice (tail withdrawal latency, Fig. 6). Very similarly to what we observed in rats, injection of fentanyl (4x60 µg/kg, sc.) displayed a strong analgesic effect followed by an hyperalgesic state that was significantly attenuated by **RF9** (5 mg/kg, sc; HI: 100 ± 10 for fentanyl vs 32 ± 9 for **RF9**, $F(2, 27) = 10$; $p < 0.001$, one way ANOVA followed by Fisher's PLSD test $p < 0.001$). Pretreatement with compound **22e** at the same dose (5 mg/kg, sc.) than

RF9 30 min before fentanyl also significantly attenuated the development of hyperalgesia (HI: 100 ± 10 for fentanyl vs 52 ± 12 for cpd **22e**, $F(2, 27) = 10$; $p < 0.001$, one way ANOVA followed by Fisher's PLSD test $p < 0.01$).

Figure 6: Effect of compound **22e** on analgesic and hyperalgesic responses of mice to fentanyl. On day 0, a single dose (5 mg/kg; sc.) of cpd **22e** or RF9 was administrated to mice 20 min before fentanyl injections (4x60µg/kg, sc; 15 min interval). Nociceptive response of the animals was measured using the tail immersion test (48°C) once daily before experiment start (d-2, d-1, d0) to verify baseline stability, every 1 h after the last fentanyl injection until return to baseline and were repeated once daily from d1 to d4. Hyperalgesia indexes (HI, %) were calculated as described in the Data and statistical analyses section and presented as histograms. Data are expressed as mean ± S.E.M, *n* = 10. **: $p < 0.01$, ***: $p < 0.001$ by Fisher's test as compared with the fentanyl-pretreated saline group. ^{ns} :$p > 0.05$ by Fisher's test as compared with the fentanyl-pretreated RF9 group.



Altogether, these results indicate that targeting NPFF receptors by AGH **1n** or its rigidified analog **22e** efficiently prevents the development of hyperalgesia induced by acute fentanyl administration in rodents. Conversely to what was originally proposed for compound **1n** described as a selective NPFF2R agonist, [29] they further suggest that the anti-hyperalgesic

activity of these compounds may not only rely on their NPFF2R character but more likely on their capacity to efficiently block NPFF1R. This hypothesis is also supported by recent data obtained with a compound showing clearly NPFF1R selectivity over NPFF2R. [26] In the future, a better understanding of the respective role of both NPFF receptor subtypes in the modulation of nociception and opioid side effects will involve the development of highly selective compounds for these two NPFF receptor subtypes with a clear *in vitro* and *in vivo* antagonistic profile. In conclusion combination of opioid analgesics (morphine, fentanyl, remifentanil)[43] with NPFF receptor antagonists may help to reduce adverse effects associated with chronic opioid treatments, including OIH and analgesic tolerance, in many clinical situations including postoperative, traumatic or tumor pain therapy.

Materials and Methods

Compound synthesis

See supporting information

Biological evaluation of compounds

Material

Fentanyl citrate, forskolin, 3-isobutyl-1-methylxanthine (IBMX) and poly(ethyleneimine) solution (PEI) were from Sigma-Aldrich (Saint Quentin Fallavier, France). [^{3}H]-FFRF-amide (13.6 Ci/mmol) was from the CEA (Saclay, France). [^{3}H]-PrRP-20 (150 Ci/mmol) was from Hartmann Analytic (Braunschweig, Germany). [^{125}I]-(D-Tyr1, N-Me-Phe3)-NPFF ([^{125}I]-1DMe-NPFF; 2200 Ci/mmol), [^{125}I]-Kp-10 (2200 Ci/mmol), [^{125}I]-QRFP43 (2200 Ci/mmol) were purchased from Perkin Elmer Life and Analytical Sciences (Courtaboeuf, France). Human RF-amide peptides were from Polypeptide (Strasbourg, France; Kp-10, NPFF, PrRP-20), Peptanova (Sandhausen, Germany, 43RFa) and Tebu-Bio (Le Perray-en-Yvelines, France

; 26RFa and RFRP-3). D-luciferine was purchased from Synchem (Felsberg-Altenberg, Germany). N-adamantanecarbonyl-Arg-Phe-NH_2 trifluoroacetate (RF9) was synthesized as reported.[22]

Receptor cDNA constructs, cell expression and membrane preparations

Human NPFF1R, NPFF2R and Kiss1R cDNAs were subcloned into the pCDNA3.1 expression vector (Invitrogen, Cergy Pontoise, France) and transfected into CHO cells before selection for stable expression, as reported.[42] CHO cells expressing human PrRPR and QRFPR were a gift from M. Parmentier (IRIBHM, Brussells, Belgium), and were grown in DMEM/Ham's F-12 Nutrient Mixture (Gibco, Fisher Scientific, Villebon sur Yvette) supplemented with 5% fetal calf serum, penicilline (100 µg/L) and streptomycine (100 U/L). HEK293 cells stably expressing the cAMP GloSensor^{TM} were a gift from J. Hanson (Molecular Pharmacology GIGA-Signal Transduction and Medicinal Chemistry Drug Research Centre, Liege, Belgium) and were grown in DMEM+4,5g/L Glucose (Gibco, Fisher Scientific, Villebon sur Yvette) supplemented with 10% fetal calf serum, penicilline (100 µg/L), streptomycine (100 U/L) and hygromycine (200 µg/mL). pCDNA3 constructs encoding human NPFF1R, NPFF2R were further transfected into these cells before G418 selection (500 µg/mL) for stable receptor expression. Membranes from CHO cells expressing RF-amide receptors were prepared as reported[42] and stored at -80°C before use.

Radioligand binding assays

Binding assay conditions were essentially as described previously.[42] Briefly, NPFF1R- or NPFF2R-containing membranes were incubated with 10 nM or 3 nM [^{3}H]-FFRF-NH_2, respectively. When [^{125}I]-1DMe-NFF was used as a radiotracer, both NPFF1R- or NPFF2R-containing membranes were incubated with 0.015 nM of this radioligand. Non-specific binding levels were determined in the presence of 1 μM RFRP-3 (NPFF1R) or 1 μM NPFF (NPFF2R). [^{3}H]-PrRP-20 (0.3 nM), [^{125}I]-Kp-10 (0.02 nM) and [^{125}I]-43RFa (0.03 nM) were

used to label PrRP, Kiss1 and QRFP receptors (respectively) and non specific binding levels were evaluated in the presence of 1 μM PrRP-20, Kp-10 and 26RFa (respectively). Competition-type experiments were performed at 25°C, under binding equilibrium conditions (30 min, 0.5 mL final volume for NPFF1R and NPFF2R; 1 h, 0.25 mL final volume for the three other receptors), in the presence of various concentrations of the compounds to be tested for their binding affinity. Membrane-bound radioactivity was separated from free radioligand by rapid filtration through 96-well GF/B unifilters presoaked in 0.1% PEI for [^{3}H]-PrRP-20, [^{125}I]-Kp-10 and [^{125}I]-43RFa and 0.5% PEI for [^{125}I]-1DMe-NPFF using a cell harvester (Perkin Elmer Life and Analytical Sciences, Courtaboeuf, France) and quantified using a TopCount scintillation counter (Perkin Elmer).

Glo Sensor cAMP assay

The fonctionnal characterization of the selected compounds was performed by Glo Sensor cAMP kinetic assay, a validated approach for measuring Gαi/s activation by GPCRs (DiRaddo et al., 2014; Gilissen et al., 2015).[44-45] HEK-Glo cells expressing either NPFF1 or NPFF2 receptors were suspended (10^6 cells per mL) in physiological Hepes buffer (10mM HEPES, 0.4mM NaH_2PO_4, 137.5mM NaCl, 1.25mM $MgCl_2$, 1.25mM $CaCl_2$, 6mM KCl, 10mM glucose and 1mg/mL bovine serum albumin, pH 7.4) supplemented with 1mM D-Luciferine. After equilibration for 2h at 25°C, luminescence levels were recorded in real time in 96-well plates using a Flexstation^{R} 3 (Molecular Devices, Sunnyvale CA, USA). Non-specific effect of the different compounds was evaluated in HEK-Glo cells without any recombinant human RF-amide receptors. To test the agonist activity on NPFF1R and NPFF2R, compounds were injected 15 minutes before addition of forskolin (0,5µM) and readings were pursued for 90 minutes. To evaluate their antagonist properties, compounds were pre-incubated with cells for 15 minutes before prototypical agonists for NPFF1R (RFRP-3) and NPFF2R (NPFF) receptors. According to their preferential coupling to $G_{i/o}$

proteins, the stimulation of NPFF1R and NPFF2R by agonists was monitored as a dose-dependent reduction in steady-state luminescence levels, reflecting the inhibition of forskolin-induced cAMP accumulation. Experiments were performed at 25°C in the presence of 0,5mM IBMX to prevent the degradation of cAMP by phosphodiesterases.

Measurement of the nociceptive threshold in rats and mice

The nociceptive mechanical response of rats was measured using the paw-pressure vocalization test adapted from the Randall–Selitto method (Elhabazi et al., 2014; Kayser et al., 1990).[46-47] Uniformly increasing pressure (in g) was applied to the hind paw until the rat squeaked. The Basile analgesimeter (Apelex, Massy, France; 1 mm stylus tip diameter) was used and a 600 g cut-off value was set to prevent tissue damage.

The nociceptive thermal response of mice was determined using the tail immersion test as previously described (Elhabazi et al., 2014; Simonin et al., 1998).[47-48] Briefly, mice were restrained in a grid pocket and their tail was immersed in a thermostated water bath. The latency (in sec) for tail withdrawal from hot water (48 ± 0.5 °C) was taken as a measure of the nociceptive response. In the absence of any nociceptive reaction, a cut-off value of 25 sec was set to avoid tissue damage.

Nociception tests were performed on male Sprague-Dawley rats (250-350 g weight; Charles River Laboratories, L'Arbresle, France) and on C57BL/6N male mice (25-30 g weight; Taconic, Denmark). Animals were housed in groups of three to five per cage and kept under a 12 h/12 h light/dark cycle at 21 ± 1°C with *ad libitum* access to food and water and were habituated to the testing room and equipment and handled for 2 weeks before starting behavioural experiments.

All experiments were carried out in accordance with the European directive for the care of laboratory animals (2010/63/EU) and approved by the local committee (CREMEAS).

Data and statistical analyses

Binding and functional data were analyzed using Prism 4.0 (GraphPad Software, San Diego, CA, USA).

Data for nociceptive tests are expressed as mean values ± S.E.M. for 8-10 animals per group. Hyperalgesia was quantified as the Hyperalgesia Index (HI) in the fentanyl induced-hyperalgesia experiment, which represents the area above the curve expressed as a mean percentage relative to the reference values for the fentanyl-preatreated saline group. Data were analyzed using one-way analysis of variance (ANOVA). Post-hoc analyses were performed with Fisher's PLSD test. The level of significance was set at $p < 0.05$. All statistical analyses were carried out using the StatView software.

Associated Content

Supporting Information: Compound synthesis and analytical characterization

Author Contributions

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# These authors participated equally to this work

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Notes

The authors declare no competing financial interest

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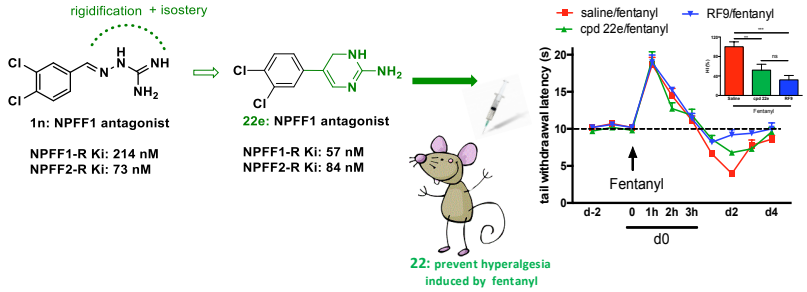
Graphical Abstract



ANNEXE II

RF313, an orally bioavailable neuropeptide FF receptor antagonist, opposes effects of RF-amide-related peptide-3 and opioid-induced hyperalgesia in rodents.

Elhabazi K, Humbert JP, Bertin I, Quillet R, Utard V, Schneider S, Schmitt, M, Bourguignon JJ, Laboureyras E, Ben Boujema M, Simonnet G, Ancel C, Simonneaux V, Beltramo M, Bucher B, Sorg T, Meziane H, Schneider E, Petit-Démoulière B, Ilien B, Bihel F, Simonin F

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RF313, an orally bioavailable neuropeptide FF receptor antagonist, opposes effects of RF-amide-related peptide-3 and opioid-induced hyperalgesia in rodents

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ABSTRACT

Although opiates represent the most effective analgesics, their use in chronic treatments is associated with numerous side effects including the development of pain hypersensitivity and analgesic tolerance. We recently identified a novel orally active neuropeptide FF (NPFF) receptor antagonist, RF313, which efficiently prevents the development of fentanyl-induced hyperalgesia in rats. In this study, we investigated the properties of this compound into more details. We show that RF313 exhibited a pronounced selectivity for NPFF receptors, antagonist activity at NPFF1 receptor (NPFF1R) subtype both *in vitro* and *in vivo* and no major side effects when administered in mice up to 30 mg/kg. When co-administered with opiates in rats and mice, it improved their analgesic efficacy and prevented the development of long lasting opioid-induced hyperalgesia. Moreover, and in marked contrast with the dipeptidic NPFF receptor antagonist RF9, RF313 displayed negligible affinity and no agonist activity (up to 100 μM) toward the kisspeptin receptor. Finally, in male hamster, RF313 had no effect when administered alone but fully blocked the increase in LH induced by RFRP-3, while RF9 *per se* induced a significant increase in LH levels which is consistent with its ability to activate kisspeptin receptors. Altogether, our data indicate that RF313 represents an interesting compound for the development of therapeutic tools aiming at improving analgesic action of opiates and reducing adverse side effects associated with their chronic administration. Moreover, its lack of agonist activity at the kisspeptin receptor indicates that RF313 might be considered

Abbreviations: AUC, area-under-the-curve; CHO, chinese hamster ovary; FSH, follicle stimulating hormone; HI, hyperalgesia index; i.c.v., intracerebroventricular; i.p., intraperitoneal; i.t., intrathecal; Kp, kisspeptin; LH, luteinizing hormone; NPFF, neuropeptide FF; OIH, opioid-induced hyperalgesia; p.o., per os; PrRP, prolactin releasing peptide; RFRP, RF-amide related peptide; s.c., subcutaneous.

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a better pharmacological tool, when compared to RF9, to examine the regulatory roles of RF-amide-related peptides and NPFF1R in reproduction.

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1. Introduction

Neuropeptide FF (NPFF) receptors belong to a subfamily of G protein-coupled receptors (GPCRs), called RF-amide receptors, that encompasses Neuropeptide FF1 (NPFF1R alias GPR147), Neuropeptide FF2 (NPFF2R alias GPR74), prolactin-releasing peptide (PrRPR alias GPR10), Kisspeptin (Kiss1R alias GPR54) and QRFP (QRFPR alias GPR103) receptors (Quillet et al., 2016). All of them bind endogenous neuropeptides that display a conserved Arg-Phe-NH_2 (RF-amide) signature at their carboxyl-terminal end. This sequence has been shown to be mandatory for the affinity and the activity of these peptides towards their receptors. Although both NPFF receptor subtypes have been shown to display promiscuous binding properties for all mammalian RF-amide peptides (Elhabazi et al., 2013; Roumeas et al., 2015), RF-amide-related peptide-1 and -3 (RFRPs), and NPFF and neuropeptide AF (NPAF), are considered as the endogenous ligands of NPFF1R and NPFF2R respectively (Ayachi and Simonin, 2014).

NPFF receptors and their endogenous ligands have been shown to modulate several functions including feeding behavior (Johnson et al., 2007; Kovacs et al., 2014; Nicklous and Simansky, 2003), reproduction (Murakami et al., 2008; Pineda et al., 2010a), arterial blood pressure (Prokai et al., 2006; Roth et al., 1987) and nociception (Ayachi and Simonin, 2014; Roumy and Zajac, 1998). The pain modulating properties of NPFF and its relationship with the opioid system were revealed very early by Yang and coworkers (Yang et al., 1985) who showed the capacity of this peptide to reverse morphine-induced analgesia. Since then, accumulating evidence support the hypothesis that NPFF belongs to an anti-opioid pronociceptive system involved in the homeostatic regulation of opioid antinociceptive activity (Ayachi and Simonin, 2014). More recently, RFRP-1 and RFRP-3 were also shown to display hyperalgesic activity on their own or to prevent the development of morphine analgesia (Elhabazi et al., 2013; Fang et al., 2011; Liu et al., 2001). In contrast, other findings support that NPFF and NPFF related peptides have a pro-opioid effect. Indeed, when these peptides are administered intrathecally they produce anti-nociception and enhance morphine analgesia (Gouarderes et al., 1993; Jhamandas et al., 2006; Kontinen and Kalso, 1995; Xu et al., 1999). Altogether, these data indicate that both NPFF1 and NPFF2 receptor subtypes and their respective peptides are involved in the modulation of nociception and pain. However, their precise roles in this function still need further investigation. In the last decade, RFRP-3 and its receptor NPFF1R have also been shown to regulate the hypothalamo-pituitary-gonadal axis although with different effects according to sex and species (Henningsen et al., 2016; Leon and Tena-Sempere, 2015).

The adamantane dipeptide derivative RF9 has been identified ten years ago as a selective NPFF receptor antagonist, with similar nanomolar affinity at NPFF1R and NPFF2R (Simonin et al., 2006). Although this compound cannot discriminate between both receptor subtypes, it proved a very useful tool to establish the roles of NPFF receptors in acute or chronic opioid-induced hyperalgesia, analgesic tolerance, dependence and hypothermia (Elhabazi et al., 2012; Simonin et al., 2006; Wang et al., 2008). The role of endogenous RFRPs in the control of the reproductive axis in mammals has also been evaluated by using the RF9 compound. Unfortunately, data were obscured by its potent gonadotropin-releasing activity *in vivo* (Pineda et al., 2010b; Quillet et al., 2016; Rizwan et al., 2012), which was later shown to rely on its agonist activity at Kiss1R (Kim et al., 2015; Min et al., 2015). Thus, the design of novel NPFF1R- or NPFF2R-selective antagonists, devoid of secondary RF-amide binding components, is of prime importance to address the various physiological functions mediated by RF-amide receptors.

Previous SAR study conducted on NPFF receptors revealed important residues involved in ligand recognition and receptor activation. Among these residues, positions 5.27 and 6.59, have a strong impact on receptor activation and were suggested to form an acidic negatively binding pocket in both NPFF receptor subtypes, while the position 7.35 was shown to play an important role in functional selectivity (Findeisen et al., 2012). On the other hand, we explored in a previous work the impact (in terms of affinity, selectivity and antagonistic nature at NPFF1 and NPFF2 receptors) of modifications introduced at the N-terminus (Gealageas et al., 2012) or at the carboxy-amidated parts of Arg-Phe-NH_2 dipeptides as well as of substitutions of either Arg or Phe residues for other aminoacids (Bihel et al., 2015). Replacement of arginine with a non-natural ornithine derivative (Schneider et al., 2015) bearing a piperidine moiety on its side chain led to the obtention of the RF313 compound, a novel antagonist of NPFF receptors referred to as compound **12e** in (Bihel et al., 2015).

In this study, we examined the pharmacological properties of RF313 into more details. We first performed *in vitro* binding experiments to examine its affinity and selectivity profile toward the five RF-amide receptor subtypes. Then, the ability of RF313 to antagonize the effects of RFRP-3 was evaluated both *in vitro* and *in vivo*. We further addressed the potential of RF313 to improve the analgesic efficacy of opiates and to prevent the development of long-lasting hyperalgesia induced by acute fentanyl or chronic morphine injections, both in rats and mice. Examination of its anti-hyperalgesic property according to the mode of administration (subcutaneous or oral route) clearly pointed out RF313 as an orally-active compound. Finally, the comparison of the effects of RF9 and RF313 on gonadotropin release in male hamster indicated that RF313, given its lack of activity at Kiss1R, is a more suitable pharmacological tool to examine the regulatory roles of RFRPs and NPFF1R in reproduction.

2. Materials and methods

2.1. Materials

Fentanyl citrate, naloxone hydrochloride, forskolin, 3-isobutyl-1-methylxanthine (IBMX) and probenecid were from Sigma-Aldrich (Saint Quentin Fallavier, France). Morphine hydrochloride was from Francopia (Paris, France). Fluo-4 acetoxymethyl ester was from Molecular Probes (Invitrogen, Cergy Pontoise, France). N-adamantane-1-carbonyl-Arg-Phe-NH_2 trifluoroacetate (RF9) was synthesized as reported (Simonin et al., 2006).

Human RF-amide peptides were from Polypeptide (Strasbourg, France; Kp-10, NPFF, PrRP-20), and Tebu-Bio (Le Perray-en-Yvelines, France; QRFP26 or 26RFa, RFRP-3). Mouse RFRP-3 was from Genecust (Luxembourg).

[^{3}H]-FFRF-amide (13.6 Ci/mmol) was from the CEA (Saclay,

France). [^{3}H]-PrRP-20 (150 Ci/mmol), [^{125}I]-(D-Tyr1, N-Me-Phe3)-Neuropeptide FF and [^{35}S]Guanosine 5′-*O*-[γ-thio] triphosphate ([^{35}S]GTPγS; 1000 Ci/mmol) were from Hartmann Analytic (Braunschweig, Germany). [^{125}I]-Kp-10 (2200 Ci/mmol) and [^{125}I]-QRFP43 (2200 Ci/mmol) were purchased from Perkin Elmer Life and Analytical Sciences (Courtaboeuf, France).

2.2. RF313 synthesis and physico-chemical properties

RF313 (Fig. 1) was synthesized in seven steps, starting from commercially available Fmoc-L-Glu (OtBu)-OH with an overall yield of 40% (Bihel et al., 2015). Purity was established as superior to 98% by reversed-phase high-performance liquid chromatography (RP-HPLC). RF313 showed a good solubility in aqueous solution with a thermodynamic solubility in water greater than 20 mM.

2.3. Receptor cDNA constructs, cell expression and membrane preparations

Human NPFF1R, NPFF2R and Kiss1R cDNAs were subcloned into the pCDNA3.1 expression vector (Invitrogen, Cergy Pontoise, France) and transfected into CHO cells before selection for stable expression, as reported (Elhabazi et al., 2013). CHO cells expressing human PrRPR and QRFPR were a gift from M. Parmentier (IRIBHM, Brussels, Belgium).

Membranes from CHO cells expressing RF-amide receptors were prepared as described (Elhabazi et al., 2013) and stored at −80 °C as aliquots (1 mg prot./mL) until use.

2.4. Radioligand binding assays

Binding assay conditions were essentially as described in (Elhabazi et al., 2013). Briefly, NPFF1R- or NPFF2R-containing membranes were incubated with 10 nM or 3 nM [^{3}H]-FFRF-NH_2, respectively. When [^{125}I]-(D-Tyr1, N-Me-Phe3)-Neuropeptide FF was used as a radiotracer, both NPFF1R- or NPFF2R-containing membranes were incubated with 0.015 nM of this radioligand. Non-specific binding levels were determined in the presence of 1 μM RFRP-3 (NPFF1R) or 1 μM NPFF (NPFF2R). Membranes of PrRPR-expressing CHO cells were incubated with 0.3 nM [^{3}H]-PrRP-20, in the absence or presence of 1 μM PrRP-20 to define total and non specific binding levels, respectively. Kiss1R-containing membranes were incubated with 0.02 nM [^{125}I]-Kp-10 using 1 μM Kp-10 to determine non specific radioligand binding. QRFPR membranes were incubated with 0.03 nM [^{125}I]-43RFa, using 1 μM 26RFa for non specific binding measurement.

Competition-type experiments were performed at 25 °C, under binding equilibrium conditions (30 min, 0.5 ml final volume for NPFF1R and NPFF2R; 1 h, 0.25 mL final volume for the three other receptor subtypes), in the presence of increasing concentrations of unlabelled peptides or compounds to be tested for their binding affinity.

Membrane-bound radioactivity was separated from free radioligand by rapid filtration through a 96-well GF/B unifilter apparatus (Perkin Elmer Life and Analytical Sciences, Courtaboeuf, France) and quantified using a TopCount scintillation counter (Perkin Elmer).

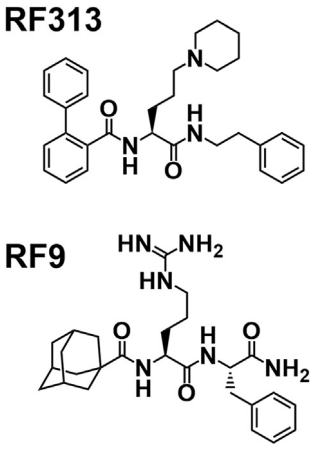

Fig. 1. Chemical structures of RF313 and RF9 compounds.

2.5. In vitro functional experiments

2.5.1. [^{35}S]-GTPγS binding

Stimulation by endogenous RF-amide peptides of [^{35}S]-GTPγS binding to membranes from CHO cells expressing each individual RF-amide receptor, and its inhibition by RF9 or RF313 compounds, were examined as reported (Simonin et al., 2006).

2.5.2. cAMP accumulation

Variations in the overall cAMP content of CHO cells expressing NPFF1 or NPFF2 receptors were monitored according to (Elhabazi et al., 2013). Cells were pre-incubated with 1 mM IBMX, then challenged for 10 min at 37 °C with various concentrations of compounds or peptides to be tested, in the absence or the presence of 10 μM forskolin. The reaction was stopped by the addition of ice-cold 0.5 M HCl and cell freezing for 1 h at −80 °C. After centrifugation at 2000 g for 15 min, cell supernatants were stored at −20 °C until quantitation of cAMP levels by radioimmunoassay.

2.5.3. Calcium mobilization

Kiss1R-expressing CHO cells were loaded with 2.5 μM Fluo-4 AM in the presence of 2.5 mM probenecid, as described (Elhabazi et al., 2013).

Compounds were serial diluted in assay buffer (in mM: 10 HEPES, 0.4 NaH_2PO_4, 137.5 NaCl, 1.25 $MgCl_2$, 1.25 $CaCl_2$, 6 KCl, 10 glucose and 1 mg/mL BSA, pH 7.4) and antagonists were allowed to pre-incubate with cells for 10 min before agonist challenge. Agonist-evoked increases in intracellular calcium were recorded over time (5 s intervals over 220 s) at 37 °C through fluorescence emission at 520 nm (excitation at 485 nm). Peak response amplitudes were normalized to basal and maximal (cells permeabilized with 20 μM digitonin) fluorescence levels.

2.6. In vivo pharmacological studies

All experiments were carried out in strict accordance with the European guidelines for the care of laboratory animals (European Communities Council Directive 2010/63/EU) and approved by the local ethical committee. All efforts were made to minimize animal discomfort and to reduce the number of animals used.

2.6.1. Animals

Nociception tests were performed on male, awake, unrestrained Sprague-Dawley rats (250–350 g weight; Charles River Laboratories, L'Arbresle, France) and on C57BL/6N male mice (25–30 g weight; Taconic, Denmark). Animals were housed in groups of three to five per cage and kept under a 12 h/12 h light/dark cycle at 21 ± 1 °C with *ad libitum* access to food and water. Experiments were performed during the light-on phase of the cycle. Rats and mice were habituated to the testing room and equipment and handled for 1–2 weeks before starting behavioral experiments. Control and treated group assignment as well as pain responses

measurements were performed in a blinded manner. Every animal was used only once. At the end of experiments, animals were euthanized with sodium pentobarbital (120 mg/kg, i.p.) or CO_2 (fill rate of 20% of the chamber volume per minute).

Adult male Syrian hamsters were bred in-house and maintained under a 14 h/10 h light/dark cycle at 22 ± 2 °C with free access to food and water.

All experiments were carried out in accordance with the european directive for the care of laboratory animals (2010/63/EU) and approved by the local commitee (C.R.E.M.E.A.S).

2.6.2. Drug administration

All drugs were dissolved in physiological saline (0.9%) and administered subcutaneously (s.c.) or orally (p.o.) at 1 mL/kg or 10 mL/kg (vol/body weight) for rats and mice, respectively.

Intracerebroventricular (i.c.v., left lateral ventricle, 5 μL total volume) injections in mice were performed using a modified Hamilton syringe as previously described (Haley and McCormick, 1957).

Male hamsters were prepared for i.c.v. injections as reported (Ancel et al., 2012). A stainless steel 30-gauge cannula was placed in the lateral ventricle and animals were allowed to recover from surgery for one week.

2.6.3. Measurement of the nociceptive threshold in rats and mice

The nociceptive mechanical response of rats was measured using the paw-pressure vocalization test adapted from the Randall–Selitto method (Elhabazi et al., 2014; Kayser et al., 1990). Uniformly increasing pressure (in g) was applied to the hind paw until the rat squeaked. The Basile analgesimeter (Apelex, Massy, France; 1 mm stylus tip diameter) was used and a 600 g cut-off value was set to prevent tissue damage.

The nociceptive thermal threshold of mice was determined using the tail immersion test as previously described (Elhabazi et al., 2014; Simonin et al., 1998). Briefly, mice were restrained in a grid pocket and their tail was immersed in a thermostated water bath. The latency (in sec) for tail withdrawal from hot water (52 ± 0.5 °C or 48 ± 0.5 °C) was taken as a measure of the nociceptive response. In the absence of any nociceptive reaction, a cut-off value of 15 s (52 °C) and of 25 s (48 °C) was set to avoid tissue damage.

2.6.4. Effect of RF313 on fentanyl-induced analgesia and hyperalgesia

Experiments were designed according to a protocol enabling the visualization of an early analgesic effect of opiates and of a delayed hyperalgesic state lasting for several days, both in rats and mice (Celerier et al., 2000; Elhabazi et al., 2012).

The impact of the dose and route (subcutaneous or oral) of administration of RF313 on the short-term analgesic response of rats to four consecutive injections (4 × 80 μg/kg, s.c., 15 min interval) of fentanyl was evaluated using the paw pressure test. RF313 was administered subcutaneously (0.05, 0.1 and 0.5 mg/kg) or orally (0.3, 1 and 3 mg/kg) 30 min before fentanyl or saline. Nociceptive responses were measured before (time 0) and every 2 h after the first fentanyl injection until return to baseline values.

Very similar experiments were performed on mice with few modifications: animals received RF313 (5 mg/kg, s.c.) 20 min before fentanyl (4 × 60 μg/kg, s.c.) and nociceptive thresholds were measured every 1 h after the last fentanyl injection using the tail immersion test (48 °C).

The impact of RF313 on long-term changes in nociceptive sensitivity induced by fentanyl injection at day 0 was evaluated once daily in rats (paw pressure test) and mice (tail immersion test, 48 °C) over a 4- to 6-days period, until recovery of the pre-drug baseline value.

2.6.5. Effect of RF313 on morphine-induced analgesia and hyperalgesia in mice

On day 0, RF313 (5 mg/kg) or saline were administered (s.c.) 20 min before morphine (5 mg/kg, s.c.) or saline. Variations of nociceptive responses were measured using the tail immersion test (at 52 °C), at 30 min-intervals, over a 0–180 min period following morphine or saline injections. The same treatment was then repeated once-daily over the next 7 days. During this period, the nociceptive threshold of the animals was measured every day, 60 min before morphine injection, using the tail immersion test (at 48 °C). On day 8, analgesia time-course experiments were performed as described for day 0.

2.6.6. Effect of RFRP-3, RF9 and RF313 on LH secretion in the male Syrian hamster

I.c.v. injections of RFRP-3 or drugs (1.5 μg in 3 μL/animal infused at a flow rate of 1 μL/min), alone or in combination, were given in the morning under light anaesthesia with isoflurane vapor. Thirty min after injection, hamsters were deeply anesthetized with CO_2 vapor and the blood was taken by intracardial puncture for subsequent LH dosage using a validated radioimmunoassay (Tena-Sempere et al., 1993).

2.7. Data and statistical analyses

Binding and functional data were analyzed using Prism 4.0 (GraphPad Software, San Diego, CA, USA) and Kaleidagraph 4.0 (Synergy Software, Reading, PA, USA).

Data for nociceptive tests are expressed as mean values ± S.E.M. for 5 to 15 animals depending on the group. Analgesia was quantified as the area-under-the-curve (AUC) calculated by the trapezoidal method (Celerier et al., 2000). Hyperalgesia was quantified either as the AUC or the hyperalgesia index (HI) in the fentanyl induced-hyperalgesia experiment, which represents the area above the curve expressed as a mean percentage relative to the reference values for the control group. Both types of data were analyzed using one-way or two-way analysis of variance (ANOVA). Post-hoc analyses were performed with Fisher's PLSD test. The level of significance was set at $p < 0.05$. All statistical analyses were carried out using the StatView software.

LH levels are mean values ± S.E.M. for 6 to 7 hamsters. Data were analyzed by one-way ANOVA, followed by Fisher's PLSD test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. In vitro characterization of RF313 pharmacological properties

In a recent study, we have identified an orally active peptidomimetic antagonist of NPFF receptors that prevents the development of fentanyl-induced hyperalgesia in rats (Bihel et al., 2015). This compound, that we called here RF313 and referred to as compound **12e** in (Bihel et al., 2015), is built on a single non-natural amino acid. As shown in Fig. 1, RF313 is a derivative of the NPFF receptor dipeptide antagonist RF9 (Simonin et al., 2006), in which the C-terminal amide function was removed, the arginine residue replaced with a non-natural ornithine derivative bearing a piperidine moiety on its side chain and the adamantine ring replaced with a biphenyl moiety.

3.1.1. Binding affinity properties of RF313

In order to further characterize RF313 properties, we performed competition experiments with cell membranes expressing each of the five RF-amide receptors (NPFF1R, NPFF2R, PrRPR, Kiss1R and QRFPR). RF313 was tested in parallel with endogenous RF-amide

peptides to verify the receptor specificity of the binding assays and with RF9 for a comparison. As shown in Table 1, RF313 exhibited a marked preference for NPFF receptors together with a moderate selectivity towards the NPFF1R subtype, while its interaction with the other RF-amide receptors was marginal (K_i > 20 μM for PrRPR and QRFPR, K_i > 100 μM for Kiss1R). As the radioligand used in this study for binding experiments with NPFF receptors ([^{3}H]-FFRF-NH_2) have not been extensively characterized yet, we further evaluated the affinity of RF313 for NPFF receptors in binding experiments with [^{125}I]-(D-Tyr1, N-Me-Phe3)-Neuropeptide FF, which is more routinely used, and found similar Ki values for NPFF1R and NPFF2R (245 ± 41 nM and 2238 ± 418 nM, respectively). RF313 binding properties resembled those of RF9 although this later compound displayed a higher affinity for both NPFF receptors and a low but significant affinity for Kiss1R (Ki = 3500 ± 740 nM). Further pharmacological profiling of RF313 at numerous receptors, channels or transporters, selected for their implication in pain modulation, indicated no significant interaction of RF313 (tested at 1 μM) with these targets (Bihel et al., 2015).

3.1.2. Examination of in vitro functional activities of RF313

We next addressed the agonist or antagonist nature of RF313 at NPFF1R through two different *in vitro* functional assays. When tested alone (up to 50 μM), RF313 did not modify basal [^{35}S]-GTPγS binding to membranes from CHO cells expressing hNPFF1R. However, as illustrated in Fig. 2A, RF313 (at 10 μM) was able to promote a rightward shift in the RFRP-3 dose-response curve indicating that this compound displayed antagonist activity at hNPFFR1. The lack of agonistic activity of RF313 at hNPFF1R was also confirmed by its incapacity (at 10 μM) to inhibit forskolin-stimulated production of cAMP in hNPFF1R expressing cells. In contrast, RF313 clearly opposed RFRP-3-mediated inhibition of this cellular response (Fig. 2B), with a pA_2 of 977 ± 145 nM. For a comparison, a pA_2 value of 340 ± 44 nM was obtained for RF9 under the same experimental conditions. These data clearly demonstrate that RF313 displays antagonist activity at hNPFF1R *in vitro*. Although this compound also showed a significant affinity for the hNPFF2 receptor subtype, we did not detect any agonist or antagonist activity in hNPFF2R-expressing CHO cells (cAMP assay). As RF9 has recently been shown to display agonist activity at Kiss1R (Liu and Herbison, 2014), we further examined the activity of both RF313 and RF9 in calcium mobilization experiments on Kiss1R expressing cells. As shown in Fig. 2C, the endogenous Kiss1R ligand Kp10 potently stimulated calcium release from those cells at subnanomolar concentrations (EC_{50} = 0.1 ± 0.025 nM). As expected, RF9 exhibited full agonism at Kiss1R but at micromolar concentration (EC_{50} 1.3 ± 0.5 μM). RF313 (up to 100 μM) did not display any agonist activity at this receptor (Fig. 2C), which is in agreement with the absence of affinity of this compound for Kiss1R observed in this study (Table 1).

3.2. In vivo evaluation of RF313

3.2.1. Evaluation of toxic and adverse effects of RF313

In a first series of experiments we examined in mice unexpected adverse effects of RF313 treatment susceptible to introduce bias in nociception measurements or to impede further use of the compound. To this end, C57BL/6N male mice received s.c. injections of RF313 (up to 30 mg/kg) and were checked for general health parameters (modified SHIRPA parameters, weight and temperature), for sensory-motor responses (muscular strength, proprioceptive, vestibular and fine-tuned motor abilities) as well as for anxiety- or depression-like behaviors. This preliminary phenotyping analysis showed no significant adverse side-effects for RF313 except a slight decrease at 10 mg/kg of the immobility time, which could reveal a potential anti-depressive effect of RF313 at this dose as evidenced from the tail suspension test (Suppl. Fig. S1).

3.2.2. RF313 prevents RFRP-3-induced hyperalgesia in mice

We then investigated in mice whether RF313 can antagonize the effect of RFRP-3, the endogenous ligand of NPFF1 receptors (Fig. 3A and B). As expected (Elhabazi et al., 2013), RFRP-3 significantly reduced the basal nociceptive threshold of the animals when injected (10 nmol, i.c.v.) alone (AUC: 181 ± 33 for RFRP-3 vs 2.2 ± 26 for saline, $F(3, 33) = 6.3$; $p < 0.01$, one way ANOVA followed by Fisher's PLSD test $p < 0.01$, Fig. 3B). RF313 (10 nmol, i.c.v.) had no significant effect on the basal nociceptive threshold of the animals when administered alone but it fully prevented RFRP-3-induced hyperalgesia (AUC: 53 ± 54 for RFRP-3 + RF313 vs - 181 ± 33 for RFRP-3. $F(1, 33) = 3.3$; $p < 0.05$, two way ANOVA followed by Fisher's PLSD test $p < 0.001$, Fig. 3B) indicating that this compound well behaves as a NPFF1R antagonist *in vivo*.

3.2.3. RF313 prevents secondary hyperalgesia induced by fentanyl in rodents when administered by subcutaneous or oral routes

We next examined the consequences of NPFF receptors blockade with RF313 on fentanyl-induced analgesia and secondary hyperalgesia in mice and rats as previously described (Celerier et al., 2000; Elhabazi et al., 2012).

As shown in Fig. 4A, fentanyl (4 × 60 μg/kg, s.c.) promoted a short lasting analgesic response in mice, which returned to basal within 3 h after the last injection of fentanyl (AUC: 807 ± 211 for fentanyl vs - 6 ± 44 for saline, $F(3, 34) = 19.8$; $p < 0.001$, one way ANOVA followed by Fisher's PLSD test $p < 0.001$), and a delayed hyperalgesic response that lasted for at least two days (HI: 100 ± 21 for fentanyl vs 21 ± 12 for saline, $F(3, 34) = 4.4$; $p < 0.01$, one way ANOVA followed by Fisher's PLSD test $p < 0.01$, Fig. 4B). When administered 20 min before fentanyl (Fig. 4A), RF313 (3 mg/kg, s.c.) significantly potentiated the amplitude and duration of fentanyl analgesia (AUC: 1578 ± 165 for RF313 + fentanyl vs 807 ± 211 for fentanyl, $F(1, 34) = 4.3$; $p < 0.05$, two-way ANOVA followed by

Table 1
Binding affinity properties of endogenous RF-amide peptides, RF313 and RF9 at the five RF-amide receptors. Binding affinity constants (K_i values in nM) of drugs are from competition experiments performed as described under Materials and methods. Mean values ± S.E.M. for at least 3 independent experiments performed in duplicate are reported.

Compound	NPFF1R	NPFF2R	PrRPR [a]	Kiss1R [a]	QRFPR [a]
Binding affinity constants (nM)					
RFRP-3	0.17 ± 0.06	4.8 ± 1.8	>20,000	>20,000	>20,000
NPFF	0.39 ± 0.15	0.14 ± 0.06	>20,000	>20,000	>20,000
PrRP-20	4.7 ± 0.3 [a]	0.7 ± 0.1 [a]	0.06 ± 0.01	>20,000	>20,000
Kp-10	0.5 ± 0.1 [a]	1.6 ± 0.3 [a]	9200 ± 1200	0.51 ± 0.04	8000 ± 120
26RFa	24 ± 1 [a]	3 ± 1 [a]	>10,000	>20,000	21 ± 1
RF313	172 ± 13	560 ± 160	>20,000	>100,000	>20,000
RF9	17 ± 8	9.3 ± 2.1	>20,000	3500 ± 740	>20,000

[a] K_i values for agonists are from (Elhabazi et al., 2013).

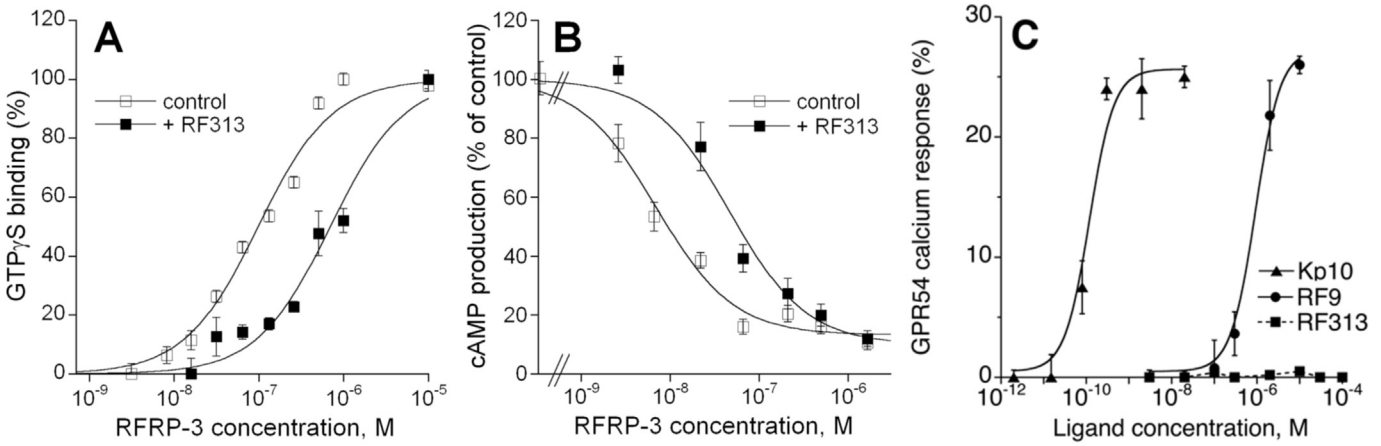

Fig. 2. Assessment of *in vitro* pharmacological properties of RF313 and RF9 on NPFF1R and Kiss1R. **A**: Stimulation of [^{35}S]-GTPγS binding by RFRP-3 on membranes from CHO cells expressing human NPFF1 receptors. RF313 (10 μM) shifted the EC_{50} values for RFRP-3 from 98 ± 10 to 695 ± 95 nM. Data are expressed as percentages of maximal [^{35}S]-GTPγS binding and are mean ± S.E. values for two separate experiments in triplicate. **B**: Reversal of RFRP-3-induced inhibition of forskolin-induced cAMP accumulation in CHO-hNPFF1R cells. RF313 (10 μM) shifted the EC_{50} values for RFRP-3 from 6.9 ± 1.1 to 47 ± 12 nM. Data are expressed as percentages of maximal cAMP levels and represent mean ± S.E.M values for four separate experiments in triplicate. **C**: Effect of Kp-10, RF9 and RF313 on calcium mobilization in Kiss1R expressing cells. Peak response amplitudes were normalized to basal and maximal (digitonin-permeabilized cells) fluorescence levels. Data points are from a representative experiment performed in duplicate. EC_{50} ± S.E. values for Kp-10 (0.1 ± 0.025 nM) and RF9 (1.3 ± 0.5 nM) are from two experiments performed in triplicate.

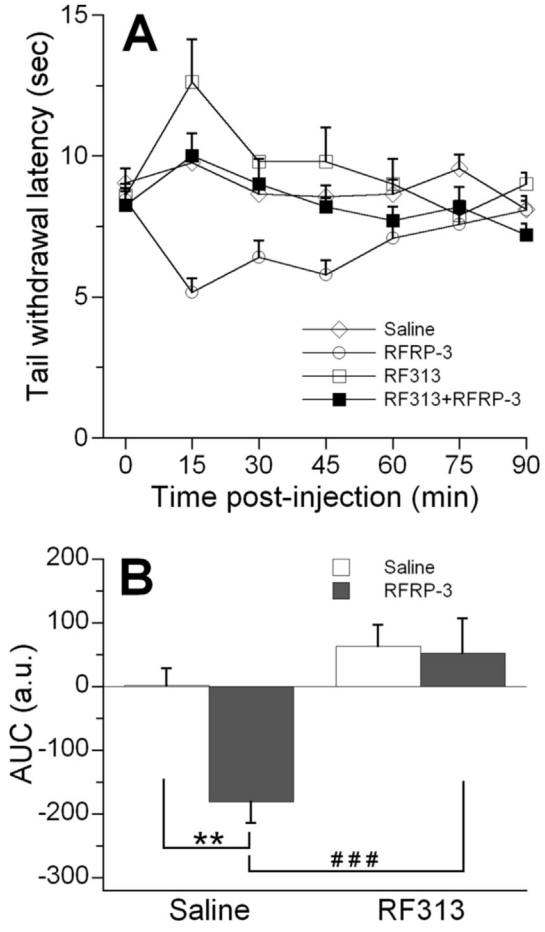

Fig. 3. RF313 blockade of RFRP-3-induced hyperalgesia in mice. **A**: Intracerebroventricular injections of RFRP-3 (10 nmol), alone or in combination with RF313 (10 nmol), were performed as described under Materials and methods. Nociceptive thresholds were measured every 15 min post-injection using the tail immersion test at 48 °C. **B**: Comparison between groups of area-under-the-curve (AUC values) over the 0–90 min time course. Data are expressed as mean ± S.E.M, $n = 6–11$. $^{**}p < 0.01$ by Fisher's test as compared with the saline group. $^{###}p < 0.001$ by Fisher's test as compared with the RFRP-3-treated group.

Fisher's PLSD test $p < 0.01$) and significantly prevented the development of hyperalgesia (HI: 36 ± 20 for RF313 + fentanyl vs 100 ± 21 for fentanyl, $F(1, 34) = 4.5$; $p < 0.05$, two-way ANOVA followed by Fisher's PLSD test $p < 0.01$, Fig. 4B). RF313 alone did not modify the basal nociceptive threshold of the animals. Interestingly, once the hyperalgesic state was already initiated, subsequent administration of RF313 (Fig. 4A, dashed arrow) was ineffective in restoring normal nociceptive baseline values. These observations suggest that NPFF receptors are involved in the onset of opioid-induced hyperalgesia but probably not in the maintenance of the hyperalgesic state.

We next examined the effect of RF313 on fentanyl-induced analgesia and hyperalgesia in rats according to its dosage and mode of administration, either subcutaneously (Fig. 4C and D) or orally (Fig. 4E and F). Higher RF313 doses were chosen for oral administration in order to account for bioavailability limitations. Whatever the route or the dose, RF313 (administered 30 min before fentanyl) did not modify the amplitude of the analgesic response after 2 h, probably because fentanyl effect was already maximal (paw pressure test, cut-off 600 g). However, we can notice that 6 h after fentanyl administration, only rats orally treated with RF313 still displayed an analgesic response. Analyses of hyperalgesia index data with one-way ANOVA showed that the development of fentanyl-induced hyperalgesia was dose-dependently prevented by RF313 when it was administered by subcutaneous ($F(3, 30) = 45$; $p < 0.001$) or oral ($F(3, 35) = 50$; $p < 0.001$) routes (Fig. 4D and F, respectively). Altogether, these results indicate that NPFF receptors blockade by RF313 (s.c. or p.o.) efficiently prevents the development of hyperalgesia induced by acute fentanyl administration in rodents.

3.2.4. RF313 improves acute morphine analgesia and reduces hyperalgesia associated with chronic morphine administration in mice

We further examined the impact of RF313 (2.5 and 5 mg/kg, s.c.) on acute morphine (5 mg/kg, s.c.) analgesia in mice (Fig. 5A). RF313 alone did not significantly modify the basal nociceptive threshold of the animals. As expected, morphine induced a strong analgesic effect as shown by the significant increase in tail immersion latencies compared with control animals (AUC: 725 ± 68 for

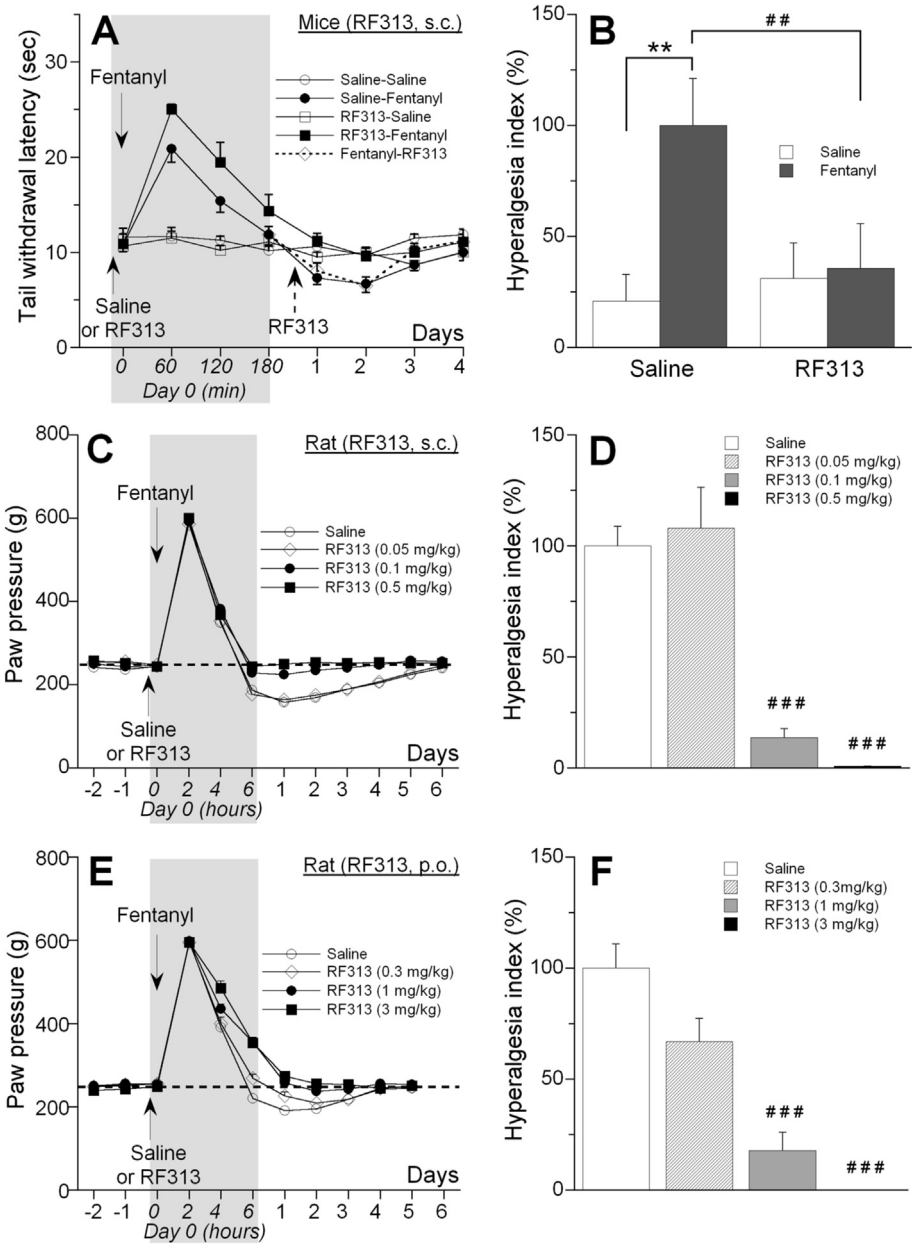

Fig. 4. Effect of RF313 on analgesic and hyperalgesic responses of mice and rats to fentanyl. **A, B**: On day 0 (grey area), a single dose of RF313 (5 mg/kg; s.c.) or saline was administered to mice 20 min before fentanyl injections (4 × 60 μg/kg; 15 min interval; s.c.). Nociceptive responses were measured using the tail immersion test (48 °C) every 1 h after the last fentanyl injection until return to baseline and were repeated once daily from d1 to d4 Panel A). In a parallel experiment, RF313 (5 mg/kg; s.c.) was injected once to fentanyl-treated mice on day 1 (arrow), 30 min before daily measurement of the nociceptive threshold (dashed line, panel A). Hyperalgesia indexes (HI) were calculated as described in the Data and statistical analyses section (Panel B). **C, D, E, F**: On day 0 (grey area), increasing doses of RF313 or saline were administered to rats either subcutaneously (0.05, 0.1 and 0.5 mg/kg; panels **C, D**) or orally (0.3, 1 and 3 mg/kg; panels **E, F**). 30 min later, animals received four consecutive fentanyl injections (80 μg/kg; 15 min interval; s.c.). Nociceptive thresholds were measured using the paw pressure test: once daily before experiment start (d-2, d-1, d0) to verify baseline stability, every 2 h after the first fentanyl injection (d0) to monitor analgesia amplitude, and once daily (d1 to d6) to follow the development of hyperalgesia. Hyperalgesia indexes (HI) are presented as histograms in panels **D** and **F**. Data are expressed as mean ± S.E.M, $n = 6–11$. $^{**}p < 0.01$, $^{***}p < 0.001$ by Fisher's test as compared with the saline group. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ by Fisher's test as compared with the fentanyl-pretreated group.

morphine vs 40 ± 18 for saline, $F(5, 54) = 33$; $p < 0.001$, one way ANOVA followed by Fisher's PLSD test $p < 0.001$; Fig. 5B). When injected 20 min before morphine, RF313 (5 mg/kg) significantly enhanced morphine-induced analgesia (AUC: 1071 ± 91 for morphine + RF313 vs 725 ± 68 for morphine, $F(1, 54) = 4.7$; $p < 0.05$, two way ANOVA followed by Fisher's PLSD test $p < 0.001$; Fig. 5B).

In a subsequent experiment, we performed daily injections of morphine (5 mg/kg, s.c.) for 8 consecutive days and the thermal nociceptive response of mice was measured every day before morphine administration. Such a daily morphine treatment led to a progressive decrease of basal nociceptive reaction latency (Fig. 6A), indicative for the development of a robust hyperalgesic state in mice (AUC: 45.2 ± 4.8 for morphine vs - 12.5 ± 4.1 for saline, $F(3, 28) = 8.6$; $p < 0.001$, one way ANOVA followed by Fisher's PLSD test $p < 0.001$; Fig. 6B). When injected before daily morphine administration (Fig. 6A), RF313 (5 mg/kg, s.c.) significantly attenuated hyperalgesia (AUC: 20.3 ± 4.3 for RF313 + morphine vs - 45.2 ± 4.8

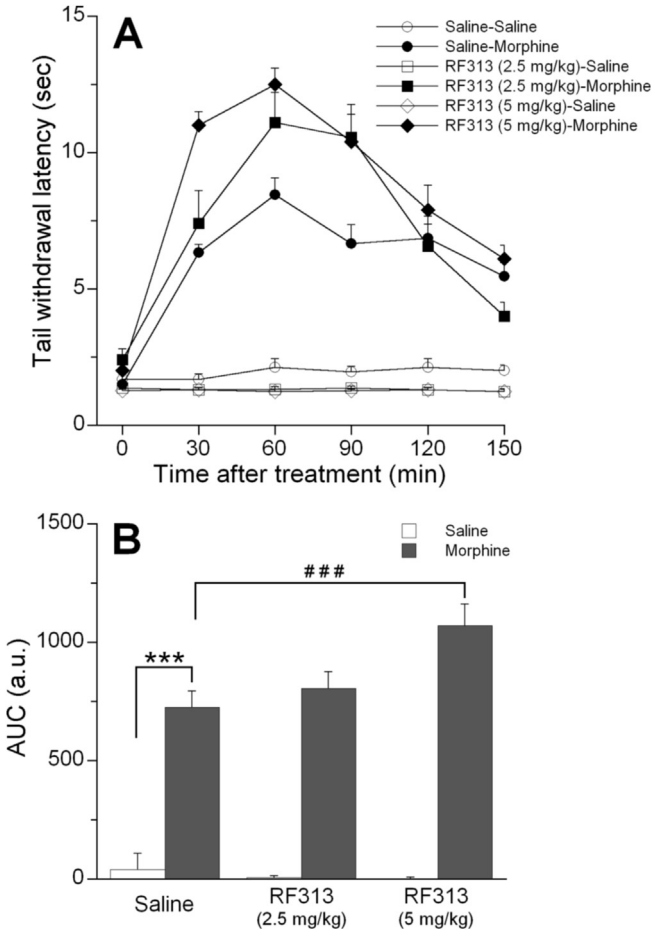

Fig. 5. Effect of RF313 on basal nociception and morphine analgesia in mice. **A**: RF313 (2.5 or 5 mg/kg, s.c.) or saline was injected to mice 20 min before morphine (5 mg/kg, s.c.) or saline. Tail withdrawal latencies were measured at 52 °C, at 30 min intervals, over a 150 min period after morphine injection. **B**: Comparison between groups of area-under-the-curve (AUC values) over the 0–150 min time course. Data are expressed as mean ± S.E.M, $n = 7–15$. $^{***}p < 0.01$ by Fisher's test as compared with the saline group. $^{###}p < 0.001$ by Fisher's test as compared with the morphine-pretreated group.

for morphine, $F(1, 28) = 4.2$; $p < 0.05$, two-way ANOVA followed by Fisher's PLSD test $p < 0.01$; Fig. 6B).

We next addressed the question whether hyperalgesia was accompanied with the development of analgesic tolerance to acute morphine and again, we considered the impact of RF313. To this end, morphine (5 mg/kg, s.c.) analgesic effect was measured in time-course experiments at day 0 (Fig. 6C, naïve mice) and at day 8 (Fig. 6D, hyperalgesic mice) on mice daily pre-treated with either saline or RF313 (5 mg/kg, s.c.). At day 0, as expected from previous experiments (Fig. 5), RF313 potentiated and prolonged the analgesic response of mice to morphine (AUC: 800 ± 62 for RF313 + morphine vs 594 ± 69 for morphine, $F(3, 36) = 12.4$; $p < 0.001$, one way ANOVA followed by Fisher's PLSD test, $p < 0.01$; Fig. 6C and E). After 8 days of treatment, the analgesic effect of morphine alone was strongly reduced (AUC: 330 ± 43 at day 8 vs 594 ± 69 at day 0, $p < 0.01$, Fisher's PLSD test; Fig. 6D and E) indicating that tolerance did develop in these animals. In mice treated with RF313 and morphine, analgesic tolerance still developed (AUC: 503 ± 39 at day 8 vs 800 ± 62 at day 0, $p < 0.001$, Fisher's PLSD test; Fig. 6D and E) but to a lower extent than in animals treated with morphine alone (AUC: 503 ± 39 for RF313 + morphine vs 330 ± 43 for morphine, $p < 0.01$, Fisher's PLSD test). Altogether, these results indicate that RF313 can both improve acute morphine analgesic effect and attenuate the development of hyperalgesia as well as of analgesic tolerance following chronic morphine administration.

3.2.5. RF313, but not RF9, antagonizes RFRP-3 stimulation of LH secretion in the hamster

Two RF-amide receptors, Kiss1R and NPFF1R (also termed GPR54 and GPR147, respectively), play essential functions in the control of reproduction in seasonal mammals (Simonneaux et al., 2013). Conversely to other species, in male hamster RFRP-3 has been shown to display stimulatory action on LH release (Ancel et al., 2012; Simonneaux et al., 2013). This species is therefore particularly well suited to study the antagonist action of RF313 on NPFF1R. As our *in vitro* experiments showed that RF9 (but not RF313) displays micromolar affinity and agonist activity at Kiss1R (Table 1 and Fig. 2C), we decided to compare the effects of these two NPFF receptor antagonists on RFRP-3-induced LH release in the male Syrian hamster. Data analyses with one way ANOVA highlighted significant differences between the tested groups ($F(4, 27) = 49$; $p < 0.001$). Post-hoc analyses with Fisher's PLSD test (Fig. 7) revealed that i.c.v. administration of the endogenous NPFF1R agonist RFRP-3 resulted in an increase in LH plasma levels (6.4 ng/ml ± 0.4 for saline vs 15.2 ng/ml ± 1.9 for RFRP-3, $p < 0.001$). RF313 did not affect basal LH levels (6.4 ng/ml ± 0.4 for RF313 vs 6.4 ng/ml ± 0.4 for saline, $p > 0.05$) but fully antagonized the stimulatory effect of RFRP-3 on LH secretion (15.2 ng/ml ± 1.9 for RFRP-3 vs 5.4 ng/ml ± 0.7 for RF313 + RFRP-3, $p < 0.001$). Conversely, RF9 exhibited *per se* a strong gonadotropin-releasing activity *in vivo* (6.4 ng/ml ± 0.4 for saline vs 27.8 ng/ml ± 1.2 for RF9, $p < 0.001$). Altogether, these results confirm previous observations linking RF9 gonadotropin-releasing activity to a possible agonist activity toward Kiss1R (Garcia-Galiano et al., 2012; Glanowska et al., 2014; Liu and Herbison, 2014; Sahin et al., 2015) and point out RF313 as a safer pharmacological tool to dissect properly the role of NPFF1R in the central control of gonadotropin release in mammals.

4. Discussion

In this paper, we provide a detailed analysis of the *in vitro* and *in vivo* pharmacological properties of RF313, a compound (referred to as **12e** in (Bihel et al., 2015)) we selected in a drug discovery program aiming at identifying orally-bioavailable antagonists of NPFF receptors. This non-natural ornithine derivative displays a high solubility in aqueous buffer, a low toxicity, a high selectivity, as well as an antagonist activity at NPFF1R both *in vitro* and *in vivo*. Moreover, it potentiates opioid analgesic effects in mice both by subcutaneous and oral routes. It completely blocks fentanyl-induced hyperalgesia and attenuates morphine-induced hyperalgesia as well and analgesic tolerance. By comparison with our previous studies conducted with RF9 (Elhabazi et al., 2012, 2013; Simonin et al., 2006), RF313 appeared to block the hyperalgesia induced by RFRP-3 and fentanyl at similar doses than RF9 but was slightly less efficient at preventing the development of hyperalgesia and analgesic tolerance induced by chronic morphine administration. Our results confirm that NPFF receptor blockade might represent an interesting strategy to improve opiates analgesic action while limiting the adverse effects associated with their chronic administration. Altogether, the data described in this study point out RF313 as an interesting lead compound for further drug development.

Despite the good *in vivo* activity displayed by RF313 at relatively low doses and by oral route, *in vitro* binding experiments showed that its affinity for NPFF1R and NPFF2R was lower than that of the dipeptide RF9 for the same receptors (Simonin et al., 2006). As

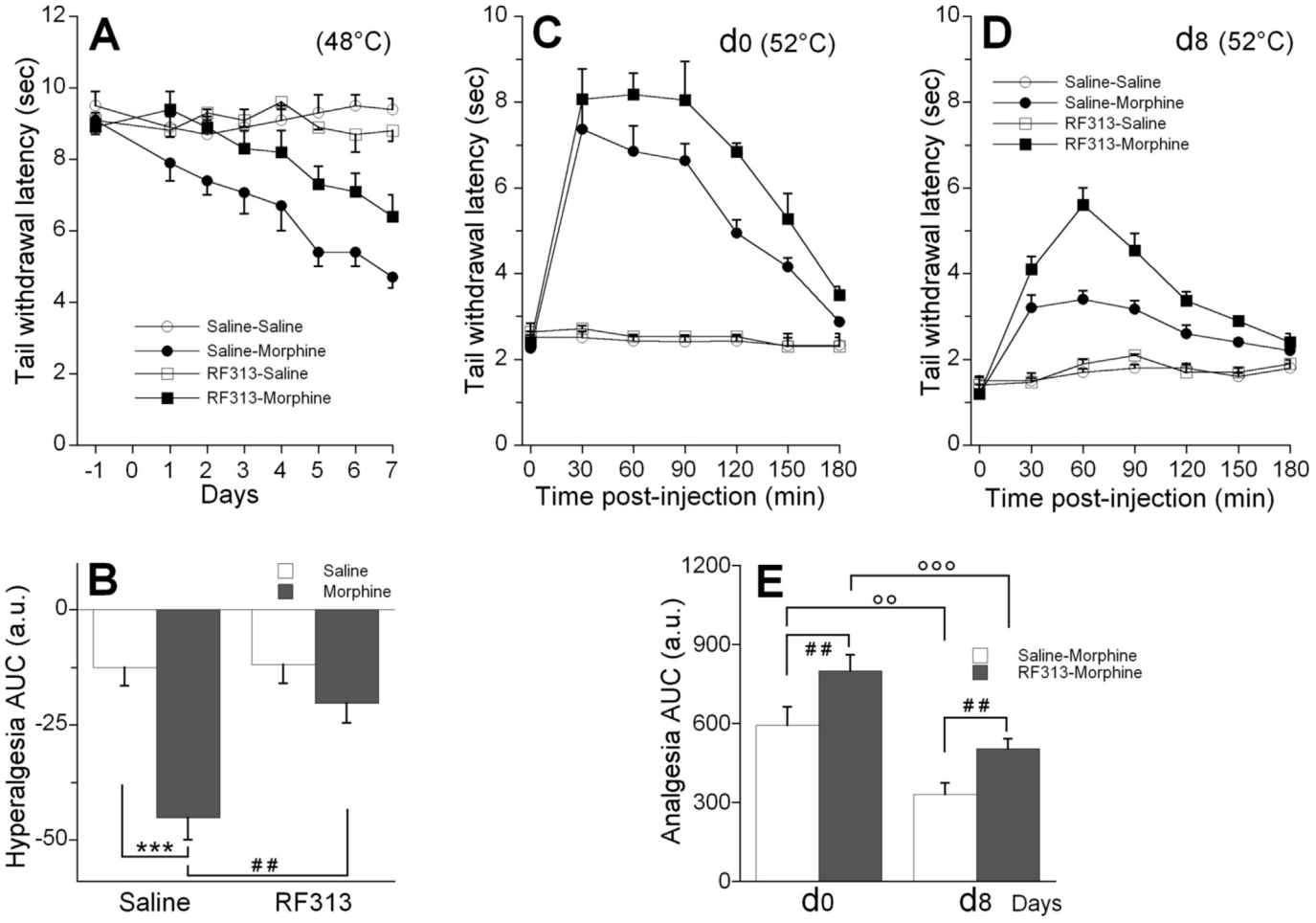

Fig. 6. Effect of RF313 on morphine-induced hyperalgesia and analgesic tolerance. **A, B**: From d0 to d7, mice received daily injections of RF313 (5 mg/kg; s.c.) 20 min prior to morphine (5 mg/kg; s.c.), as reported under Materials and methods. Saline replaced either RF313 or morphine, according to groups. Basal nociceptive values were measured once daily before experiment start (d-1) and every day before treatment (d1 to d7), using the tail immersion test (48 °C). B: Comparison between tested groups of AUC values calculated from d-1 to d7. **C**: On day 0, the analgesic effect of the first morphine injection (5 mg/kg; s.c.) was monitored every 30 min over a 3 h period. Mice that received RF313 (5 mg/kg; s.c.) 20 min prior to morphine were similarly examined for their nociceptive responses, using the tail immersion test at 52 °C. **D**: On day 8, 24 h after the end of the chronic RF313/morphine treatment, mice received either saline or RF313 (5 mg/kg; s.c.) 20 min prior to saline or morphine (5 mg/kg; s.c.) injections. Nociceptive responses were determined using the tail immersion test (set at 52 °C), according the time-course paradigm already defined in **C**. **E**: Comparison between groups of calculated AUC values from **C** and **D**. Data are expressed as mean ± S.E.M, $n = 5–10$. ***$p < 0.001$ by Fisher's test as compared with the saline group. ##$p < 0.01$ by Fisher's test as compared with the morphine-pretreated group. °°$p < 0.01$, °°° $p < 0.001$ by Fisher's test as compared with the AUC value of the same group at d0.

molecular rigidification has been described as a standard approach for the development of GPCR antagonists, a lead-optimization process with particular focus on a decrease in the flexibility of the RF313 backbone could represent an alternative strategy to improve its affinity for NPFF receptors.

Binding experiments also indicated that RF313 displayed a slight preference (3-fold selectivity) for the NPFF1R subtype compared to NPFF2R. When testing *in vitro* the nature of the interaction of RF313 at NPFF receptors, we were not able to detect any agonist or antagonist activity of this compound toward NPFF2R while it clearly blocked NPFF1R-mediated cellular responses to RFRP-3. These data suggest that the functional selectivity of RF313 toward NPFF1R is probably greater than that afforded from binding experiments. Although one cannot completely rule out a role of NPFF2R, our *in vivo* data point out NPFF1R as an important actor in the development of secondary hyperalgesia induced by opiates. These results are in agreement with previous observations made with NPFF1R/NPFF2R compounds with varying degrees of functional activity, which suggest that these two receptors display opposing pro- and anti-nociceptive roles, respectively, in various models of pain (Lameh et al., 2010). However, several studies have shown that neuropeptide FF, the endogenous ligand of NPFF2R, can display both pro- or anti-nociceptive actions depending on its site of administration i.t. (Gouarderes et al., 1993) versus i.c.v. (Journigan et al., 2014; Oberling et al., 1993). Thus, despite the recent design of several peptidic and non peptidic NPFF receptors ligands (Gaubert et al., 2009; Journigan et al., 2014; Lameh et al., 2010; Mazarguil et al., 2012), the development of highly selective ligands still represents an important challenge that should greatly help clarifying the respective contribution of NPFF1/2 receptor subtypes in the modulation of nociception.

In this study, we also compared the *in vitro* binding and activity profiles of RF313 and RF9, a dipeptidic compound we identified previously as a NPFF receptor antagonist (Simonin et al., 2006). We observed that RF9 displayed a low but detectable affinity for Kiss1R, as well as full agonist activity, while RF313 neither binds nor activates this receptor at concentrations up to 100 μM. Kiss1R has been shown to play a key role in reproduction in numerous species including rodents and human (Goodman and Lehman, 2012). It is expressed in GnRH neurons of the hypothalamus and its activation

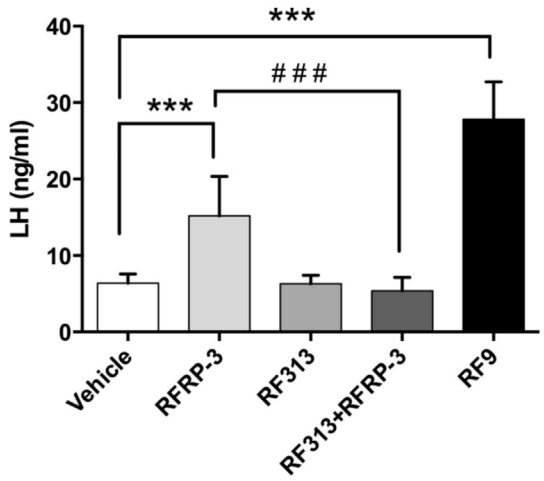

Fig. 7. Effects of central injection of RFRP-3 and of NPFF antagonists on LH secretion in the male Syrian hamster. Drugs (1.5 μg) were injected i.c.v., alone or in combination, in a 2 μL final volume. 30 min later, the blood was sampled and assayed for LH content (ng/ml plasma) as described under Materials and methods. Mean ± S.E.M. values for 6 to 7 animals are presented as histograms. ***$p < 0.001$ by Fisher's test as compared with the saline group. ###$p < 0.001$ by Fisher's test as compared with RFRP-3 treated group.

by kisspeptin has been shown to strongly stimulate GnRH release, which controls the release of sexual hormones. More recently, NPFF1R and its endogenous ligands RFRP-1 and RFRP-3 have been proposed to play an inhibitory role on GnRH release, thus counteracting the stimulatory role of the kisspeptin/Kiss1R system (Kriegsfeld, 2010). This hypothesis was based in part on results showing that RF9 (when administered i.c.v. in rodents or ewes) is a potent stimulator of LH and FSH release (Caraty et al., 2012; Pineda et al., 2010b; Rizwan et al., 2012) suggesting the existence of an endogenous tone of RFRPs that exerts a negative feedback on GnRH neurons (Pineda et al., 2010a, b). However, several studies also suspected RF9 impact on gonadotropin release to rely on an off-target action on kisspeptin receptors (Garcia-Galiano et al., 2012; Glanowska et al., 2014; Liu and Herbison, 2014; Sahin et al., 2015), an issue that was later elucidated with the demonstration of RF9 agonist activity at Kiss1R, both *in vitro* and *in vivo* (Kim et al., 2015; Min et al., 2015). As RF9 effect was absent in Kiss1R KO mice but well preserved in NPFF1R KOs (Kim et al., 2015; Liu and Herbison, 2014; Min et al., 2015), one may conclude that this compound modulates gonadotropin release primarily through Kiss1R activation rather than via blockade of NPFF1R. We further extended this observation in male hamsters, in which RF9 strongly increased LH levels while RF313 had no activity *per se* but efficiently blocked RFRP-3 stimulation of LH release. Altogether, these results indicate that RF313, despite its lower affinity at NPFF1R than RF9, may be regarded as a more suitable pharmacological tool to study the involvement of NPFF1R in the central control of reproduction, owing to its lack of Kiss1R agonist activity. Moreover, our data also indicate that an important clue to developing high affinity NPFF1R antagonists will be the careful investigation of their selectivity toward other RF-amide receptors and particularly Kiss1R.

5. Conclusion

In conclusion, we characterized RF313 as a novel antagonist of the NPFF1 receptor. It displays many characteristics for a lead compound amenable to further therapeutic development in order to improve analgesic action of opiates and reduce adverse side effects associated with their chronic administration. Moreover, and in contrast with RF9, it is devoid of agonist activity at Kiss1R and may therefore be used as a safer pharmacological tool to study the role of NPFF1R in the central control of reproduction. The development of highly selective compounds for both NPFF1 and NPFF2 receptors will be mandatory in the next future to dissect their own roles in the modulation of essential physiological functions such as nociception, feeding and reproduction.

Disclosures

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2017.03.012.

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ANNEXE III

Neuropeptide FF increases M2 activation and self renewal of adipose tissue macrophages.

Waqas FSH, Hoang AC, Lin YT, Ampem G, Azgrouz H, Balogh L, Thuroczy J, Chen JC, Gerling, IC, Nam, S, Lim, JS, Martinez-Ibanez J, Real JT, Paschke S, Quillet R, Ayachi S, Simonin F, Schneider EM, Brinkman JA, Lamming DW, Seroogy CM, Röszer T

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Neuropeptide FF increases M2 activation and self-renewal of adipose tissue macrophages

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The quantity and activation state of adipose tissue macrophages (ATMs) impact the development of obesity-induced metabolic diseases. Appetite-controlling hormones play key roles in obesity; however, our understanding of their effects on ATMs is limited. Here, we have shown that human and mouse ATMs express NPFFR2, a receptor for the appetite-reducing neuropeptide FF (NPFF), and that NPFFR2 expression is upregulated by IL-4, an M2-polarizing cytokine. Plasma levels of NPFF decreased in obese patients and high-fat diet–fed mice and increased following caloric restriction. NPFF promoted M2 activation and increased the proliferation of murine and human ATMs. Both M2 activation and increased ATM proliferation were abolished in NPFFR2-deficient ATMs. Mechanistically, the effects of NPFF involved the suppression of E3 ubiquitin ligase RNF128 expression, resulting in enhanced stability of phosphorylated STAT6 and increased transcription of the M2 macrophage–associated genes IL-4 receptor α (*Il4ra*), arginase 1 (*Arg1*), IL-10 (*Il10*), and alkylglycerol monooxygenase (*Agmo*). NPFF induced ATM proliferation concomitantly with the increase in N-Myc downstream-regulated gene 2 (*Ndrg2*) expression and suppressed the transcription of *Ifi200* cell-cycle inhibitor family members and MAF bZIP transcription factor B (*Mafb*), a negative regulator of macrophage proliferation. NPFF thus plays an important role in supporting healthy adipose tissue via the maintenance of metabolically beneficial ATMs.

Introduction

Adipose tissue macrophages (ATMs) are immune cells of adipose tissue (AT) stroma with key roles in AT development and metabolism (1). ATMs accumulate in AT in obesity and undergo so-called classical M1 polarization to a metabolically harmful proinflammatory phenotype, which drives obesity-associated metabolic diseases (2). M1-activated ATMs produce inflammatory cytokines, NO, and ROS that induce AT inflammation, leading to insulin resistance (IR) and contributing to comorbidities of obesity (2). Given the near-pandemic incidence of obesity and associated metabolic diseases, there is a clear need for a better understanding of how the quantity and activation of ATMs are controlled (1).

M1-activated ATMs are replenished from circulating monocytes that infiltrate obese AT (3). ATMs also proliferate locally in obese AT, increasing the quantity of M1-activated ATMs and exacerbating AT inflammation (4). ATMs can also adopt an alternative or M2 activation state, which supports the resolution of inflammation and is hence metabolically beneficial; the prevalent ATM activation state in lean AT is M2 (2). Th2 cytokines, lipid metabolites, neurotransmitters, and hormones can shift ATMs into an M2-activated state (5); however, how these signals function to control the replenishment of metabolically beneficial ATMs is unknown.

Neuropeptide FF (FLFQPQRFamide, abbreviated herein as NPFF) is a member of the evolutionarily conserved RF-amide neuropeptide family (6). NPFF has been principally studied in mammals as a pain modulator and in the adaptive responses to opiate treatment, but it can also reduce food intake in rodents by acting at hypothalamic nuclei (6, 7). Appetite control is key in obesity development, and in vitro studies have shown that NPFF inhibits both human and murine adipocyte development (8, 9). Peripheral tissues have negligible NPFF receptor expression, with the exception of AT, in which NPFF receptor 2 (NPFFR2, also known as GPR74, HG31, and NPFF2) is expressed (10, 11). Genetic variance of *NPFFR2* is closely associated with obesity status (12). Human plasma contains NPFF (13), and it is postulated that NPFF may be released from nerve endings in AT (11). However, the in vivo role of NPFF in AT function is unknown.

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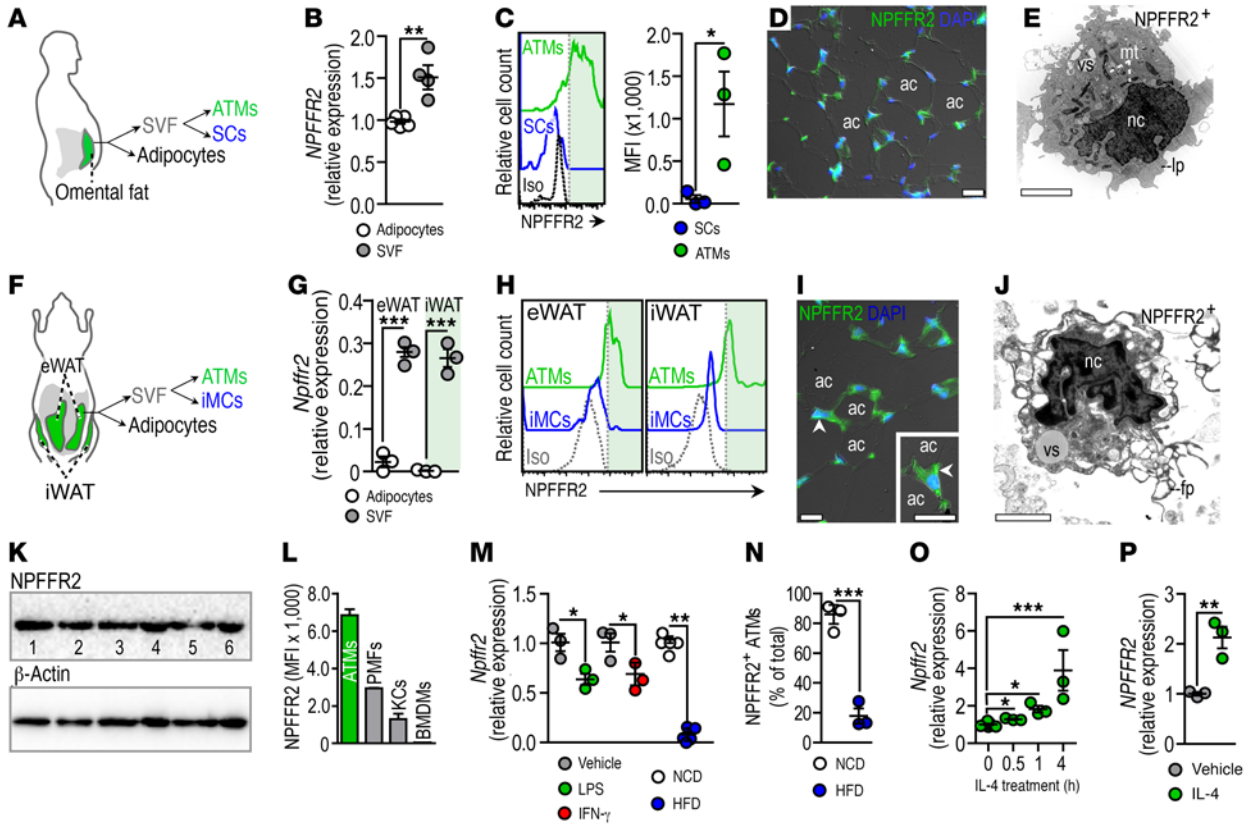

Figure 1. Human and mouse ATMs express NPFFR2, which increases after IL-4 exposure. (**A**) Diagram of human cell types of the omental fat depot analyzed in this study. ATMs, $CD68^+CD14^+$ ATMs; SCs, $CD68^-CD14^-$ stromal cells. (**B**) *NPFFR2* transcription in SVF and adipocytes of human omental fat ($n = 4$). (**C**) FACS analysis of NPFFR2 expression in human ATMs and SCs. (**D**) NPFFR2 immunostaining in human omental fat. Scale bar: 25 μm. (**E**) TEM image of an $NPFFR2^+$ human ATM. Scale bar: 0.5 μm. (**F**) Diagram of the analyzed mouse fat depots. (**G**) Relative transcription of *Npffr2* in SVF of lean mice; each data point shows pools of cells from 3 mice. (**H**) FACS analysis of NPFFR2 expression in mouse ATMs and iMCs. (**I**) Immunostaining of NPFFR2 in mouse eWAT. Arrowheads indicate the cell border. Scale bars for both images: 25 μm. (**J**) TEM image of an $NPFFR2^+$ mouse ATM. Scale bar: 0.5 μm. (**K**) Western blot of NPFFR2 in mouse ATMs. Lanes 1–3: ATMs from eWAT; lanes 4–6: ATMs from iWAT. (**L**) FACS analysis of NPFFR2 expression in mouse macrophages. PMFs, peritoneal macrophages; KCs, Kupffer cells; BMDMs, nonstimulated bone marrow–derived macrophages ($n = 3$). (**M**) *Npffr2* transcription in mouse ATMs after 100 ng/ml LPS or 2.5 ng/ml IFN-γ treatment for 4 hours in vitro or after 3 months on a NCD or HFD ($n = 3$ and 5, respectively). (**N**) $NPFFR2^+$ ATMs in NCD- or HFD-fed mice. Each data points represent pooled ATMs from 2 mice. The experiment was conducted 2 times. (**O** and **P**) *Npffr2* and *NPFFR2* transcription after 10 ng/ml IL-4 treatment of mouse (**O**) and human (**P**) ATMs ($n = 3$). Human ATMs were treated for 4 hours. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, by unpaired, 2-tailed Student's *t* test (**B**, **C**, **G**, **M**, **N**, and **P**) and 1-way ANOVA with Dunnett's post-hoc test (**O**). ac, adipocyte; fp, filopodia; iMCs, immature myeloid cells; iso, isotype; lp, lamellipodia; MFI, mean fluorescence intensity; mt, mitochondria; nc, nucleus; vs, vesicle.

The mechanisms governing macrophage proliferation and M1/M2 polarization are key to defining the metabolic effects of ATMs (2). We show here that NPFF has metabolically beneficial effects on ATMs by increasing M2-macrophage activation and proliferation. Thus, NPFF appears to play an important role in the maintenance of healthy AT.

Results

NPFF receptor expression is restricted to ATMs in AT, and its expression is upregulated by IL-4. NPFFR2 is the principal receptor for NPFF in mammalian cells, and its gene expression has been demonstrated in AT (10). To study the cell-autonomous function of NPFFR2 in AT, we first examined its expression in human omental fat (Figure 1A). We found that *NPFFR2* mRNA transcription and NPFFR2 protein expression were restricted to ATMs in the stromal vascular fraction (SVF) (Figure 1, B–E; details of ATM isolation are provided in Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI90152DS1). Like human AT, we observed that *Npffr2* mRNA and NPFFR2 protein were expressed only by ATMs in mouse epididymal white adipose tissue (eWAT) and inguinal WAT (iWAT) (Figure 1, F–J). The expression of NPFFR2 was equivalent in ATMs of eWAT and iWAT (Figure 1K). Because eWAT from lean mice contained more ATMs than did iWAT (Supplemental Figure 1, B and C), we used ATMs isolated from eWAT for in vitro assays throughout the study. Other potential receptors of NPFF, such as *Npffr1* and acid-sensing ion channel 1 (*Asic1a*), were not expressed by ATMs (Supplemental Figure 2, A and B).

We next compared NPFFR2 expression levels between ATMs and other macrophage types in mice. Peritoneal macrophages, Kupffer cells, and bone marrow–derived macrophages had minor or negligible NPFFR2 protein and *Npffr2* mRNA expression levels relative to levels detected in ATMs (Figure 1L and Supplemental Figure 2, C and D), whereas the murine macrophage line J774A.1 had more detectable expression of *Npffr2* (Supplemental Figure 2E). Moreover, *NPFFR2* transcription was undetectable in human peripheral blood monocytes and in nonadherent, monocytic THP1

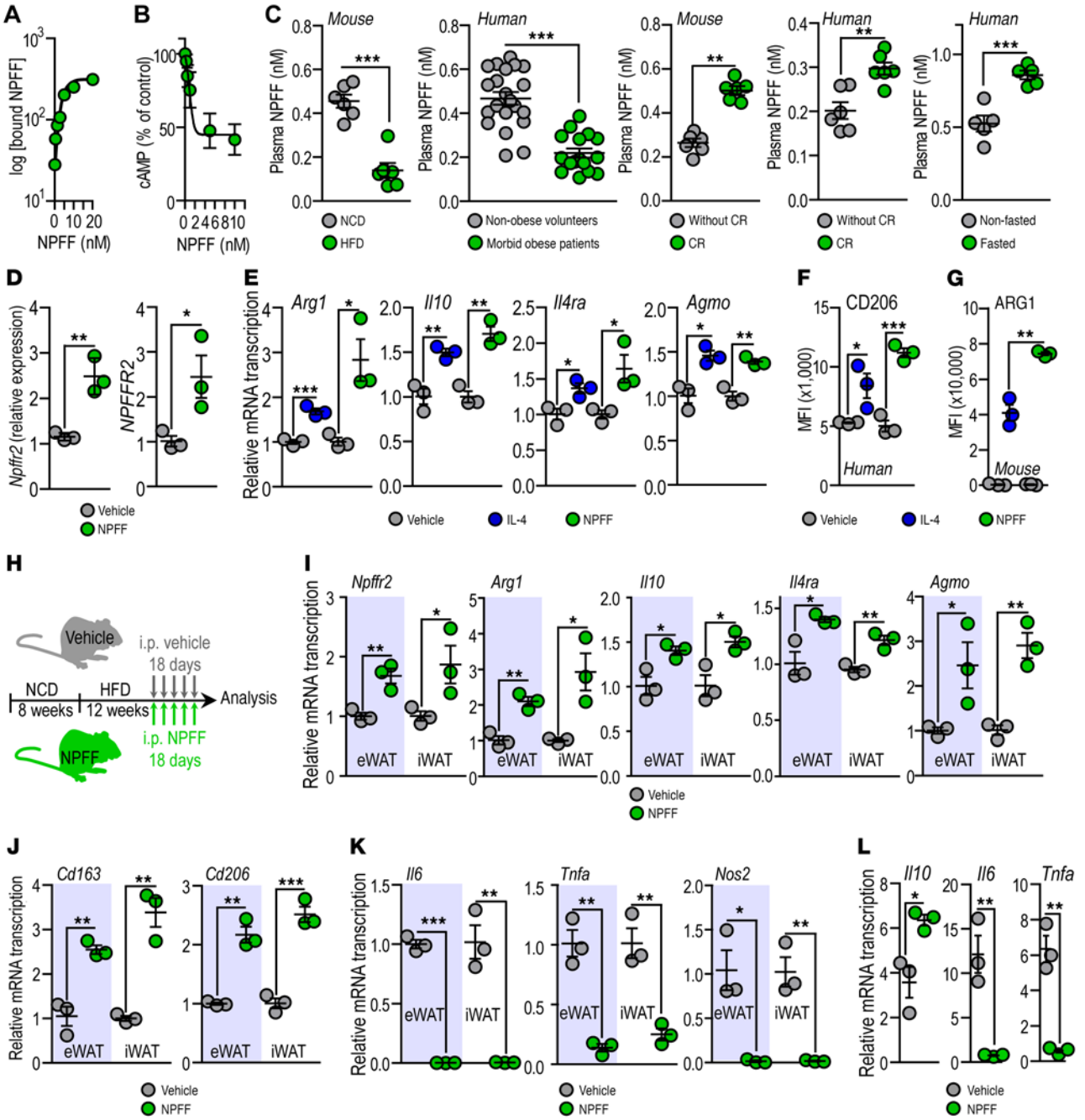

Figure 2. Human and mouse plasma contains sufficient NPFF to provoke M2 activation of ATMs. (**A**) Binding of FITC-conjugated NPFF to mouse ATMs. (**B**) cAMP levels in mouse ATMs treated with NPFF. (**C**) Plasma NPFF levels were measured in NCD- and HFD-fed mice (n = 6); in nonobese volunteers (n = 21) and morbidly obese patients (n = 16); in obese mice without or with 14 days of CR (n = 6); in morbidly obese patients with or without a CR diet (n = 6); and in nonobese, nonfasted volunteers and nonobese volunteers following 28 days of intermittent fasting (n = 5). (**D**) *Npffr2* and *NPFFR2* transcription in response to treatment of mouse and human ATMs for 4 hours with 0.5 nM NPFF (n = 3). (**E**) Transcription of M2 macrophage activation genes following a 30-minute treatment of mouse ATMs with 10 ng/ml IL-4 or 0.5 nM NPFF. (**F**) FACS analysis of human ATMs treated with 0.5 nM NPFF for 4 hours. Each data point represents pooled ATMs from 2 donors. (**G**) Arginase 1 (ARG1) expression of mouse ATMs treated with 10 ng/ml IL-4 or 0.5 nM NPFF for 24 hours. Each data point represents pooled ATMs from 3 to 5 mice. (**H**) Scheme of NPFF treatment of HFD-fed mice. (**I**–**K**) Relative transcription of *Npffr2*, M2, and M1 genes in ATMs from HFD-fed mice, without or with NPFF treatment (n = 6; each point represents pooled ATMs from 2 mice). (**L**) Effect of NPFF on palmitic acid–treated ATMs. ATMs were pooled from 3 to 5 mice and treated in triplicate in panels **D**, **E**, **G**, and **L**. *P < 0.05, **P < 0.01, and ***P < 0.001, by unpaired, 2-tailed Student's t test.

cells (Supplemental Figure 2F). Overall, these results suggest that ATMs are potential targets of NPFF.

The metabolic effects of ATMs are determined by their activation state (2). M1 activation of ATMs contributes to AT inflammation, which can exacerbate IR in obese AT. M1 activation can be triggered in vitro by treatment with various agents such as LPS, IFN-γ, or palmitic acid (5). IL-4 induces M2 activation, which is considered metabolically beneficial in AT, since it triggers a

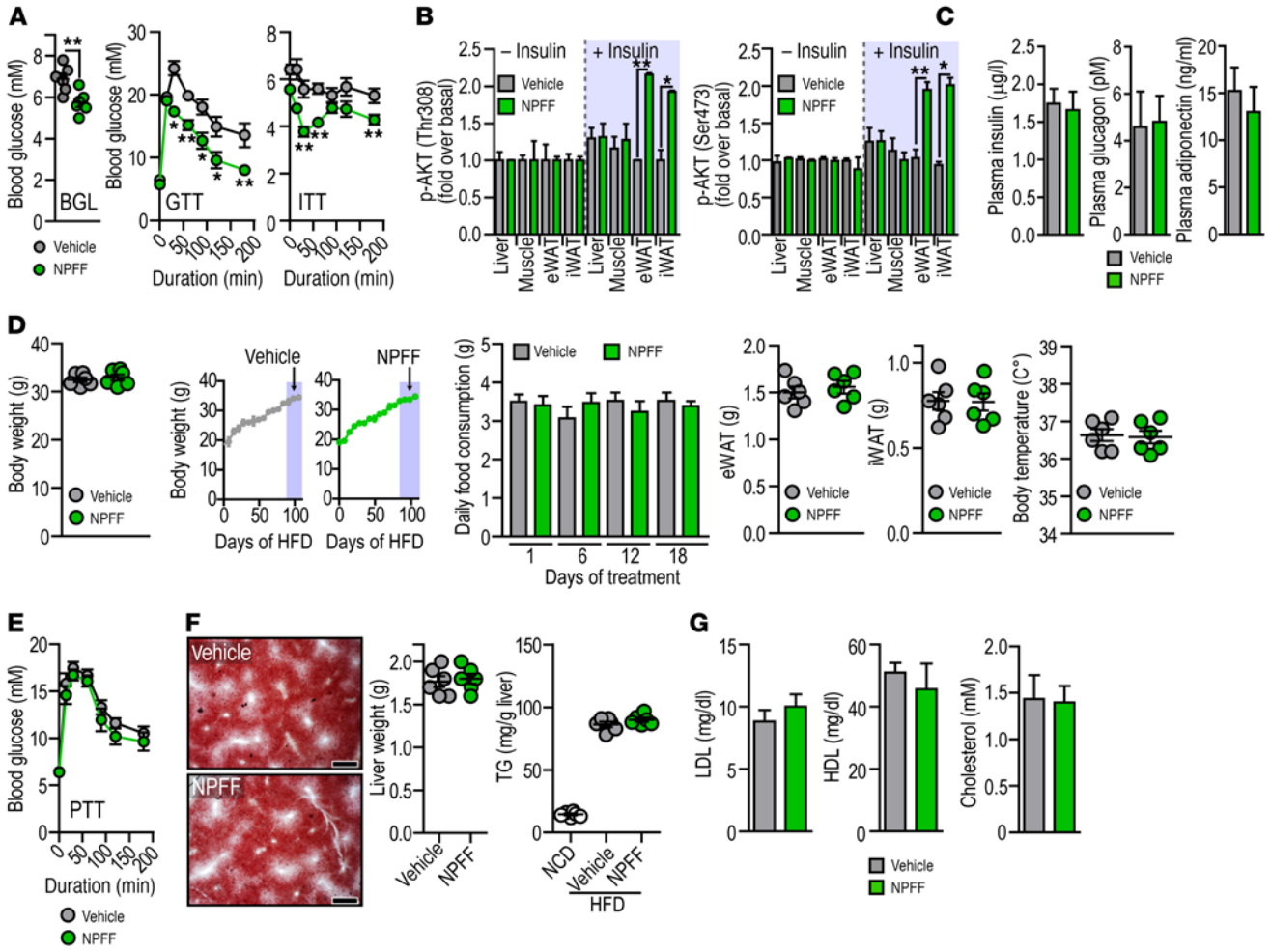

Figure 3. Metabolic phenotyping of HFD-fed mice after NPFF treatment. (**A**) HFD-fed mice were treated with vehicle or NPFF daily, as shown in Figure 2H. Basal blood glucose level (BGL) after a 4-hour fast, glucose tolerance test (GTT), and insulin sensitivity test (ITT) (n = 6). (**B**) Insulin-induced AKT phosphorylation in liver, quadriceps muscle, eWAT, and iWAT. Total AKT and p-AKT (Ser308 and Thr473) levels were measured by ELISA (n = 6). (**C**) Plasma insulin, glucagon, and adiponectin levels (n = 6). (**D**) Body weight, weight gain, food consumption, eWAT and iWAT weight, and body temperature (measured at 9:00 am) (n = 6). (**E**) PTT (n = 6). (**F**) Oil red O staining of liver sections. Scale bars: 100 μm. Liver weights and liver triglycerol (TG) content. As a comparison, NCD liver TG content is also shown (n = 6). (**G**) Plasma lipid profile (n = 6). *P < 0.05 and **P < 0.01, by unpaired, 2-tailed Student's t test and 2-way ANOVA (**A**) and 1-way ANOVA with Dunnett's post-hoc test (**B**).

pro-resolving ATM phenotype that can impede AT inflammation and IR (2). Thus, to question whether *Npffr2* transcription was dependent on the ATM activation state, we treated murine ATMs with LPS or IFN-γ and found that *Npffr2* transcription was diminished in response to either treatment (Figure 1M). Obese AT is rich in M1-activated ATMs (2). To test whether obesity-induced ATM activation modulated *Npffr2* transcription, we rendered C57BL/6 mice obese by placing them on a high-fat diet (HFD) for 12 weeks (body and AT weights are shown in Supplemental Figure 3A). When compared with normal diet–fed mice, the total ATM population from HFD-fed mice had diminished *Npffr2* transcription (Figure 1M) and reduced NPFFR2 protein levels (Supplemental Figure 2G), which was reflected by the reduced number of NPFFR2$^+$ ATMs (Figure 1N). Murine ATMs treated in vitro with IL-4 increased their expression of *Npffr2* (Figure 1O), which was proportional to the duration of IL-4 exposure. Human ATMs also had upregulated *NPFFR2* transcription following IL-4 treatment (Figure 1P), and IL-13 had a similar effect (Supplemental Figure 2H).

Plasma levels of NPFF in mice and humans are sufficient for M2 activation of ATMs. Since *Npffr2* expression was upregulated by IL-4, we questioned whether NPFF impacted the IL-4–induced transcriptional changes in ATMs. Data on NPFF bioavailability and receptor binding are scarce. Thus, we first determined the effective dose of NPFF that could activate NPFFR2 signaling in ATMs. NPFF bound to murine ATMs with a K_D of 0.37 nM ± 0.01 nM (Figure 2A and Supplemental Figure 3B). NPFFR2 belongs to the family of G_i protein–linked neuropeptide receptors (14), and, accordingly, NPFF treatment strongly reduced cAMP levels in ATMs, with a half-maximal effective concentration (EC_{50}) of 0.40 ± 0.01 nM (Figure 2B and Supplemental Figure 3C).

To estimate the NPFF concentration to which ATMs can be exposed in vivo, we determined its concentration in mouse and human plasma (details of the mass spectrometric analysis and ELISA for plasma NPFF levels can be found in Supplemental Figure 4, A–D). The plasma levels of NPFF were 0.53 nM ± 0.02 nM in lean 8-week-old male C57BL/6 mice and 0.4 8 nM ± 0.03 nM

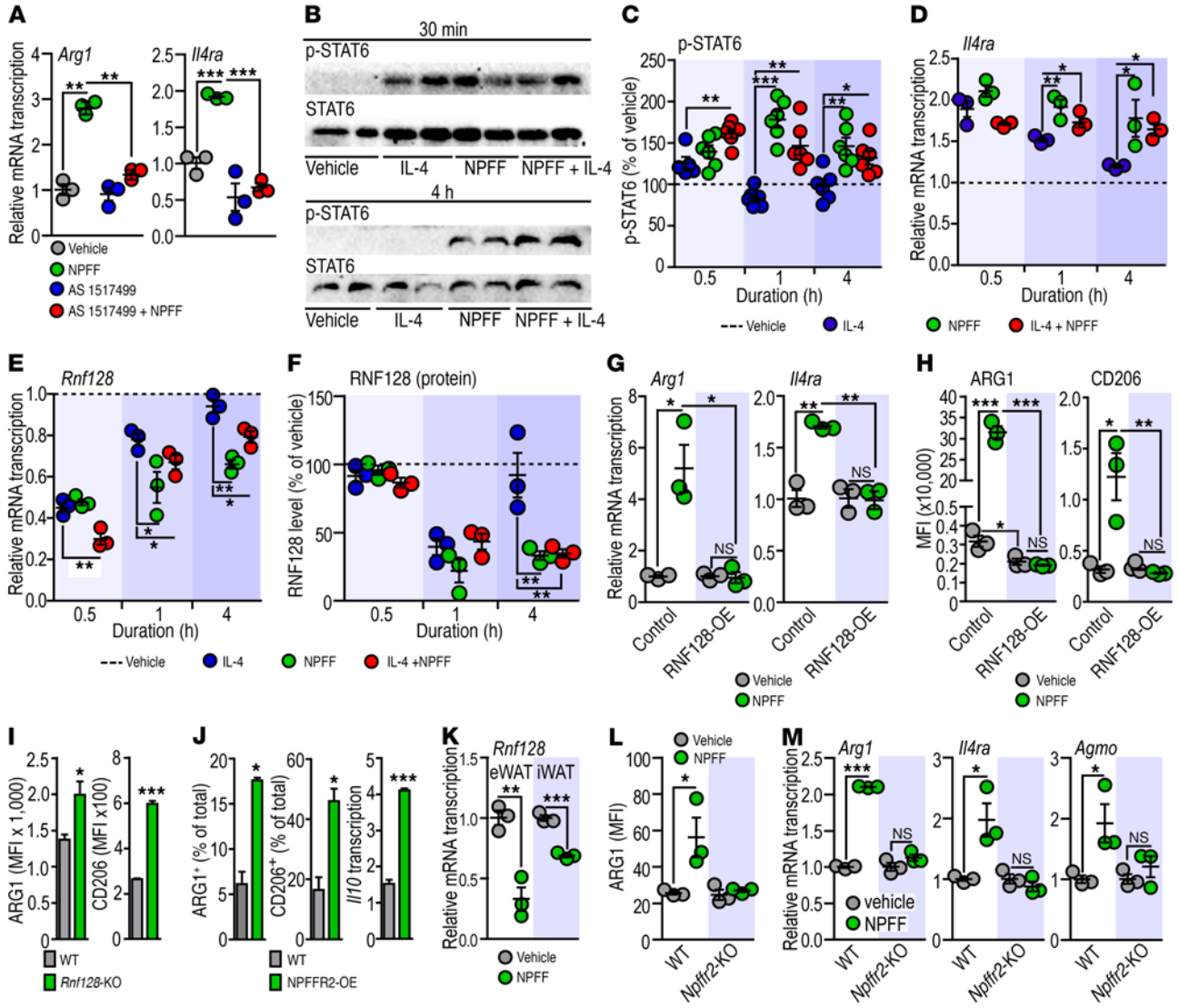

Figure 4. NPFF inhibits p-STAT6 decay and triggers M2 activation of ATMs. (**A**) Effect of the STAT6 inhibitor AS1517499 (200 nM) on NPFF-induced *Arg1* and *Il4ra* transcription. ATMs were treated with 0.5 nM NPFF for 1 hour. (**B**) Western blot of p-STAT6/STAT6 in ATMs treated with 100 ng/ml IL-4 or 0.5 nM NPFF. Each lane corresponds to pooled ATMs from 3 to 5 mice. Results are representative of 3 independent experiments. (**C**) p-STAT6 levels following 10 ng/ml IL-4 and 0.5 nM NPFF treatment, as measured by in-cell ELISA. (**D**) Effect of 10 ng/ml IL-4 and 0.5 nM NPFF on the transcription of *Il4ra* in ATMs. (**E**) Effect of 10 ng/ml IL-4 and 0.5 nM NPFF on the transcription of *Rnf128* in ATMs. (**F**) RNF128 levels in lysates of ATMs treated with 10 ng/ml IL-4, 0.5 nM NPFF, or their combination. In **C**–**F**, the dotted lines represent the levels with vehicle treatment. (**G** and **H**) Relative transcription and MFI of M2 markers in control and RNF128-overexpressing (RNF128-OE) J774A.1 macrophages after a 4-hour treatment with vehicle or 0.5 nM NPFF. (**I**) MFI of M2 markers in ATMs from WT and *Rnf128*-KO mice. Data points represent pooled ATMs from 2 mice. The experiment was conducted 2 times. (**J**) Percentage of M2 ATMs and relative transcription of *Il10* in ATMs from WT and NPFFR2-overexpressing (NPFFR2-OE) mice ($n = 6$). (**K**) Relative transcription of *Rnf128* in ATMs from vehicle-treated and NPFF-treated HFD-fed mice (treatment scheme as in Figure 2H). Data points represent pooled ATMs from 2 mice. The experiment was conducted 2 times. In **A** and **C**–**F**, the data points represent pooled ATMs from 3 mice. The experiment was conducted 3 times. (**L** and **M**) Arginase 1 expression (**L**) and transcriptional changes (**M**) in WT and *Npffr2*-KO ATMs evoked by 0.5 nM NPFF within 1 hour. ATMs harvested from 6 mice and treated in triplicate. The experiment was conducted 6 times. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by 1-way ANOVA with Dunnett's post-hoc test (**A** and **C**–**H**) and unpaired, 2-tailed Student's *t* test (**I**–**M**).

in nonobese volunteers (Figure 2C). These values fit well with the EC_{50} of NPFFR2 in ATMs. We found no correlation between age and sex and human plasma NPFF levels (Supplemental Figure 4E). By contrast, in HFD-fed mice and morbidly obese patients, the plasma concentrations of NPFF were below the EC_{50} for NPFFR2 (Figure 2C; see the Supplemental Information for a summary of the patients' metabolic parameters). This reduction in plasma NPFF levels was reversed by caloric restriction (CR) in both HFD-fed mice and morbidly obese patients (Figure 2C). We found that intermittent fasting also increased plasma NPFF levels in lean volunteers (Figure 2C). Indeed, plasma glucose levels negatively correlated with plasma NPFF levels in both mice and humans (Supplemental Figure 4F).

NPFF is released from the hypothalamus and from peripheral nerves within the adrenal gland (13). In addition, we identified the pancreas as a site of *Npff* expression (Supplemental Fig-

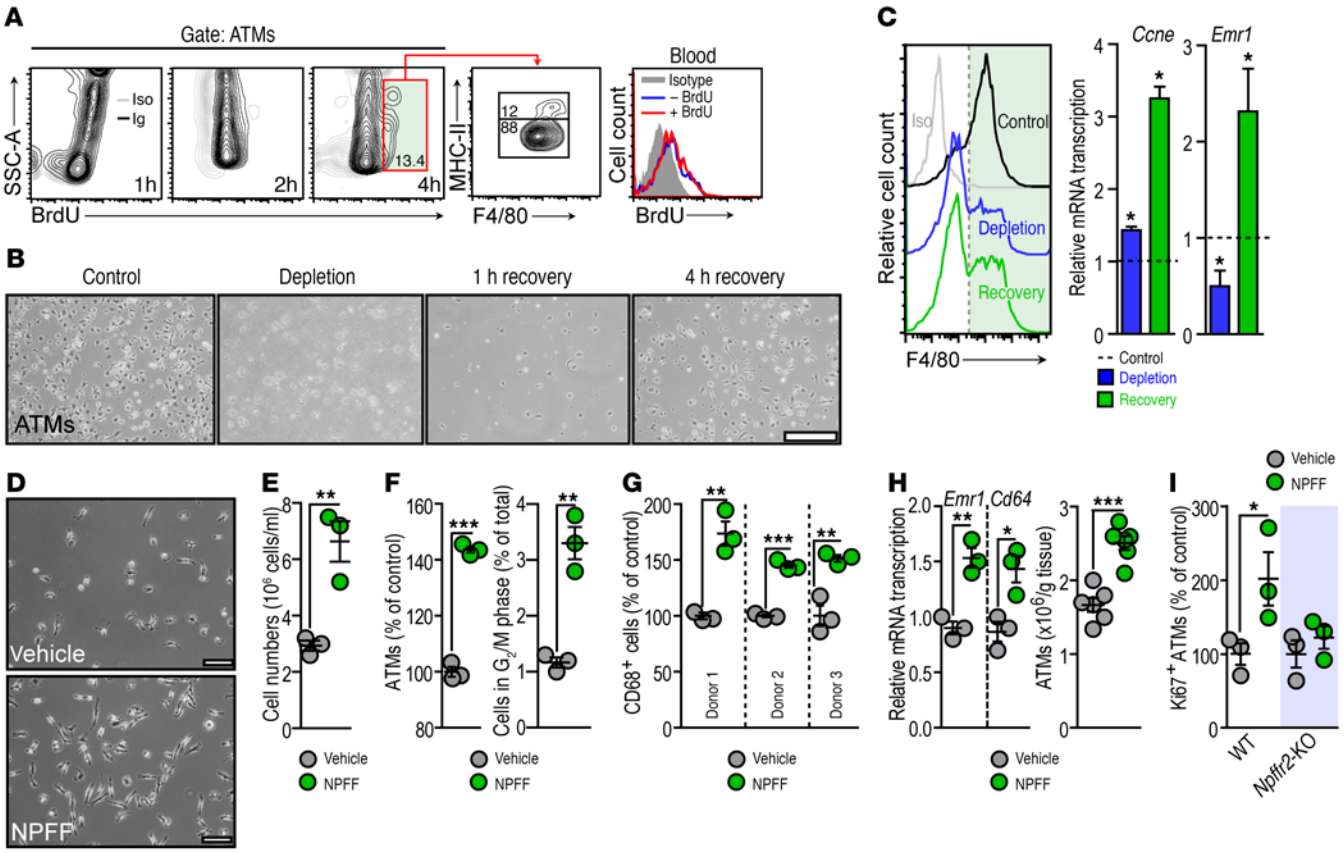

Figure 5. NPFF increases the proliferation of ATMs. (**A**) Mice were injected with BrdU, and ATMs were isolated after BrdU administration as shown. FACS analysis of BrdU$^+$ ATMs from eWAT. BrdU incorporation was also assessed in blood cells after 4 hours. SSC-A, side scatter area. (**B**) SVF cells pooled from eWAT from 6 lean mice were cultured in vitro (Control). SVF cells were incubated with clodronate liposomes for 1 hour (Depletion). Apoptotic cells were removed by washing, and surviving cells were allowed to recover in fresh medium for 1 hour and 4 hours. Scale bar: 100 μm. (**C**) FACS analysis of cells from the experiment shown in **B** (left). Relative transcription of *Ccne* and *Emr1* in SVF cells treated with clodronate liposomes (blue bars) and allowed to recover for 2 hours (green bars) (right). Dashed lines indicate gene transcription level in control cells. (**D**) Overview of ATM cultures treated with vehicle or 0.5 nM NPFF (18 hours). Scale bars: 50 μm. (**E**) Number of ATMs after treatment with vehicle or 0.5 nM NPFF for 18 hours. (**F**) Proliferation of mouse ATMs cultured in vitro and treated for 18 hours with 0.5 nM NPFF. ATMs were pooled from 3 to 5 mice and treated in triplicate in **D**–**F**. (**G**) Proliferation of human ATMs in response to treatment with 0.5 nM NPFF for 18 hours. ATMs were isolated and cultured in triplicate from each donor. (**H**) HFD-fed mice were treated with vehicle or NPFF as shown in Figure 2H. Relative transcription of the macrophage markers *Emr1* and *Cd64* (also known as *Fcgr1a*) was measured in SVF. Each data point represents pooled ATMs from eWAT of 2 mice. The experiment was conducted 2 times. Quantity of ATMs in eWAT after treatment with vehicle or NPFF (n = 6). The values for iWAT are shown in Supplemental Figure 16G. (**I**) Proliferation of in vitro cultured WT and *Npffr2*-KO ATMs treated with 0.5 nM NPFF for 4 hours. ATMs were pooled from 6 mice and treated in triplicate. The experiment was conducted 6 times. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, by unpaired, 2-tailed Student's t test.

ure 5). In both mice and humans, the majority of the pancreatic polypeptide- and somatostatin-containing endocrine cells of the islets contained NPFF-immunoreactive material (Supplemental Figure 6). Of note, pancreatic transcription of *Npff* was reduced in HFD-fed mice, whereas hypothalamic *Npff* levels remained unchanged (Supplemental Figure 6I).

We treated ATMs with 0.5 nM NPFF, a concentration that was within the plasma level range in lean mice and humans and an effective dose for reducing intracellular cAMP levels (Figure 2, A–C). This concentration of NPFF increased the transcription of *Npffr2* and *NPFFR2* in mouse and human ATMs, respectively (Figure 2D), and mimicked the effect of IL-4 (Figure 2, E–G, and Supplemental Figure 7A).

In mouse ATMs, both NPFF (0.5 nM) and IL-4 (10 ng/ml) increased the transcription of arginase 1 (*Arg1*), IL-10 (*Il10*), and IL-4 receptor α (*Il4ra*), all of which are effectors of M2 macrophages (Figure 2E). These transcriptional changes were concomitant with reduced transcription of the M1 markers IL-6 (*Il6*) and TNF-α (*Tnfa*) (Supplemental Figure 7A). IL-4 and NPFF also upregulated *Agmo* (Figure 2E), which encodes alkylglycerol monooxygenase (AGMO, EC 1.14.16.5). AGMO has antiinflammatory potential (15, 16), and *Agmo* transcription was reduced in response to LPS and IFN-γ (Supplemental Figure 7B). In human ATMs, NPFF treatment increased the expression of CD206 (also known as MRC-1) (Figure 2F), which is associated with M2-like, pro-resolving activation of human macrophages (17, 18) (Supplemental Figure 7C). Moreover, in human ATMs, NPFF increased the transcription of *IL4RA* and *AGMO*, the human homologs of *Il4ra* and *Agmo* (Supplemental Figure 7C). Prolonged exposure to NPFF (24 h) led to an increase in arginase 1 expression in mouse ATMs, which was more robust than that provoked by IL-4 (Figure 2G). Peritoneal macrophages and Kupffer cells, which have moderate or negligible NPFFR2 expression when

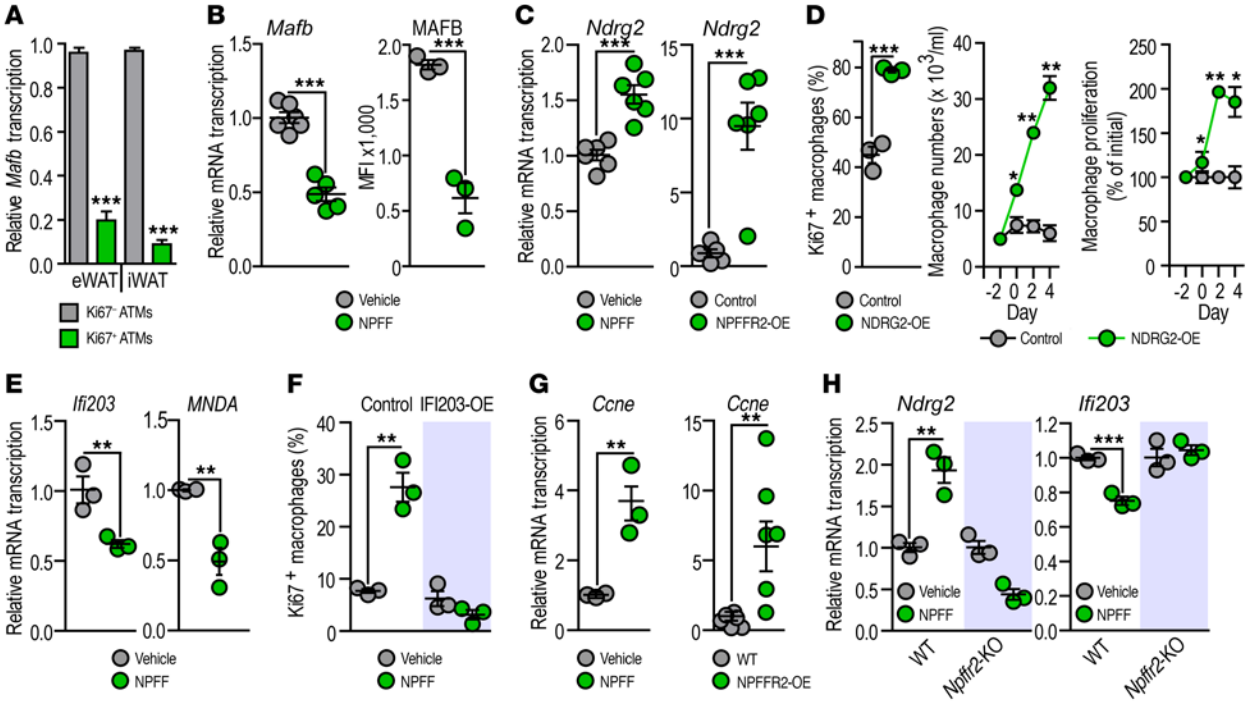

Figure 6. NPFF suppresses the cell cycle inhibiting gene transcription in ATMs. (**A**) Relative transcription of *Mafb* in Ki67$^-$ and Ki67$^+$ ATMs (n = 3). (**B**) Relative transcription of *Mafb* and MFI of MAFB protein in mouse ATMs treated with vehicle or 0.5 nM NPFF for 4 hours (n = 5 and 3, respectively). (**C**) Relative transcription of *Ndrg2* in ATMs treated with 0.5 nM NPFF for 0.5 hours (n = 5) and in freshly isolated ATMs from WT or NPFFR2-overexpressing (NPFFR2-OE) mice (n = 6). (**D**) Cell numbers and proliferation of control and NDRG2-overexpressing (NDGR2-OE) U937 macrophages (n = 3). Quantity of Ki67$^+$ cells in control and NDRG2-overexpressing U937 macrophage cultures after 4 days of culture (n = 3). (**E**) Relative transcription of *Ifi203* in mouse ATMs in response to treatment for 0.5 hours with 0.5 nM NPFF. Relative transcription of *MNDA* in human ATMs in response to a 4-hour treatment with 0.5 nM NPFF (n = 3). (**F**) Effect of a 4-hour treatment with 0.5 nM NPFF on the proliferation of control and IFI203-overexpressing (IFI203-OE) J774A.1 macrophages (n = 3). (**G**) Relative transcription of *Ccne* in ATMs treated with 0.5 nM NPFF for 4 hours and in WT and NPFFR2-overexpressing ATMs (n = 3 and 7, respectively). (**H**) Effect of 0.5 nM NPFF on *Ndrg2* and *Ifi203* transcription in WT and *Npffr2*-KO ATMs. ATMs were pooled from 3 mice and treated in triplicate. The experiment was conducted 3 times. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, by unpaired, 2-tailed Student's t test.

compared with ATMs (Figure 1L and Supplemental Figure 2C), failed to upregulate *Arg1*, *Il4ra*, or *Agmo* or reduce *Tnfa* following 0.5 nM NPFF treatment in vitro (Supplemental Figure 7D). To evaluate whether the plasma levels of NPFF found in obesity could effect changes in ATMs, we treated mouse ATMs with 0.2 nM NPFF; however, this concentration failed to induce M2 activation (Supplemental Figure 7E), corroborating the notion that plasma levels of NPFF in obesity are insufficient to affect ATM activation.

NPFF causes M2 activation of ATMs in vivo. Our finding that diet-induced obesity reduced NPFF plasma levels and diminished the prevalence of *Npffr2*-expressing ATMs suggested that the M2-activating effect of NPFF is lost in this setting. This prompted us to explore whether NPFF replacement could induce M2 activation and yield metabolic benefits in diet-induced obesity. Accordingly, HFD-fed mice were treated with 4 nmol/kg/day NPFF for 18 days (Figure 2H), which increased plasma levels of NPFF to 0.494 nM ± 0.074 nM versus 0.109 nM ± 0.019 nM in vehicle-treated mice at the end of the study (Supplemental Figure 8A) and was within the range observed in lean mice (Figure 1C). We analyzed ATMs in both eWAT and iWAT, since AT depots can have a specific impact on metabolism and some hormones or drugs can have adipose depot–specific effects (1, 2). The inflammatory state was comparable between the 2 adipose depots after HFD feeding (Supplemental Figure 7F and Supplemental Figure 8, B–D). The level of transcription of *Npffr2*, *Arg1*, *Il10*, *Il4ra*, *Agmo*, *Cd163*, and *Cd206* was higher in ATMs of both depots from NPFF-treated mice than in ATMs from vehicle-treated mice, with a concomitant reduction of *Il6*, *Tnfa*, and *Nos2* transcription (Figure 2, I–K, and Supplemental Figure 8, B–D). These transcriptional changes are characteristic of diminished AT inflammation and have been shown to be protective against the development of IR (19, 20). Obese AT accumulates CD11c$^+$ ATMs, which are associated with the dominance of M1-ATM activation (20). We found that NPFF treatment reduced *Cd11c* (also known as *Itgax*) transcription and the amount of CD11c$^+$ ATMs in eWAT and iWAT (Supplemental Figure 7F). Indeed, glucose tolerance as well as AT insulin sensitivity were increased after NPFF treatment (Figure 3, A and B), whereas plasma insulin, glucagon, and adiponectin levels (Figure 3C), as well as body weight, food intake, AT weight, and body temperature (Figure 3D) were unaffected by NPFF treatment. Moreover, gluconeogenesis measured by a pyruvate tolerance test (PTT) (Figure 3E), the degree of change in fatty liver (Figure 3F), and the plasma lipid profile (Figure 3G) were unaffected by NPFF. NPFF treatment reduced AT inflammation (Supplemental Figure 7G), however, liver and muscle inflammatory states were unchanged, and NPFF had negligible effects on the transcription of *Il6*, *Tnfa*, *Nos2*, and *Arg1* in Kupffer cells and skeletal muscle (Supplemental Figure 7H), which is in striking contrast to its effect on ATMs in vivo (Figure 2, I–K). These findings suggest that

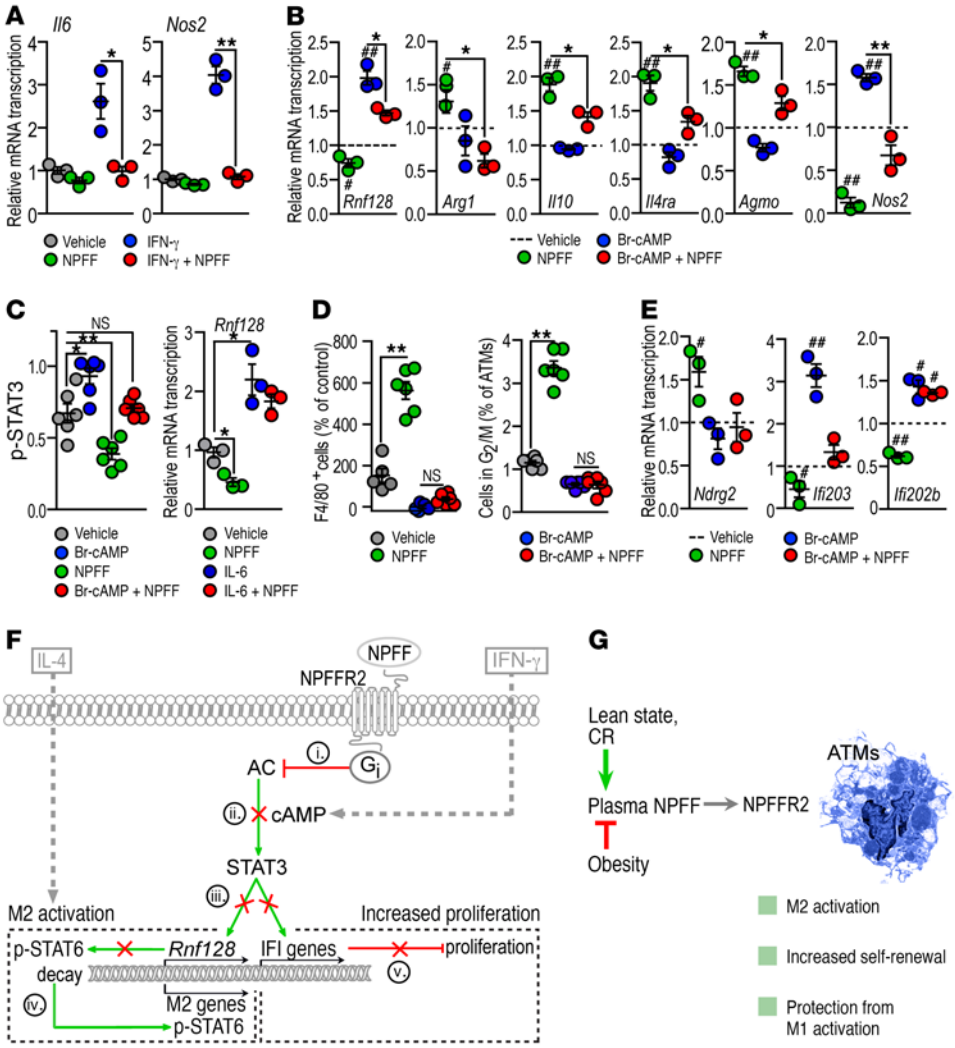

Figure 7. NPFF/NPFFR2 inhibits IFN signaling. (**A**) Effect of a 4-hour treatment with 0.5 nM NPFF, 2.5 ng/ml IFN-γ, or their combination on *Il6* and *Nos2* transcription in mouse ATMs. (**B**) Effect of 500 μM Br-cAMP on the transcriptional changes in mouse ATMs evoked within 0.5 hours by treatment with 0.5 nM NPFF. (**C**) STAT3 phosphorylation within 20 minutes in mouse ATMs following treatment with 0.5 nM NPFF and 500 μM Br-cAMP (left). Effect of a 1-hour treatment with 0.5 nM NPFF and 100 ng/ml IL-6 on *Rnf128* transcription in mouse ATMs. The effect of NPFF was also tested following a 20-minute preincubation with IL-6 (right). (**D**) Proliferation of ATMs treated for 18 hours with 0.5 nM NPFF, 500 μM Br-cAMP, or their combination. (**E**) Effect of 500 μM Br-cAMP and 0.5 nM NPFF on *Ndrg2* and *Ifi203* transcription in ATMs and *Ifi202b* transcription in J774A.1 macrophages. In **A**–**E**, each data point represents pooled ATMs from 3 to 5 mice. In **E**, triplicates of J774A.1 cultures were used. $^{*}P < 0.05$ and $^{**}P < 0.01$ for the indicated comparisons and $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus vehicle control; 1-way ANOVA with Dunnett's post-hoc test. (**F**) NPFF action in macrophages (simplified scheme): (i) NPFF/NPFFR2 blocks adenylate cyclase (AC); (ii) NPFF reduces cAMP levels; (iii) STAT3 signaling is inhibited; (iv) Reduced *Rnf128* transcription delays p-STAT6 decay; and (v) Inhibition of IFN-inducible (IFI) genes. (**G**) Role of NPFF/NPFFR2 signaling in ATMs. Morbid obesity reduces plasma NPFF to levels below the effective dose of NPFF in ATMs.

ATMs are the major targets of NPFF. To further confirm the direct antiinflammatory effect of NPFF on ATMs, we treated them in vitro with palmitic acid to mimic M1 activation caused by lipid overload. We observed that NPFF exposure increased *Il10* transcription and abolished *Il6* and *Tnfa* transcription in palmitic acid–treated ATMs (Figure 2L). NPFF treatment also inhibited nuclear translocation of NF-κB in an IL-10–dependent manner (Supplemental Figure 9).

NPFF inhibits phosphorylated STAT6 decay in ATMs. It is known that IL-4 exerts its effects on macrophage activation via STAT6 signaling (21). We found that the effect of NPFF on macrophage activation was also dependent on STAT6, as shown by the reduction of *Arg1* and *Il4ra* expression in NPFF-treated ATMs cotreated with the STAT6 inhibitor AS1517499 (Figure 4A; further details on the inhibitory effect of AS1517499 on STAT6 signaling are provided in Supplemental Figure 10A). Accordingly, ATMs cotreated with AS1517499 lost their capacity to phosphorylate STAT6 following NPFF treatment (Supplemental Figure 10B). The level of phosphorylated STAT6 (p-STAT6) was sustained by NPFF for a longer period than was observed with IL-4 (Figure 4, B and C), and, as a consequence, transcription of the STAT6 target *Il4ra* remained elevated for an extended period (Figure 4D). This result is consistent with the finding that arginase 1 expression was higher in NPFF-treated ATMs after 24 hours than in IL-4–treated ATMs (Figure 2G).

These findings led us to investigate whether NPFF inhibited the inactivation of STAT6 signaling. While little is known about the mechanisms that inactivate IL-4/STAT6 signaling in macrophages, proteasomal degradation of p-STAT6 is thought to be an important switch-off mechanism (22). It has been recently demonstrated that the E3 ubiquitin–protein ligase RNF128 (EC 6.3.2.), also known as GRAIL, is responsible for p-STAT6 ubiquitination and proteasomal degradation in Th2 cells (23, 24). The effect of NPFF on transcription of the STAT6 target *Il4ra* was similar to that of RNF128 deficiency in Th2 cells, which increases the transcription of *Il4ra* (24). This prompted us to postulate that the reduced level of RNF128 was responsible for the effect of NPFF in ATMs. Therefore, we measured *Rnf128* transcription after IL-4 and NPFF treatment in ATMs. IL-4 treatment reduced *Rnf128* transcription within 30 minutes, but this effect was lost at longer (1- or 4-h) treatment durations (Figure 4E). Protein expression of RNF128 was strongly decreased by IL-4 within 1 hour; however, the initial level of RNF128 was restored in ATMs treated for 10 hours with IL-4 (Figure 4F). By contrast, in NPFF-treated ATMs, restoration of *Rnf128* mRNA and RNF128 protein levels did not occur if the ATMs were treated with NPFF (Figure 4, E and F).

The above findings suggest that reduced RNF128 is responsible for the maintained STAT6 phosphorylation observed in NPFF-treated ATMs. Supporting this possibility, NPFF failed to maintain STAT6 phosphorylation in J774A.1 macrophages overexpressing RNF128 (Supplemental Figure 10C) and did not induce M2 activation (Figure 4, G and H). In turn, ATMs from RNF128-deficient mice showed increased p-STAT6 levels (Supplemental Figure 11A) and expression of arginase 1 and CD206, both of which are hallmarks of M2 activation (Figure 4I and Supplemental Figure 11B; details on the generation and phenotype of RNF128-deficient mice are provided in Supplemental Figure 11, C–E). Similar to RNF128 deficiency, ATMs overexpressing NPFFR2 had increased expression of arginase 1 and CD206 and transcription of *Il10* (Figure 4J, and Supplemental Figure 12A; details on the generation and phenotype of NFPFFR2-overexpressing mice are provided in Supplemental Figure 12, B–E). When HFD-fed mice were treated with NPFF (Figure 2H), the transcription of *Rnf128* was reduced in ATMs (Figure 4K). Human ATMs also had reduced *RNF128* transcription following treatment with 0.5 nM NPFF in vitro (Supplemental Figure 7C).

The M2-activating effects of NPFF were mediated by NPFFR2, since ablation of NPFFR2 abolished NPFF-induced p-STAT6 (Supplemental Figure 10D) and NPFF-induced transcriptional changes (Figure 4, L and M, and Supplemental Figure 8E; details on the generation and phenotype of NPFFR2-deficient mice are provided in Supplemental Figure 13, A–F). Similarly, pharmacological inhibition of NPFFR2 abolished NPFF-induced transcriptional changes (Supplemental Figure 14, A and B).

NPFF increases ATM self-renewal. It has been reported that ATMs can proliferate (4), and recent studies suggest that IL-4 promotes tissue-resident macrophage proliferation (25, 26). Nevertheless, increased ATM proliferation contributes to the accumulation of M1-ATMs in obese AT (1, 4); however, signals controlling self-renewal of ATMs are poorly understood (1). Whether local proliferation is relevant for ATM replenishment in lean AT is also unknown. Nonetheless, this possibility was supported by our findings that $Ki67^+$ ATMs were present in both eWAT and iWAT (Supplemental Figure 15), and BrdU was incorporated into ATMs (Figure 5A). The time window for BrdU incorporation into ATMs matched the ATM proliferation rate reported for obese AT in mice (27). BrdU was not incorporated into blood monocytes (Figure 5A). Altogether, these findings suggest that a proliferating ATM population exists in lean AT, which replenishes ATMs. To study this possibility in more detail, isolated ATMs were maintained in vitro and challenged with clodronate liposomes. Under this condition, blood monocytes cannot be recruited, and new ATMs should be generated from a bone marrow–independent stem cell pool. Shortly after removal of the clodronate liposomes, we found that newly formed ATMs were present in the culture (Figure 5B), as demonstrated by the increased number of $F4/80^+$ cells and increased *Emr1* (encoding F4/80 antigen) transcription (Figure 5C). ATM recovery was associated with increased cyclin E (*Ccne*) transcription (Figure 5C). We observed that replenishment of ATMs was also increased in vitro by NPFF treatment (Figure 5, D and E).

To corroborate the finding that NPFF increases ATM self-renewal, we treated mouse and human ATMs in vitro with 0.5 nM NPFF. The number and proliferation of both mouse and human ATMs were increased by NPFF treatment (Figure 5, F and G). Similarly, the number of ATMs was increased following NPFF treatment of mice (Figure 5H, Supplemental Figure 14C, and Supplemental Figure 16G). ATMs lacking NPFFR2 expression failed to upregulate proliferation in response to NPFF (Figure 5I), and pharmacological inhibition of NPFFR2 abolished the effect of NPFF on macrophage proliferation (Supplemental Figure 14C and Supplemental Figure 16, A–F). Accordingly, NPFFR2-overexpressing mice had increased ATM numbers and proliferation (Supplemental Figure 12F), while NPFFR2-deficient mice had reduced ATM numbers and proliferation (Supplemental Figure 13, G and H), without an effect on peripheral blood hematology (Supplemental Figure 12G and Supplemental Figure 13I).

Proliferating ATMs in both eWAT and iWAT had lower expression levels of the transcription factor MAF bZIP transcription factor B (*Mafb*) than did quiescent ATMs (Figure 6A). Reduced *Mafb* transcription allows macrophage proliferation (25, 28, 29), and we have previously demonstrated the impact of low *Mafb* transcription levels on the proliferation of macrophage precursors (28). When mouse ATMs were treated with NPFF, mRNA and protein expression levels of *Mafb* were decreased (Figure 6B).

NPFF treatment or NPFFR2 overexpression increased the transcription of N-Myc downstream-regulated gene 2 (*Ndrg2*) in ATMs (Figure 6C). NDRG2 can increase cell proliferation in specific cell types (30); however, its impact on macrophage proliferation is not known. Overexpression of NDRG2 in U937 macrophages (Supplemental Figure 13J) led to increased proliferation, mimicking the effect of NPFF (Figure 6D). Furthermore, NPFF treatment decreased the transcription of IFN-inducible 200 (IFI200) family members, which inhibit proliferation and suppress growth in various cell types including macrophage progenitors (31). *Ifi203* was the relevant IFI200 family member expressed in C57BL/6-derived ATMs, whereas *Ifi202b*, *Ifi204*, and *Ifi205* were expressed in BALB/c-derived J774A.1 macrophages (Figure 6E and Supplemental Figure 17A), in agreement with a previous study on strain-specific expression of *Ifi200* genes (32). We found that overexpression of IFI203 (Supplemental Figure 13K) abrogated the effect of NPFF on macrophage proliferation (Figure 6F). Moreover, in human ATMs, myeloid cell nuclear differentiation antigen (*MNDA*), a homolog of the murine IFI200 family, was suppressed by NPFF (Figure 6E).

NPFF-treated ATMs and ATMs overexpressing NPFFR2 had increased *Ccne* transcription, further confirming the notion that NPFF/NPFFR2 signaling increases ATM proliferation (Figure 6G). The absence of NPFFR2 abolished the effect of NPFF on *Ndrg2* and *Ifi203* transcription in ATMs (Figure 6H). Pharmacological inhibition of NPFFR2 had a similar effect (Supplemental Figure 16F).

NPFF inhibits IFN-γ signaling, which is key for ATM activation and proliferation. IFN-γ treatment reduced *Npffr2* transcription in ATMs (Figure 1M), and, in turn, NPFF treatment abolished the effect of IFN-γ on the transcription of *Il6* and *Nos2* (Figure 7A). We found that NPFF also inhibited IFN-inducible gene transcription in J774A.1 macrophages (Supplemental Figure 17, B and C). The NPFF-regulated genes ring finger protein 128 (*Rnf128*), *Ndrg2*, and *Ifi200* family members are IFN-responsive genes, and IFN-γ had an effect opposite that of NPFF on their transcription (Supplemental Figure 17C).

NPFF triggers a strong reduction of intracellular cAMP, contrasting with the effect of IFN-γ, which increases cAMP levels (33–35). Thus, we hypothesized that the antagonistic effects of IFN-γ and NPFF on cAMP synthesis were key for NPFF-induced M2 activation and proliferation of ATMs. As with IFN-γ, the cAMP analog Br-cAMP provoked a pronounced increase in *Rnf128* transcription and thus had an effect opposite that of NPFF (Figure 7B). Br-cAMP abolished the inhibitory effect of NPFF on the transcription of *Rnf128* and impeded M2 activation provoked by NPFF (Figure 7B). A recent study has shown that cAMP is involved in STAT3 signaling (35), and a STAT3 response element is present in the *Rnf128* promoter (Supplemental Figure 17D). We found that Br-cAMP increased STAT3 phosphorylation in ATMs, and this effect was antagonized by NPFF (Figure 7C). When STAT3 was phosphorylated by IL-6 before treatment, NPFF failed to reduce *Rnf128* transcription (Figure 7C).

Like M2-activation, increased ATM proliferation was dependent on the reduction of intracellular cAMP levels (Figure 7D). Br-cAMP abolished the effect of NPFF on the proliferation of ATMs (Figure 7D). Further, Br-cAMP abolished the inhibitory effect of NPFF on the transcription of IFI200 family members and abrogated the NPFF-induced upregulation of *Ndrg2* transcription (Figure 7E).

Discussion

This study shows that NPFF is a potent regulator of ATM quantity and quality. We demonstrate here that NPFF/NPFFR2 signaling in macrophages augments STAT6 signaling, which mimics M2 activation and enhances macrophage self-renewal. NPFF/NPFFR2 signaling is thus a common hub for the control of macrophage activation and proliferation.

It has been known for more than 20 years that human plasma contains levels of NPFF one order of magnitude higher than the levels found in cerebrospinal fluid, and, consequently, it has been hypothesized that NPFF is distributed in the bloodstream and acts as a hormone (13, 36). This possibility, however, has not been systematically investigated. Moreover, the known effects of NPFF on metabolism, such as appetite control and thermogenesis, were traditionally associated with its neurotransmitter function in the nervous system (7). We show for the first time to our knowledge that the peripheral effects of NPFF are relevant.

Discrepancies exist between the reported plasma and cerebrospinal fluid levels of NPFF in humans and experimentally used concentrations of NPFF (8, 9, 13, 36–38), making it challenging to distinguish physiological and pharmacological effects of NPFF and to define signaling pathways activated by this neuropeptide. For instance, the inhibitory effect of micromolar amounts of NPFF on NO synthesis in macrophages has been interpreted as an antiinflammatory potential of NPFF (38). However, the NPFF concentration used was orders of magnitude higher than its physiological level, making it conceivable that toxic or nonspecific effects of high-dose NPFF occurred. Our findings show that mouse and human plasma NPFF levels match the EC_{50} for NPFFR2 determined in our study and by others (39) and that this concentration is effective for inducing ATM proliferation and M2 activation, with concomitant protection from M1 activation. Thus, it is conceivable that plasma NPFF has the potential to control the content and activation state of ATMs.

We show that plasma NPFF levels are reduced in human and murine obesity, together with decreased numbers of *Npffr2*-expressing ATMs in obese AT in mice. Thus, obesity can lead to the loss of the effect of NPFF on ATMs, which is consistent with the current paradigm for the relationship between obesity and dysregulated ATM proliferation and activation (1). NPFF is known as an anorexigenic factor in rodents that acts at hypothalamic nuclei (6, 7). The balance between anorexigenic and orexigenic signals in the hypothalamic center of appetite control is key in obesity development (40). A reduction in plasma NPFF levels may reflect impaired NPFF release from the hypothalamic nuclei in obesity. In addition, diminished *Npff* transcription in the endocrine pancreas can also account for the reduced plasma NPFF pool in obesity.

As shown here, NPFF shifts macrophages to an M2 activation state. IL-4 increases NPFFR2 expression, clearly demonstrating the role of NPFF signaling in M2 macrophages. In HFD-fed mice, NPFF treatment increased *Npffr2* transcription in ATMs and triggered M2 activation of ATMs, and this effect was associated with improved glucose tolerance. Since ATMs are prominent NPFFR2-expressing macrophages, there is the potential for NPFF to reduce AT inflammation. NPFFR2 expression was also detectable at lower levels in other macrophage types, such as Kupffer cells, and NPFF exerted a mild antiinflammatory effect on Kupffer cells. This, however, would augment the beneficial effect of NPFF and not challenge the role of NPFF in M2 activation of ATMs.

The mechanistic basis of NPFF action in macrophages involves diminished cAMP synthesis, which suppresses the transcription of genes involved in the IFN response (Figure 7F). NPFF suppresses the expression of the IFN-responsive gene *Rnf128*. Reduced *Rnf128* transcription delays p-STAT6 decay in NPFF-treated macrophages, leading to the transcription of M2 activation genes such as *Arg1, Il10*, *Il4ra*, and *Agmo*. Furthermore, NPFF increases the transcription of *Ndrg2*, a known inhibitor of STAT3 signaling (41). In addition to the augmented IL-4 signaling, NPFF also increases *Il10* transcription. It is well established that IL-10 suppresses the expression of proinflammatory cytokines as well as antigen-presenting and costimulatory molecules in macrophages (42).

NPFF also suppresses the transcription of members of the IFI200 family. The IFI200 family includes proteins that accumulate in the nucleus during the IFN response and interact with various transcription factors (32, 43). They control the cell cycle in a wide range of cell types including macrophage progenitors (44, 45). IFNs cause growth arrest, and IFI200 family members are involved in this effect (32, 44). While it is known that the *Ifi200* gene family inhibits immune cell proliferation and affects inflammation (32, 44), little is known about how IFI200 expression is controlled. Our study raises the possibility that NPFF exerts hormonal control of IFI200 transcription. This has relevance in various disease conditions such as autoimmunity, in which macrophages, IFN response, and IFI200 family members play key roles (32).

NPFF exerts its effects through NPFFR2 both in vitro and in vivo. We show that NPFF/NPFFR2 signaling is a common hub for M2 activation and increased macrophage proliferation (Figure 7F), thus enriching AT with M2-activated ATMs and impeding AT inflammation (Figure 7G). The impact of macrophage self-renewal has been recently recognized (4, 25, 27); however, the underlying mechanisms that are decisive for ATM proliferation

remain enigmatic. Our findings identify an endogenous hormonal signal mechanism that impacts on ATM self-renewal.

In conclusion, this study shows that NPFF controls ATM self-renewal and M2 activation. NPFF has the unique ability to increase ATM numbers and maintain the newly formed ATMs in an M2-activated state. NPFF can also protect against AT inflammation, the main predisposing factor for IR. Thus, NPFF may have utility as a tool in the future treatment of obesity-associated dysregulation of ATM activation and proliferation.

Methods

Animals. We used C57BL/6 male mice (Charles River Laboratories), NPFFR2-deficient mice, NPFFR2-overexpressing mice, and RNF128-deficient mice on a C57BL/6 background. All mice were 8 weeks of age at the beginning of the experiments. The generation of transgenic mouse lines is described in the Supplemental Methods. Male mice were rendered obese on a 12-week-long HFD consisting of 45% of calories from fat (RD formula; SSNIFF). The control group was fed a normal chow diet (NCD) for the same period. For CR experiments, the test group received a low-calorie diet (SSNIFF) for 2 weeks. For NPFF replacement, HFD-fed mice were injected daily with 4 nmol/kg NPFF (Phoenix Pharmaceuticals Inc.) for 18 days. Each experimental group was established from 6 control and 6 treated mice.

Human samples. Plasma and fat samples were obtained between June 2015 and January 2016 at the Hospital Clínico Universitario de València. The metabolic parameters for patients and volunteers are summarized in Supplemental Table 3. Plasma samples were obtained from healthy volunteers ($n = 32$), morbidly obese patients ($n = 18$), and patients with type 1 diabetes mellitus ($n = 6$). Patient selection was based on the NIH's recommendation of a BMI above 40 kg/m^2. The nonobese volunteers had a BMI of 20 to 25 kg/m^2. To test the effect of intermittent fasting, we recruited 10 nonobese volunteers, 5 of whom followed a regime of daily 16-hour fasting for 28 days, while the other 5 volunteers did not fast. Blood samples were collected in EDTA-coated hemotubes and centrifuged, and the plasma samples were stored at –80°C until further analysis. Blood glucose, insulin, and NPFF levels were assayed using commercial ELISA kits from Sigma-Aldrich and Cloud-Clone Corporation. AT samples were collected from the omental fat depot of morbidly obese patients ($n = 16$) during routine bariatric surgical intervention at the Hospital Clínico Universitario de València between June 2015 and January 2016. Eight patients followed a CR diet for 1 month before blood sampling and surgery. These patients were allowed to drink a specially formulated balanced energy drink (Vegenat SA) and other calorie-free drinks such as water, tea, coffee, and nonfat broth. Omental fat from 3 nondiabetic obese donors without CR was used for isolation and in vitro culturing of ATMs. Pancreas samples were collected from deceased organ donors (Network of Pancreatic Organ Donors, University of Florida, Gainesville, Florida, USA), fixed in paraformaldehyde (PFA), and processed for histological and gene expression analysis. Samples from 2 nondiabetic donors were used for histology. Gene expression analysis was performed for 18 control donors with no autoantibodies and no diabetes and 20 donors with type 1 diabetes mellitus (for details, see the Supplemental Information). One patient underwent radical pancreas resection, and plasma samples were collected prior to and after surgery.

Assessment of plasma NPPF levels. Plasma NPFF levels in human and murine plasma were assayed using commercial ELISA kits (Cloud-Clone Corp.). Details of the mass spectrometric data on plasma NPFF levels and quality control for the ELISA are provided in Supplemental Figure 3.

Isolation and in vitro culture of ATMs. ATMs were isolated from eWAT and iWAT depots from mice and omental fat from human donors using collagenase digestion as described previously (3). ATMs were suspended in selection medium consisting of 2 mM EDTA and 0.5% BSA in PBS, pH 7.4, and used for flow cytometry, transmission electron microscopy (TEM), preparative cell sorting, and RNA extraction, or were cultured in vitro (Supplemental Figure 1). For in vitro culturing, CD11b$^+$F4/80$^+$ mouse ATMs or CD14$^+$CD68$^+$ human ATMs were retrieved with preparative cell sorting as described below. ATMs were allowed to adhere to 6-well plates in RPMI 1640 medium with 10% FCS and were treated with NPFF (Tocris Bioscience; Phoenix Pharmaceuticals Inc.), RF9 (14), 100 ng/ml LPS from *E. coli*, 10 ng/ml mouse or human IL-4, 20 ng/ml human IL-13, 5 μg/ml pI:pC, 2.5 ng/ml IFN-γ, 500 μM Br-cAMP (all from Sigma-Aldrich), and 100 ng/ml IL-6 (ImmunoTools) or 200 nM AS 1517499 (Axon Chemicals). NPFF purity was controlled by HPLC.

FACS analysis and preparative cell sorting. Cell density was adjusted to 10^6 cells/ml, and analysis was performed on a BD LSR II Cell Cytometer (BD Bioscience). The antibodies used are listed in Supplemental Table 1. After a 2-hour incubation, the cells were washed in selection buffer and used for analysis. For preparative cell sorting of CD11b$^+$, F4/80$^+$; CD14$^+$, CD68$^+$; NPFFR2$^+$; and Ki67$^+$ or Ki67$^-$ ATMs, we used the Aria Cell Sorter. CD11b, F4/80, CD14, CD68, and NPFFR2 staining was performed on live cells. The sorted cells were collected for Western blotting, RNA extraction, or for further TEM analysis. Ki67 staining was carried out on cells fixed with 2% PFA and permeabilized with 0.05% Triton X-100 to ensure recognition of the nuclear antigen Ki67 by the antibody. In this case, CD11b and F4/80 staining was performed on fixed cells. Sorted Ki67$^-$ and Ki67$^+$ ATMs were used for RNA extraction. For cell-cycle analysis, the in vitro cultured ATMs were fixed with 2% PFA. Cells were washed with selection buffer and treated with RNAase, and the DNA was stained with propidium iodide (Sigma-Aldrich).

RNA extraction and quantitative PCR. Total RNA was isolated from tissues, adipocytes, and macrophages with TRI Reagent (Sigma-Aldrich). RNA integrity was controlled by denaturing agarose gel electrophoresis. Only nondegraded RNA samples were used for cDNA synthesis, as described previously (46). Sorting of ATMs for Ki67 antigen required prior fixation with PFA, therefore, the RNeasy FFPE Kit (QIAGEN) was used to extract RNA from Ki67$^-$ and Ki67$^+$ ATMs. The ViiA 7 (Thermo Fisher Scientific) quantitative PCR platform was used for analysis, and gene expression values were expressed as relative mRNA levels according to dCt and ddCt methods, using *Actb* and *Ppia* (also known as *Cypa*) as reference genes for murine samples and *ACTB* and *PPIA* as reference genes for human samples. The primer sequences are provided in Supplemental Table 2.

Supplemental methods. The assessment of glucose homeostasis in mice, TEM of ATMs, analysis of NPFF distribution in pancreas, isolation of resident macrophages, cell culturing of J774A.1 macrophages, Western blot analysis, cell-based ELISA, generation of transgenic mice and macrophages, NPFFR2-binding assay, intracellular cAMP measurement, and Affymetrix gene expression analysis are described in the Supplemental Methods. See complete unedited blots in the supplemental material.

Statistics. All data represent the mean ± SEM. For statistical analysis, an unpaired, 2-tailed Student's *t* test, 1-way ANOVA with Dunnett's post-hoc test for multiple comparisons, or 2-way ANOVA was applied as appropriate. A *P* value of less than 0.05 was considered significant. GraphPad Prism 5 (GraphPad Software) and MATLAB (MathWorks) statistical software programs were used.

Study approval. All animal experiments were carried out in accordance with local laws and were approved by local authorities (State of Baden Württemberg, 1206; Chang Gung University, CGU13-014; the IACUC of the University of Wisconsin; National Research Institute for Radiobiology and Radiohygiene Budapest; the University of Strasbourg, PEI/001/2073-6/2014; and the IACUC of the William S. Middleton Memorial Veterans Hospital, 02963.02). Human studies were approved by the local ethics commissions (University of Ulm, 121/16 22050 and 112/2003; University of Tennessee Health Science Center Institutional Review Board, 10-00848-XM; and the University of Valencia, PI 12/01978). None of the patients had clinical symptoms of systemic inflammation, and all provided written informed consent prior to their participation in the study. The patients were identified by numbers.

Author contributions

JCC, JSL, LB, JT, FS, JTR, EMS, DWL, and CMS provided methodology and designed experiments. SFHW, ACH, YTL, GA, HA, LB, JT, ICG, SN, JMI, SP, RQ, SA, and JAB performed experiments and analyzed data. TR conceived the project, designed experiments, and wrote the manuscript.

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ANNEXE IV

Le neuropeptide FF, aiguilleur des macrophages du tissue adipeux.

Quillet R, Simonin F.

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Le neuropeptide FF, aiguilleur des macrophages du tissu adipeux

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> Les chiffres de l'Organisation mondiale de la santé concernant l'obésité sont alarmants. Depuis plus de 30 ans, cette pathologie est apparue progressivement au sein de notre société, favorisée par des régimes caloriques et composés de lipides en excès. En 2014, 13 % de la population mondiale étaient considérés comme obèses, et le développement des maladies métaboliques et cardiovasculaires associées n'a pas cessé de croître.

L'obésité est une maladie du tissu adipeux. Ce tissu est constitué d'adipocytes, dont le rôle est de stocker des réserves énergétiques, mais également de cellules du système immunitaire, appelées macrophages du tissus adipeux (ATM pour *adipose tissue macrophage*) [1] (➜).

(➜) Voir la Synthèse de E. Delmas *et al.*, *m/s* n° 11, novembre 211, page 993

Les cellules immunitaires du tissu adipeux jouent un rôle majeur dans le développement et le métabolisme de ce tissu [2]. En cas d'obésité, les macrophages s'y accumulent et se différencient en un type spécifique, appelé M1 (pour macrophage de type 1, en référence aux lymphocytes Th[*helper*] 1), caractérisé par un fort potentiel pro-inflammatoire [3]. L'inflammation, qui est liée à l'infiltration du tissu adipeux par ces macrophages, est l'une des altérations majeures que l'on observe chez les patients souffrant d'obésité. Elle est responsable des maladies métaboliques concomitantes à l'évolution de la pathologie, comme le diabète de type 2 et les dyslipidémies. Les macrophages de type 1 produisent des cytokines inflammatoires, de l'oxyde d'azote (NO) et des espèces réactives de l'oxygène (ROS) qui sont à l'origine de l'inflammation du tissu et conduisent à une résistance à l'insuline [4, 5]. *In vitro*, les macrophages M1 sont activés par différents traitements comme le LPS (lipopolysaccharide) bactérien, l'interféron-γ (IFN-γ), ou l'acide palmitique.

Les macrophages du tissu adipeux, ATM, peuvent également adopter un état d'activation de type 2, ou M2. Par leur action anti-inflammatoire, ces macrophages M2 sont capables de limiter l'inflammation et la résistance à l'insuline. Cet état, qui est bénéfique d'un point de vue métabolique, est donc favorable. Il peut être induit par différentes molécules, parmi lesquelles les cytokines de type Th2 (*T helper 2*), les métabolites lipidiques, les neurotransmetteurs ou encore les hormones [4]. Les mécanismes impliqués dans cette transition d'état d'activation des macrophages restent cependant encore inconnus.

Le neuropeptide FF (NPFF) est un membre de la famille des peptides RF-amides[1]. Il a été principalement étudié chez les mammifères pour son rôle modulateur de la douleur dans les réponses adaptatives aux traitements par les opiacés. Certains éléments indiquent qu'il pourrait également être impliqué dans la réduction de la prise alimentaire chez les rongeurs, *via* une action hypothalamique [6]. Des études menées *in vitro* ont montré qu'il inhibait le développement des adipocytes chez la souris et l'Homme [7]. Le récepteur du NPFF, NPFFR2 (*NPFF receptor 2*), est très faiblement exprimé dans les tissus périphériques, à l'exception du tissu adipeux [8, 9]. Dans ce tissu, NPFF serait libéré à partir des terminaisons nerveuses [9], mais son action sur les adipocytes reste inconnue. Des variants génétiques du récepteur NPFFR2 ont cependant été associés à l'obésité, ce qui renforce l'hypothèse de l'existence d'un lien entre NPFF et cette maladie [10].

Le NPFF est présent dans la circulation sanguine, en concentration suffisante pour activer les ATM en macrophages M2

L'étude que nous avons menée au sein du consortium international coordonné par le Dr Tamas Röszer a été publiée récemment dans le *Journal of Clinical Investigation* [11]. Dans cet article, nous avons montré que le neuropeptide FF (NPFF) avait un effet bénéfique sur le métabolisme : il amplifie en effet, chez l'Homme et la souris, l'activation des macrophages de type 2 (M2) et leur prolifération, ce qui permet le maintien d'un tissu adipeux sain *(Figure 1)*. Chez la souris, nous avons montré que le NPFF se liait à ses récepteurs présents à la surface des macrophages du tissu adipeux (ATM) avec une constante de dissociation (K_D) de 0,37 nM, et qu'il induisait une diminution de l'accumulation d'AMP cyclique en activant la sous-unité inhibitrice de l'adénylate cyclase G_i. Nous avons aussi montré que sa concentration, dans les plasmas murin et humain, était compatible avec son action sur son récepteur. Elle est diminuée en cas d'obésité, mais une restriction calorique permet d'inverser cette situation.

[1] La famille des peptides RF-amides regroupe des neuropeptides qui partagent la même séquence N-terminale à savoir Arg(R)-Phe(F)-NH_2.

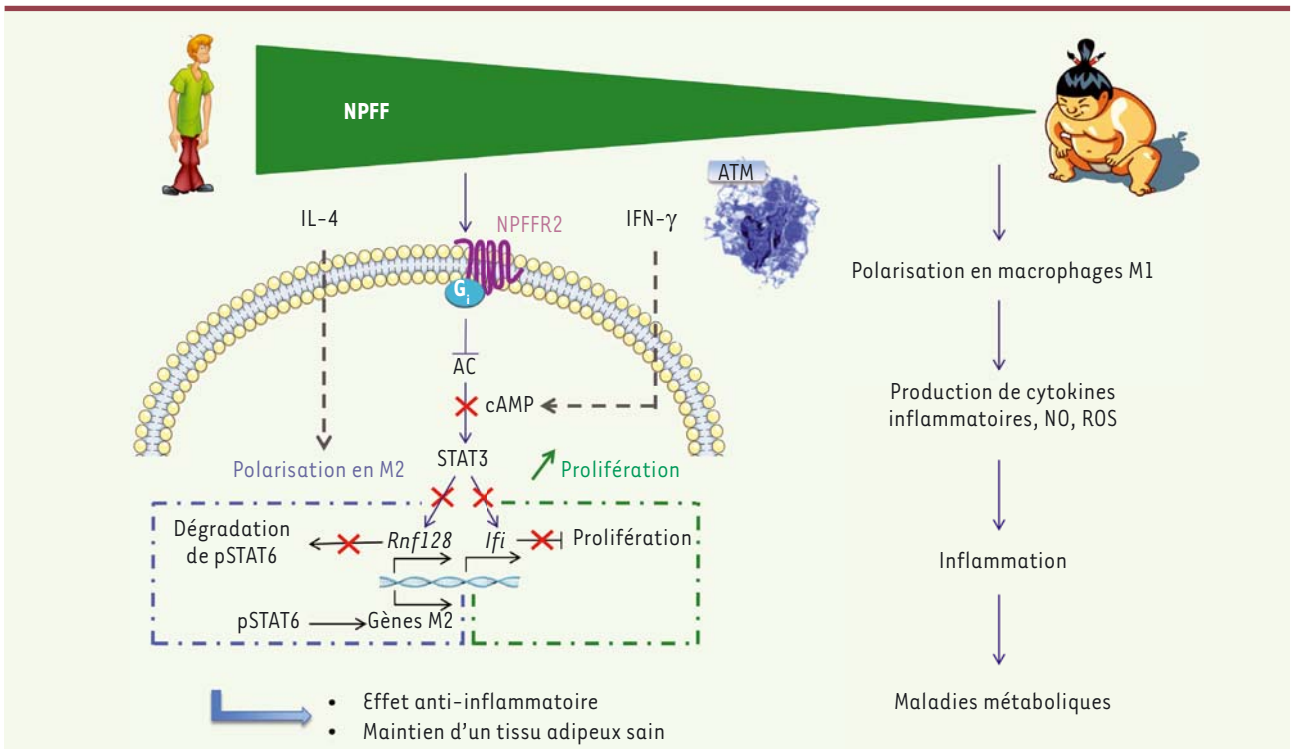

Figure 1. ***Le système NPFF/NPFFR2 inhibe la signalisation IFN-γ et empêche l'inflammation du tissu adipeux.*** La liaison du NPFF au récepteur NPFFR2 bloque l'adénylate cyclase (AC) et, ainsi, la production d'adénosine monophosphate cyclique (AMPc), ce qui inhibe la voie dépendante de STAT3 (*signal transducer and activator of transcription 3*). Une diminution de la transcription de *Rnf128* codant une E3-ubiquitine ligase retarde la dégradation de phospho-STAT6 et permet la transcription des gènes induisant un phénotype M2. L'inhibition des gènes inductibles par l'IFN-γ (interféron-gamma) favorise la prolifération des macrophages. Cette cascade conduit à l'action anti-inflammatoire du NPFF et au maintien d'un tissu adipeux sain. En revanche, le faible taux de NPFF retrouvé chez les patients obèses entraîne la polarisation des macrophages en macrophages de type M1, qui produisent des cytokines inflammatoires, de l'oxyde nitrique (NO) et des espèces réactives de l'oxygène (ROS). L'inflammation des tissus qui en résulte peut conduire à l'apparition de maladies métaboliques et contribuer aux comorbidités associées à l'obésité.

Le NPFF stimule, chez la souris, la transcription de gènes qui codent des molécules impliquées dans les fonctions des macrophages M2, comme AGMO (alkylglycérol monooxygénase, connue pour son potentiel anti-inflammatoire), le récepteur α de l'IL-4 (*interleukin-4 receptor,* IL4Ra), l'Arg1 (arginase-1) et l'IL-10. Chez l'Homme, il induit également la transcription de l'IL4Ra et de AGMO mais aussi du *macrophage mannose receptor* (MR ou CD206). Ces activations transcriptionnelles, spécifiques des macrophages M2, coïncident avec la diminution de l'expression des marqueurs caractéristiques des macrophages M1 : l'IL-6 et le TNF-α (*tumor necrosis factor-alpha*). Le traitement des ATM murins et humains par le NPFF mime ainsi l'effet de l'IL-4 en favorisant l'orientation M2. Cependant, le traitement des ATM murins, avec une dose de NPFF correspondant à la concentration plasmatique trouvée chez des patients obèses, ne permet pas d'observer d'activation des macrophages en macrophages de type M2. Les taux plasmatiques de NPFF qui sont détectés chez ces patients sont donc insuffisants pour activer les macrophages. À noter que les ATM de souris soumises à un régime riche en graisses durant 12 semaines (correspondant à un contexte d'obésité) ont des taux de récepteurs NPFFR2 qui sont diminués par rapport à ceux des souris nourries sans excès. Ceci suggère que le défaut d'orientation en macrophages M2 aurait pour origine un déficit du système NPFF.

Le NPFF inhibe la dégradation de phospho-STAT6 dans les ATM

De même que l'IL-4 active les macrophages par l'intermédiaire de STAT6 (*signal transducer and activator of transcription-6)* [12], la transition d'état induite par le NPFF dépend de la voie de signalisation impliquant STAT6. L'expression des gènes activés par l'IL-4 et qui sont impliqués dans l'activation des macrophages anti-inflammatoires (de type M2) est également observée après stimulation par le NPFF *(Figure 1)*. En particulier, la transcription de *RNF128*, qui code une E3 ubiquitine-ligase responsable de la dégradation de la forme phosphorylée de STAT6 (phospho-STAT6), est diminuée par le NPFF comme par l'IL-4. La réduction de RNF128, dans les ATM traités avec le NPFF, apparaît donc responsable du maintien

de la phosphorylation de STAT6. Dans des cellules macrophagiques de souris J774A.1 surexprimant RNF128, le NPFF est incapable d'induire l'orientation des cellules en macrophages M2. En revanche, des ATM de souris déficientes en RFN128 présentent des taux élevés de phospho-STAT6 et expriment fortement les marqueurs d'activation de cellules M2 (comme Arg-1 et CD206), à l'instar de cellules surexprimant le récepteur NPFFR2. Le taux de RNF128 dans les ATM humains, et chez les souris nourries avec un régime riche en graisse, diminue après traitement avec le NPFF. Ces effets ne sont pas retrouvés chez des souris génétiquement modifiées déficientes en NPFFR2, ce qui confirme son rôle dans la polarisation des macrophages et la spécificité de la cible d'action du NPFF.

Le NPFF favorise l'auto-renouvellement des ATM

Nos études démontrent que le NPFF induit la prolifération des macrophages du tissu adipeux chez la souris et l'Homme. Cet effet n'est pas observé pour les ATM provenant de souris déficientes en NPFFR2 ou dans des expériences de blocage pharmacologique du récepteur. Le NPFF réduit en effet les niveaux de transcription de *Mafb* (*musculoaponeurotic fibrosarcoma bZIP transcription factor B*) et ceux des gènes codant la famille des IFI200 (*IFN-inducible 200*), des régulateurs négatifs de la prolifération des macrophages. En revanche, le traitement par le NPFF, ou la surexpression du récepteur NPFFR2, augmente la transcription de *Ndrg2* (*N-myc downstream-regulated gene 2*) qui code un facteur impliqué dans la prolifération de différents types cellulaires.

NPFF inhibe la signalisation IFN-γ, une clé pour l'activation et la prolifération des ATM

L'interféron-γ (IFN-γ) est à l'origine d'une diminution de la transcription de NPFFR2. Il active des facteurs pro-inflammatoires comme l'IL-6 et la Nos2 (*nitric oxide synthase 2*) dans les ATM. À l'inverse, le NPFF inhibe la Nos2 et la transcription de gènes inductibles par l'IFN-γ dans la lignée J774A.1. À noter que le traitement par l'IFN-γ induit des effets opposés à ceux du NPFF sur les gènes qu'il régule, comme *RNF128*, *Ndrg2* et les membres de la famille *IFI200*. Ces effets antagonistes entre IFN-γ et NPFF s'observent également sur la production d'AMP cyclique intracellulaire, qui diminue sous l'action du NPFF, ce qui conduit à la polarisation des macrophages en M2 et à la prolifération des ATM.

Conclusion

Notre étude révèle que le NPFF est un puissant régulateur de la qualité et de la quantité des macrophages résidents du tissu adipeux. Dans les macrophages, la liaison de NPFF à son récepteur, NPFFR2, active la voie de signalisation dépendant de STAT6, qui stimule leur polarisation en macrophages de type M2, et provoque leur auto-renouvellement. Actuellement, les effets du NPFF sur le métabolisme et, notamment, sur le contrôle de l'appétit et la thermogenèse, sont classiquement associés à ses fonctions de neurotransmetteur dans le système nerveux central. Notre étude démontre, pour la première fois, que ce neuropeptide a également de puissants effets périphériques, en agissant comme une hormone, et que le pancréas est un site d'expression du gène codant le NPFF, *Npff*. Une diminution de sa traduction pourrait ainsi être à l'origine de la réduction du taux plasmatique de NPFF observée chez la personne obèse. L'expression de NPFF et de son récepteur, par les macrophages du tissu adipeux, est réduite chez les patients obèses, en comparaison à celle des individus sains. Cela pourrait conduire à la perte de l'effet du peptide sur les macrophages, ce qui est en accord avec le paradigme actuel décrivant une relation entre obésité et dérégulation de l'activation et de la prolifération des ATM *(Figure 1)*. Chez les rongeurs, le NPFF est un facteur anorexigène. Il agit en effet sur les noyaux hypothalamiques. Or, la balance entre les signaux orexigènes et anorexigènes dans les centres de l'hypothalamus contrôlant l'appétit est une clé dans le développement de l'obésité.

En conclusion, cette étude montre que le NPFF contrôle l'auto-renouvellement des macrophages du tissu adipeux et leur polarisation en macrophages M2. Il protège de l'inflammation du tissu adipeux, principal facteur de prédisposition au syndrome de résistance à l'insuline. Le NPFF et son récepteur NPFFR2 représentent donc des cibles thérapeutiques potentielles et prometteuses pour les futurs traitements des défauts de régulation de la prolifération et de l'activation des ATM associés à l'obésité. ◊

The neuropeptide FF: a signal for M1 to M2 type switching in macrophages from the adipose tissue

LIENS D'INTÉRÊT

Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

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Raphaëlle QUILLET

Identification et caractérisation de nouveaux outils pharmacologiques sélectifs pour les différents récepteurs à peptides RF-amides.

Résumé

Les RF-amides (RFRPs, NPFF, QRFP, PrRPs, Kisspeptines) sont une famille de peptides caractérisée par une signature Arg-Phe-NH_2 à l'extrémité C-terminale très conservée au cours de l'évolution. Ils exercent leurs effets *via* 5 récepteurs couplés aux protéines G (NPFF1R, NPFF2R, QRFPR, PrRPR, Kiss1R), et plusieurs études soutiennent leur implication dans la modulation de nombreuses fonctions physiologiques telles que la douleur et la nociception, la reproduction, ou encore le métabolisme. Néanmoins, l'étude de ces systèmes est entravée par l'absence d'outils pharmacologiques tels que des antagonistes sélectifs. C'est pourquoi mon travail de thèse a consisté au développement d'outils pharmacologiques permettant de répondre à ces attentes, particulièrement sur les récepteurs NPFF1R, NPFF2R et Kiss1R. Des études de relation structure-activité nous ont amenés à considérer l'importance de la signature Arg-Phe-NH_2 dans l'activité des peptides RF-amides sur leurs récepteurs. L'introduction de modifications au niveau N-terminal ou C-terminal du dipeptide Arg-Phe-NH_2 nous a conduit à l'identification d'un antagoniste hautement affin et sélectif pour le récepteur NPFF1R *in vitro* et *in vivo*. Ce dernier nous a permis d'identifier le rôle du récepteur NPFF1R dans les effets secondaires liés à l'administration d'opiacés, tels que l'hyperalgésie et la tolérance morphinique. La responsabilité du récepteur NPFF1R dans ces phénomènes a été par la suite confirmée chez des souris KO NPFF1R. De plus, des données plus récentes suggèrent l'importance de ce dernier au niveau de l'axe hypothalamo-hypophyso-gonadotropique (HHG) des animaux saisonniers, et en particulier du hamster. Notre antagoniste nous a permis de déterminer le rôle du récepteur NPFF1R dans la libération de LH induite par le RFRP-3. Pour la première fois, nous avons également mis en évidence des molécules hautement affines et sélectives pour le récepteur NPFF2R, pour lequel nous avons révélé une activité agoniste partielles *in vitro*. Mon travail a également mené à la caractérisation *in vitro* d'un antagoniste sélectif du récepteur Kiss1R, qui vient compléter l'ensemble des outils disponibles pour l'étude des fonctions physiologiques modulées par ce récepteur et son ligand, la kisspeptine.

Dans l'ensemble, mon travail de thèse a permis de caractériser plusieurs ligands affins et sélectifs des récepteurs à peptides RF-amides, et de mettre en lumière le rôle du système RFRP-3/NPFF1R dans les effets à long-terme liés aux opiacés.

Mots-clés : peptides RF-amides, RFRP-3, NPFF, NPFF1R, NPFF2R, Kiss1R, nociception, hyperalgésie, antagoniste, axe HHG

Abstract

RF-amides (RFRPs, NPFF, QRFP, PrRPs, Kisspeptins) belong to a family of peptide characterized by a Arg-Phe-NH_2 C-terminus highly conserved throughout the evolution. They target 5 G-protein coupled receptors (NPFF1R, NPFF2R, QRFPR, PrRPR, Kiss1R) and most studies highlight their roles in the modulation of various functions as pain and nociception, reproduction or metabolism. Nonetheless, the study of these systems is severely limited by the absence of pharmacological tools as selective antagonists. Thus, my PhD project consisted in the development of selective ligands to answer these questions, notably on NPFF1R, NPFF2R and Kiss1R receptors. Structure-Activity relathionship studies highlighted the importance of Arg-Phe-NH_2 signature for the activity of RF-amide peptides on their receptors. Introduction of modifications at N or C-terminus of Arg-Phe-NH_2 dipeptide led us to the identification of a compound displaying high affinity, selectivity and antagonist activity for NPFF1R both *in vitro* and *in vivo*. This compound allowed us to identify the critical role played by NPFF1R in the secondary effects related to opiates administration, as hyperalgesia and analgesic tolerance. The involvement of NPFF1R was then confirmed in KO NPFF1R mice. Moreover, recent data suggest the importance of NPFF1R on hypothalamo-pituitary gonadotropic (HPG) axis of seasonnal animals, and particularly of hamsters. Our antagonist allowed us to decipher the role of NPFF1R in RFRP-3-induced-LH release. For the first time, we also have characterized high affinity and selective compounds for NPFF2R that display partial agonist activity *in vitro*. Moreover, our work led to the *in vitro* characterization of a selective antagonist for Kiss1R, that complete the available tools to study the physiological functions modulated by this receptor and its ligand, the kisspeptin.

Overall, my PhD thesis led to the characterization of several selective ligands for RF-amide receptors, and highlight the role of RFRP-3/NPFF1R system in the long-term effects associated to opiates.

Key-words : RFamide peptides, RFRP-3, NPFF, NPFF1R, NPFF2R, Kiss1R, nociception, hyperalgesia, antagonist, HPG axis.