



Effets anxiolytiques/antidépresseurs et neurogéniques des ligands du récepteur 5-HT₄ chez la Souris : rôle de la protéine β -arrestin 1

Indira Mendez-Martinez Mendez Martinez-David

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Effets anxiolytiques/antidépresseurs et neurogéniques des ligands
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Composition du jury :

Directeurs de thèse :

Professeur Alain GARDIER
Professeur René HEN

Université Paris-Sud, Châtenay-Malabry, France
Columbia University, New York, USA

Rapporteurs :

Professeur Catherine BELZUNG
Professeur Joël BOCKAERT

Université François Rabelais, Tours, France
Institut de Génomique Fonctionnelle, Montpellier, France

Examineur :

Docteur Etienne SIBILLE

University of Pittsburgh, Pittsburgh, USA

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Publications

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Review papers

-**Mendez-David I**, Hen R, Gardier AM, David DJ (2013) Adult hippocampal neurogenesis: an actor in the antidepressant-like action. *Ann Pharm Fr.* May;71(3):143-9. **Article 2.**

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Original papers

-**Mendez-David I**, David DJ, Darcet F, Wu MV, Kerdine-Romer S, Gardier AM, Hen R, Rapid anxiolytic effects of a 5-HT₄ receptor agonist are mediated by a neurogenesis-independent mechanism. Accepted for publication in *Neuropsychopharmacology*, manuscript NPP-13-1008RR. **Article 4.**

-**Mendez-David I**, El Ali Z, Hen R, Falissard B, Corruble E, Gardier AM, Kerdine-Romer S, David DJ (2013) A method for biomarker measurements in peripheral blood mononuclear cells isolated from anxious and depressed mice: β -arrestin 1 protein levels in depression and treatment. *Frontiers in Pharmacology*:(4):124. **Article 5.**

Others published papers not related to the thesis

-David DJ, Klemenhagen KC, Holick KA, Saxe MD, **Mendez I**, Santarelli L, Craig DA, Zhong H, Swanson CJ, Hegde LG, Ping XI, Dong D, Marzabadi MR, Gerald CP, Hen R (2007) Efficacy of the MCHR1 antagonist N-[3-(1-{[4-(3,4-difluorophenoxy)phenyl]methyl}(4-piperidyl))-4-methylphenyl]-2-methylpropanamide

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-Xia L, Delomé C, **David I**, Rainer Q, Marouard M, Delacroix H, David DJ, Gardier AM, Guilloux JP (2012) Ventral hippocampal molecular pathways and impaired neurogenesis associated with 5-HT(1)A and 5-HT(1)B receptors disruption in mice. *Neurosci Lett.* Jul 11;521(1):20-5.

-Quesseveur G, David DJ, Gaillard MC, Pla P, Wu MV, Nguyen HT, Nicolas V, Auregan G, **David I**, Dranovsky A, Hantraye P, Hen R, Gardier AM, Déglon N, Guiard BP (2013) BDNF overexpression in mouse hippocampal astrocytes promotes local neurogenesis and elicits anxiolytic-like activities. *Transl Psychiatry.* Apr 30;3.

-Guilloux JP, **Mendez-David I**, Pehrson A, Guiard BP, Repérant C, Orvoën S, Gardier AM, Hen R, Ebert B, Miller S, Sanchez C, David DJ (2013) Antidepressant and anxiolytic potential of the multimodal antidepressant vortioxetine (Lu AA21004) assessed by behavioural and neurogenesis outcomes in mice. *Neuropharmacology.* May 28;73C:147-159.

Posters

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Résumé

Les inhibiteurs sélectifs de recapture de la sérotonine (ISRS), agonistes indirects des récepteurs de la sérotonine (5-HT), ont un début d'effet antidépresseur retardé de plusieurs semaines. Des travaux antérieurs suggèrent que le récepteur 5-HT₄ de la sérotonine serait une cible directe pour traiter la dépression et un nouvel espoir pour traiter plus rapidement ces pathologies anxio-dépressives. Toutefois, l'hypothèse « 5-HT₄ » doit encore être validée dans des modèles animaux d'anxiété/dépression. Les questions posées étaient : la stimulation des récepteurs 5-HT₄ centraux est-elle nécessaire aux effets comportementaux des ISRS ? la neurogenèse hippocampique adulte contribue-t-elle à ces effets ? En utilisant le modèle de stress chronique à la corticostérone (CORT) chez la souris, nous avons évalué les effets sur ces paramètres d'un traitement chronique avec un agoniste du récepteur 5-HT₄ (RS67333, 1,5 mg/kg/jour pendant 4 semaines) comparé à un traitement à la fluoxétine (18 mg/kg/jour). Nous avons ensuite utilisé ce modèle murin combiné à l'ablation de la neurogenèse hippocampique par rayons-X afin d'examiner si la neurogenèse est nécessaire aux effets comportementaux d'un traitement subchronique (7 jours) ou chronique (28 jours) avec le RS67333. Nous avons également évalué le blocage des effets de la fluoxétine par un antagoniste du récepteur 5-HT₄ (GR125487, 1 mg/kg/jour). Le traitement chronique avec RS67333, comme celui de la fluoxétine, induit une activité anxiolytique/antidépresseive et stimule la neurogenèse hippocampique adulte. Cependant, contrairement à la fluoxétine, les effets anxiolytiques du RS67333 sont déjà présents après 7 jours de traitement, sans nécessité l'activation de la neurogenèse. Le traitement chronique avec le GR125487 empêche les deux effets anxiolytique/antidépresseur et neurogénique de la fluoxétine, indiquant que l'activation du récepteur 5-HT₄ est nécessaire à ces effets de l'ISRS. Nous avons ensuite cherché à savoir si le court délai d'action antidépresseur du RS67333 peut être prédit par l'expression d'un biomarqueur périphérique. Des données de la littérature indiquent que la cascade de signalisation de β -arrestine 1 (impliquée dans la désensibilisation et l'internalisation du récepteur 5-HT₄) serait un biomarqueur potentiel pré-clinique/clinique des états dépressifs et des effets d'un traitement antidépresseur. À cette fin, nous avons développé une nouvelle méthode d'évaluation des taux de protéines circulantes grâce à une analyse par immunoblot des leucocytes (PBMC) isolés à partir du sang total de souris. Les taux de β -arrestine 1 sont diminués dans les leucocytes des souris pré-traitées à la CORT. Il faut 7 jours de traitement avec le RS67333, mais 28 jours avec la fluoxétine chez ces animaux pour restaurer un taux de β -arrestine 1 comparable à celui des animaux contrôles. Ces résultats suggèrent que le taux sanguin de β -arrestine 1 est un biomarqueur de la rapidité de la réponse antidépresseive. Enfin, l'activation du récepteur 5-HT₄ dans le cerveau peut représenter une approche thérapeutique innovante d'apparition pour traiter plus rapidement des symptômes dépressifs associés à l'anxiété.

Abstract

Selective serotonin reuptake inhibitors (SSRIs) display a delayed onset of antidepressant action of several weeks. Past work demonstrated evidence that the 5-HT₄ receptor may be a direct target for treating depression and a new hope for fast acting antidepressant treatment. However, the 5-HT₄ hypothesis still needs to be validated in models of anxiety/depression.

We decided to investigate whether 5-HT₄ receptor stimulation was necessary for the effects of SSRIs in a mouse model of anxiety/depression and whether hippocampal neurogenesis contributed to these effects. Using the mouse corticosterone (CORT) model of anxiety/depression, we assessed whether chronic treatment with a 5-HT₄ receptor agonist (RS67333, 1.5 mg/kg/day) had effects on anxiety and depression-related behaviors as well as on hippocampal neurogenesis in comparison to chronic fluoxetine treatment (18 mg/kg/day). Then, using our model combined with ablation of hippocampal neurogenesis, we investigated whether neurogenesis was necessary for the behavioral effects of subchronic (7-days) or chronic (28-days) RS67333 treatment. We also assessed whether a 5-HT₄ receptor antagonist, (GR125487, 1 mg/kg/day) could prevent the behavioral and neurogenic effects of fluoxetine in CORT pre-treated mice. Chronic treatment with RS67333, similar to fluoxetine, induced anxiolytic/antidepressant-like activity and stimulated adult hippocampal neurogenesis. However, unlike fluoxetine, the anxiolytic effects of RS67333 were already present after 7 days and did not require hippocampal neurogenesis. Chronic treatment with GR125487 prevented both anxiolytic/antidepressant-like and neurogenic effects of fluoxetine in CORT pre-treated mice, indicating that 5-HT₄ receptor activation is necessary for these effects of SSRIs.

We then explored whether the fast onset of action of the 5-HT₄ receptor agonist RS67333 could be predicted by expression of a peripheral biomarker. The β -arrestin-signaling cascade which is involved in 5-HT₄ receptor desensitization and internalization, has recently gained attention as a potential pre-clinical/clinical bridging biomarker for depressive states and treatment effects. To this end, we developed a new method to assess levels of circulating proteins through immunoblot analyses of mouse peripheral blood mononuclear cells (PBMCs) isolated from whole blood of anesthetized animals. β -arrestin 1 levels were decreased in CORT pre-treated mice. RS67333 restored the level of this protein to control levels after only 7 days of treatment, while fluoxetine did it after 28 days. These results suggest that blood levels of β -arrestin 1 may be a useful biomarker to predict rapid antidepressant/anxiolytic activities.

Finally, the activation of 5-HT₄ receptors in the brain may represent an innovative and rapid onset therapeutic approach to treat depressive symptoms with comorbid anxiety.

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Abréviations

5-HT : sérotonine/serotonin

5-HT1A, 1B, 2C, 3, 4, 5, 6, 7 : récepteurs de la sérotonine 1A, 1B, 2C, 3, 4, 5, 6, 7/ 1A, 1B, 2C, 3, 4, 5, 6, 7 serotonergic receptor

5-HTT/SERT : transporteur de la sérotonine/serotonergic transporter

AC : Adenylate cyclase/adenylyl cyclase

Ach : acetylcholine

AMPA : acide 2-amino-3-(5-méthyl-3-hydroxy-1,2-oxazol-4-yl)propanoïque/ 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid

ARN/ARNm/mRNA : acide ribonucléique/acide ribonucléique messenger/messenger ribonucleic acid

β-CD : β-cyclodextrine/β-cyclodextrin

BDNF : brain derived neurotrophic factor

CA : corne d'ammon/ammon's horn

cAMP : Adénosine monophosphate cyclique/cyclic adenosine monophosphate

CNS : système nerveux central/central nervous system

CMS : stress chronique modéré/chronic mild stress

CORT : corticostérone/corticosterone

CREB : C-AMP Response Element-binding protein

CT : thérapie cognitive/cognitive therapy

DA : dopamine

DAT : inhibiteur de recapture de la dopamine/dopamine reuptake inhibitor

DCX : doublecortine/doublecortin

DG : gyrus dentelé/dentate gyrus

DRN : noyaux du raphe dorsal/dorsal raphe nucleus

DSM : the diagnostic and statistical manual of mental disorders

ECT : electro-convulsivo therapie/electroconvulsive therapy

EPM : labyrinthe en croix surélevée/elevated plus maze

FDA : food and drug administration

FST : test de la nage forcée/ forced swim test

GIP : protéine interagissant avec les récepteurs couplés aux protéines G/GPCR interacting proteins

GPCR : récepteurs couplés aux protéines G/G-protein coupled receptor

GRK : kinase des récepteurs couplés aux protéines G/G-protein-regulated kinase

HPA : hypothalamo-hypophyso-surrénalien/ hypothalamic-pituitary-adrenal axis

HRSD : Echelle d'évaluation d'Hamilton/Hamilton rating scale for depression

IMAO : inhibiteur de la monoamine oxydase/monoamine oxydase inhibitor

IP : intrapéritonéale/intraperitoneal

ISRN : inhibiteur sélectif de recapture de la noradrénaline/selective norepinephrin reuptake inhibitor

ISRS/SSRI : inhibiteur sélectif de recapture de la sérotonine/selective serotonin reuptake inhibitor

LTP : potentialisation à long terme/long term potentiation

KO : knock out

MASSA : agoniste mélatoniergiques/antagoniste des récepteurs sérotoninergiques/melatonin agonist and selective serotonin antagonist

MDD : major depressive disorders

NA : noradrénaline/norepinephrin

NATNET : transporteur noradrénergique/norepinephrine transporter

NASSA : antagoniste des récepteurs noradrénergiques et sérotoninergiques/noradrenergic and specific serotonergic antidepressants

NDMA : N-methyl-D-aspartate receptor

NDRI : inhibiteur de recapture de la noradrénaline et de la dopamine/norepinephrine and dopamine reuptake inhibitor

NRI : inhibiteur de recapture de la noradrénaline/Norepinephrin reuptake inhibitor

NSF : novelty suppressed feeding

OF : test du champ ouvert/open field

OMS/WHO : Organisation mondiale de la Santé/world health organization

PCR : réaction en chaîne par polymérase/polymerase chain reaction

PBMC : cellules sanguines mononuclées/peripheral blood mononuclear cells

PFC : cortex préfrontal/prefrontal cortex

sAPP α : soluble amyloid precursor protein

SARI : antagoniste des récepteurs 5-HT₂ et inhibiteur de recapturee/Serotonin 2 receptor antagonist and reuptake inhibitor

SRE : promoteur sélectif de la recapture de la sérotonine/serotonin reuptake enhancer

TCA : antidépresseur tricycliques/tricyclic antidepressant

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INTRODUCTION

Introduction

1-Les Episodes dépressifs Majeurs

I. Généralités sur les maladies dépressives

Les troubles dépressifs et l'anxiété sont un lourd fardeau pour la société. L'Organisation Mondiale de la Santé (OMS) prévoit que d'ici 2030 la dépression, une maladie potentiellement mortelle, sera la deuxième cause d'invalidité dans le monde entier (Kessler et al., 2003). Les troubles de l'humeur touchent 7% de la population mondiale, tandis que les formes sévères de dépression ont une incidence sur 2-5% de la population américaine (Kessler et al., 2005). En outre, environ 32 à 35.000.000 adultes dans la population américaine (16%) connaîtront un épisode dépressif majeur au cours de leur vie. En Europe, une méta- analyse fondée sur 27 études cliniques, comprenant plus de 150.000 sujets de 16 pays européens, a estimé la prévalence de la dépression entre 3 à 10 % au cours des 12 derniers mois. Un individu sur six environ succombera à une des formes de dépression au cours de sa durée de vie (Kessler et al., 2005). En 2000, le fardeau économique américain des troubles dépressifs était estimé à 83,1 milliards de dollars, dont près d'1/3 sont attribuables à des frais médicaux (Greenberg et al., 2003). Des pertes de productivité de la main-d'œuvre liés à la dépression seraient de 24 milliards de dollars par an (Birnbaum et al., 2009). La comorbidité des troubles dépressifs avec des troubles anxieux, puisqu'on rapporte que 50-60% des patients souffrant de ces troubles ont aussi un historique de trouble anxieux qui précèdent souvent l'apparition des symptômes dépressifs (Kaufman et Charney, 2000). Ces résultats soulèvent la question de savoir si les troubles anxieux et dépressifs, malgré les distinctions faites de leur diagnostic clinique, partagent une physiopathologie commune.

Malgré une certaine variabilité, un élément commun à ces deux pathologies est que la dépression majeure est presque deux fois plus fréquente chez les femmes que chez les hommes (Burt et Stein, 2002; Waraich et al., 2004). Il faut également tenir compte de 1.3 à 4.4% de toutes les incapacités et des décès prématurés dans le monde.

La dépression se caractérise par plusieurs symptômes biologiques et psychologiques qui affectent de nombreux aspects du quotidien. Selon le Manuel diagnostique et statistique des troubles mentaux (DSM-V), le trouble dépressif majeur est plus que de la tristesse occasionnelle, mais comprend plutôt une humeur dépressive ou une perte de plaisir ou d'intérêt dans les activités quotidiennes pendant au moins deux semaines, et ces épisodes sont souvent récurrents. Les liens sociaux, professionnels et personnels du patient sont altérés par ces symptômes. La dépression se caractérise par des changements dans les fonctions biologiques de base y compris le sommeil et l'alimentation, ainsi qu'un dynamisme réduit. D'un point de vue cognitif, les patients décrivent souvent des sentiments d'inutilité ou de culpabilité, et peuvent envisager le suicide, si oui ou non il existe effectivement un plan en place pour tenter de passer à l'acte (**Tableau 1**). La dépression est clairement un changement dans la biologie normale d'une personne. Ces symptômes de l'humeur existent sur un continuum, et dans les cas graves, décrivent une pathologie qui doit être diagnostiquée et traitée.

Tableau 1 : DSM-V Criteria for Major Depressive Disorder (adapted from Care Management Program Clinical Guidelines).

Major depressive episode criterion:

A. At least five of the following symptoms have been present during the same 2-week period and represent a change from previous functioning: at least one of the symptoms is either 1) depressed mood or 2) loss of interest or pleasure.

1. Depressed mood most of the day, nearly every day, as indicated either by subjective report (e.g., feels sad or empty) or observation made by others (e.g., appears tearful)
2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated either by subjective account or observation made by others)
3. Significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day
4. Insomnia or hypersomnia nearly every day
5. Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down)
6. Fatigue or loss of energy nearly every day
7. Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick)
8. Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others)
9. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or specific plan for committing suicide

B. The symptoms do not meet criteria for a mixed episode.

C. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

D. The symptoms are not due to the direct physiological effects of a substance (e.g. a drug of abuse, a medication) or a general medical condition (e.g., hypothyroidism).

E. The symptoms are not better accounted for by bereavement, i.e., after the loss of a loved one, the symptoms persist for longer than 2 months or are characterized by marked functional

impairment, morbid preoccupation with worthlessness, suicidal ideation, psychotic symptoms, or psychomotor retardation

II. La Dépression, une pathologie hétérogène

La dépression est une pathologie hautement héréditaire : 40-50% du risque de dépression serait d'origine génétique, mais les gènes spécifiques qui sous-tendent ce risque restent inconnus. Les 50-60% de risques restants, non génétiques, sont également mal définis : des hypothèses mettent en cause des événements tels que des traumatismes de la petite enfance, un stress émotionnel, une maladie physique, et même des infections virales, seraient (**Figure 1**) (Hasin et al., 2005; Berton et Nestler, 2006; Leonardo et Hen, 2006; Tanti et Belzung, 2010). Élucider les bases neurobiologiques de la dépression et l'anxiété est l'un des défis majeurs pour la société d'aujourd'hui. De même, l'imagerie humaine et les études post-mortem de personnes qui ont souffert de dépression ont servi de contribution à la connaissance croissante des structures cérébrales impliquées dans la diversité des symptômes dépressifs. (Drevets, 2001; Drevets, 2003; Drevets et al., 2002; Nestler et al., 2002; Rajkowska, 2003; Morilak et Frazer, 2004).

Plusieurs arguments suggèrent qu'il existe des circuits neuronaux spécifiques du système cortico-limbique intervenant dans la réactivité au stress, l'humeur et la régulation émotionnelle. Le cortex préfrontal et le cortex cingulaire ainsi que l'hippocampe ont reçu le plus d'attention, en particulier dans les études animales de la dépression. On suppose que ces régions du cerveau sont plus particulièrement associées à des anomalies cognitives et à des déficits de mémoire observés chez de nombreux patients souffrant de dépression (Berton & Nestler, 2006). D'autres régions cérébrales jouent un rôle important dans la dépression comme l'amygdale pour son implication dans les stimuli aversif ou enrichissant, et le noyau accumbens qui joue un rôle important dans les niveaux de récompense, le plaisir, la motivation et l'énergie qui sont parmi les anomalies qu'on retrouve chez les personnes qui souffrent de dépression (Hasler et al., 2004; Sibille et al., 2009) . Ensemble, ces régions du cerveau participent à une série de circuits neuronaux hautement interactifs impliqués dans la

dépression (Zhu et al., 1999; Drevets, 2001; Liotti et Mayberg, 2001; Manji et al., 2001; Nestler et al., 2002; Krishnan et Nestler, 2008).

La dépression est également associée à des facteurs environnementaux, et en particulier à l'exposition au stress. Il est bien connu que, outre l'hippocampe, l'axe hypothalamo-hypophyso-surrénalien joue un rôle important (Nestler et al., 2002; Dranovsky et Hen, 2006). L'hétérogénéité de la dépression implique que des facteurs biologiques, psychologiques et sociaux soient des éléments pivots de la pathophysiologie et du développement de nouveaux traitements.

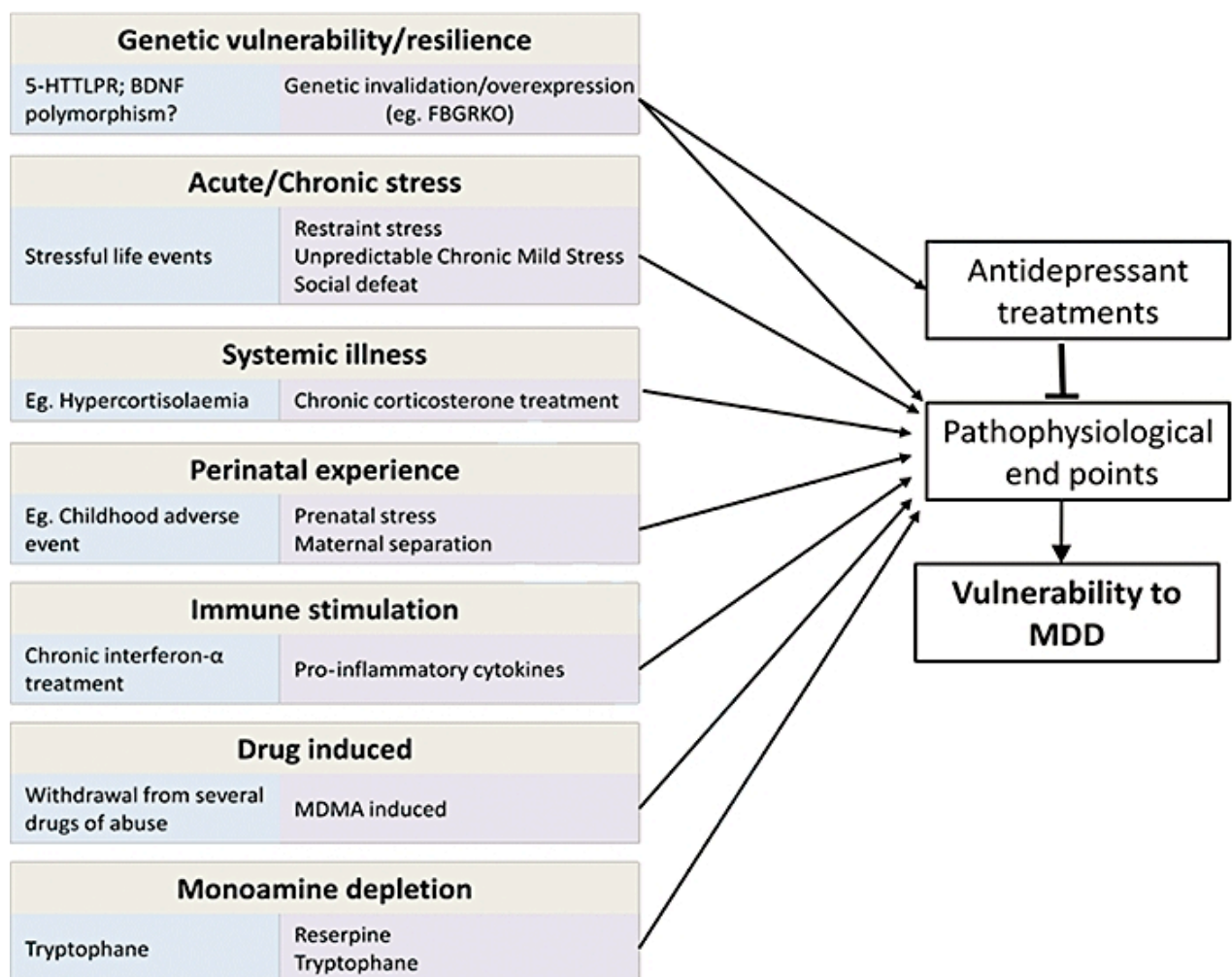


Figure 1 : Representation of aetiological factors involved in depression.

Examples of triggers in humans are given in blue boxes. Examples of experimental models used in animals to reproduce these triggers are given in purple boxes. These aetiological factors are thought to induce changes in pathophysiological end points precipitating a depression. Antidepressant treatments may act by reversing these changes. BDNF, brain-derived neurotrophic factor; MDD, major depressive disorder; MDMA, methylenedioxymethamphetamine (from Tanti and Belzung, 2010).

2- Les traitements antidépresseurs & leurs limitations

I. Traitements Antidépresseurs

Des traitements efficaces de la dépression sont disponibles, y compris une variété d'antidépresseurs, la thérapie par électrochocs (électro-convulsivo thérapie ou ECT), et certains types de psychothérapie ciblée comme la thérapie cognitivo-comportementale et interpersonnelle (Schatzberg, et Nemeroff, 2009). L'objectif d'un traitement antidépresseur est de soulager les symptômes. Cependant, d'autres traitements qui n'impliquent pas des antidépresseurs sont également efficaces pour soulager la dépression. Ceux-ci incluent la thérapie cognitive (CT) et d'autres formes de psychothérapie, comme la thérapie par électrochocs et la stimulation électrique du nerf vague (**Tableau 2**). Des interventions plus récentes, telles que la stimulation chronique de la région cingulaire subgénéral (aire 25 de Brodmann) ont également montré des effets prometteurs lors d'essais cliniques récents (Mayberg et al., 2005).

L'hypothèse monoaminergique de la dépression et de son traitement a été à la base de la recherche sur les troubles d'humeur au cours des 50 dernières années. Les antidépresseurs tricycliques (par exemple, l'imipramine), qui inhibent la recapture de la sérotonine et de la noradrénaline, et les Inhibiteurs de la MonoAmine Oxydase (IMAO comme par exemple l'iproniazide), les premiers médicaments utilisés en clinique pour traiter la dépression, sont apparus dans les années 1960 (Nestler et al., 2002). Ces médicaments ont été depuis remplacés par de nouveaux antidépresseurs ayant une tolérance supérieure et un meilleur profil de sécurité. Malgré les progrès indéniables qui ont été réalisés concernant la sélectivité d'effet et le rapport bénéfices/risques, d'importantes questions demeurent sans réponse concernant tous les antidépresseurs :

- leur efficacité limitée (30% de patients déprimés résistants),
- leur trop long délai d'action (*voir ci-dessous*) avant d'observer un bénéfice clinique
- les raisons des rechutes et récidives.

Même parmi les patients qui reçoivent un traitement adéquat et étroitement surveillé avec la psychothérapie ou des antidépresseurs, tous ne répondent pas complètement à ce traitement. Dans le traitement de la dépression, une réponse complète ou une rémission, est définie comme la résolution complète des symptômes dépressifs et un retour complet de fonctionnement, généralement définie comme la réalisation d'un score <8 sur l'échelle standardisée « Hamilton Rating Scale for Depression » (HRSD).

Tableau 2: Recommended modalities for acute phase treatment of Major Depressive Disorders (MDD) (adapted from Care Management Program Clinical Guidelines).

Modality				
Severity	Pharmacotherapy	Depression-Focused Psychotherapy	Pharmacotherapy in combination With Depression-Focused Psychotherapy	Electroconvulsive Therapy
Mild to Moderate	Yes	Yes	May be useful for patients with psycho-social or interpersonal problems, intrapsychic conflict, or co-occurring Axis II disorder	Yes, for certain patients
Severe Without Psychotic Features	Yes	NO	Yes	Yes
Severe With Psychotic Features	Yes, provide both antidepressant and antipsychotic medication	NO	Yes, provide both antidepressant and antipsychotic medication	Yes

La découverte des effets antidépresseurs des premiers antidépresseurs et de leurs cibles moléculaires a conduit à la conception de médicaments de deuxième et troisième génération, tels que les Inhibiteurs Sélectifs du Recapture de la Sérotonine (ISRS) et les Inhibiteurs Sélectif du Recapture de la noradrénaline (ISRN). Depuis cette découverte, la dépression a été associée à une atteinte de la neurotransmission sérotoninergique,

noradrénergique et, dans une moindre mesure, dopaminergique centrale. Aujourd'hui, la plupart des médicaments utilisés pour augmenter les concentrations cérébrales de sérotonine et traiter la dépression, y compris les médicaments les plus couramment prescrits, les ISRS (comme par exemple, la fluoxétine), sont efficaces pour traiter les deux pathologies, l'anxiété et la dépression. (Schatzberg et Nemeroff, 2009; Samuels et al., 2011) (**Figure. 2**).

II. Limites des traitements antidépresseurs

Au cours des dernières décennies, des milliards de dollars ont été dépensés en efforts de la recherche pour cibler de nouveaux médicaments plus sélectifs des récepteurs de la sérotonine ou de la noradrénaline, agonistes ou antagonistes ayant des effets semblables aux médicaments antidépresseurs déjà sur le marché, mais avec une réponse plus rapide et présentant moins d'effets indésirables. Malheureusement, en dépit de la recherche et des connaissances acquises sur le mécanisme d'action des antidépresseurs classiques et de leurs propriétés thérapeutiques, de nombreuses questions restent sans réponse. Le traitement pharmacologique de la dépression reste peu satisfaisant. Bien que les antidépresseurs actuellement disponibles aient une réelle efficacité curative (démontrée par des études cliniques « *contre placebo* » ou en comparaison à la référence, l'ECT) dans le traitement de cette pathologie, il faut attendre plusieurs semaines pour qu'ils démontrent une pleine efficacité, de nombreux patients répondent peu au traitement, les symptômes concomitants sont souvent peu contrôlés et certains antidépresseurs peuvent engendrer des problèmes de tolérance ou de dépendance (pour revue, Martinowich et al., 2013). Environ 60% des patients souffrant de dépression ne répondent pas de façon adéquate aux antidépresseurs ou sont résistantes à ces médicaments. Moins de 50 % des patients souffrant de dépression montrent une complète amélioration (Trivedi et al., 2006; Samuel et al., 2011). Les effets indésirables des ISRS sont fréquemment décrits lors d'un traitement chronique, notamment l'insomnie,

somnolence, sensation vertigineuse, akathisie et dysfonction sexuelle à long terme (par exemple, diminution de la *libido*, l'éjaculation retardée, etc...).

Comme les antidépresseurs de première génération, les ISRS nécessitent au moins 2 à 4 semaines d'administration avant l'obtention de bénéfices thérapeutiques (Wong et Licinio, 2001). Le fait que la réponse au traitement antidépresseur soit si imprévisible chez un individu et qu'il soit souvent nécessaire d'essayer plusieurs antidépresseurs pour obtenir un effet optimal, peut causer de la frustration chez le patient, favoriser une mauvaise observance de ce traitement, ce qui en limitera son efficacité et involontairement servira à renforcer la dépression (Fava, 2000; Trivedi et al., 2006). L'écart entre les effets aigus des ISRS (*in vitro*, le blocage des transporteurs SERT et/ou NET est rapide) et l'apparition tardive de leurs effets *in vivo* après administration chronique chez l'animal et chez l'Homme suggèrent un mécanisme d'action complexe (cf *Chapitre 3, Articles 1 et 2 concernant le rôle de la neurogenèse hippocampique adulte*). Cette distinction apparente entre les effets aigus et chroniques des ISRS a fait émerger l'hypothèse de la nécessaire activation de plusieurs mécanismes pharmacologiques dans le cerveau lors d'un traitement chronique avec des ISRS.

L'efficacité modeste des antidépresseurs conventionnels tels que les ISRS appelle à de nouvelles approches pour traiter les différentes formes légères, modérée ou sévères des épisodes dépressifs associés ou non à des troubles anxieux. Des études cliniques mettant en oeuvre des thérapies pharmacologiques combinées, telles que le blocage des récepteurs monoaminergiques centraux en plus de l'inhibition d'un des transporteurs des monoamines, ont déjà été proposées afin de raccourcir le délai d'apparition de l'effet antidépresseur et/ou d'augmenter l'efficacité de ces médicaments.

Tableau 3 : Antidepressant classification.

Class of Antidepressants			Drugs	Mechanism of Action	References
Reuptake Inhibitors	Tricyclics (TCAs)		Imipramine, Desipramine, Trimipramine, Clomipramine, Amytriptyline, Nortriptyline, Protriptyline, Doxepin, Amoxepin, Maprotiline	TCAs inhibit the NAT and the SERT, respectively	(Gillman, 2007)
	Selective Serotonin Reuptake Inhibitors (SSRIs)		Fluoxetine, Paroxetine, Citalopram, Escitalopram, Sertraline, Fluvoxamine	SSRIs selectively inhibit the SERT	(Vaswani et al., 2003)
	Noradrenaline and Dopamine Reuptake Inhibitor (NDRI)		Bupropion	NDRI block the action of the NAT and the DAT,	(Preskorn and Othmer, 1984)
	Dual Serotonin and Noradrenaline Reuptake Inhibitor (SNRI)		Venlafaxine	SNRIs inhibit the NAT and the SERT	(Gorman and Kent, 1999)
	Noradrenaline Specific Reuptake Inhibitor (NRI)		Reboxetine	NRIs inhibit the NAT but not the SERT	(Brunello et al., 2002)
	Serotonin 2 Antagonist and Reuptake Inhibitors (SARIs)		Nefazodone, Trazodone	SARIs act by antagonizing 5-HT receptors (5-HT _{2A} receptor) and inhibiting the SERT, the NAT, and/or the DAT. Additionally, most also act as α 1-adrenergic receptor antagonists	(Nemeroff, 1994)
Enzyme Inhibitors	Monoamine Oxidase Inhibitors (MAOIs)		Phenelzine, Tranylcypromine, Moclobemide	MAOIs prevent the breakdown of monoamine neurotransmitters, such as 5-HT and NA	(Tollefson, 1983)
Multimodal Antidepressants	Noradrenergic and Specific Serotonergic Antidepressant (NASSA)		Mirtazapine	NASSAs block the noradrenaline α 2 autoreceptors and α 2 heteroreceptors. In addition, mirtazapine blocks postsynaptic 5-HT ₂ and 5-HT ₃ receptors.	(Stimmel et al., 1997)
	Melatonin Agonist and Selective Serotonin Antagonist (MASSA)		Agomelatine	MASSAs antidepressants act as a melatonergic agonist (at both MT ₁ and MT ₂ receptors) and 5-HT _{2C} receptor antagonist	(Le Strat and Gorwood, 2008)
	Multimodal antidepressant		Vortioxetine	Vortioxetine is a 5-HT ₇ , 5-HT ₃ and 5-HT _{1D} receptor antagonist and 5-HT _{1B} partial agonist, a 5-HT _{1A} receptor agonist and SERT inhibitor	(Stahl et al., 2013)
Other Antidepressants	Serotonin Reuptake Enhancer (SRE)		Tianeptine	SRE enhances the reuptake of 5-HT,	(McEwen et al., 2010)

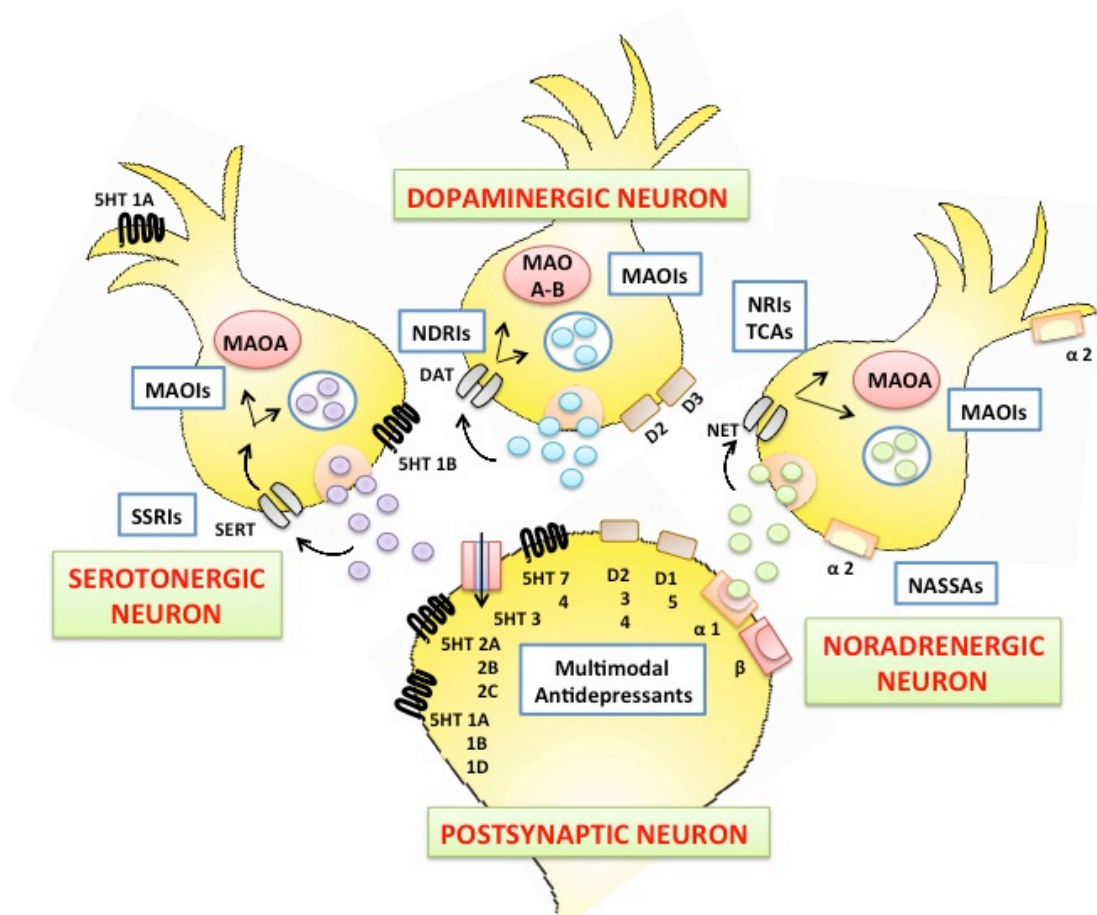


Figure 2: Mechanisms of action of antidepressants available in the market.

(MAOI: monoamine oxidase inhibitor; MASSA: Melatonin Agonist and Selective Serotonin Antagonist; NASSA: Noradrenergic and specific serotonergic antidepressants; NDRI: Norepinephrine Reuptake Inhibitor; SSRI: Selective Serotonin Reuptake Inhibitor; TCA: Tricyclic Antidepressant) (Figure from David and Felice).

III. Nouveaux traitements antidépresseurs

Malgré l'accumulation de preuves indiquant la pertinence d'augmenter la fonction du système sérotoninergique central dans le traitement des épisodes dépressifs, de nombreuses lacunes subsistent concernant l'aspect pharmacologique. Les efforts actuels de développement de médicaments visent à découvrir de nouvelles cibles et de nouvelles classes d'antidépresseurs dans l'espoir d'identifier de nouveaux composés ayant une efficacité plus large et / ou d'apparition des effets plus rapide avec un meilleur profil d'effets indésirables (Tableau 1). Pour exemples: les inhibiteurs de recapture doubles ou triples, des molécules

nouvelles capables d'agir sur plusieurs systèmes de neurotransmetteurs à la fois (Roose et al., 1994).

Ainsi, la vortioxetine (Lu AA21004; 1-[2-(2,4-diméthyl-phénylsulfanyl)-phényl]-pipérazine) est un nouvel antidépresseur ayant une activité antidépressive « multimodale » développé par des Laboratoires Lundbeck (**Figure 3**). Dans des cellules recombinantes exprimant soit des récepteurs humains, soit SERT, la vortioxetine agit comme un inhibiteur du transporteur de la 5-HT (SERT, $K_i = 1,6$ nM). C'est également :

- un antagoniste des récepteurs 5-HT₃, 5-HT₇ et 5-HT_{1D} ($K_i = 3,7$, 19 et 54 nM, respectivement),
- un agoniste partiel des récepteurs 5-HT_{1B} ($K_i = 33$ nM),
- un agoniste du récepteur 5-HT_{1A} ($K_i = 15$ nM) (Bang-Andersen et al., 2011; Mork et al., 2012; Westrich et al., 2012).

Chez le rat, les affinités de liaison sont $K_i = 1,1$, 200, 3.7, 16 et 230 nM respectivement pour les récepteurs 5-HT₃, 5-HT₇, 5-HT_{1D}, 5-HT_{1B} et 5-HT_{1A}, et un K_i de 8,6 nM pour le SERT (Mork et al., 2012; Westrich et al., 2013).

Récemment, explorant l'activité comportementale chez la souris adulte, nous avons montré que l'administration aiguë et répétée de vortioxetine produit un effet anxiolytique/antidépresseur plus prononcé que celui de la fluoxétine, associé à une augmentation de la prolifération cellulaire et de l'arborisation dendritique (Guilloux et al., 2013).

Fin Septembre 2013, la « Food and Drug Administration » (FDA) américaine a approuvé la mise sur le marché de la vortioxetine (BRINTELLIX®) avec pour indication le traitement des adultes souffrant de trouble dépressif majeur. Six études cliniques dans lesquelles les adultes ayant de tels troubles ont été randomisés pour recevoir soit un placebo,

soit la vortioxétine ont démontré son efficacité supérieure à celle du placebo pour traiter la dépression (Communiqué de presse de la Food Drug Administration, 30 septembre 2013).

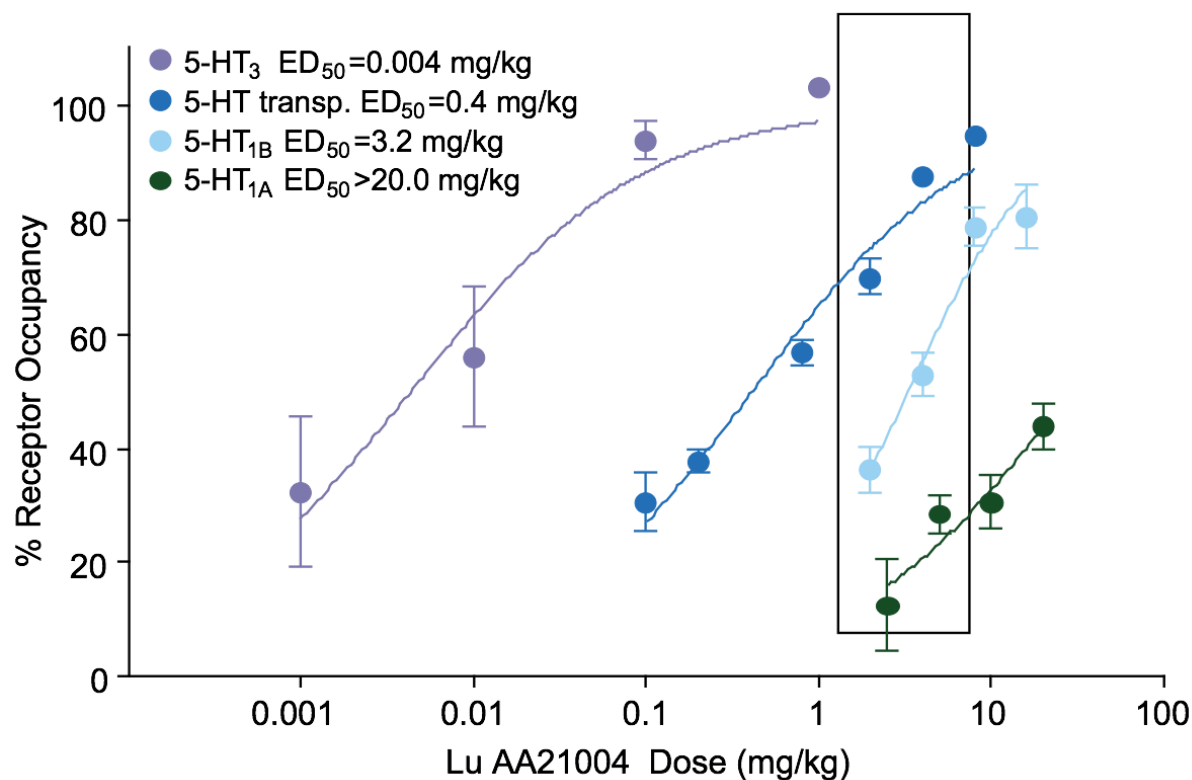


Figure 3: Lu AA21004 binding for the SERT and various 5-HT receptors after systemic administrations in rat (From Mork et al., ECNP Philadelphia 2012).

Autre exemple, le développement de l'agomélatine, médicament antidépresseur ayant à la fois des propriétés agoniste mélatonergique et antagoniste du récepteur 5-HT_{2C} est prometteur car les troubles affectifs sont caractérisés par des rythmes circadiens anormaux (pour revue, voir De Bodinat et al., 2010). L'agomélatine (S20098, N-[2 - (7-méthoxynaphthalén-1-yl) éthyl] acétamide) est nouveau antidépresseur (agissant comme un agoniste sur les récepteurs mélatonergiques et antagonistes des récepteurs 5-HT_{2C} Après évaluation pharmacologique complète et étendue des essais cliniques. agomélatine (VALDOXAN®/ THYMANAX® développé par les Laboratoires IRIS) a obtenu l'autorisation

de commercialisation en 2009 pour le traitement des épisodes dépressifs majeurs en Europe, devenant ainsi un des premiers antidépresseurs approuvés avec un mécanisme d'action non exclusivement monoaminergique.

Les antagonistes du récepteur ionotropique glutamatergique NMDA, en particulier la kétamine, sont apparus comme des candidats à la prochaine génération d'antidépresseurs d'action rapide (pour revue, voir Martinowich et al., 2013; Maeng et al., 2008). De façon étonnante, il semble que les effets de la kétamine sur le comportement reposent sur l'activation du récepteur AMPA, un autre type de récepteur canal ionique du L-glutamate et, par conséquent, des médicaments capables d'activer directement le récepteur AMPA *ou un de ses sites modulateurs allostériques* pourraient produire des actions antidépresseurs rapides et de longue durée.

Malgré les énormes progrès réalisés en recherche, l'utilisation de traitements physiques ou somatiques comme la psychothérapie, la thérapie par électrochocs (ECT) et la pharmacologie des antidépresseurs visant à élucider la physiopathologie des troubles anxio-dépressifs, de nombreuses questions restent en suspens concernant le mécanisme d'action des antidépresseurs et leur lien avec la dépression (Nestler et al., 2002). Ainsi, il est essentiel d'avoir une compréhension de ce qui sous-tend le retard du délai d'action des antidépresseurs pour être en mesure d'avoir un traitement bénéficiant à chaque patient déprimé.

3- La Neurogenèse Hippocampique Adulte: un acteur de l'activité de type antidépressive

Une hypothèse récente concernant le rôle de la neurogenèse dans l'origine des troubles dépressifs et/ou dans le mécanisme d'action des antidépresseurs gagne du terrain. Des arguments convaincants ont été apportés surtout quant à la capacité d'un traitement antidépresseur chronique à augmenter:

- l'expression du BDNF et celle de son récepteur TrkB de haute affinité dans les neurones hippocampiques (Nibuya et al., 1995),
- la neurogenèse hippocampique, processus nécessaire pour observer une réponse bénéfique dans certains paradigmes comportementaux, suite à un traitement antidépresseur chronique chez les Rongeurs (van Praag et al., 1999; Malberg et al., 2000; Santarelli et al., 2003).

Un aspect particulier de la plasticité cérébrale a donc reçu beaucoup d'attention ces 20 dernières années : il s'agit de la neurogenèse hippocampique adulte, ou la capacité de l'hippocampe à générer de nouveaux neurones tout au long de la vie. Il est maintenant bien établi que chez les Mammifères, dont les humains, de nouveaux neurones sont générés et intégrés dans le réseau neuronal fonctionnel du cerveau adulte en continu (Eriksson, 1998). Très récemment, une équipe Suédoise dirigée par Jonas Frisen, réussi à prouver, en mesurant, dans les cerveaux de 55 personnes décédées en Suède, âgées de 19 à 92 ans, le rapport entre le carbone 12 (le carbone normal) et le carbone 14 (son isotope radioactif), que les neurones de l'hippocampe humain se renouvellent durant toute la vie. Cette études montrent aussi que le renouvellement des neurones de l'hippocampe chez l'homme était d'environ 1,8 % par an, ce qui correspond à la production moyenne de 1 400 nouveaux neurones par jour dans

l'hippocampe d'un adulte. Ces recherches ont également révélé, de manière surprenante, que ce taux de renouvellement restait constant tout le long de la vie (Spalding et al., 2013).

On sait que l'hippocampe joue un rôle essentiel dans l'apprentissage et la mémoire. De même, cette région cérébrale est l'une des structures limbiques dans laquelle la neurogenèse se produit. Ceci a été également étudié chez des individus souffrant de troubles psychiatriques et neurologiques (Boldrini et al., 2009, 2012, 2013). En effet, au fil du temps, un grand nombre de publications ont montré que ces diverses pathologies cérébrales sont marquées par une diminution du volume et de la fonction de l'hippocampe. Par exemple, lors d'épisodes dépressifs majeurs, dans la schizophrénie, la maladie d'Alzheimer. L'atrophie de l'hippocampe, les difficultés d'apprentissage et de mémorisation, et des dérèglements de l'humeur sont des facteurs qui favorisent l'apparition de troubles mentaux (Sapolsky, 2000; Nestler et al., 2002).

L'**Article 1** qui suit est une revue des principaux modèles animaux d'anxiété/depression comme le stress modéré chronique, la défaite sociale ou l'administration chronique de corticostérone chez les Rongeurs et leurs conséquences sur la neurogenèse hippocampique chez l'adulte avec ou sans traitement antidépresseur.

Article 1: Role of hippocampal neurogenesis in the mechanism of action of antidepressant/anxiolytic drugs in animal model of depression and anxiety.

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ROLE OF HIPPOCAMPAL NEUROGENESIS IN THE MECHANISM OF ACTION OF ANTIDEPRESSANT/ ANXIOLYTIC DRUGS IN ANIMAL MODEL OF DEPRESSION AND ANXIETY

BENJAMIN ADAM SAMUELS, INDIRA DAVID, QUENTIN RAINER,
ALAIN MICHEL GARDIER, RENÉ HEN, DENIS JOSEPH DAVID

Abstract: Depression and anxiety are psychiatric illnesses that are major burdens on society and affect as much as 7% of the world's population. Here we review common approaches used to model depression in rodents such as chronic mild stress (CMS), social defeat, and chronic corticosterone treatment. We discuss the pros and cons of these different approaches. Furthermore, we provide a detailed review of adult hippocampal neurogenesis, including the distinct phases that a cell passes through when transitioning from precursor to neuron. Finally, we discuss at length the experiments that have related adult hippocampal neurogenesis to treatments of depression and anxiety, and why neurogenesis might be necessary for these treatments. We end by revisiting the neurogenesis hypothesis of depression and by providing suggestions for future research directions.

INTRODUCTION

Depressive and anxiety disorders are a major burden on society. Mood disorders affect 7% of the world's population, while severe forms of depression impact 2-5% of the US population [1, 2]. Furthermore, approximately 32-35 million adults in the US population [16%] experience an episode of major depression in their lifetime [3]. The heterogeneous nature of depression suggests an involvement of multiple distinct brain regions, which may be responsible for the diverse symptoms. Human imaging and post-mortem studies have supported this hypothesis, implicating brain areas including the prefrontal and cingulate cortex, hippocampus, striatum, amygdala, and thalamus [4]. Together, these brain regions operate a series of highly interacting circuits that forms a neural circuitry involved in depression [5]. The hippocampus is one of several limbic structures that have been extensively studied in individuals with psychiatric and neurologic disorders in the last decade [5, 6]. Besides its critical role in learning and memory, the hippocampus is one of only two areas in mammalian brain where adult neurogenesis occurs [6]. Adult hippocampal neurogenesis is therefore defined as the progression from neural stem cell to mature dentate granule neuron.

Moreover, while many classes of drugs with antidepressant activity have been developed and approved [7], many patients do not respond to treatment [8]. Therefore it is critical for basic research to develop animal models that present behavioral, neurochemical and brain morphological phenotypes reminiscent of depression and anxiety. Given that anxiety and depression have a high comorbidity with co-occurrence rates up to 60% in patients [9, 10], animal models that present signs of both diseases could potentially be the most useful.

To fully understand the pathophysiology and treatment of depression, it is essential to delineate molecular, cellular and circuit-level determinants of chronic antidepressant action in addition

BENJAMIN ADAM SAMUELS, INDIRA DAVID, QUENTIN RAINER,
ALAIN MICHEL GARDIER, RENÉ HEN, DENIS JOSEPH DAVID (Authors)

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Bruno P Guiard, Eliyahu Dremencov/BMC Netherlands

to behavioral models. Of the current leading hypotheses of the pathophysiology and treatment of depression, the Neurogenesis Hypothesis of Depression deserves particular attention because it allows the characterization because changes in neurogenesis are only seen after chronic, but not acute, antidepressant treatment. This review revisits the role of adult hippocampal neurogenesis in the pathophysiology of mood disorders, especially anxiety/depression, and also in the antidepressant responses, especially in non-stressed and stressed rodents.

HIPPOCAMPAL NEUROGENESIS

Neurogenesis refers to the production of new neurons in the brain. Originally, it was only described during development. The dogma of Ramon y Cajal (1913) that the adult brain was unable of generating new neurons was first questioned by the work of Altman in the 1960s, who revealed the genesis of new cells in the brain of adult rat and cat by autoradiography with tritiated thymidine [11]. Unfortunately, it was uncertain whether the new cells were actually neuronal cells. The work of Kaplan and Hinds (1977) [12] confirmed this by using an ultrastructural analysis of the labeled cells. Many years later, a combination of specific neuronal markers and an analogue of thymidine, 5-bromo-2'-deoxyuridine (BrdU), confirmed the neuronal phenotype [13]. The process of adult neurogenesis is located in two discrete brain regions: the subventricular zone (SVZ) and subgranular zone (SGZ) of dentate gyrus of the hippocampus. In this chapter, only the hippocampal neurogenesis and its involvement in depression will be presented.

PRODUCTION OF NEW NEURONS IN THE SGZ

Hippocampal neurogenesis is possible in the SGZ of the dentate gyrus of the hippocampus because of to the presence of stem cells. These stem cells evolve into neural progenitor cells that can produce multiple cell types in the central nervous system such as neurons, astrocytes, oligodendrocytes, or microglial cells [14]. In rodents, the duration of the mitotic cycle of proliferating precursors is approximately 12 to 24 hours, leading to the production of about 8,000 to 10,000 new neurons per day [15]. Given that the dentate gyrus consists of approximately one million granule cells, this phenomenon is capable of generating a little less than 1% of total granule cells each day. However, the proportion of new neurons that survive beyond one month is less than 50%, and the production of new cells is offset by the daily loss of mature granule cells. The surviving cells are predominantly a neuronal phenotype (75%), mainly glutamatergic granule cells, but also some are GABAergic interneuron basket cells. A smaller proportion of cells differentiate into astrocytes (15%), oligodendrocytes or microglia [16].

NEUROGENIC NICHE

Adult hippocampal neurogenesis, therefore, mainly refers to the production of a single neuronal type: the granule cells of the dentate gyrus. These cells are the principal excitatory neurons of the dentate gyrus (DG), receiving connections from the entorhinal cortex and sending projections along mossy fibers into the CA3 subfield of the hippocampus, where they terminate in structures rich in synapses and interneurons. Several projections are also sent into the hilus. The precursors are located between the hilus and the granular zone, in a niche called the subgranular zone (SGZ). The SGZ provides a microenvironment that promotes neuronal development. This neurogenic niche includes precursors, immature neurons, glial and endothelial cells, microglia and some immune cells. The niche is surrounded by a basement membrane. Because of the important role of vascularization in this region, this niche is also called a vascular niche [17]. In all cases, the niche provides a unique environment for neuronal development. Studies suggest that astrocytes have a local key role in neurogenesis. In vivo,

the development of cells has a preferred spatial relationship with astrocytes. Ex vivo, astrocytes and astrocyte-derived factors are potent inducers of hippocampal neurogenesis for the precursors [18]. The SGZ is also special because it receives connections from several brain regions: dopamine fibers in the ventral tegmental area, projections of serotonergic raphe nuclei, septum acetylcholine connections, and connections of GABAergic local interneurons. Manipulations of these neurotransmitter systems has also demonstrated regulatory effects on neurogenesis [19] [20]. The fact that these neurotransmitter systems regulate neurogenesis is of particular interest because antidepressants mainly act by changing monoamine levels [see later section entitled ANTIDEPRESSANTS AND ADULT HIPPOCAMPAL NEUROGENESIS]. A prominent example is that mice deficient in the Serotonin 1A receptor do not show a neurogenic response to chronic fluoxetine treatment [21].

CELLULAR STAGES OF HIPPOCAMPAL NEUROGENESIS

Neurogenesis can be divided into four phases: a step in which precursor cells are dividing, then surviving, a post mitotic maturation phase, and a late phase of survival.

a. The stage of precursor cell

The precursors have properties reminiscent of glial cells, including the morphology of radial glia. The cell body is located in the subgranular zone and the dendrites extend into the molecular layer. The nature of hippocampal astrocyte precursors was first revealed by [22]. Type 1 precursor cells yield intermediate progenitor cells, or type-2 cells, with a high proliferation activity. A subset of these cells continue to express glial markers, but lack the morphological characteristics of radial cells (type-2a). The type-2 cells that express nestin filaments (such as cell type-1) also express markers such as NEUROD1 and Prox1. This particular phenotype is used to classify cells as type-2b [16, 23]. Prox1 is specific to the development of granule cells. In type 2 cells, the developing cells receive GABAergic innervation [24], and are more sensitive to GABAergic stimulation. Similarly, type-1 cells respond to these stimuli by increasing cell proliferation [25]. Among the early expressed neuronal markers, doublecortin (DCX) is expressed on the type-2b cells. The expression of DCX extends from the proliferation phase to post mitotic maturation, which lasts about 2-3 weeks [26].

b. The phase of survival

Soon after the release of the cell cycle, new neurons express markers, such NeuN [“Specific neuronal nuclear protein”] and the transient marker calretinin [27]. A majority of cells fail to establish connections with the molecular layer and die. In contrast, cells that establish stable connections develop a stable dendritic arborization that extends in a specific way in time. In the days following the exit of the cell cycle, new cells emanate their axons to their target region CA3 to form appropriate synapses. This period is marked by the expression of a protein mediator, such as TOAD-64 or TUC-4, implicated in axon guidance. The first GABAergic innervations are excitatory and then become inhibitory when glutamatergic connections are established [24]. The GABAergic action leads cells to mature into glutamatergic cells and to develop synaptic integration [28] Most regulatory elements and processes occur at the stage of neuronal development, and rarely during the expansion phase [27, 29]

c. The maturation step; from early to late stage

Doublecortin is expressed in granule cells that range from 1 day to about 4 weeks of age, with 20% of its total population being proliferating neuroblasts and the rest being post-mitotic neurons [30]. Another marker of maturation, called calretinin, is also used as a marker for immature GCs at the early postmitotic stage. The period of expression of calretinin (3-4 weeks), is closely linked to dendritic maturation. However, using a lentiviral approach expressing the Green Fluorescent protein, it has been shown that the axonal growth to CA3 precedes the development of dendritic arborization [14]. After full integration to existing circuits, the new cells move from the calcium-binding protein calretinin to

calbindin [27]. It is several weeks before the cells are no longer distinguishable electrophysiologically from mature neurons of the granule cells [31]. It has been postulated that the time needed to complete the functionality of these young neurons is related to the formation and storage of new elements of memory [32]. Finally, calbindin-like immunoreactivity has been described as a marker for mature granule cells.

QUANTITATIVE ANALYSIS OF PROLIFERATION, SURVIVAL, MATURATION AND DIFFERENTIATION, NEWBORN CELLS.

For a full characterization of the neurogenic effects of new compounds, all the steps of neurogenesis, including proliferation, survival, maturation, and differentiation have to be completed. So far, no specific and exclusive marker has been identified that would allow for prospective studies of neurogenesis. As a result, detection of neurogenic steps depends on a combination of labeling approaches. Thus, proliferation, differentiation and survival steps each require a specific protocol using the administration of a synthetic thymidine analogue, “5-bromo-3'-deoxyuridine” (BrdU) that substitutes for thymidine incorporation into DNA synthesized during the S phase of the cycle. Quantitative analysis of proliferation, differentiation, and survival of newborn cells is made by varying the time interval between the pulse administration of BrdU and the sacrifice of animals [33].

Main methods for a Detection of cell proliferation

For the quantification of rate of cell division, animals are administered BrdU [between 50 to 150 mg/kg 2h before sacrifice [34]. Proliferation is quantified by counting BrdU-positive cells. To simplify the experiment, quantification of BrdU-positive clusters can also be performed to measure proliferation since a positive correlation exists between BrdU-positive clusters and BrdU-positive cells. Since the quantification of BrdU-positive clusters is much less time consuming than counting BrdU-positive cells, this method can be used as a rapid indicator of the neurogenic effect of drugs or other manipulations [33]. This is important also because BrdU immunostaining has been used not only to test whether new drugs affect adult hippocampal neurogenesis, but also whether the anxiety/depressive-like state has been related to changes in hippocampal neurogenesis.

Endogenous markers of cell proliferation

Endogenous cell cycle proteins are expressed at different stages of cell cycle progression. For example the “Proliferating Cell Nuclear Antigen, PCNA” is expressed throughout the cell cycle and provide low temporal resolution. Ki-67 closely corresponds to BrdU as it is expressed both during S phase and through the remainder of the cell cycle (figure 1).

Survival and fate of the newly generated cells

Usually, for the quantification of rate of cell survival, animals are administered BrdU (100 to 150 mg/kg) twice a day during three days, 3 to 4 weeks before sacrifice [35]. The reason to wait for sacrifice is that the fate of the newly generated cells can only be determined three to four weeks later, once neuronal migration has occurred [13, 36]. Once progenitor cells reach maturity they begin expressing neuronal markers such as neurofilaments, NeuN, and Calcium-binding proteins or astrocyte markers such as glial fibrillary acidic protein (GFAP). Calcium-binding proteins such as calbindin are produced when cells become electrophysiological active. It is not until 4 weeks after birth that newly generated granule cells have acquired the typical features of mature granule cells and cease to express immature neuronal markers.

Markers of maturation

Several markers have been used to assess maturation such as microtubule-associated protein [doublecortin]. In the adult dentate gyrus, DCX is exclusively expressed in immature neurons [26,

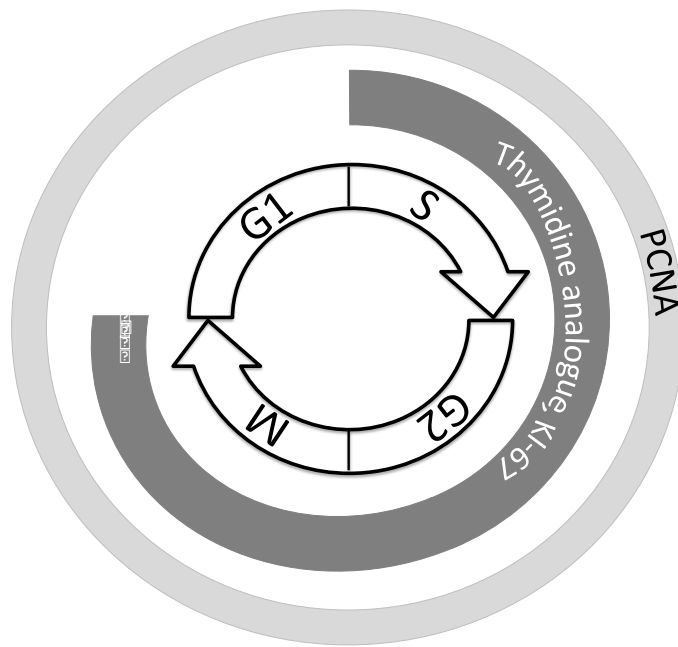


Figure 1: Schema representing the endogenous or exogenous markers used to study adult neurogenesis

37], and thus has been widely used as an immature neuronal marker that reliably reflects the level of neurogenesis and its modulation [26]. To assess the impact of antidepressants on dendritic maturation, the morphology of cells that express doublecortin (DCX) are examined. DCX-positive cells with tertiary dendrites, which display more complex dendritic arborization, can be distinguished from others DCX cells. The ratio of DCX-positive cells with tertiary dendrites over total DCX-positive cells is informative of the rate of maturation. The polysialylated form of the neural cell adhesion molecule (PSA-NCAM) also has a transient expression pattern that marks neuronal progenitors. However, it should be noted that, in contrary to DCX, PSA-NCAM has also been detected in glial cells.

METHODS USED TO ABLATE OR INCREASE NEUROGENESIS

The analysis of the causal relationship between neurogenesis and behavior came from the removal of progenitor cells. Several methods to date have been developed to reduce or abolish neurogenesis (table 1):

- An X-irradiation of either the whole brain or locally in the hippocampus [21];
- A systemic treatment with an anti-mitotic agent such as methylazoxymethanol acetate [MAM] [38];
- By genetic manipulation, such as GFAP-TK mice, in which the GFAP + progenitor cells die following treatment with ganciclovir [39].

In a contrary experiment, Sahay and colleagues [40] developed a genetic gain-of-function strategy to inducibly augment the survival of adult-born neurons in a cell-autonomous manner [40]. Because 60–80% of young adult-born neurons undergo programmed cell death, for which the pro-apoptotic gene Bax is required, they used a transgenic mouse line in which the tamoxifen (TAM)-regulatable

Pharmacological manipulation	Cranial Irradiation	Genetic manipulation
5-fluorouracil [5-Fu], methylazoxymethanol [MAM], temozolomide [TMZ] cytosine arabinoside [Ara-C]	X-irradiation, gamma-rays	GFAP-TK, Nestin-TK, Nestin-Bax, TrkB ^{lox/lox} -Cre ERT2 Cyclin D2 transgenic
[38] [96] [115]	[21, 39, 52, 69, 73, 97, 116-118]	[6, 51, 100, 101, 114, 119];

Table 1: Methods used to ablate or increase neurogenesis

recombinase CreERT2 is expressed under the control of a 5.26-kilobase fragment of the rat nestin (Nes) gene promoter [41] together with a floxed Bax mouse line to ablate Bax selectively in neural stem cells in the adult brain and promote survival.

HIPPOCAMPAL NEUROGENESIS AND MAJOR DEPRESSION AND ITS TREATMENT

THE ANXIETY / DEPRESSION STATE AND HIPPOCAMPAL NEUROGENESIS

The neurogenic hypothesis of depression postulates that decreased production of new granule cells in the dentate gyrus of the hippocampus is linked to the pathophysiology of depression and that the increase in hippocampal neurogenesis is required for the behavioral effects of antidepressant treatment [42]. The few studies of hippocampal neurogenesis in depressed patients published to date have mainly relied on histological examinations of post-mortem brain tissue and studies of magnetic resonance imaging. Thus, a reduction in hippocampal volume in depressed patients is somewhat established, and the contribution of two meta-analysis confirmed this reduction in hippocampal volume in patients with depression [43]. The frequency of depressive episodes and the period during which they are not treated coincide with the severity of the decline in hippocampal volume. However, pathophysiological studies on post-mortem brain tissue indicates that the change in the number of neuropil and glial cells may be responsible for reducing the volume of the hippocampus [44]. It is highly unlikely that loss of hippocampal neurogenesis can account for the decreased volume. These imaging results, involving hippocampal neurogenesis in the pathophysiology of major depressive episodes, have been challenged by a recent histology study [45]. This study did not actually detect the difference in stem cell proliferation [by Ki-67] in the hippocampus between patients with depression and healthy volunteers. The results of this study are limited, however, since the patients were on antidepressant medication at the time of death, which could mask the specific effects of depression on cell proliferation. In addition, in the absence of toxicology studies, it is not clear that the patients observed treatment. Moreover, given the diversity in the stages of neurogenesis, it is difficult to conclude any involvement of neurogenesis after only observation of cell proliferation. In contrast, a more recent study actually showed a 50% decrease in proliferation [by Ki-67] in patients with depression than in controls [46], [47]. However, this difference did not reach statistical significance.

Preclinical studies are useful to prove causal links between hippocampal neurogenesis and behavior. Using exposure to different types of stress, like chronic stress or social submission, causes a

decrease in cell proliferation in the hippocampus. It is important to keep in mind the disadvantages of these methods, such as non-specific effects of ablation that can involve structures other than the hippocampus, and therefore other functions. Dysfunction of hippocampal neurogenesis is only hypothesized to be part of the pathogenesis of major depressive disorders [48], [23]. In reality, the suppression of hippocampal neurogenesis in mice does not alter anxiety behavior in the open field or the light/dark paradigms, the elevated plus maze, or novelty suppressed feeding [49], [21], [39]. Thus, the X-irradiation in the hippocampus has no effect in the previously mentioned paradigms, suggesting that the loss of hippocampal neurogenesis is not sufficient for a behavioral phenotype of anxiety/depression, and does not exacerbate those induced by stress. Similarly, ablation of neurogenesis by MAM, a pharmacological agent, is not sufficient to induce an anhedonic behavior in rats [38]. Airan et al [50] explored in more detail the link between depression and hippocampal neurogenesis. It is clear from their study that chronic stress in rats is not associated with a decrease in neurogenesis, and that the removal of neurogenesis does not induce a depressive-like behavior [50]. However, other studies suggest that the mechanisms are more complex. Recently it was shown that adult hippocampal neurogenesis plays an important role in the regulation of affective states [51]. Indeed, transgenic mice that overexpress the protein Bax in apoptotic progenitor cells and thus have a deficit in neurogenesis, have an anxiety phenotype. In sum, there is evidence indicating that neurogenesis is not a major factor in the development of depression, but may be necessary for the behavioral effects of antidepressants [2].

ANTIDEPRESSANTS AND ADULT HIPPOCAMPAL NEUROGENESIS

IN PHYSIOLOGICAL CONDITIONS.

To study the effects of antidepressants in unstressed animals, the choice of the strain is essential. It should be noted that 129SvEv mice express a low basal level of proliferating cells in the SGZ, and therefore are more relevant to study an induced increase in antidepressant treatment in conditions without stress than BALB / cJ or C57Bl / 6 mice, which express a higher cell proliferation [52]. Instead, the study of the impact of stress on hippocampal neurogenesis is more relevant in these last two strains.

Effects of antidepressants on the proliferation and cell survival. In addition to the effects of fluoxetine [18 mg/kg] in increasing cell proliferation in 129/SvEvTAc mice [21] [table 2], it also increases the survival of postmitotic granule cells. These effects of SSRIs on proliferation and cell survival were also found in rats [53, 54]. It is important to note that the neurogenic effects of antidepressants are only seen with chronic treatment [21, 49, 54-56]. In addition, other treatments such as the atypical antidepressant tianeptine, electroconvulsive therapy, mood stabilizers such as lithium, and new antidepressants such as agomelatine, increase proliferation and cell survival in the adult hippocampus [57]. Furthermore, agomelatine selectively alters neurogenesis in the ventral hippocampus, a region more involved in the emotional response [58]. Therefore, it seems that proliferation and survival are regulated by distinct mechanisms. For example, an enriched environment increases the survival of immature cells without affecting proliferation [59]. In contrast, voluntary exercise increases the proliferation and survival, without affecting the maturation [30] or dendritic morphology of newborn neurons [60]. Finally, a recent study showed that fluoxetine targets a class of neural progenitor cells by directly increasing symmetric divisions [53]. Effects of antidepressants on the maturation. Until recently, it was not clear that SSRIs also targeted immature neurons by affecting their maturation and functional integration to the network. Wang and colleagues [2008] demonstrated that chronic treatment with fluoxetine stimulates maturation of young neurons [56]. Indeed, they have a dendritic

Table 2: Effects of chronic antidepressant treatment on hippocampal neurogenesis in rodents

Antidepressant	Specie	Dose (mg/kg)	Length of Treatment	Prolif.	Diff.	Survival	References
			25 days	=	∅	=	(120)
			21 days	↑	∅	∅	(121)
			7 days	=	∅	∅	
	Sprague	5	1 day	=	∅	∅	
	Dawley Rat		5 days	=	∅	∅	(54)
			14 days	↑	∅	∅	
			28 days	↑	∅	∅	
		0.6	28 days	↑	∅	∅	(122)
	Wistar Rat	10	28 days	↑	↑	∅	(123)
	Lister Rat	7	14 days	↑	↑	∅	(124)
			21 days	↑	∅	∅	
	(BALB/c * DBA/2) mice	10	10 days	↑	∅	∅	(125)
			5 days	=	∅	∅	
Fluoxetine		?	?	↑			(126)
			5 days	=	∅	∅	
	S129 SvEv mice	10	11 days	↑	∅	∅	(21)
			28 days	↑	∅	↑	
		18	5 days	=	=	=	(56)
			28 days	↑	↑	↑	
	129Sl mice	18	21 days	↑	∅	∅	(127)
	A/J mice	18	21 days	=	∅	∅	
		18	28 days	=	=	∅	(52)
	BALB/cJ mice	10	28 days	=	=	=	(68)
		16	24 days	=	=	=	(128)
	C57BL/6 mice	16	24 days	=	=	=	
	CD1 mice	10	28 days	↑	=	↑	(129)
	DBA/2 mice	18	21 days	↑	∅	∅	(127)
	SWR/J mice	18	21 days	=	∅	∅	
			21 days	↑	↓	↑	
Agomelatine	Wistar Rat	40	7 days	=	∅	∅	
			1 day	=	∅	∅	(57)
AMPA	Rodents	?	?	↑	∅	∅	(130)
Glutamatergic antagonist	Rodents	?	?	↑	∅	∅	(131)
Interleukine beta 1receptor antagonist	Rodents	?	?	↑	∅	∅	(132)
Citalopram	Wistar Rat	10	28 days	=	=	=	(133)
ECT	Wistar Rat	1 fois par jour	5 days	↑	↑	∅	(134)

Legend : ↑, increase; ↓, decrease; =, no change; ∅, not study

Antidepressant	Species	Dose (mg/kg)	Length of Treatment	Prolif.	Diff.	Survival	References
Fluoxétine/adolescent	BALB/cJ mice	16	24 days	↑	=	=	(128)
	C57BL/6 mice	16	24 days	↑	↑	↑	
	Wistar Rat (BALB/c *)	5	56 days	↑	∅	∅	(135)
Imipramine	DBA/2) mice	30	21 days	↑	∅	↑	(125)
	129 SvEv mice	20	28 days	↑	∅	↑	(21)
	Wistar Rat	2,5	14 days	↑	↑	↑	(136)
Lithium	C57BL/6 mice	2.4 g/kg	28 days	↑	=	↑	(137)
	Sprague	2	21 days	↑	∅	∅	(121)
Olanzapine	Dawley Rat	2	7 days	=	∅	∅	
	Rat Sprague	20 (2/day)	21 days	↑	∅	∅	(54)
Reboxétine	Dawley Rodents	?	?	↑	∅	∅	(138)
Riluzole	129 SvEv mice	?	?	↑		∅	(139)
Tesofensine	Wistar Rat	3	5 days	=	∅	=	(140)
		3	14 days	↑	∅	↑	
Tianeptine	Tree Shrews	50	28 days	=	∅	∅	(141)
		7.5, then					
Tranylcypromine	Sprague	10 (14 last days)	21 days	↑	∅	∅	(142)
	Dawley Rat						
Venlafaxine	Wistar Rat	10	14 days	=	∅	∅	(143)
		40		↑	∅	∅	

Legend : ↑, increase; ↓, decrease; =, no change; ∅, not study

arborization more complex than those present in untreated animals, suggesting an accelerating effect of fluoxetine on hippocampal neuronal maturation [56]. The duration of onset of fluoxetine effects on maturation also coincides with the time required for the onset of behavioral effects of this treatment in mice. Similarly, electroconvulsive therapy (ECT), which provides a very rapid therapeutic effect, stimulates neurogenesis faster than fluoxetine [61]. In addition, ECT stimulates dendritic development and maturation [62]. After ECT, young neurons express an increase in dendritic growth, and begin to receive glutamatergic synaptic connections early. These results suggest that molecules that increase the maturation of new neurons are targets for the development of future treatments.

a. Effects of antidepressants on the differentiation

Four weeks after birth, progenitor cells acquire properties of mature granule cells. For example, the new cells no longer express the immature neuronal markers DCX or PSA-NCAM, and receive the same GABAergic and glutamatergic afferents as mature granule cells of the dentate gyrus [14, 63, 64]. However, these new cells continue to mature morphologically and physiologically. The dendritic spines of a neuron at four weeks old are more associated with multiple synaptic boutons than older neurons and the density continues to increase even after 8 weeks [63]. In addition, neurons aged 2 to 4 weeks exhibit an increase in excitability and a lower threshold for induction of long-term potentiation [LTP], while at 4 to 6 weeks, new neurons exhibit greater LTP amplitude [65]. In addition, a specific form of LTP [ACSF-LTP] requires the hippocampal neurogenesis, as this form of LTP is blocked after ablation of neurogenesis [39]. This critical period

in young neurons coincides with the development of receptor expression of NMDA NR2B [28]. At any given time following differentiation, young neurons can be found across stages of development and with distinct morphological and physiological characteristics. Similar to new neurons in the developing brain, the newborn granule cells depolarize in response to GABA because of their high intracellular concentration of chloride ions [66]. Somewhere between 2 to 4 weeks after birth the response to GABA changes from depolarization to hyperpolarization as the young cells develop their dendritic spines and receive glutamatergic connections. In addition, new granule cells begin to make connections with the hilus and CA3 region while synaptic complexity is similar to that of mature neurons [67].

b. Dependent and independent neurogenic effects in the action of antidepressants

Recently Boldrini and colleagues [2009] suggested that future studies in humans should determine at which level antidepressants should affect neurogenesis to maximize the response [46]. Preclinical studies in rodents, using approaches that override functional neurogenesis, are used to establish the relationship between neuronal activity and the contribution of hippocampal neurogenesis in the behavioral effects of antidepressants in animals that are either stressed or not. The study by Santarelli and colleagues [2003] has raised many questions about the contribution of hippocampal neurogenesis in the behavioral improvement induced by antidepressants, unstressed mice [21]. From this study it was shown that the behavioral effect of SSRIs is dependent on the presence of hippocampal neurogenesis [56]. However, in BALB / cJ mice, abolishing neurogenesis by x-ray does not block the behavioral response induced by SSRIs in different behavioral paradigms related to anxiety / depression, especially the NSF [52]. Furthermore, chronic treatment with fluoxetine did not increase hippocampal neurogenesis [52, 68]. Likewise, the beneficial contributions of an enriched environment, physical exercise, or learning behavior related to anxiety appear to be independent of increased neurogenesis in adult mice [69].

To examine whether the increase induced by antidepressant treatment may simply be an epiphenomenon, the study of animal models of anxiety / depression in which hippocampal neurogenesis is reduced appears to be a good alternative.

ANIMAL MODELS OF DEPRESSIVE PHENOTYPES

Since no genetic variants with high penetrance that cause depression are known, animal models have mainly relied on different means of chronically exposing rodents to stressful experiences, or sensory tract lesions such as in olfactory bulbectomy, to induce behavioral states that present depression-like signs and are responsive to chronic antidepressant treatment.

The oldest most commonly used paradigm to induce a depression-like state is chronic mild stress (CMS). Initial observations suggested that animals subjected to multiple stressors over a prolonged period of time reduced their intake of saccharine or sucrose, a potential behavioral model of anhedonia [70]. Furthermore, this effect was selectively reversed by chronic treatment with the TCA imipramine [70]. Further work was able to repeat this result using more mild stressors, such as periods of food and water deprivation, small temperature reductions and changes of cage mates [71, 72]. Following these studies the CMS procedure, and modified versions such as chronic unpredictable stress (CUS or UCMS), became commonly used and much work demonstrated that other depression-like changes were induced in animals, such as decreased sexual and aggressive behavior, decreased self-care, and altered sleep patterns [71]. Furthermore these behaviors are all reversible by chronic, but not acute, treatment using multiple classes of antidepressants [73]. While historically potential pitfalls of the

CMS procedure are that it is notoriously labor intensive, and that there has been some difficulty in getting the procedure established and the results replicated across laboratories [74], the modified versions of the CMS have proven more useful.

Recently, there have been some reports using CMS or variants to model treatment resistance in rodents. In one study, CMS significantly decreased sucrose consumption and the proliferation of adult hippocampal neural progenitors [75]. Following chronic treatment with a SSRI (escitalopram), the subjects were retested for sucrose consumption. A bimodal distribution was found where one group recovered [increased sucrose consumption] while another refracted treatment [no increase in sucrose consumption]. Interestingly, there was a correlation between the animals in the group that recovered with a reversal of the decreased proliferation that was absent in the group resistant to treatment [75]. More recently, follow-up work has taken a proteomic approach in an attempt to find molecular differences in the ventral hippocampus between responders and non-responders [76]. Another study demonstrated that if animals are on a high fat diet during multiple UCMS procedures they become resistant to treatment with a SSRI (fluoxetine) [77].

A distinct procedure that has gained traction is the usage of a social defeat model. In this paradigm a mouse is forced into the territory of a mouse from a larger, more aggressive strain leading to an interaction resulting in intruder subordination. Repeated defeats over 10 days can result in a long lasting reduced social interaction, sexual dysfunction, sleep dysregulation, anxiety, metabolic deficits and anhedonia [78-81]. Interestingly, following the social defeat procedure there remains a large variance in behavior outcomes in spite of using an inbred mouse strain (C57BL/6). Some animals display a resistance to social defeat (resilience) while others are susceptible (determined by interaction with a social target relative to an empty enclosure). If animals are separated based on this measure, susceptible mice demonstrate decreased sucrose intake, a blunted circadian rhythm, and conditioned place preference to cocaine [80]. Furthermore, phenotypes induced by social defeat in susceptible mice can be reversed by antidepressant treatment [79]. Given that molecular mechanisms for resilience to the stressful procedure are now being worked out [80, 82], it would be intriguing to see if similar pathways are necessary for mediating response to antidepressants.

A third procedure for inducing a depression-like state in animals is administration of chronic glucocorticoids in order to mimic the effects of chronic stress. A significant proportion of depressed patients display altered activity of the HPA axis, and stress generally leads to hypersecretion of corticosteroids, which imposes an increased risk for depression [10, 37, 83-88]. Chronic treatment of rodents with corticosterone effectively induces multiple anxiety- and depression-like changes in behavior, neurochemistry and brain morphology [89-92]. Behaviorally, depression-related changes include suppression of sucrose intake and decreased self-care [90, 93], while anxiety-related changes include increased latency to emerge into the light compartment in the light/dark test, decreased time, entries and percent distance in the center of an open field and increased latency to take a bite of food in the novelty suppressed feeding (NSF) test [90, 91].

ADULT HIPPOCAMPAL NEUROGENESIS IN PATHOLOGICAL CONDITIONS

One of the main findings of the role of adult hippocampal neurogenesis in depression is the observation that antidepressants confer behavioral effects by stimulating neurogenesis in rodents and humans. A recent study showed for the first time in the dentate gyrus in humans that there are more nestin-expressing progenitor cells, and increased dividing cells after treatment with an SSRI antidepressant (sertraline, fluoxetine) or tricyclic (nortriptyline, clomipramine) in patients with depression compared to untreated patients [46]. Another study also showed that antidepressants increase human hippocampal

neurogenesis by activating the glucocorticoid receptor [94]. In nonhuman primates, repeated separation stress resulted in depression-like behaviors [anhedonia and subordination] accompanied by reduced hippocampal neurogenesis [95]. Treatment of the nonhuman primates with fluoxetine stimulated neurogenesis and prevented the emergence of depression-like behaviors. Furthermore, ablation of neurogenesis with irradiation of the nonhuman primates abolished the therapeutic effects of fluoxetine.

To address whether altered neurogenesis is important for the treatment of depression, Deisseroth's group used voltage sensitive dye imaging to probe hippocampal activity in the CMS in Rat and specifically the role of neurogenesis in depression-relevant neurophysiology and behavior [50]. Using irradiation to ablate neurogenesis, Airan and colleagues also found that antidepressant behavioral efficacy in the Forced Swim Test in Rat required intact neurogenesis. Overall, antidepressant treatment was sufficient to transiently increase neurogenesis and exert behavioral effects long after drug clearance from the system, and this effect was absent in animals lacking neurogenesis (X-Ray). Recently, an elegant study in Rat confirmed Deisseroth's study by showing that antidepressants retain some but not all their therapeutic efficacy in reducing measured indices of anxiety/depression-like behavior when hippocampal neurogenesis was blocked by a cytostatic agent [96]. Indeed, using CMS and the antimitotic agent MAM, authors showed that the various antidepressants ameliorated CMS-induced behavioral signs of depression to the same extent in vehicle and MAM-treated animals. Conversely, using the NSF paradigm, they found that the antidepressant drugs studied (imipramine, fluoxetine) reduced the hyperanxious state observed in CMS-exposed rats even though neurogenesis was blocked. Overall, authors concluded that antidepressants re-established neuronal plasticity in hippocampus. In the "CORT model", using X-irradiated mice, in which hippocampal neurogenesis was abolished, we demonstrated that antidepressant treatment still elicits some anxiolytic/antidepressant-like effects. Specifically we found that antidepressant effects in the Open Field and Forced Swim Test were neurogenesis independent, while effects in the Novelty Suppressed Feeding Test or on coat state were neurogenesis dependent. As such, our study reveals that the behavioral effects of fluoxetine are mediated through both neurogenesis-dependent and -independent actions [97]. Previously, Surget and colleagues [73] presented important evidence for both neurogenesis-dependent and -independent mechanisms for the reversal of stress-induced behaviors by antidepressant drugs, including fluoxetine [73]. Our paper, using a different model of stress, extends this study by utilizing a mechanistic approach to propose a neurogenesis-independent pathways for mediating the effects of SSRIs, namely the β -arrestin signaling pathway.

ADULT HIPPOCAMPAL NEUROGENESIS IN DIFFERENT GENDERS

Taking into consideration that depression is twice as common in women as in men, it is important to also consider sex differences in the effects of depression models and antidepressants on adult neurogenesis. In MRL/MpJ mice treated with fluoxetine (10 mg/kg b.i.d.) for 21 days, cell proliferation was increased in both genders, but females produced more new cells than males [95]. Furthermore, while fluoxetine did not alter survival in males, 10 mg/kg reduced survival in females. Another study showed that while acute stress reduced cell proliferation in males, it did not affect proliferation in the female hippocampus [98]. Repeated training with controllable stress did not influence proliferation in females and under all conditions, males were more likely than females to express helplessness behavior. This was true even for males that were not previously stressed. Another study also showed that female rats learn trace memories better than male rats and consequently retain a greater proportion of new neurons in their hippocampi [99]. Therefore, it is critical to also consider gender when planning experiments to study adult neurogenesis.

POTENTIAL MECHANISMS UNDERLYING THE REQUIREMENT OF NEUROGENESIS IN MEDIATING THE ANTIDEPRESSANT RESPONSE

While much work has been done that has laid a foundation for the understanding of how antidepressants increase neurogenesis, much less is known about why the increase in neurogenesis is required for the antidepressant response [2]. One likely mechanism would be negative feedback regulation of the HPA axis and the stress response. Consistent with this hypothesis, recent studies demonstrated that in mice with ablation of neurogenesis there was an increased HPA axis response to an acute stress [100, 101]. Since stimulation of the subiculum, CA3 or DG can yield an inhibitory effect on the HPA axis [102, 103] through well-described circuitry [103-105], it is possible that young neurons may contribute to hippocampal-dependent negative feedback of the HPA axis. One recent study even suggests that chronic stress severely impairs HPA axis activity and the ability of the hippocampus to modulate downstream brain areas involved in the stress response [73]. Chronic antidepressant treatment can restore the relationship between the hippocampus and the HPA axis, but only in the presence of an intact neurogenic niche. Another study also demonstrates that adult-born hippocampal neurons are required for normal expression of the endocrine and behavioral components of the stress response [106]. Future studies will need to use genetic methods to more directly determine if young neurons impact the negative feedback circuit to the HPA axis.

Another hypothesis, which is not mutually exclusive, that has gained traction is whether neurogenesis in different areas of the SGZ play distinct roles in the regulation of mood. Due to participation in different circuitry, it has been suggested that the dorsal and ventral hippocampus may be distinct structures [58]. In the hippocampus, the dorsal dentate gyrus receives inputs from lateral and caudomedial entorhinal cortex and medially located cells of the medial septal nucleus [107]. Outputs of the dorsal hippocampus are to the mammillary complex, dorsal lateral septum and lateral entorhinal cortex. In contrast ventral dentate gyrus receives inputs from the rostromedial entorhinal cortex and laterally located cells of the medial septal nucleus while ventral hippocampus outputs are to the prefrontal cortex, amygdala, nucleus accumbens, hypothalamus, medial entorhinal cortex, bed nucleus of stria terminalis and rostral and ventral lateral septum [108]. This different circuitry may suggest that the dorsal hippocampus is more important for learning and memory while the ventral hippocampus is more involved in emotion [58, 108]. Some lesion studies have supported this hypothesis [109, 110]. Based on this work, it has been proposed that neurogenesis along the dorsal-ventral axis may also play distinct roles in learning and mood [108]. In this idea, the main effect of neurogenesis in the antidepressant response would be on circuitry through ventral structures. Genetic models and ablation techniques that are restricted to dorsal or ventral SGZ need to be developed in order to test this hypothesis.

Much work has been done to advance the understanding of the synaptic and physiological properties of the young neurons and these unique properties allow for distinguishing young neurons from their mature granule neuron counterparts [111, 112]. Of particular relevance to antidepressant treatment is a form of long-term potentiation derived from a weak stimulation paradigm in the absence of GABA blockers [ACSF-LTP] that is sensitive to manipulations that block hippocampal neurogenesis [39, 56]. After chronic, but not acute, fluoxetine treatment, ACSF-LTP is enhanced in sham animals and completely blocked in animals subjected to X-irradiation [56], suggesting an effect of fluoxetine on the electrophysiological properties of young neurons that have integrated into the hippocampal circuitry.

Relatively little work has addressed the function of young neurons in an intact hippocampal circuit in vivo. In hippocampal slice work, it has been demonstrated that fluoxetine treatment enhances activity of the dentate gyrus relative to CA1 in a neurogenesis-dependent manner [50], suggesting a network effect of the young neurons. Furthermore, one very recent study used multiple methods to ablate adult

neurogenesis in vivo and assessed hippocampal activity [113]. In anesthetized mice after X-irradiation or thymidine kinase mediated pharmacogenetic ablation, perforant-path evoked responses were reduced in magnitude. In striking contrast, there was an increase in the amplitude of spontaneous gamma-frequency bursts in the dentate gyrus and hilus, as well as increased synchronization of dentate neuron firing to these bursts. This striking result may suggest that the young neurons can serve to modulate activity in the much larger population of mature granule cells rather than acting solely as independent encoding units [113]. One could imagine that antidepressant treatment may modulate hippocampal circuitry by enhancing this effect of the young neurons on the mature granule neurons, but this possibility remains to be tested.

REVISITING THE NEUROGENESIS HYPOTHESIS OF DEPRESSION

The neurogenesis hypothesis of depression postulated that a decrease in the production of newborn granule cells in the dentate gyrus is related to the pathophysiology of depression while enhanced hippocampal neurogenesis is required for the beneficial effects of antidepressant treatment. With few exceptions [51, 114], in most studies ablation of hippocampal neurogenesis alone is not sufficient to induce a phenotype reminiscent of either anxiety or depression [14, 21, 39, 50, 52, 69, 73, 107]. It is also unlikely that decreased neurogenesis could account for the volumetric decreases seen in the hippocampus of depressed patients, as X-irradiation of mouse hippocampus does not yield a significant reduction [21]. Whether specific manipulations that increase hippocampal neurogenesis alone result in a “non-depressed” phenotype remains to be tested. However, evidence is strong that neurogenesis is required for at least some of the beneficial effects of antidepressant treatment. It will be critical for future work to determine how the addition of new units to the dentate is involved in mediating the effects of antidepressants.

It will also be critical for future work to validate the importance of antidepressant-induced neurogenesis in translational studies in humans. It will be important to test if biomarkers (such as CBV and MRS) are increased in patients treated with antidepressants. Furthermore, it may be interesting to correlate rates of neurogenesis as measured by these biomarkers with improvement of depressive signs and symptoms.

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L'Article 2 ci-dessous intitulé «*Neurogenèse hippocampique adulte: Un acteur dans l'action antidépresseur*» revisite le rôle de la neurogenèse hippocampique adulte dans des modèles animaux physiopathologiques de troubles de l'humeur.

Article 2: **Adult hippocampal neurogenesis: an actor in the antidepressant-like action.**

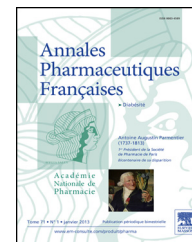
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UPDATE

Adult hippocampal neurogenesis: An actor in the antidepressant-like action

La neurogenèse hippocampique adulte : un acteur dans le mécanisme d'action des antidépresseurs

I. Mendez-David^a, R. Hen^{b,c}, A.M. Gardier^a,
D.J. David^{a,*,1}

^a EA 3544 « pharmacologie des troubles anxio-dépressifs et neurogenèse », faculté de pharmacie, université Paris-Sud, Tour D1, 2^e étage, 5, rue J.-B.-Clement, 92296 Chatenay-Malabry cedex, France

^b Department of psychiatry, Columbia university, New York, 10032, USA

^c Department of neuroscience, Columbia university, New York, 10032, USA

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Summary Depression and anxiety are psychiatric illnesses that are major burdens in society and affect as much as 7% of the world's population. The heterogeneous nature of depression suggests an involvement of multiple distinct brain regions including amygdala, prefrontal cortex and the hippocampus, which may be responsible for the diversity of the symptoms. Besides its critical role in learning and memory, the hippocampus is one of only two areas in mammalian brain where adult neurogenesis occurs. Of the current leading hypotheses of the pathophysiology and treatment of depression, the neurogenesis hypothesis of depression deserves particular attention because changes in neurogenesis are only seen after chronic, but not acute, antidepressant treatment. This review revisits the role of adult hippocampal neurogenesis in the pathophysiology of mood disorders, especially anxiety/depression, and also in the antidepressant-like responses, especially in stressed rodents.

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* Corresponding author.

E-mail address: denis.david@u-psud.fr (D.J. David).

¹ <http://www.neuropharmacologie.u-psud.fr/>.

MOTS CLÉS

Dépression ;
Antidépresseur ;
Hippocampe ;
Neurogenèse adulte

Résumé La dépression et l'anxiété sont des maladies psychiatriques qui touchent plus de 7% de la population mondiale. Le caractère hétérogène de la dépression suggère une implication de plusieurs régions cérébrales distinctes dans la physiopathologie (comme l'amygdale, le cortex préfrontal ou hippocampe), qui peuvent être responsables de symptômes divers. En plus de son rôle essentiel dans l'apprentissage et la mémoire, l'hippocampe est l'une des deux seules régions du cerveau des mammifères où se produit la neurogenèse adulte. Parmi les principales hypothèses actuelles concernant la physiopathologie et le traitement de la dépression, l'hypothèse neurogénique mérite une attention particulière en raison des changements observés uniquement après un traitement chronique par antidépresseur, et non pas après l'administration d'une dose unique. Cette revue revient sur le rôle à proprement parlé de la neurogenèse hippocampique adulte dans la physiopathologie des troubles de l'humeur, notamment l'anxiété/la dépression, mais aussi dans les réponses antidépresseurs à travers des études précliniques effectuées chez le rongeur soumis à un stress chronique.

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Introduction

Depressive and anxiety disorders are a major burden in society. Mood disorders affect 7% of the world's population, while severe forms of depression impact 2–5% of the US population [1–3]. Furthermore, approximately 32–35 million adults in the US population (16%) experience an episode of major depression in their lifetime [1]. In Europe, a meta-analysis based on 27 eligible studies including over 150,000 subjects from 16 European countries estimated the prevalence of depression between 3 to 10% during the last 12 months [4]. The heterogeneous nature of depression suggests an involvement of multiple distinct brain regions, which may be responsible for the diverse symptoms. Human imaging and post-mortem studies of the brain have supported this hypothesis, implicating brain areas including the prefrontal and cingulate cortex, hippocampus, ventral striatum, amygdala, and thalamus [5]. Together, these brain regions operate a series of highly interacting circuits that forms a neural circuitry involved in depression [6]. The hippocampus is one of several limbic structures that have been extensively studied in individuals with psychiatric and neurologic disorders in the last decade [6,7]. Besides its critical role in learning and memory, the hippocampus is one of the only two areas in mammalian brain where adult neurogenesis occurs [7]. Adult hippocampal neurogenesis is therefore defined as the progression from neural stem cell to mature dentate granule neuron.

To fully understand the pathophysiology and treatment of depression, it is essential to delineate molecular, cellular and circuit-level changes in depressive state and also after chronic antidepressant treatment. Of the current leading hypotheses of the pathophysiology and treatment of depression, the neurogenesis hypothesis of depression deserves particular attention because changes in neurogenesis are only seen after chronic, but not acute, antidepressant treatment. This review revisits the role of adult hippocampal neurogenesis in the pathophysiology of mood disorders, especially anxiety/depression, and also in the antidepressant-like responses measured especially in stressed rodents.

Hippocampal neurogenesis

Neurogenesis refers to the production of new neurons in the brain. Originally, it was only described during development of the central nervous system. Ramon y Cajal (1913) stated that the adult brain was unable to generate new neurons. This dogma was first questioned by Altman in the 1960s, who revealed the genesis of new cells in the brain of adult rat and cat by autoradiography with tritiated thymidine [8]. Unfortunately, it was uncertain whether the new cells were actually neuronal cells. Many years later, a combination of specific neuronal markers with an analogue of thymidine, 5-bromo-2'-deoxy-uridine (BrdU), confirmed the neuronal phenotype [9]. The process of adult neurogenesis is located in two discrete brain regions: the subventricular zone (SVZ) and subgranular zone (SGZ) of dentate gyrus of the hippocampus. In this review, only the hippocampal neurogenesis and its involvement in depression will be presented.

Production of new neurons in the subgranular zone of the dentate gyrus

Hippocampal neurogenesis is possible in the SGZ of the dentate gyrus of the hippocampus because of the presence of stem cells. These stem cells evolve into neural progenitor cells that can produce multiple cell types in the central nervous system such as neurons, astrocytes, oligodendrocytes, or microglial cells. In rodents, the duration of the mitotic cycle of proliferating precursors is approximately 12 to 24 hours, leading to the production of about 8000 to 10,000 new neurons per day [10]. Given that the dentate gyrus consists of approximately one million granule cells, this phenomenon is capable of generating a little less than 1% of total granule cells each day. However, the proportion of new neurons that survive beyond 1 month is less than 50%, and the production of new cells is offset by the daily loss of mature granule cells. The surviving cells have predominantly a neuronal phenotype (75%), mainly glutamatergic granule cells, while very few are GABAergic interneuron basket cells.

Brain-derived neurotrophic factor (BDNF), depression and adult hippocampal neurogenesis

Neurotrophins, including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), are important regulators of neuronal activity, influencing a plethora of events such as neuronal differentiation, maintenance, survival, and synaptic plasticity throughout life. BDNF, a secretory glycoprotein of the neurotrophin family, is widely expressed in the adult central nervous system [11]. Like other neurotrophins, BDNF is synthesized and released in an activity-dependent manner. Studies have shown lower BDNF levels in post-mortem hippocampus of depressed patients, but higher levels in patients who are taking antidepressants at the time of death [12]. Stronger evidence for a causal role of hippocampal BDNF in the action of antidepressants comes from animal studies [13]. Direct infusion of BDNF into the dentate gyrus of the hippocampus exerted potent antidepressant effects in rodents. Furthermore, animal studies indicated that stress and chronic antidepressant treatments have opposing actions on hippocampal BDNF levels [13]. Specifically, antidepressants increase hippocampal BDNF mRNA levels, and this increase is dependent upon chronic treatment, which is consistent with the delayed onset of the therapeutic effects of antidepressants. Conversely, numerous studies have shown that different forms of stress decrease hippocampal BDNF mRNA levels [13]. Given the key role of BDNF/TrkB in mood regulation and the mechanism of action of antidepressants as reviewed earlier, a number of studies have investigated the role of BDNF/TrkB signaling in hippocampal neurogenesis. Experimental conditions that induce BDNF expression, such as physical exercise, enriched environment, and chronic antidepressant treatment, also increase hippocampal neurogenesis [14]. The survival of newborn cells is decreased in both BDNF \pm mice and TrkB dominant-negative mice. In addition, enriched environment failed to increase the survival of newborn cells in BDNF \pm mice [15]. These results suggest a role for BDNF/TrkB signaling in the survival of newborn hippocampal neurons. In contrast, there have been mixed results on the role of BDNF/TrkB signaling on hippocampal cell proliferation.

Hippocampal neurogenesis and major depression

Revisiting the neurogenesis hypothesis of depression

The neurogenic hypothesis of depression postulates that decreased production of new granule cells in the dentate gyrus of the hippocampus is linked to the pathophysiology of depression and that the increase in hippocampal neurogenesis is required for the behavioral effects of antidepressant treatment [16,17]. The few studies of hippocampal neurogenesis in depressed patients published to date have mainly relied on histological examinations of post-mortem brain tissue [18,19]. The main findings in one of these post-mortem studies are that antidepressant treatment

results in an increase in the number of neural progenitors in the anterior dentate gyrus [18,20]. This was not observed in another study but these authors did not examine specifically the anterior hippocampus [19]. In both studies there was no difference between control and untreated depression. A reduction in hippocampal volume in depressed patients is somewhat established, and two meta-analyses confirmed this reduction in hippocampal volume in patients with depression using magnetic resonance imaging [21]. The length of depressive episodes coincides with the severity of the decline in hippocampal volume. However, pathophysiological studies on post-mortem brain tissue indicate that changes in the neuropil, a possible consequence of a decrease in connectivity, and glial cells may be responsible for reducing the volume of the hippocampus [22]. However, it is unlikely that such change in hippocampal volume will be difficult to determine according to the ratio between numbers of neuron provided by neurogenesis according to the total number of hippocampal neurons.

Preclinical studies in rodents, using approaches that manipulate neurogenesis, are used to establish the relationship between hippocampal neurogenesis and the behavioral effects of antidepressants in animals that are either stressed or not. Several methods to date have been developed to reduce or increase neurogenesis in rodents (Table 1):

- an X-irradiation of either the whole brain or locally in the hippocampus [16,23,24];
- asystemic treatment with an anti-mitotic agent such as methylazoxymethanol acetate (MAM) [25];
- a genetic manipulation, such as GFAP-TK mice, in which the glial fibrillary acidic protein (GFAP)-positive progenitor cells die following treatment with ganciclovir [23,26] or the inducible over-expression of pro-apoptotic gene Bax specifically in neural precursors [27].

The suppression of hippocampal neurogenesis in mice does not alter anxiety behavior in the open field or the light/dark paradigms, the elevated plus maze, or novelty suppressed feeding tests [16,23]. Thus, except one study [28], the X-irradiation in the hippocampus has no effect in the previously mentioned paradigms suggesting that the loss of hippocampal neurogenesis is not sufficient to induce a behavioral phenotype of anxiety/depression in mice, and does not exacerbate those induced by stress. Similarly, ablation of neurogenesis by a subchronic treatment with the toxin MAM, is not sufficient to induce an anhedonic behavior in rats [25].

Sahay et al. [29] developed a genetic gain of function strategy to inducibly augment the survival of adult-born neurons in a cell-autonomous manner [29]. Because 60–80% of young adult-born neurons undergo programmed cell death, for which the pro-apoptotic gene Bax is required, they used a transgenic mouse line in which the tamoxifen (TAM)-regulatable recombinase CreERT2 is expressed under the control of a 5.26-kilobase fragment of the rat nestin (Nes) gene promoter [30] together with a floxed Bax mouse line to ablate Bax selectively in neural stem cells in the adult brain and promote survival of these cells. Using this strategy, increasing adult hippocampal neurogenesis alone

Table 1 Methods used to ablate or increase neurogenesis.
Méthodes permettant la manipulation de la neurogenèse.

Pharmacological manipulation	Cranial irradiation	Genetic manipulation
5-fluorouracil (5-Fu), methylazoxymethanol (MAM), temozolomide (TMZ), cytosine arabinoside (Ara-C) [23,25,35,36]	X-irradiation, gamma-rays [16,23,24,37]	GFAP-TK, Nestin-TK, Nestin-Bax, Nestin ^{Cre} ERT2-Bax, TrkB ^{lox/lox} -Cre ERT2 Cyclin D2 transgenic [26,27,29]

does not produce an anxiolytic or antidepressant-like response. However, it is important to note that this induction of neurogenesis was performed in “non-stressed animals”. Further studies will be necessary to assess whether genetic strategies designed to specifically increase adult hippocampal neurogenesis are sufficient to reverse the effects of chronic stress.

In sum, there is evidence indicating that a decreased neurogenesis is not a major factor in the development of depression, but that an increase in neurogenesis may be necessary for the behavioral effects of antidepressants [3].

Antidepressant and adult hippocampal neurogenesis in pathological conditions

Animal models of depressive phenotypes

Since no genetic variants with high penetrance that cause depression are known, animal models have mainly relied on different means of chronically exposing rodents to stressful experiences, or sensory tract lesions such as in olfactory bulbectomy, to induce behavioral states that present depression-like symptoms and are responsive to chronic antidepressant treatment.

A recent procedure for inducing a depression-like state in animals is chronic glucocorticoid administration in order to mimic the effects of chronic stress. A significant proportion of depressed patients display altered activity of the hypothalamic-pituitary-adrenal (HPA) axis, and stress generally leads to hypersecretion of corticosteroids, which imposes an increased risk for depression [31]. Chronic treatment of rodents with corticosterone effectively induces multiple anxiety- and depression-like changes in behavior, neurochemistry and brain morphology [24,32,33]. Behaviorally, depression-related changes include suppression of sucrose intake and decreased self-care [33], while anxiety-related changes include:

- increased latency to emerge into the light compartment in the light/dark test;
- decreased time, entries and percent distance in the center of an open field;
- increased latency to take a bite of food in the novelty suppressed feeding (NSF) test.

Adult hippocampal neurogenesis in pathological conditions

Using a model based on the exogenous elevation of glucocorticoids (named the “CORT-model”), a reduction in the proliferation of progenitor cells after chronic corticosterone treatment was observed, demonstrating a role for glucocorticoids in the regulation of the proliferation stage of the neurogenic process [18]. The effects of corticosterone on neurogenesis are limited to the proliferation stage and do not involve the survival or maturation of newborn neurons. Interestingly, the effects of fluoxetine on all stages of neurogenesis (proliferation, differentiation, maturation and survival) were more pronounced in corticosterone-treated mice than in controls. It is possible that our model of corticosterone-induced stress may increase the dynamic range in which fluoxetine exerts its effects on different stages of adult hippocampal neurogenesis [24].

Agomelatine is an antidepressant drug that has a different mechanism of action from currently available antidepressants (melatonergic agonist and 5-HT_{2C} antagonist properties). Thus, we assessed its effects on neurogenesis in the “CORT-model” of anxiety/depression [34]. Similarly to fluoxetine, chronic administration of agomelatine reversed the decrease in cell proliferation in the hippocampus without any discrimination between the dorsal and the ventral hippocampus (Table 2).

Neurogenesis-dependent and independent effects of antidepressants

The most compelling evidence to link adult hippocampal neurogenesis with antidepressants comes from our laboratory, in a study demonstrating that neurogenesis is necessary for the effects of imipramine, a tricyclic antidepressant, and fluoxetine, a selective serotonin reuptake inhibitor, in two mouse behavioral screens for antidepressant activity [16]. To address whether altered neurogenesis is important for the treatment of depression, Deisseroth's group used voltage sensitive dye imaging to probe hippocampal activity in the Chronic Mild Stress in Rat. They specifically studied the role of neurogenesis in depression-relevant neurophysiology and behavior [35]. Using irradiation to ablate neurogenesis, Airan et al. also found that antidepressant behavioral efficacy in the Forced Swim Test in Rat required intact hippocampal neurogenesis. Overall, antidepressant treatment was sufficient to transiently increase

Table 2 Behavioral and neurogenic effects of various chronic antidepressant treatments in the “CORT”-model in mice. *Effets comportementaux et neurogéniques de différents traitements chroniques par antidépresseurs dans le modèle « CORT » chez la souris.*

Drugs	Pharmacological target	Phenotype	Neurogenic effects	References
Fluoxetine	Serotonin reuptake inhibitors	Reversed anxiogenic/depressive-like phenotype No effect on the flattened circadian rhythm induced by chronic corticosterone	Reversed the decrease in cell proliferation induced by chronic corticosterone Increased all steps of adult hippocampal neurogenesis For all neurogenic parameters in the hippocampus: effects more pronounced in corticosterone-treated mice	[24,34]
Imipramine	Tricyclics	Reversed anxiogenic/depressive-like phenotype	Not tested	[24]
Reboxetine	Norepinephrin reuptake inhibitors	Reversed anxiogenic/depressive-like phenotype	Not tested	[24]
Agomelatine	MT1/MT2 agonist and 5-HT _{2C} antagonist	Reversed anxiogenic/depressive-like phenotype Reversed the flattened circadian rhythm induced by chronic corticosterone	Reversed the decrease in cell proliferation induced by chronic corticosterone Reversed (ventral effects for maturation)	[34]

neurogenesis and exert behavioral effects long after drug clearance from the system. This effect was absent in animals lacking neurogenesis (X-ray). Recently, an elegant study in rat showed that antidepressants retain some, but not all, of their therapeutic efficacy in reducing indices of anxiety but not depression-like behavior when hippocampal neurogenesis was blocked by a cytostatic agent [36]. Indeed, using chronic mild stress (CMS) and the anti-mitotic agent MAM, authors showed that various antidepressants ameliorated CMS-induced behavioral signs of depression to the same extent in vehicle and MAM-treated animals. Conversely, using the NSF paradigm, they found that the antidepressant drugs studied (imipramine, fluoxetine) reduced the hyper-anxious state observed in CMS-exposed rats even though neurogenesis was blocked. Overall, authors concluded that antidepressants re-established neuronal plasticity in the hippocampus [36]. In the “CORT-model”, using X-irradiated mice, in which hippocampal neurogenesis was abolished, we demonstrated that antidepressant treatment still elicits some anxiolytic/antidepressant-like effects. Specifically, we found that antidepressant effects in the Open Field and Forced Swim Test were neurogenesis independent, while effects in the Novelty Suppressed Feeding Test

or on coat state were neurogenesis-dependent. As such, our study revealed that the behavioral effects of fluoxetine are mediated through both neurogenesis-dependent and -independent actions [24]. Previously, Surget et al. [37] presented important evidence for both neurogenesis-dependent and -independent mechanisms for the reversal of stress-induced behaviors by antidepressant drugs, including fluoxetine [37].

Potential mechanisms underlying the requirement of neurogenesis in mediating the antidepressant response

Despite all the work done that has laid a foundation for the understanding of how antidepressants increase adult hippocampal neurogenesis, much less is known about why the increase in neurogenesis is required for the antidepressant response [3]. One likely mechanism would be negative feedback regulation of the HPA axis and the stress response. It is possible that young neurons may contribute to hippocampal-dependent negative feedback of the HPA axis. One recent

study suggests that chronic stress severely impairs HPA axis activity and the ability of the hippocampus to modulate downstream brain areas involved in the stress response [37]. Chronic antidepressant treatment can restore the relationship between the hippocampus and the HPA axis, but only in the presence of an intact neurogenic niche. Another study also demonstrates that adult-born hippocampal neurons are required for normal expression of the endocrine and behavioral components of the stress response [38]. Future studies will need to use genetic methods to determine more directly if young neurons impact the negative feedback circuit to the HPA axis.

Another hypothesis, which is not mutually exclusive, that has gained attraction is whether neurogenesis in different areas of the SGZ play distinct roles in the regulation of mood. Due to participation in different circuitries, it has been suggested that the dorsal and ventral hippocampus may have distinct roles [39,40]. In the hippocampus of Rodents, the dorsal dentate gyrus receives inputs from lateral and caudomedial entorhinal cortex and medially located cells of the medial septal nucleus [41]. Outputs of the dorsal hippocampus are to the mammillary complex, dorsal lateral septum and lateral entorhinal cortex. In contrast, ventral dentate gyrus receives inputs from the rostromedial entorhinal cortex and laterally located cells of the medial septal nucleus, while ventral hippocampus outputs are to the prefrontal cortex, amygdala, nucleus accumbens, hypothalamus, medial entorhinal cortex, bed nucleus of stria terminalis and rostral and ventral lateral septum [42]. These different anatomical circuitries suggest that the dorsal hippocampus is a more important brain area for learning and memory, while the ventral hippocampus is more involved in emotion [39,42]. In this idea, the main effect of neurogenesis in the antidepressant response would be on circuitry through ventral structures. Genetic models and ablation techniques that are restricted to dorsal or ventral SGZ need to be developed in order to test for this hypothesis.

Conclusions

The neurogenic hypothesis of mood disorders remains promising for conceptualizing depression mechanisms, which may lead to novel avenues for treatments of these psychiatric diseases [43]. Clinically, we need more information on the level and regulation of human adult hippocampal neurogenesis. Interestingly, neurogenesis decreases with age in humans and animals (Gould, 1999), whereas depression prevalence increases with age. As recently suggested by Eisch et al., more research is warranted to examine to what extent the age-induced increase in depression is due to life experience, age-induced increase in medical burden, or possibly age-induced decrease in neurogenesis [4,5,12,25]. Also, in vivo imaging of correlates of human neurogenesis is difficult, and greater technical advances are needed before we can conclude what aspects of neurogenesis structure and function are shared between humans and laboratory animals, and modulated by stress or antidepressants. Thus, it will be important to test if potential biomarkers (such as cerebral blood volume and magnetic resonance spectroscopy) [44,45] are increased in patients treated with antidepressants. Furthermore, it will

be interesting to correlate rates of neurogenesis as measured by these biomarkers with improvement in depressive symptoms.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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4- Le récepteur 5-HT₄ et son implication dans la dépression

The current classification of serotonin receptors reveals multiple subtypes (Filip and Bader, 2009; Fink, and Gothert, 2007). Apart from the 5-HT₃ receptor which is a ligand-gated cation channel, all the other 5-HT receptors are metabotropic receptors coupled to G proteins and containing a predicted seven-transmembrane domain structure. The different subtypes of serotonin receptors can be divided in seven families: 5-HT₁ (1A, 1B, 1D, 1E, 1F) 5-HT₂ (2A, 2B, 2C), 5-HT₃ (3A, 3B, 3C, 3D, 3E), 5-HT₄, 5-HT₅ (5A, 5B), 5-HT₆ and 5-HT₇ (**Figure 4**).

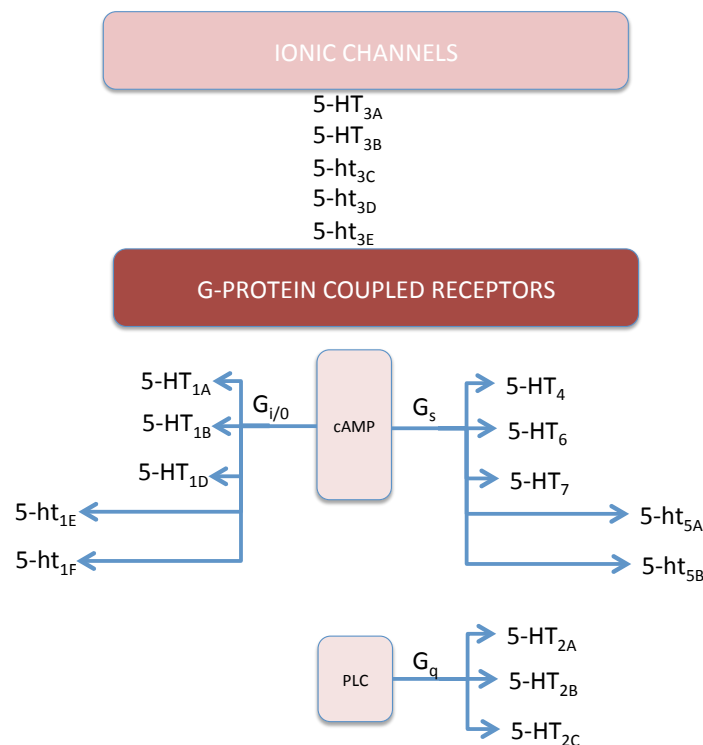


Figure 4 : Classification of 5-HT receptor (adapted from Hoyer et al., 2002).

The classification shows the different subtypes of 5-HT receptor and their coupling.

The 5-HT₅ receptor is at present not yet functionally identified and its physiological role remains to be defined; thus, it can only be provisionally classified retaining its lower case appellation (Hannon and Hoyer, 2008).

The present study is focused on the 5-HT₄ receptor subtype. The 5-HT₄ receptor is one of 14 serotonin receptors in the mammalian nervous system (Barnes and Sharp, 1999). Along with 5-HT₆ and 5-HT₇ receptors, these metabotropic receptors are positively coupled to adenylyl cyclase, and thus stimulate the production of the second messenger cAMP (Gerald et al., 1995).

I. Généralités

The 5-HT₄ receptor was originally identified by its pharmacology that was unique among the serotonin receptor subtypes known at the time. Specifically, in the late 1980's there was speculation about a novel 5-HT receptor subtype that was present in neurons of the colliculus and hippocampus that stimulated adenylyl cyclase activity and increased cAMP production, but was insensitive to known antagonists of the 5-HT₁, 5-HT₂ and 5-HT₃ receptor subtypes (Dumuis et al. 1988; Bockaert et al. 1990; Bockaert et al., 2004). For a long time, the 5-HT₃ and 5-HT₄ receptors seemed closely related due to their similar pharmacological profile. Indeed, the first ligands discovered for the 5-HT₄ receptor had also a high affinity for the 5-HT₃ receptor (Dumuis et al., 1988; Eglen et al., 1995; Bockaert et al., 2004). In 1992, the 5-HT₄ receptor was finally recognized as a new serotonergic receptor subtype and subsequent development of ligands with affinity and/or selectivity for this receptor has grown exponentially throughout the years (Bockaert et al., 2004; Dumuis et al., 1988; Eglen et al., 1995; Bockaert et al., 2004). In the late 1990's, the gene coding for the 5-HT₄ receptor, which is exceptionally large and complex (700kb, 38 exons), was simultaneously cloned in two different species mouse and rat (Gerald et al., 1995; Claeysen et al., 1996; Bockaert et al., 2004).

The 5-HT₄ receptor possesses three intracellular loops and three extracellular loops. The amino terminus is oriented toward the extracellular space, whereas the carboxyl terminus (C-terminus) is oriented toward the cytoplasm. Primary sequences of the different isoforms are identical throughout the first 358 residues but divergences at their C-terminal end suggest differences in G protein coupling (Claeysen et al., 1999; Bockaert et al., 2004) (**Figure 5**).

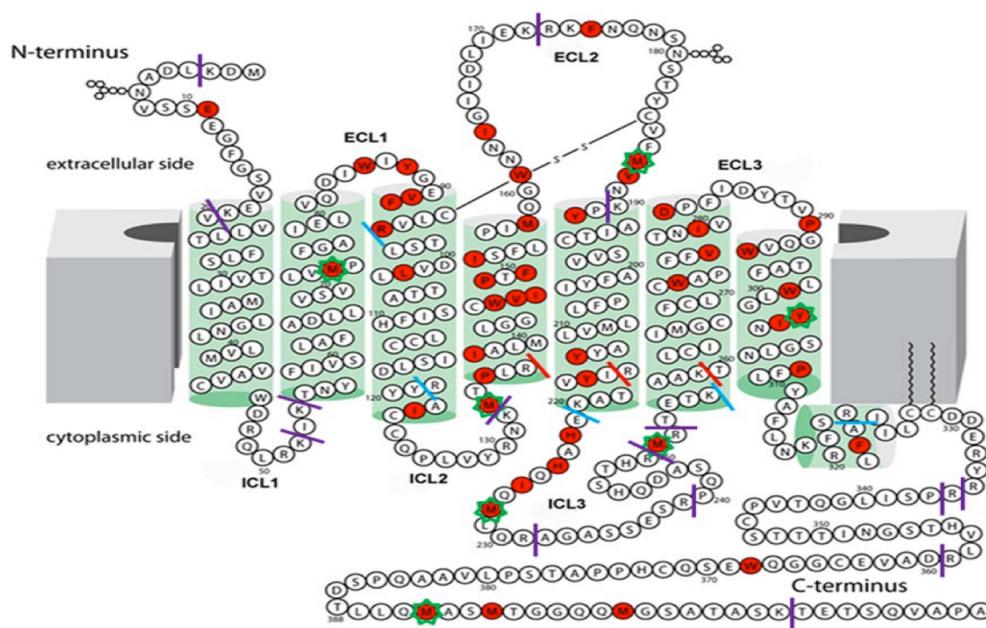


Figure 5 : 5-HT₄ receptor sequence (Padayatti et al., 2013).

Two-dimensional representation of the 5-HT₄ receptor sequence.

To date, it is well known that 5-HT₄ receptors can exist in multiple isoforms with distinct functional properties (Claeysen et al., 1999; Pindon et al., 2002; Ponimaskin et al., 2002; Bockaert et al 2004). Alternative splice variants of the 5-HT₄ receptor have been identified in both rodent and human totalling nine identified variants, though all variants do not appear in every species. Humans have at least five of these variants, whereas mice only have four (Claeysen et al., 1999) (**Figure 6**). The functional differences in the alternative forms have yet to be firmly established, though in general, differences in the intracellular loops (i3 in particular) and the carboxyl termini can result in differences in G protein coupling. These isoforms can be further modified by phosphorylation and palmitoylation at

their carboxyl-terminal or intracellular loops that can influence signaling and regulation (Barthet et al., 2005).

C-termini of 5-HT ₄ R splice variants			
	358		
m5-HT ₄ R	(a)	LRYTVLHSGHHQELEKLPIHNDPESLESCF	387 *
	(b)	LRDAVECGGQWESRCHLTATSPLVAAQPSDT	388 *
	(e)	LSFPLLFRNRVPVPV	371 *
	(f)	LRPVPV	363 *
r5-HT ₄ R	(a)	LRYTVLHSGQHQELEKLPIHNDPESLESCF	387 †
	(b)	LRDTVECGGQWESRCHLTATSPLVAAQPSDT	388 †
	(e)	LSFPLLFCNRVPVPV	371 *
h5-HT ₄ R	(a)	LRYTVLHRGHHQELEKLPIHNDPESLESCF	387 *
	(b)	LRDAVECGGQWESQCHPPATSPLVAAQPSDT	388 §
	(c)	LSSGTETDRRNFGIRKRRLTKPS	380 ‡
	(d)	LRF	360 ‡
	(e)	LSGCSPVSSFLLFCNRVPVPV	378 *

Figure 6 : C-terminal amino acid sequences of the 5-HT₄ receptor splice variants (Claeyssen et al., 1999).

All of the cloned 5-HT₄ receptors share the same amino acid sequence up to Leu 358 and differ from Leu 358 on (boxed). *Receptors cloned by (Claeyssen et al., 1996; Claeyssen et al., 1997). †Receptors cloned by Gerald et al. (1995). ‡Receptors previously cloned by Blondel et al. (1998). §Receptors cloned by Van den Wyngaert et al. (1997); m stands for mouse, r for rat and h for human. Note that the amino acid sequences after Leu 358 of newly cloned m5-HT₄(e), m5-HT₄(f), r5-HT₄(e), and h5-HT₄(e) receptors are shaded. The EMBL Nucleotide Sequence Database accession numbers for mouse 5-HT₄(a), 5-HT₄(b), 5-HT₄(e) and 5-HT₄(f) receptors are Y09587, Y09585, Y09588, and AJ011369, respectively. For r5-HT₄(a), r5-HT₄(b), and r5-HT₄(e) receptors, the numbers are U20906, U20907, and AJ011370, respectively, and for h5-HT₄(a), h5-HT₄(b), h5-HT₄(c), h5-HT₄(d) and h5-HT₄(e) receptors, Y09586, Y10437, Y12506, Y12507, and AJ011371, respectively.

Through the use of PCR and quantitative analysis of mRNA levels, the researchers found a strong expression of isoforms a, b, c, g and n in the central nervous system (CNS) (Vilaro et al 2002, 2005; Bockaert et al., 2004). Isoform (a) appears to be highly expressed in the amygdala, hippocampus, nucleus accumbens and caudate nucleus; some expression was also found in the small intestine, the atrium, and pituitary gland. Isoform (b) appears to be the

most predominant form in the CNS and periphery such as the caudate nucleus, putamen, amygdala, the pituitary gland and the small intestine. As for isoform (g), it seems to be highly expressed in the hypothalamus and cortex; then isoform (c) is highly expressed in the pituitary gland and small intestine, but it is also found at some lower levels in the caudate nucleus, hippocampus, and putamen. Conversely, isoform (d) was not detected in the CNS and is present only in the small intestine (Vilaro et al 2002, 2005 ; Bockaert et al., 2004). Alternative splicing of the receptor results in almost identical pharmacological properties, but with distinct functional profiles.

The emergence of functional studies with transfected 5-HT₄ receptor splice variants showed interesting differences in efficacy and potency as well as sensitivity toward homologous agonist-induced desensitization (Mialet et al., 2003; Bockaert et al., 2004; Ponimaskin et al., 2005). In addition, several studies indicate that differences between splice variants in intrinsic activity suggest a role of the carboxyl terminus in maintaining an inactive state of the receptor (Mialet et al., 2003). Further investigations have shown that progressive C-terminal deletions resulted in greater constitutive activity, which goes in line with previous findings that demonstrated that the shorter variants display greater production of cAMP in the absence of ligand (Claeysen et al., 1999).

The study of signaling and trafficking of 5-HT₄ receptors is a relatively new area of research when compared to other GPCR's such as opioid or dopamine receptors. However, numerous studies suggest that isoform-specific difference in 5-HT₄ receptors might impact the overall coupling and regulation of this receptor and their potential as future targets for therapeutic intervention (Barthet et al., 2005; Vilaro et al., 2005; Bohn and Schmid, 2010; Marin et al 2012 and Mnie-Filali and Pineyro, 2012).

II. Localisation et fonctionnalité du récepteur 5-HT₄

The 5-HT₄ receptors are expressed in a wide variety of tissues of vertebrates including brain, esophagus, ileum, colon, adrenocortical cells, urinary bladder, and heart (Eglen et al., 1995).

A. Localisation et fonctionnalité du récepteur 5-HT₄ à la périphérie

The 5-HT₄ receptor has been shown to play important roles in the heart, gastrointestinal tract, adrenal gland, and urinary bladder, as well as in the central nervous system (Hegde and Eglen, 1996) (**Figure 7**). The cardiac effects mediated by 5-HT₄ receptors are present in the atrium with tachycardia and increased myocardial contractility. In the gastrointestinal tract, activation of 5-HT₄ receptors has been found to facilitate acetylcholine (ACh)-induced motility (Eglen et al., 1990; Kilbinger and Wolf, 1992), to mediate peristalsis and secretion of electrolytes (Craig and Clarke, 1991). 5-HT₄ receptors have also been shown to modulate ACh-induced bladder contractions (Hegde and Eglen, 1996). In the adrenal gland, it was shown that secretion of corticosteroids, and regulation of the production of aldosterone and cortisol is modulated by 5-HT₄ receptor (Lefebvre et al., 1992) and in the urinary bladder 5-HT₄ receptors modulate cholinergic/purinergic transmission (Boyd & Rohan, 1994). Moreover, these receptors are involved in the control of nausea and vomiting as well as smooth muscle tone, mucosal electrolyte secretion and peristaltic reflex (Hegde and Eglen, 1996).

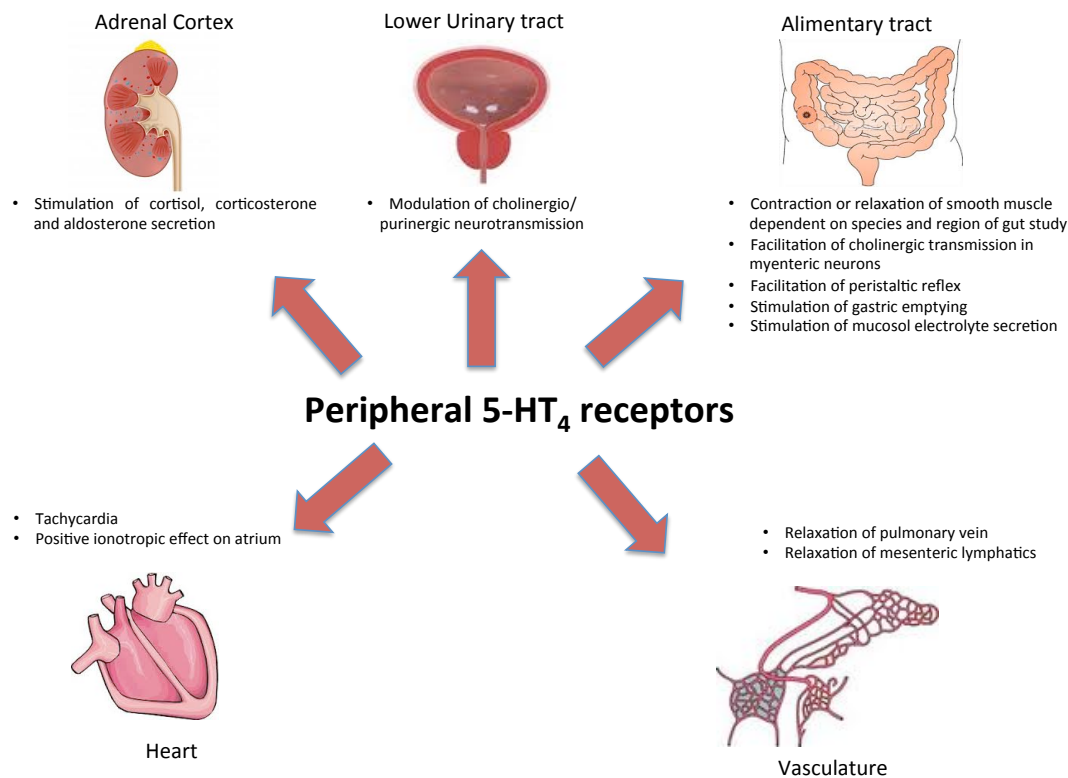


Figure 7: Localization and major effect of 5-HT₄ receptors in various peripheral organs (adapted from Hedge and Eglen 1996).

B. Localisation et fonctionnalité du récepteur 5-HT₄ au niveau du système nerveux central

1. Localisation au niveau du système nerveux central

The development in 1993, of two new specific radioligands of the 5-HT₄ receptor, the antagonists [³H]-GR 113808 and [¹²⁵I]-SB 207710, revolutionized the study of this receptor. Their use in biochemical assays and autoradiography of different species allowed an accurate determination of the regional distribution of 5-HT₄ receptor in the brain (Grossman et al., 1993).

The vast majority of 5-HT₄ receptors are expressed in the hypothalamus, hippocampus, nucleus accumbens, the ventral pallidum, amygdala, the basal ganglia, olfactory bulbs, frontal cortex, the septal area, the substantia nigra, the fundus striatus (**Figure 8**) (Bockaert et al., 2004; Eglen et al, 1995; Vilario et al, 1996; Vilario et al., 2005; Waeber et

al., 1993). The localization of these receptors in the limbic system suggests a role in controlling emotions.

5-HT₄ receptors are heteroreceptors, located in the somatodendritic compartment and axon terminals of the spiny efferent neurons of the striatum containing γ -aminobutyric acid (GABA) (Compan et al., 1996; Cai et al., 2002; Bockaert et al, 2004; King et al., 2008). They are also expressed in glutamatergic pyramidal neurons in the medial prefrontal cortex and hippocampus (CA1, CA3) and granule cells of the dentate gyrus (Roychowdhury et al., 1994; Bockaert et al, 2004 ; Vilaro et al., 2005; King et al., 2008). In the cortex, hippocampus and amygdala, 5-HT₄ receptors are also localized on cholinergic neurons where they stimulate the release of acetylcholine (Waeber et al., 1994; Bockaert et al, 2004; King et al., 2008). Recently, it has been shown that 5-HT₄ receptors are likely expressed by efferent neurons of the nucleus accumbens projecting to the lateral hypothalamus (Jean et al., 2012 and Bockaert et al., 2011).

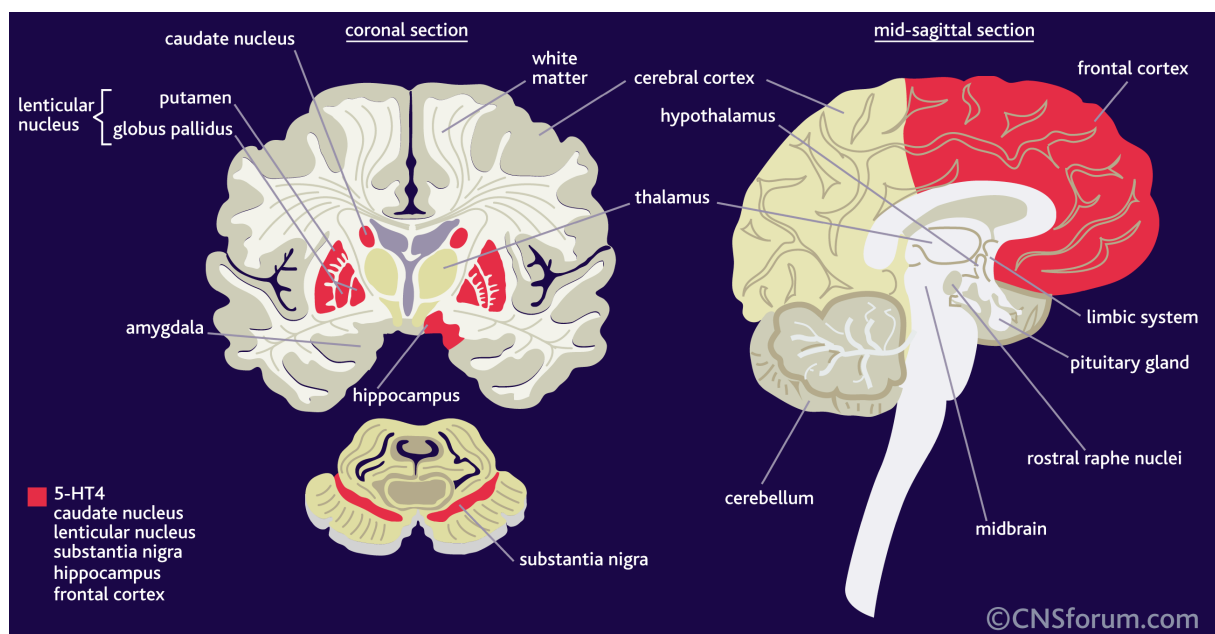


Figure 8: Localization of 5-HT₄ receptors in the human brain (Reynolds et al., 1994).

A large body of evidences has been gathered over the past decades in regard to the importance of 5-HT₄ receptor isoforms a and b (Vilaro et al, 2002; Pindon et al., 2002;

Qanbar and Bouvier, 2003; Ponimaskin et al., 2002, 2005; Mnie-Filali et al., 2010; Bockaert et al., 2008; Marin et al. 2012). These isoforms have a high expression in the limbic system such as the nucleus accumbens, hippocampus and the striatum, structures which are responsible for monitoring responses of motivation and pleasure affected in depression. (Vilaro et al., 2002, 2005.) These isoforms have also contributed to potential targets for the development of novel, rapid antidepressant (Lucas et al., 2007; Pascual-Brazo 2012). However, due to a lack of antibody and specific ligands to each of the two isoforms, it has been an arduous task to distinguish and characterize their properties in signaling and regulation functions, which are important aspect for the development of antidepressants with an effective response and faster onset of action (Lucas et al., 2007; Pascual-Brazo et al., 2012).

2. Fonctionnalité au niveau du système nerveux central

Several studies showed that agonist activation of the 5-HT₄ receptors, leads to a cascade of signaling events such as production of cyclic AMP (cAMP), activation of a protein kinase A which results in the phosphorylation of cAMP response element binding protein (CREB) and as a consequence the expression of a number of genes involved in cell survival (**Figure 9**) (Bockaert et al., 2006; Bockaert et al., 2008; Ahamad and Nirogi, 2011). In addition, all 5-HT₄ receptor splice variants are able to stimulate adenylyl cyclase (AC) activity and raise intracellular cAMP levels in the presence of 5-HT or agonists.

Based on a diversity of methodological approaches such as 5-HT₄ receptor binding, single cell PCR studies, autodiography, electrophysiology, microdialysis, in situ hybridization, and many other techniques, we know the cellular and subcellular localization of the 5-HT₄ receptors (Waeber et al. 1993, 1994; Compan et al., 1996; Bonaventure et al., 2000; Bockaert et al., 2008; Lucas, 2009). Likewise, an impressive number of articles highlighted the prominent role of the 5-HT₄ receptors in brain functions such as memory and cognition, mood disorders and feeding disorder (Bockaert et al, 2004; Compan et al., 2004; King et al.,

2008; Bockaert et al., 2008; Lucas et al., 2007; Lucas, 2009; Ahamad and Nirogi, 2011; Pascua-Brazo et al., 2012).

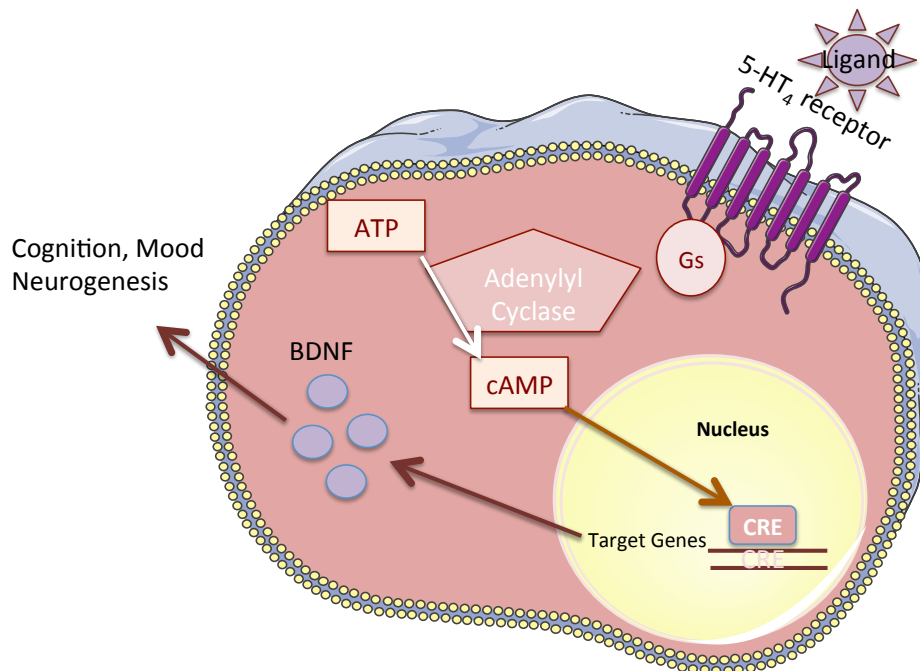


Figure 9: 5-HT₄ receptor activation leading to various cellular events (adapted and modified from Ahamad and Nirogi, 2011).

Activation of 5-HT₄ receptor leads to acetylcholine release, which coupled with the release of BDNF, may help in memory/mood. The activation of the receptor is also reported to enhance the release of BDNF-induced neurogenesis.

a) Le récepteur 5-HT₄ et la prise alimentaire

As previously addressed, 5-HT₄ receptors expressed in the central nervous system have been associated with limbic/affective functions as well as food intake. The first evidence that demonstrated the implication of 5-HT₄ receptors in food intake was obtained using the 5-HT₄ receptor knockout mice (Compan et al., 2004). Indeed, the defective mutation of the mHtr4 gene encoding 5-HT₄ receptors reduced hypophagia following restraint stress and novelty-induced exploratory activity a possible animal model for anorexia nervosa (Compan et al., 2004; Bockaert 2008, 2011). It is also known that 5-HT₄ receptors control mRNA expression of CART (cocaine- and amphetamine-regulated transcript), an anorectic peptide, via a signaling pathway involving cAMP/PKA (Compan et al., 2004; Jean et al., 2007).

Further study also showed that the rewarding drug MDMA (3,4-N-methylenedioxymetham-phetamine) reduces feeding via 5-HT₄ receptor activation through the same mechanism (Jean et al., 2007). Interestingly, in a recent study, Compan's group demonstrated that when motivation comes into play, the NAc-5-HT₄ receptor/5-HT_{1B} receptor/CART pathway might prevail over the autonomic nervous control of feeding. The NAc-5-HT₄ receptor/CART surplus makes the brain "silent" to energy loss (Jean et al., 2012).

b) Le récepteur 5-HT₄ et la cognition

5-HT₄ receptors expressed in the central nervous system have been involved in memory and cognitive functions. It has been established that activation of CREB protein is an important mediator of memory formation and pro-cognitive effects (Bockaert et al., 2004; Bockaert et al., 2008). Several signaling pathways leading to cAMP accumulation in neurons are being investigated as prospective candidates for therapeutic interventions of cognitive deficits linked with various neurological disorders (Ahamad and Nirogi, 2011; Marin et al., 2012). Additionally, pharmacological and behavioral essays have shown improvement in memory acquisition induced by 5-HT₄ receptor in rodents and primates (King et al., 2008; Bockaert et al., 2008; Marchetti et al., 2008). Indeed, in various animal models, partial 5-HT₄ receptor agonists were reported to promote learning in a variety of behavioral tests (Bockaert et al., 2004, 2008 ; Marchetti et al., 2008) (**tables 4, 5, 6 and 7**). Activation of this receptor inhibits neuronal potassium channels and subsequent mobilization of calcium ions, resulting in increased neuronal excitability (Bockaert et al., 2004, 2008). It also improves neurotransmission and enhances the release of acetylcholine in the frontal cortex and hippocampus and promotes an increase in Long Term Potentiation (LTP), in hippocampus and amygdala.

Consolo and colleagues were the first to demonstrate by *in vivo* microdialysis studies

that agonists BIMU1 and BIMU8 increase acetylcholine. Further studies confirmed this results, but it also demonstrated that 5-HT₄ agonists facilitates hippocampal Ach outflow (Consolo et al., 1994; Yamaguchi et al., 1997; Letty et al., 1997; Orsetti et al., 2007). Thus, 5-HT₄ receptors may improve learning and memory formation by enhancing the synaptic release of acetylcholine in the brain. (Bockaert et al., 2004, 2008; King et al., 2008; Lucas et al., 2009). The putative role of 5-HT₄ receptors in learning and memory has also been investigated in animal studies. Pre-training administration with 5-HT₄ agonists BIMU1 or BIMU8, has been shown to enhance acquisition, but impair the consolidation of learning in an autoshaping paradigm in rats (Meneses and Hong, 1997). In addition, 5-HT₄ receptor agonists enhanced spatial navigation in rats. Specifically in the Morris Water maze, 5-HT₄ receptor agonists were also found to reduce escape latency (Fontana et al., 1996), and to reverse atropine-induced deficits (Fontana et al., 1997). In the Y Maze, the agonist RS-67333 was also able to enhance place recognition as evidenced by increased visits to the novel arm of the maze (Orsetti et al., 2003).

The role of the 5-HT₄ receptor in synaptic plasticity was also demonstrated in freely moving rats with implanted microelectrodes by agonist stimulation with RS67333, which led to increased synaptic transmission in hippocampus (Kulla et al., 2002). Similar effects were observed in anesthetized rats using another 5-HT₄ selective agonist SC 53116 (Matsumoto et al., 2001). Lastly, in both young and old macaques, the agonist RS 17107 induced improvements in a delayed matching-to-sample task in which the monkeys had to learn to correctly match colors to receive a food reward (Terry et al., 1998). Efficacy of 5-HT₄ receptor agonists in the older monkeys lends further support to the potential use of these compounds in the treatment of memory disorders.

Furthermore, 5-HT₄ receptors also activates the exchange factor Epac, which in turn stimulates an α -secretase activity and the release of soluble amyloid precursor protein

(sAPP α), which has neuroprotective and memory-enhancing properties (Bockaert et al., 2008; King et al., 2008; Marin et al., 2012). These findings in correlation with other studies showing a loss of 5-HT₄ receptor binding sites in cortical and hippocampal regions in the Alzheimer brain suggest that 5-HT₄ agonists may be used in the treatment of Alzheimer's disease.

Tableau 4 : Revue des effets des ligands du récepteur 5-HT₄ dans les processus cognitifs – part 1.

References	Name	Pharmacological properties	Doses	Species	Paradigms	Effects
Fontana et al., 1995	(R)-Zacopride , 4-amino-5-chloro-2-methoxy-N-(quinuclidin-3-yl)benzamide	agonist (also 5-HT ₃ receptor antagonist)	0.001-100 µg/kg, i.p, acutely	rat	Morris water in atropine-treated rat	No effect
	(S)-Zacopride , 4-amino-5-chloro-2-methoxy-N-(quinuclidin-3-yl)benzamide	agonist (also 5-HT ₃ receptor antagonist)	0.001-100 µg/kg, i.p, acutely			Reduction of cognitive deficit at 0.001-1 µg/kg, i.p,
Fontana et al., 1997	RS 67333 , (1-[4-Amino-5-chloro-2-methoxyphenyl]-3-[1-butyl-4-piperidinyl]-1-propanone)	agonist	0.1, 10 and 1000 µg/kg, i.p, acutely	rat	Morris water in atropine-treated rat	Reduction of cognitive deficit at 0.001-1 µg/kg, i.p,
	RS 67506 , (1-(4-amino- 5-chloro-2-methoxyphenyl)-3-[1-[2-[(methylsulfonyl)amino]ethyl]-4- piperidinyl]-1-propanone)	agonist	0.1, 10 and 1000 µg/kg, i.p, acutely			No effect
	RS 67532 1-(4-amino-5-chloro-2-(3, 5-dimethoxy benzyloxyphenyl)-5-(1-piperidinyl)-1-propanone)	antagonist	0.1, 10 and 1000 µg/kg, i.p, acutely			No effect
	BIMU 1 , (3-ethyl-2,3-dihydro-N-[endo-8-methyl-8-azabicyclo (3.2.1)oct-3-yl]-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride	agonist	5-20 mg/kg, i.p, acutely			Increase conditioned response at 10 and 20mg/kg
Meneses and Hong, 1997	BIMU8 , 2,3-Dihydro-N-[(3- <i>endo</i>)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl]-3-(1-methylethyl)-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride	agonist	10-30 mg/kg, i.p, acutely	rat	Autoshaping Training	Increase conditioned response at 20mg/kg and decreased at 5 mg/kg
	SDZ 205557 , 2-methoxy-4-amino-5-chloro-benzoic acid 2-(diethylamino ester)	antagonist	1.0-10.0 mg/Kg, i.p, acutely			No effect
	GR 125487D , [1-[2-[(Methylsulfonyl)amino]ethyl]-4-piperidinyl]methyl 5-fluoro-2-methoxy-1H-indole-3-carboxylate	antagonist	0.39-1.56 mg/kg, i.p, acutely			No effect
	BIMU 1 , (3-ethyl-2,3-dihydro-N-[endo-8-methyl-8-azabicyclo (3.2.1)oct-3-yl]-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride	agonist	10-25 mg/kg, i.p, acutely			prevented scopolamine and dicyclomine amnesia at 10 mg/kg
Galeotti et al., 1998	BIMU8 , 2,3-Dihydro-N-[(3- <i>endo</i>)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl]-3-(1-methylethyl)-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride	agonist	10-30 mg/kg, i.p, acutely	mice	Passive avoidance in scopolamine or dicyclomine-treated mice	prevented scopolamine and dicyclomine amnesia at 30 mg/kg
	GR 125487 , [1-[2-[(Methylsulfonyl)amino]ethyl]-4-piperidinyl]methyl 5-fluoro-2-methoxy-1H-indole-3-carboxylate	antagonist	5-30 mg/kg, i.p, acutely			amnesic effect at 10 mg/kg
	SDZ 205557 , 2-methoxy-4-amino-5-chloro-benzoic acid 2-(diethylamino ester)	antagonist	5-30 mg/kg, i.p, acutely			amnesic effect at 10 mg/kg

Tableau 5 : Historical review of the effects of 5-HT₄ receptors ligand in learning – part 2.

References	Name	Pharmacological properties	Doses	Species	Paradigms	Effects
Terry et al., 1998	RS 17017 , 1-(4-amino-5-chloro-2-methoxyphenyl)-5-(piperidin-1-yl)-1-pentanone hydrochloride	agonist	0.3, 3.0, 30, 300, 3000, and 10 000 µg/kg orally	Young and Old macaques	Computer-assisted delayed matching-to-sample task	improvements in DMTS in both younger and older monkeys
	RS 17017 , 1-(4-amino-5-chloro-2-methoxyphenyl)-5-(piperidin-1-yl)-1-pentanone hydrochloride	agonist	1 mg/kg, i.p, acutely	rat	Olfactory associative discrimination task	Improved learning and memory performance
	RS 67333 , (1-[4-Amino-5-chloro-2-methoxyphenyl]-3-[1-butyl-4-piperidinyl]-1-propanone)	Partial agonist	1 mg/kg, i.p, acutely			Improved learning and memory performance
Marchetti et al., 2000	RS 67532 1-(4-amino-5-chloro-2-(3, 5-dimethoxy benzyloxyphenyl)-5-(1-piperidinyl)-1-pentanone)	antagonist	1 mg/kg, i.p, acutely			Deficit in associative memory
	RS 67333 , (1-[4-Amino-5-chloro-2-methoxyphenyl]-3-[1-butyl-4-piperidinyl]-1-propanone)	Partial agonist	40-500 ng/0.5µl in nucleus basalis magnocellularis, acutely	rat	Y-maze	enhances the acquisition (200–500 ng/0.5 µL) and the consolidation (40–200 ng/0.5 µL)
	RS 39604 , (1-[4-Amino-5-chloro-2-(3,5-dimethoxyphenyl)methyloxy]-3-[1-{2-methylsul-phonylamino}ethyl]piperidin-4-yl)propan-1-one)	antagonist	300 ng/0.5µl in nucleus basalis magnocellularis, acutely			No effect
Lelong et al., 2003	RS 67333 , (1-[4-Amino-5-chloro-2-methoxyphenyl]-3-[1-butyl-4-piperidinyl]-1-propanone)	Partial agonist	0.25, 0.5, 1 mg/kg, i.p, acutely	mouse	Y-maze	prevented the scopolamine-induced alternation deficits
	BIMU 1 , (3-ethyl-2,3-dihydro-N-[endo-8-methyl-8-azabicyclo (3.2.1)-oct-3-yl]-2-oxo-1H) benzimidazole-1-carboxamide hydrochloride	agonist	1, 3, 10 mg/kg, i.p, acutely			prevented the scopolamine-induced alternation deficits

Tableau 6 : Historical review of the effects of 5-HT₄ receptors ligand in learning – part 3.

References	Name	Pharmacological properties	Doses	Species	Paradigms	Effects
Lamirault et al., 2003	RS 67333 , (1-[4-Amino-5-chloro-2-methoxyphenyl]-3-[1-butyl-4-piperidinyl]-1-propanone)	Partial agonist	0.01, 1, 10 mg/kg i.p. acutely	rat	Place or Object recognition	improved performance in both place and object recognition tasks in young adult as well as in old rats
Mohler et al., 2007	PRX 03140 , (formerly VRX-03011), 4-hydroxy-7-isopropyl-6-oxo-N-[3-(1-piperidinyl)propyl]-6,7-dihydrothieno[2,3-b]pyridine-5-carboxamide	agonist	0.1, 1, 5, or 10 mg/kg, i.p.	rat	Spontaneous alternation (no delay/30-s delay)	enhanced delayed spontaneous alternation performance at 1, 5 and 10 mg/kg
Sunyer et al., 2008	RS 67333 , (1-[4-Amino-5-chloro-2-methoxyphenyl]-3-[1-butyl-4-piperidinyl]-1-propanone)	agonist	1 mg/kg, i.p. acutely	mice	Morris Water maze	No effect
Hille et al, 2008	SL65.0155 , 5-(5-amino-6-chloro-2,3-dihydro-1,4-benzodioxin-8-yl)-3-(1-phenethyl-4-piperidyl)-1,3,4-oxadiazol-2-one hydrochloride	agonist	0.1 or 1 mg/kg s.c	rat	five-choice serial reaction time task	increasing % correct trials
Marchetti et al., 2008	SL65.0155 , 5-(5-amino-6-chloro-2,3-dihydro-1,4-benzodioxin-8-yl)-3-(1-phenethyl-4-piperidyl)-1,3,4-oxadiazol-2-one hydrochloride	agonist	0.01 mg/kg, i.p., acutely	Rat pre-treated with colchicine	olfactory associative discrimination task	Complete recovery of associative learning performance in the lesioned rats
Levallet et al., 2009	RS 67333 , (1-[4-Amino-5-chloro-2-methoxyphenyl]-3-[1-butyl-4-piperidinyl]-1-propanone)	agonist	1 mg/kg, i.p. acutely	rat	object recognition memory task	Increase in time exploring the novel object

Tableau 7 : Historical review of the effects of 5-HT₄ receptors ligand in learning – part 4.

References	Name	Pharmacological properties	Doses	Species	Paradigms	Effects
Restivo et al., 2008	SL 65.0155 , 5-(5-amino-6-chloro-2,3-dihydro-1,4-benzodioxin-8-yl)-3-(1-phenethyl-4-piperidyl)-1,3,4-oxadiazol-2-one hydrochloride	agonist	0.01 mg/kg, i.p., acutely	mice	olfactory associative discrimination task	enhances simultaneous olfactory discrimination performances
	RS 39604 , (1-[4-Amino-5-chloro-2-{3,5-dimethoxyphenyl}methoxy]-3-[1-{2-methylsulfonylamino}ethyl]piperidin-4-yl]propan-1-one)	antagonist	0.5 mg/kg, i.p., acutely	mice	Morris Water maze	No effect
Cachard-Chastel et al., 2008	RS 67333 , (1-[4-Amino-5-chloro-2-methoxyphenyl]-3-[1-butyl-4-piperidyl]-1-propanone)	agonist	1 mg/kg, i.p., acutely	Mice	Morris Water maze in scopolamine-treated mice	Reduction of cognitive deficit
Marchetti et al., 2011	SL 65.0155 , 5-(5-amino-6-chloro-2,3-dihydro-1,4-benzodioxin-8-yl)-3-(1-phenethyl-4-piperidyl)-1,3,4-oxadiazol-2-one hydrochloride	agonist	0.1 mg/kg, i.p., acutely	Aged rat	olfactory associative discrimination task	Improved memory performance

c) Le récepteur 5-HT₄ et la régulation des émotions

The expression of the 5-HT₄ receptors in the limbic system also suggests a role of this receptor in mood regulation. It has been reported that 5-HT₄ agonists exert stimulatory effect in firing activity on 5-HT neurons in the dorsal raphe nucleus (Lucas and Debonnel 2002), suggesting a role for this receptor in depression. It is also well known that activation of 5-HT₄ receptor leads to cAMP formation, which through cAMP Response Element-binding protein (CREB) phosphorylation initiates a cascade of signaling such as induction of neurogenesis (Lucas et al., 2005; Pascual-Brazo et al., 2012).

5-HT₄ receptor binding in different brain regions, electrophysiology studies and behavioral studies support a role of the 5-HT₄ receptor in mood disorders (Compan et al., 2004, Lucas et al 2007; Lucas, 2009; Licht et al., 2009; Bockaert et al., 2011; Pascual Brazo et al., 2012). Further explanation will be given in chapter IV.

III. La protéine β -arrestin 1, le récepteur 5-HT₄ et leurs interactions

A. La protéine β -arrestine 1, un exemple de protéine interagissant avec les récepteurs couplés aux protéines G

Although coupling to G proteins remains a central event in 5-HT₄ receptor-mediated signaling, evidence indicates that the regulation of their functional status also depends on interactions with a variety of additional protein partners named GPCR interacting proteins (GIPs) (Bockaert et al., 2006, 2011). These interactions create additional opportunities for not only G-protein-dependent signaling, but also for G-protein-independent signaling (Lefkowitz and Whalen, 2004). The GIPs have three main roles: First, they allow receptor targeting to appropriate subcellular compartments such as dendrites, axons, spines in which they have their specific function. Second, control receptor trafficking in and out of the plasma membrane, and third, ensure a fine-tuning of receptor signaling (Bockaert et al., 2004; Barthet

et al., 2009; Marin et al., 2012).

β -arrestins, is a GPCR interacting protein that belong to the arrestin family. They are ubiquitously expressed in the brain and interact specifically with GPCRs, including the 5-HT₄ receptor, following their activation by agonists and subsequent phosphorylation by GRKs (Bockaert et al., 2004). To date, four members of the arrestin gene family have been cloned (Freedman and Lefkowitz, 1996). Arrestin1 and arrestin 4, also known as visual arrestin and cone arrestin, which are expressed exclusively in the retina where they regulate photoreceptors functions. By contrast, β -arrestin 1 and β -arrestin 2, also known as arrestin 2 and arrestin 3 respectively, are ubiquitously expressed and regulate GPCRs.

A growing body of evidences have revealed that β -arrestins play a key role in the signaling, desensitization and internalization of GPCR's, including the majority of 5-HT receptors (Vilaro et al., 2005), and are particularly well positioned to play a significant role in ligand-directed functional signaling. The role of β -arrestins in the desensitization and internalization of certain 5-HT receptors has been intensively investigated (Barthet et al, 2005; Bohn et al., 2010; Mnie-Filali and Pineyro, 2012). It has been established that β -arrestins in their inactive form are phosphorylated cytosolic proteins. In many cases, receptor phosphorylation on serine and threonine residues present in the carboxyl-terminal domain or intracellular loops of the receptor appears to play a major role in initiating the desensitization process which can be homologous or heterologous (Mialet et al., 2003). For intance, β -arrestin 1 undergoes phosphorylation in serine-412 in the carboxyl-terminal (Lin et al., 1997), by extracellular signal regulated kinase (ERK1/2). Following agonist stimulation, β -arrestin 1 is translocated to the plasma membrane where it is dephosphorylated, a process that is not required for receptor binding and desensitization but is obligatory for receptor internalization.

B. La protéine β -arrestine 1 et les troubles anxio/dépressifs

Recently, a substantial body of evidence indicates that β -arrestins (β -arrestin-1 and β -

arrestin-2), proteins that regulate G-protein receptor coupling, play a role in the pathophysiology of mood disorders and in the mechanisms underlying antidepressant actions (Avissar et al., 2004; Schreiber and Avissar, 2009; Matuzany-Ruban et al., 2005; Beaulieu et al., 2008; David et al., 2009; Schreiber et al., 2009; Golan et al., 2010). The β -arrestin-signaling cascade has recently gained attention as a potential pre-clinical/clinical bridging biomarker for depressive states and treatment effects. In naïve rats, SSRI, SNRI and non-selective reuptake inhibitor antidepressants significantly elevate β -arrestin-1 levels in the cortex and the hippocampus (Avissar et al., 2004; Beaulieu and Caron, 2008; Beaulieu et al., 2008; David et al., 2009). Similarly, β -arrestin 1 expression is decreased in the hypothalamus and hippocampus in anxious/depressed mice exposed to glucocorticoid elevation, and is restored by chronic fluoxetine treatment (David et al., 2009). Moreover, β -arrestin 1 and 2 signaling is involved in mediating the response to fluoxetine and lithium (Beaulieu et al., 2008; David et al., 2009).

Clinical data from Avissar's group (Avissar et al., 2004) suggest that β -arrestin-1 mRNA and protein levels are highest in peripheral blood leukocytes of MDD patients. Therefore, β -arrestin-1 may be a putative candidate biochemical marker in clinical practice for depressive pathophysiology and the response to antidepressants (for review see (Schreiber et al., 2009). β -arrestin mRNA levels and β -arrestin-1 protein levels in mononuclear leukocytes of untreated patients with MDD are lower than the levels found in healthy subjects. Furthermore, reduced levels of β -arrestin-1 protein and mRNA are significantly correlated with the severity of depressive symptoms (Avissar et al., 2004; Schreiber et al., 2009). However, the low β -arrestin-1 protein and mRNA levels are alleviated by antidepressant treatment. Therefore, these low levels may predict clinical improvement (Avissar et al., 2004; Golan et al., 2010).

C. Le récepteur 5-HT₄ et la protéine β -arrestine 1

In 2005, Barthet and colleagues identified GRK2 as the major GRK responsible for the desensitization of the 5-HT₄ receptor mediated Gs pathway (**Figure 10A**). They demonstrated by in vitro studies using primary colliculi neurons or HEK293 cells that uncoupling of 5-HT₄ receptors from Gs proteins and β -arrestins-dependent receptor endocytosis are distinct molecular events with different GRK requirements (**Figure 10**) (Barthet et al., 2007; Bohn et al., 2010; Marin et al., 2012). For instance, uncoupling of 5-HT₄ receptors from G α_s proteins required the presence of GRK2 but did not depend on its kinase activity (Dumuis et al., 1988; Barthet et al, 2005) (**Figure 10A**). In contrast, recruitment of β -arrestins, which allows receptor internalization, required receptor phosphorylation by GRK2 of a cluster of serines and threonines located in the receptor C-terminus. This mechanism also implies that the phosphorylation process of the 5-HT₄ receptor C-terminal domain and its Serr/thr clusters seems to be absolutely necessary for β -arrestin recruitment (**Figure 11**).

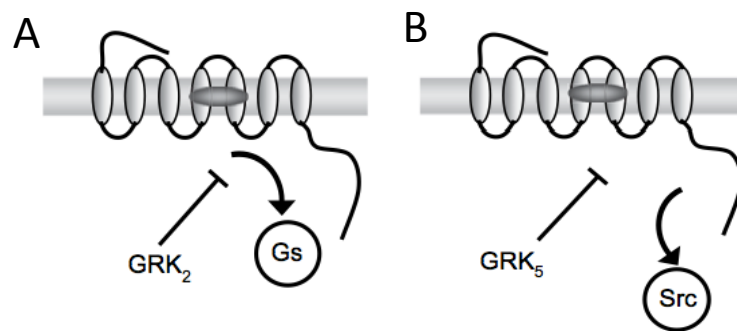


Figure 10: A general schema of Gs-dependent (A) and Gs-independent signaling (B) and desensitization of 5-HT₄ receptors (Bockaert et al. 2008).

Further characterization by Barthet and colleagues showed a constitutive interaction of 5-HT₄ receptors and GRK5, and claimed that effect contributes to the basal “constitutive activity” of the receptor as previously reported (**Figure 10B**) (Claeysen et al., 1999; Barthet et al., 2009). These studies were based on the role of GRK5 in the desensitization of G-protein-independent pathway and identified β -arrestin1 as the protein responsible for the negative regulation of 5-HT₄ receptor-mediated Src/ERK pathway. This desensitization process required two complex molecular events such as a recruitment of β -arrestin1 to a phosphorylated serine/threonine cluster located in the receptor C-terminal domain (**Figure 11A**) and a direct phosphorylation (at Ser⁴¹²) in which β -arrestin1 phosphorylation at Ser⁴¹² is essential for GRK5-mediated inhibition of the 5-HT₄ receptor-operated Src/ERK pathway (**Figure 11B**).

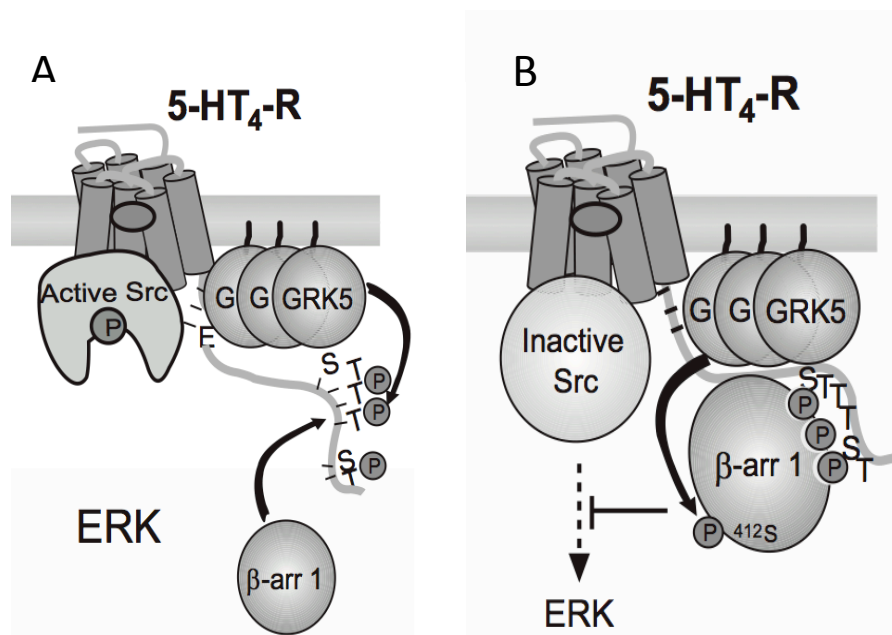


Figure 11: Model of desensitization by GRK5 of 5-HT₄ receptor-operated ERK signaling (Bockaert et al., 2008).

These studies demonstrated for the first time a role of a GRK family member (GRK5) in the desensitization of G protein-independent pathway and identified β -arrestin1 as a novel

substrate of GRK5 in addition of its well described GPCR substrates. They also showed earlier that engagement of the ERK1,2 pathway (implicated in modulation of cognitive functions) by 5-HT₄ receptors and its regulation are critically dependent of a complex “receptosome” including at least Src, GRK5 and β -arrestin1 (Barthet et al 2009; Marin et al., 2012). As expression of both GRK5 and β -arrestin1 increases considerably during development (Gurevich et al, 2004), it is likely that the negative regulation of the 5-HT₄ mediated activation of the G protein-independent Src/ERK pathway will be higher in adult neurons. Therefore, GRK5 and β -arrestin1 play a critical role in determining the nature of the signaling pathway (G_s or ERK) predominantly engaged upon activation of 5-HT₄ receptor which could be critical for 5-HT₄-dependent synaptic plasticity and memory consolidation (Kemp and Manahan-Vaughan, 2004, 2005; Marchetti et al, 2004; Micale et al, 2006).

D. Les variants du récepteur 5-HT₄ et la protéine β -arrestine 1

A better understanding of the consequences of the 5-HT₄ receptor activation is linked to isoforms differences, which seems to play an important part in regard to the coupling of the receptor. For instance, the internalization process of the 5-HT₄ receptor is dependent on the two isoforms of the receptor, 5-HT_{4a} and 5-HT_{4b}. Serotonin-induced internalization of the 5-HT_{4a} receptor in HEK293 cells was inhibited by expression of dominant negative GRK2 and β -arrestin1 (β arr1₃₁₉₋₄₁₈), while 5-HT_{4b} receptor internalization was only partially attenuated by β arr1₃₁₉₋₄₁₈. This study suggests that although β -arrestins are involved in the trafficking of 5-HT₄ receptors, there are isoform-specific differences that may impact on the overall regulation of these receptors (Mnie-Filali et al., 2010; Bockaert et al., 2008; Marin et al., 2012).

Furthermore, other studies have shown that isoform 5-HT_{4a} is accumulated in a perinuclear compartment location while isoform 5-HT_{4b} is widely distributed in the cytosol of

the cell and these differences have an impact in post-endocytic distribution of each isoform, which is also consistent with the different requirements GRK2 and β -arrestin for their internalization process. The internalization and post-endocytosis distribution of GPCRs distribution is determined by several factors including the activity of kinases that phosphorylate Ser / Thr residues intracellular (Ferguson et al, 1996; Ferguson et al., 1998), and the ability of β -arrestin to interact with the receptor (Lefkowitz, 1998; Lohse et al, 1990).

Ponimaskin and colleagues have shown that 5-HT₄ receptor contains potential sites for posttranslational modifications within their cytoplasmic carboxyl terminal. They identified three palmitoylation sites located at the extremity of the C-terminal of the 5-HT_{4(a)} isoform and demonstrated that 5-HT_{4(a)} receptor palmitoylation is dynamically regulated upon agonist stimulation of the receptor (Ponimaskin et al., 2001). In addition, mutation studies at the palmitoylation sites of the 5-HT_{4(a)} isoform have decreased the rate and the extent of β -arrestin recruitment to this receptor. Likewise, receptor palmitoylation was also found to be required for ligand-induced β -arrestin recruitment to the cell membrane and endocytosis of the thyrotropin-releasing hormone receptor (Groarke et al., 2001). Further studies also demonstrated that mutation of the membrane proximal palmitoylation site facilitates β -arrestin-mediated internalization of 5-HT_{4(a)} receptors.

Therefore understanding the consequences of 5-HT₄/ β -arrestin interaction could aid in the development of pharmacotherapies to selectively target the activation or the inhibition of specific serotonin receptor signaling cascades.

IV. Le récepteur 5-HT₄ et ses ligands et leurs implication dans la dépression

Human and animal studies have suggested that receptors 5-HT₁ and 5-HT₂ receptors play a critical role in anxiety/depression and antidepressant response (Barnes and Sharp, 1999; Zhuang et al 1999; Cryan and Lucki 2000). However, recent evidences also suggested

that 5-HT₄ receptors may be implicated in depression and in antidepressant treatment (Lucas et al., 2005, 2007; Pascual-Brazo et al., 2012). The 5-HT₄ receptor has been identified as a positive regulator of 5-HT neuron firing in the dorsal raphe nucleus, suggesting a role for this receptor in depression and antidepressant treatment (Lucas et al., 2005; Lucas and Debonnel, 2002). There are two clinical studies, which suggest a role of the 5-HT₄ receptor in clinical depression. One study reported polymorphisms in the splice variant region of the serotonin 4 receptor gene, which have been associated with unipolar depression (Ohtsuki et al. 2002). The second study revealed alterations of both 5-HT₄ receptor binding sites and cAMP concentration levels in several brain regions of depressed violent suicide victims (Rosel et al., 2004).

A. Les ligands du récepteurs 5-HT₄ et leurs conséquences neurochimiques

By in vivo extracellular recordings in the DRN of rats, where nearly all of the serotonergic cell bodies are found in the brain, acute systemic administration of cisapride, a 5-HT₄ receptor agonist, and GR125487, a 5-HT₄ receptor antagonist, was found to increase and decrease, respectively, the firing rate of dorsal raphe neurons (Lucas and Debonnel, 2002), suggesting both a tonic and phasic influence of 5-HT₄ receptor activity on serotonergic neuron activity. More recently, it has been shown that 5-HT₄ agonists increase the firing of serotonergic neurons, achieving their maximal effect within about 3 days and these effects persist after 21 days in rats (Lucas and Debonnel, 2002; Lucas et al., 2005). For instance, SSRIs increase synaptic levels of 5-HT relatively quickly (hours or days), but there is a delay in therapeutic response (weeks or months) due to the likely requirement for adaptations at both pre- and postsynaptic site (Blier et al., 1990; Blier, 2003; Czachura and Rasmussen, 2000). Alternatively, 5-HT₄ receptor agonists may cause a more rapid desensitization of 5-HT_{1A} autoreceptors to permit such a fast increase in DRN firing rates. Thus, 5-HT₄ receptors

may mediate antidepressant-like effects via increased serotonergic release at postsynaptic sites.

In support of a central role of the 5-HT₄ receptor in 5-HT system control, 5-HT₄ receptor activation increased the effect of acute SSRI (paroxetine) administration on 5-HT levels in the ventral hippocampus, both acutely and after three days of administration with the 5-HT₄ receptor partial agonist RS67333 (Licht et al., 2009). This effect is most likely due to the acute stimulation of 5-HT neuron firing rate by 5-HT₄ receptor partial agonism, which has been reported by others. While acute administration of RS67333 did not affect extracellular 5-HT levels in the ventral hippocampus, only three days of RS67333 administration was required to elevate 5-HT levels. As an increase in extracellular 5-HT levels is associated with antidepressant effects of drug treatment, these reports suggest that 5-HT₄ receptor partial agonism have antidepressant potential, in particular in combination with SSRI administration.

B. Les ligands du récepteurs 5-HT₄ et leurs effets comportementaux

Recent findings indicate that mental disorders such as anxiety and/or depression could benefit from modulation of the 5-HT₄ receptor signaling (Compan et al. 2004; Lucas et al., 2007; Lucas 2009; Compan et al., 2004; Pascual-Brazo et al., 2012).

1. Les ligands du récepteurs 5-HT₄ et leurs effets de type anxiolytiques

Behavioral tests and the use of experimental animals have been very useful for the study of pharmacological compounds to investigate the impact of 5-HT₄ receptors in anxiety/depression phenotypes. Furthermore, studies have also focused on whether 5-HT₄ receptors may mediate anxiolytic behavioral effects of SSRIs. From experimental animal studies, the role of 5-HT₄ receptors in anxiety is unclear. For instance, in the light/dark choice paradigm a test to measure anxiolytic activity, mice treated with an anxiolytic (diazepam) spend more time in the light compartment than vehicle-treated mice. This effect of diazepam has been

shown to be dose-dependently inhibited by antagonists of the 5-HT₄ receptor (GR 113808, SB 204070, and SDZ 205-557) (Costall and Naylor, 1997) suggesting a role of the 5-HT₄ receptor in mediating these anxiolytic effects (**table 7**). Although a direct effect of the 5-HT₄ receptor antagonists on anxiety-like behavior in the light/dark choice test was not detected (Costall and Naylor, 1997), others report an anxiolytic effect of 5-HT₄ receptor antagonists SB 204070, GR 113808 (Silvestre et al., 1996) and SB 207266A (Silvestre et al., 1996; Kennett et al., 1997) in rats in the elevated plus maze, another test of anxiety-like behavior. Rats treated with SB 204070 or GR 113808 displayed increases in the percent of total time spent in the open arms, which would suggest reduced anxiety-like behavior. However, one study did not detect an effect of the antagonists SB 204070 and GR 113808 on the number of open arm entries with a 10 minute pre-test injection interval (Silvestre et al., 1996), whereas another study reported an increase in percent open arm entries after injections of SB 204070 or SB 207266A one hour prior to testing (Kennett et al., 1997). Whether 5-HT₄ receptor antagonists cause anxiolytic effects remains unclear since reports indicate a lack of an effect in the light/dark choice paradigm, and anxiety-reducing effects in the elevated plus maze.

Further investigations have shown that stimulation of 5-HT₄ receptor inhibits the anxiolytic effect of diazepam (an enhancer of GABA response), particularly under conditions of high serotonergic tone (Costall et al., 1993). Since GABA_A receptor-mediated inhibition of synaptic transmission is highly involved in controlling neuronal excitability, and GABA_A receptors have been implicated in the pathogenesis of anxiety disorders (Macdonald and Olsen, 1994; Cai et al 2002), these lines of evidence prompt the speculation that 5-HT₄ receptors may exert some of their functions by acting on GABAergic signaling in PFC neurons.

2. Les ligands du récepteurs 5-HT₄ et leurs effets de type antidépresseur

Studies have investigated whether a 5-HT₄ receptor antagonist can exert antidepressant-like effects independently, or whether it can alter acute fluoxetine effects in the rat forced swimming test (FST) (Cryan and Lucki, 2000). The fluoxetine-induced decreases in immobility in the FST were not affected by co-administration of the 5-HT₄ receptor antagonist SB 204070A, and the antagonist had no independent effects in the FST either (Cryan and Lucki, 2000). Conversely, studies by Lucas and colleagues showed that with 5-HT₄ agonists, RS 67333 and prucalopride in the rat forced swimming test (FST) significantly reduced the time of immobility by about 50% compared with controls, whereas citalopram reduced immobility time by about 23%. (**tables 7 and 8**). Additional behavioral experiments were in agreement with this result showing that 5-HT₄ receptor agonist RS 67333, was more effective than citalopram (Lucas et al., 2007, 2010). In addition, Lucas and colleagues demonstrated that cellular and behavioral responses that require 2–3 weeks of antidepressant treatment in rodents would occur after only 1–3 d of treatment with a selective 5-HT₄ receptor agonist (RS67333) (Lucas et al., 2007). Moreover, signaling molecules that interact with the 5-HT₄ receptor such as P11 (S100A10) (Egeland et al., 2011; Warner-Schmidt et al., 2009) may represent novel targets for fast-acting anxiolytic/antidepressant treatments. There is indeed recent evidence that cortical neurons that express both P11 and 5-HT₄ receptors are involved in the behavioral effects of SSRIs (Schmidt et al., 2012) and that chronic treatment with fluoxetine results in an increase in 5-HT₄ receptor expressions in cortical neurons (Schmidt et al., 2012). Finally, in behavioral paradigms such as the FST and the Tail Suspension Test (TST), the antidepressant activity of RS67333 was absent in P11 knockout mice when compared with wild-type littermate controls (Warner-Schmidt et al., 2009). All these studies open the door to the link between the 5-HT₄ receptor and depression and provide an encouraging pharmacological strategy to obtain a fast antidepressant response.

Tableau 8 : Effects of 5-HT₄ receptors ligand on anxiety/depression-like phenotype – part 1.

References	Name	Pharmacological properties	Doses	Species	Paradigms	Effects
Silvestre et al., 1996	SB204070 , 8-amino-7-chloro-(N-butyl-4-piperidyl)-methylbenzo-1,4-dioxan-5-carboxylate hydrochloride	antagonist	0.3-3 mg/kg, s.c., acutely	rat	EPM	Exhibits an anxiolytic-like profile
	GR 113808 , 1-[2-methylsulphonylamino]ethyl-4-piperidyl-[methyl-1-methyl-1H-indole-3-carboxylate maleate	antagonist	0.3-3 mg/kg, s.c., acutely			Exhibits an anxiolytic-like profile
Kennet et al., 1997	SB204070 , 8-amino-7-chloro-(N-butyl-4-piperidyl)-methylbenzo-1,4-dioxan-5-carboxylate hydrochloride	antagonist	0.01 and 10 mg/kg p.o 0.01 and 1 mg/kg s.c., acutely	rat	Social interaction /EPM	Increased time spent in social interaction / induced anxiolysis
	SB 207266A , 2H-(1,3)Oxazino(3,2-a)indole-10-carboxamide, N-((1-butyl-4-piperidyl)methyl)-3,4-dihydro-, monohydrochloride	antagonist	0.001 and 0.1 mg/kg s.c 0.01 and 1 mg/kg s.c., acutely		Social interaction / elevated x-maze	Increased time spent in social interaction / induced anxiolysis
	SDZ205-557 , 2-methoxy-4-amino-5-chloro-benzoic acid 2-(diethylamino ester)	antagonist	0.001–100 µg/kg, i.p., acutely		Light/Dark	No effect by itself, but reduced the disinhibitory effect of diazepam
	GR113808 , 1-[2-methylsulphonylamino]ethyl-4-piperidyl-[methyl-1-methyl-1H-indole-3-carboxylate maleate	antagonist	0.001–10 µg/kg, i.p., acutely			No effect by itself, but reduced the disinhibitory effect of diazepam
Schreiber et al., 1998	SB204070 , 8-amino-7-chloro-(N-butyl-4-piperidyl)-methylbenzo-1,4-dioxan-5-carboxylate hydrochloride	antagonist	0.001–10 µg/kg, i.p., acutely	rat	ultrasomic vocalization	No effect
	GR 125487 , [1-[2-[(Methylsulfonyl)amino]ethyl]-4-piperidyl-[methyl 5-fluoro-2-methoxy-1H-indole-3-carboxylate	antagonist	3 mg/kg, s.c., acutely		FST	No effect
Cryan and Lucki, 2000	SB204070 , 8-amino-7-chloro-(N-butyl-4-piperidyl)-methylbenzo-1,4-dioxan-5-carboxylate hydrochloride	antagonist	1.5 mg/kg, i.p. during 3 days 1.5 mg/kg, i.p. during 3 and 14 days	rat	FST	Decrease immobility duration and increase climbing duration
	RS 67333 , (1-[4-Amino-5-chloro-2-methoxyphenyl]-3-[1-butyl-4-piperidyl]-1-propanone)	agonist		OBX rat	Locomotor activity	Reversed OBX-induced increase in locomotor activity after 14 days
Lucas et al., 2005				CMS rat	Sucrose consumption	Reversed CMS-induced decrease in sucrose consumption after 14 days

Tableau 9 : Effects of 5-HT₄ receptors ligand on anxiety/depression-like phenotype – part 2.

References	Name	Pharmacological properties	Doses	Species	Paradigms	Effects
Pascual-Brazo et al., 2012	RS 67333 , (1-[4-Amino-5-chloro-2-methoxyphenyl]-3-[1-butyl-4-piperidiny]-1-propanone)	agonist	1.5 mg/kg, i.p. during 3/7 days 1.5 mg/kg, i.p. during 3/7 days	Rat	FST / NSF	Decrease immobility duration at 3/7 days in the FST and decrease latency to feed after 7 days
				Cort-treated rat	Sucrose intake	Increase sucrose intake at 3/7 day
Gomez-Lazaro et al., 2012	RS 67333 , (1-[4-Amino-5-chloro-2-methoxyphenyl]-3-[1-butyl-4-piperidiny]-1-propanone)	agonist	1.5 mg/kg, i.p. during 5 days	Social stress in rat	FST	Increase swimming

C. Les ligands du récepteurs 5-HT₄ et la neurogenèse hippocampique chez l'adulte

In addition to behavioral data, several hippocampal neuroplasticity changes have been related to antidepressant efficacy. One of the most remarkable features of 5-HT₄ receptor agonists is their ability to induce neurogenesis in hippocampus as well as enteric system in rodents (Lucas et al., 2007, 2009; Liu et al., 2009; Pascual-Brazo et al., 2012). In addition to previous behavioral data, and in agreement with a previous report from Lucas and colleagues, a recent study performed in naïve rats confirmed that three days of treatment with the 5-HT₄ agonist (RS67333) significantly promoted neurogenesis in the subgranular zone of the dentate gyrus of the hippocampus, an effect normally seen after a minimum of two weeks of treatment with classical antidepressants such as SSRIs (Pascual-Brazo et al., 2012). These results are interesting because hippocampal neurogenesis has been implicated in some of the behavioral effects of antidepressants in adult rodents (David et al., 2009; Santarelli et al., 2003). However, no direct evidence has yet linked the antidepressant-like effects of 5-HT₄ receptor activation and its neurogenic effects. Further support for an antidepressant-like effect of 5-HT₄ receptor activation is based on analysis of signal transduction. 5-HT₄ receptors are G(s)-coupled GPCRs that activate adenylyl cyclase, and thus increase production of cAMP (Dumuis et al., 1989; Torres et al., 1995), which could activate protein kinase A, and in turn phosphorylate the transcription factor CREB. Interestingly, chronic antidepressant treatment has been shown to activate the same signal transduction machinery (Nibuya et al., 1996). It is thought that the activation of CREB into pCREB constitutes a key step for the facilitation of hippocampal neurogenesis, another characteristic property of antidepressants (Malberg et al 2000; Duman et al., 2001; Castren, 2004).

The second-messenger cAMP has also been strongly suggested to be involved in the mechanism of action of antidepressants (Donati and Rasenick, 2003). Interestingly, a

significant decrease in 5-HT₄ receptor-dependent cAMP production has been reported after chronic treatment within 3 weeks of fluoxetine, venlafaxine (Vidal et al. 2010) or imipramine (Reierson et al. 2009), probably reflecting a desensitization process, due to a less efficient coupling to Gs proteins. A study also reported that 7 days stimulation with the 5-HT₄ agonist is required to fully desensitize the post-receptor signaling pathway associated to 5-HT₄ receptors, in agreement with the results obtained in behavioral tests predictive of chronic response (Pascual-Brazo et al., 2012).

BDNF has a role in neurite outgrowth and synaptic plasticity (McAllister, 1999). Several studies have shown that antidepressants up-regulate hippocampal BDNF (Duman and Monteggia, 2006), although the temporal pattern of these changes is a matter of debate. While some authors have reported an induction of BDNF expression after subchronic treatment (Larsen et al., 2008; Musazzi et al., 2009), other studies only show up-regulation of BDNF after chronic antidepressant administration (De Foubert et al., 2004; Nibuya et al., 1995). It appears that the long-term effect of antidepressants to modulate behavior and survival of new neurons is mediated, at least in part by BDNF. Therefore, since 5-HT₄ receptors activation could also enhance BDNF expression, its activation can lead to antidepressant-like effects on behavior and neuroplasticity. A study by Pascual-Brazo and colleagues reported an increase in expression of BDNF after 3 days treatment with the 5-HT₄ receptor agonist RS67333, and a significant increase in the expression of BDNF protein in whole hippocampal homogenates following 7 day treatment (Pascual-Brazo et al., 2012). Another study also reported an increase in the expression of BDNF following acute administration of another 5-HT₄ agonist (SL65.0155) (Tamburella et al. 2009).

Problématique du travail de thèse

Despite side effects, Selective Serotonin Reuptake Inhibitors continue to be leader for the treatment of depression and several anxiety disorders. SSRIs such as fluoxetine (Prozac[®]) and paroxetine (Paxil[®]) are highly prescribed in France for the treatment of mood disorders such as depression and anxiety. However, the delayed onset of action (2-4 weeks) is a main concern. High suicidal rates among patients combined with a significant amount of patients who do not respond to the first treatment demand an urgent need for faster acting and more effective antidepressants. Thus, the development of new antidepressants is of considerable importance as well as understanding the mechanisms underlying the delayed onset of action.

Past relevant work discussed in this thesis demonstrated evidence that 5-HT₄ receptor may be a direct target for treating depression and a new hope for fast acting antidepressant treatment. Administration of 5-HT₄ agonists induced similar molecular and behavioral changes as common antidepressants in rodents. In addition to behavioral data, it has been proposed that a short period of treatment with 5-HT₄ agonists increased the number of newborn cells in the dentate gyrus (DG), one of the steps of adult hippocampal neurogenesis. These results are interesting because hippocampal neurogenesis has been implicated in some of the behavioral effects of antidepressants in adult rodents. However, no direct evidence has yet linked the antidepressant-like effects of 5-HT₄ receptor activation and its neurogenic effects.

During my PhD work, I have endeavored to investigate both antidepressant and anxiolytic-like effects of either subchronic or chronic administration of a 5-HT₄ receptor agonist in a model of anxiety/depression based on the elevation of glucocorticoids in mice (CORT model). We also assessed whether chronic 5-HT₄ receptor stimulation can affect proliferation of newborn cells and maturation of newborn neurons. Lastly, using our mouse model of anxiety/depression combined with ablation of hippocampal neurogenesis by X-

irradiation, we assessed whether the anxiolytic/antidepressant action of RS67333 after 7-days and 28-days of treatment recruits a neurogenesis-dependent mechanism.

Another interesting point covered during my thesis work was to investigate whether the fast onset of anxiolytic/antidepressant action of 5-HT₄ receptor agonist treatment can be predicted by measuring β -arrestin 1 levels in Peripheral Blood Mononuclear Cells (PBMC). I was particularly interested in β -arrestin 1 expression due to its potential as a biochemical diagnostic tool. As previously described, β -arrestin 1 is known to mediate receptor desensitization, endocytosis and G protein-independent signaling. However, it is also recognized as a protein involved in the pathophysiology of depression and in antidepressant mechanism of action. Assessing peripheral protein levels in PBMCs is an attractive method because PBMCs are circulating cells that can be easily collected and monitored.

Therefore, I developed a method to screen putative biomarkers of the pathophysiology of mood disorders and the response to antidepressants by measuring and assessing circulating proteins, especially β -arrestin 1 in PBMCs. First, I was interested in investigating whether changes in β -arrestin 1 levels in mouse PBMCs were observed in a model of anxiety/depression and secondly, whether these levels could be corrected by chronic treatment with the SSRI fluoxetine or/and by a 5-HT₄ agonist.

Lastly, I wanted to investigate the effects of a chronic treatment with RS67333 on the β -arrestin 1 expression levels in the mouse brain treated with chronic corticosterone in two specific regions: the hippocampus and the cortex, two key brain structures possibly involved in the antidepressant response. Recent reports previously described showed that chronic treatment with various classes of antidepressants induced desensitization and/or downregulation of the 5-HT₄ receptors in cerebral regions implicated in depression such as the hippocampus, probably reflecting a desensitization process, due to a less efficient coupling to Gs proteins. Another study showed that a short exposure with the 5-HT₄ agonist is

required to fully desensitize the signaling pathway associated to 5-HT₄ receptors. Therefore, I investigated whether in our CORT model there was a restoration of β -arrestin 1 expression in the hippocampus and cortex after RS67333 or fluoxetine chronic treatment.

TRAVAIL EXÉRIMENTAL

Matériel & Méthodes

Article 3: **5-HT₄ receptor subtype, β -arrestins pathways and rapid onset effects of antidepressant drugs.**

Indira Mendez-David, Denis J David, Jean-Philippe Guilloux S, René Hen , Alain M Gardier,

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5-HT₄ receptor subtype, β -arrestins pathways and rapid onset effects of antidepressant drugs

Indira Mendez-David¹, Denis Joseph David¹, Jean-Philippe Guilloux¹, René Hen² and Alain Michel Gardier^{1*}

¹EA 3544, Lab. Neuropharmacologie, Faculté de Pharmacie, Université Paris-Sud, Châtenay-Malabry F-92296, France.

²Departments of Neuroscience and Psychiatry, Columbia University, New York, NY, 10032, USA; Department of Integrative Neuroscience, New York State Psychiatric Institute, New York, NY 10032, USA

*To whom correspondence should be addressed.

Pr Alain M. Gardier

Univ Paris-Sud

Fac Pharmacie

5, rue J-B Clement, Tour D1, 2e etage

EA 3544 "Serotonine et Neuropharmacologie"

F-92296 Chatenay-Malabry cedex

Tel: (33) 1 46 83 54 16

Fax: (33) 1 46 83 53 55

E-mail: alain.gardier@u-psud.fr

1. Abstract

Understanding the pathophysiology of affective disorders and their treatment relies on the availability of experimental models that accurately mimic aspects of the disease. The use of exogenously administered corticosterone (CORT model) has validity as an animal model to study chronic stress and depression, displaying some hallmark characteristics of anxiety and depression observed in patients.

Recently, we have adapted the CORT model protocol to screen a rapid onset drug to treat anxiety/depression disorders. In spite of the fact that selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed drugs for the treatment of depression and several anxiety disorders, the onset of action of SSRIs is often delayed by 3 to 6 weeks. The existence of this delayed action combined with the fact that one third of patients do not respond to treatment emphasizes the need for faster acting and more effective antidepressants. This chapter gives laboratory protocols including step-by-step recommendations to explain how the CORT model in mice can be used to screen a rapid onset drug to treat anxiety/depression. For this purpose we took as an example, the behavioral and cellular effects of a 5-HT₄ receptor ligand, RS67333 in comparison to the classical Selective Serotonin Reuptake Inhibitors, fluoxetine. Likewise, we emphasize how proteins levels, including **β-arrestin 1** measurements, in peripheral blood mononuclear cells (PBMCs) isolated from whole blood in corticosterone-treated mice could provide one of the markers of a biological signature of treatment response and predict a fast onset action in the mouse CORT model.

Keywords: anxious/depression-model, 5-HT₄ receptor, corticosterone, β-arrestin 1

2. Background and Historical Overview

In order for basic research to provide potential advances, a critical first step is to create useful animal models with relevant phenotypic features of the pathophysiology of depression and their treatment response[1]. Therefore it is critical for basic research to develop animal models that present behavioral, neurochemical and brain morphological phenotype reminiscent of depression and anxiety. Given that anxiety and depression have a high comorbidity with co-occurrence rates up to 60% in patients [2], animal models that present signs of both diseases could potentially be the most useful. Historically, since very few genetic variants with high penetrance (Brain Derived Neurotrophic Factor with the Val66Met, Serotonergic transporter with the short and the long variant) that cause depression are known, animal models have mainly relied on different means of chronically exposing rodents to stressful experiences, or sensory tract lesions such as in olfactory bulbectomy, to induce behavioral states that present depression-like signs and are responsive to chronic antidepressant treatment. Previously, drugs with putative anxiolytic/antidepressant properties were screened in naïve animals using behavioral paradigms predictive to anxiolytic (Open Field, Elevated Plus Maze, Light/Dark paradigm) or antidepressant-like activity (Forced Swim Test, tail Suspension test) sensitive to acute administration.

The oldest most commonly used paradigm to induce a depression-like state is chronic mild stress (CMS). Initial observations in 1982 suggested that rats subjected to multiple stressors over a prolonged period of time reduced their intake of saccharine or sucrose, a potential behavioral model of anhedonia [3]. Furthermore, this effect was selectively reversed by chronic treatment with the tricyclic antidepressant drugs (TCA) such as imipramine [3]. Further work was able to repeat this result using more mild stressors, such as periods of food and water deprivation, small temperature reductions and changes of cage mates [4,5]. Following these studies, the CMS procedure, and modified versions such as

chronic unpredictable stress (CUS or UCMS), became commonly used and much work demonstrated that other depression-like changes were induced in animals, such as decreased sexual and aggressive behaviors, decreased self-care, and altered sleep patterns [5]. Furthermore, these behaviors were all reversible by chronic, but not acute, treatment using multiple classes of antidepressants [6]. While historically potential pitfalls of the CMS procedure are that it is notoriously labor intensive, and that there has been some difficulty in getting the procedure established in mice of various backgrounds and the results replicated across laboratories [7], the modified versions of the CMS have proven more useful.

A distinct procedure that has gained traction is the usage of a social defeat model. In this paradigm a mouse is forced into the territory of a mouse from a larger, more aggressive strain leading to an interaction resulting in intruder subordination. Repeated defeats over 10 days can result in a long lasting reduced social interaction, sexual dysfunction, sleep dysregulation, anxiety, metabolic deficits and anhedonia [8-10]. Interestingly, following the social defeat procedure there remains a large variance in behavior outcomes in spite of using an inbred mouse strain (C57BL/6). Some animals display a resistance to social defeat (resilience) while others are susceptible (determined by interaction with a social target relative to an empty enclosure). Similar validity as the social defeat has been established for early life stress, such as maternal separation, which induces life-long behavioral and neuroendocrine abnormalities in the pups, some of which can be reversed by antidepressant medications [11,7]

Another procedure for inducing a depression-like state in Rodents is administration of chronic glucocorticoids in order to mimic the effects of chronic stress. A significant proportion of depressed patients display altered activity of the HPA axis, and chronic stress generally leads to hypersecretion of corticosteroids, which imposes an increased risk for

depression [12].

Among these possibilities to induce depression with co-morbid anxiety in Rodents, we choose to set up a model based on chronic corticosterone because the procedure is easy to implement compared to the CMS and easy to replicate across laboratories. Previous protocols have implemented the administration of corticosterone using pellets [13] or chronic injections [14]. The protocol described in this chapter is a method based on chronic corticosterone administration in the drinking water. This protocol provides an alternative to chronic injections preventing to dissolve CORT in sesame oil or EtOH. Chronic corticosterone administration has already been used in both C57BL/6 and CD-1 mice [15] but also in Rat [16]. It effectively induces multiple anxiety- and depression-like changes in behavior, neurochemistry and brain morphology [17,15,18,19]. Behaviorally, depression-related changes include suppression of sucrose intake and decreased self-care [15,16], while anxiety-related changes include increased latency to emerge into the light compartment in the light/dark test, decreased time, entries and percent distance in the center of an open field and increased latency to take a bite of food in the novelty suppressed feeding (NSF) test [19,15,20]. Furthermore, these effects are reversible by chronic, but not acute, antidepressant treatment [19,15,16]. Importantly, using X-irradiation of a restricted region of mouse brain containing the hippocampus, we demonstrated that some but not all of the effects of antidepressant treatment were neurogenesis-dependent [21]. More specifically, X-irradiation prevented the neurogenic and behavioral effects of selective serotonin reuptake inhibitors (SSRIs), fluoxetine in the NSF test, which is a neurogenesis-dependent test; conversely, fluoxetine-induced reversal of anxiety measures in the open field is neurogenesis-independent. Finally, this corticosterone paradigm is a useful paradigm not only to screen new antidepressant drugs [19], but also to investigate the mechanism of action of antidepressant drugs [15,22].

Recently, we have adapted the CORT model protocol to screen a rapid onset of action of drugs to treat anxiety/depression disorders. In spite of the fact that SSRIs are the most commonly prescribed drugs for the treatment of depression and several anxiety disorders, the onset of action of these antidepressant drugs is often delayed by 3 to 6 weeks [23]. The existence of this delayed action combined with the fact that one third of patients do not respond to treatment emphasizes the need for faster acting and more effective antidepressants [1]. This chapter gives a detailed overview of how the CORT model can help, at least in part, to solve this problem. For this purpose, we will take as an example, the effect of a 5-HT₄ receptor ligand, RS67333, in comparison to the classical SSRI, fluoxetine. Indeed, past works performed in naïve, non-stressed rats pointed out 5-HT₄ receptor agonists as a putative class of antidepressants with a rapid onset of action on depression-related behaviors and on hippocampal neurogenesis [24]. Indeed, a 3-day regimen with such compounds was sufficient to reverse CMS-induced decrease in sucrose intake in rats. Furthermore, several pre-clinical [15] and clinical [25-28] studies indicating the role of β -arrestin1, a protein involved in G protein receptor coupling, in the pathophysiology of mood disorders as well as in the mechanism of action of antidepressant [29,25,30,26,28]. So we also emphasize in this chapter how proteins levels, including β -arrestin 1 measurements in peripheral blood mononuclear cells (PBMCs) isolated from whole blood in corticosterone-treated mice, could provide one of the markers of a biological signature of treatment response.

3. Procedure

A classical protocol design to screen a rapid onset of action of drugs to treat anxiety/depression phenotype requires a 12 weeks study from the reception of the animals to their sacrifice (table 1). The corticosterone treatment will begin a week after animals are identified (the method used ear punch). The drug treatment will follow 4-weeks after the start of corticosterone treatment. Interestingly, in comparison to our original work [15], animals

are tested at two different time points throughout the protocol to compare subchronic (7 days) effects to chronic (28days) effects of drugs. The behavioral sequences are determined from the least to the most stressful events (i.e., from the open field to the FST). In addition, to address inherent difficulties in behavioral phenotyping in mice over time and to summarize results over tests and studies results, we use a method based on z-normalization principles also known as the emotionality score [31]. This test allowed the quantification of our behaviors results in an integrative manner along coherent dimensions, such as shown here for emotionality. Indeed, it is often difficult to reconcile positive or intermediate findings across tests, especially for behavioral measures that are subject to known variability in Rodents [31]. Thus, it is crucial to keep a record of behavioral data related to each animal across paradigm. It will allow calculating the emotionality score and/or performing correlation between behavior responses in anxiety/depression tests and changes in putative biomarkers. Blood collection and PBMCs isolation occurs the day after the last behavioral test is performed (table 1).

3.1. Subjects

Historically we have been using adult male C57BL/6Ntac mice purchased from Taconic Farms (Lille Skensved, Denmark and Germantown, NY, USA). The choice of the adult male C57BL/6Ntac mice is based on the fact that most of C57BL/6Ntac, and more generally C57BL/6 mice, are non-responders at baseline, i.e., chronic antidepressant treatment has little effect on neurogenesis or behavioral tests in this mouse background. However, when C57BL/6 mice are exposed to chronic corticosterone paradigms, a depression-like state is induced as determined by multiple behavioral tests [15]. Chronically stressed adult C57BL/6 mice also display a reduction in proliferation of progenitor cells [15,19]. Subsequent chronic antidepressant treatment will rescue the behavioral and neurogenesis effects of chronic stress

in C57BL/6 mice [15]. In other words, chronic stress paradigms can convert C57BL/6 mice from non-responders to responders (for review see [32]).

For a classical screening of a compound, experimenters should consider using 15 animals per group. A classical study includes a corticosterone-treated group, a corticosterone-treated group in the presence of fluoxetine and the corticosterone-treated group with the tested drugs at 2-3 different doses. A control group (without CORT) should also be included. It will allow the comparison with the corticosterone-treated group, pointing out the anxiety/depression-like state in this latest group.

Thus, to screen a putative rapid onset of action of a drug to treat anxiety/depression, at least 60 animals should be included in an experiment. All mice are 7-8 weeks old, weighed 23-25g at the beginning of the treatment, and are maintained on a 12L:12 D schedule (lights on at 0600). They are housed in groups of five (table 1). Food and water are provided *ad libitum*. A week after their reception, animals are identified by ear punch. This identification is crucial and will allow a monitoring of each animal across time and across behavioral paradigms. We recommend monitoring body weights once a week during all the experiment. In our hands, male C57BL/6Ntac exposed to chronic corticosterone (5 mg/kg/day for 4 weeks) might gain weight in comparison to controls animals (table 1). All testings must be conducted in compliance with the laboratory animal care guidelines and with protocols approved by the Institutional Animal Care and Use Committee.

3.2. Corticosterone preparation

Our designed protocol consists in chronic administration of corticosterone in the drinking water. For this purpose, corticosterone (4-pregnen-11b-DIOL-3 20-DIONE 21-hemisuccinate (Sigma-Aldrich Saint-Quentin Fallavier, France) is dissolved in vehicle (0.45% hydroxypropyl- β -cyclodextrin, Sigma-Aldrich Saint-Quentin Fallavier, France). The dose and duration of corticosterone treatment are selected based on previous studies [15,33]. For a

cohort of 60 animals corticosterone (35 µg/ml/d, equivalent to about 5 mg/kg/d) is available *ad libitum* in the drinking water in (table 2). Corticosterone powder begins to degrade once in solution (table 1). Thus, to protect corticosterone from light, opaque bottles should be used. Control animals (vehicle group) should receive the vehicle (0.45 % β -cyclodextrin, β -CD).

3.3. Drugs treatment to assess rapid onset of action of 5-HT₄ receptor agonist, RS67333

While administration with β -CD or corticosterone continued, mice are treated with vehicle (0.45% β -CD), fluoxetine hydrochloride (Anawa Trading, Zurich, Switzerland), RS67333 hydrochloride (1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-butyl-4piperidinyl)-1-propanone hydrochloride) (Tocris Bioscience, Bristol, United Kingdom) (Fig. 1). RS67333 is delivered by osmotic mini-pumps at a dose of 1.5 mg/kg/day [34]. Fluoxetine (18 mg/kg per day) is delivered in the drinking water as previously described [15]. Osmotic minipumps (42 days mini-pumps, 2006 model, Alzet, Cupertino, CA) is implanted subcutaneously under light anesthesia (ketamine/xylazine; (75/20 mg/kg) from Sigma Aldrich (Sigma-Aldrich Saint-Quentin Fallavier, France). Treatment is always maintained until the end of the experiments (figure 1).

3.4. Behavioral tests

The same cohort of animals is tested in five different behavioral tests of anxiety and depression. The originality of our protocol consists of successively testing after 7 days and 28 days of treatment, each animal, over a week in the Open Field (OF), Elevated Plus Maze (EPM), Novelty Suppressed Feeding (NSF), Splash Test (ST) and Tail Suspension Test (Fig 1). Behavioral testing occurred during the light phase between 07:00 a.m. and 07:00 p.m.. Behavioral paradigms occur after 7 or 28 days of drug treatment. Other behavioral paradigms could also be processed such as the light/dark paradigm for anxiety or the sucrose preference

for depression. Experimenters should keep in mind that they should always start behavioral analyses from the less to the most stressful paradigm, i.e., Open Field, Elevated Plus Maze, Novelty Suppressed Feeding, Forced Swim Test/Tail Suspension Test, Splash Test).

3.1.1. Open Field (OF)

This test was initially described by Hall and Ballachey (1932) [35] and is performed in our laboratory as described by David and colleagues [15]. Motor activity is quantified in four 43 x 43 cm² Plexiglas open field boxes (MED Associates, Georgia, VT). Two sets of 16 pulse-modulated infrared photo beams are placed on opposite walls 2.5-cm apart to record x–y ambulatory movements. Activity chambers are computer interfaced for data sampling at 100-ms resolution. The computer defines grid lines dividing each open field into center and surround regions, with each of four lines being 11 cm from each wall. Dependent measures in the center are the total time and the number of entries over a 30-min test period. The activity in the center is quantified as distance traveled in the center divided by total distance traveled.

3.1.2. Elevated Plus Maze (EPM)

This test was initially described by Pellow and colleagues (1985) [36] and is performed in our laboratory as described by David and colleagues [15]. The maze is a plus-shaped apparatus, with two open arms and two arms closed by walls linked by a central platform 50 cm above the floor. Mice are individually placed in the center of the maze facing an open arm and are allowed to explore the maze during 5 min. The time spent in the open arms as well as the numbers of entries into the open arms are used as an anxiety index. All parameters are measured using a video tracker (EPM3C, Bioseb, Vitrolles, France).

3.1.3. Novelty-Suppressed-Feeding (NSF)

The NSF in mice was initially described by Santarelli and colleagues (2003) [21]. It is a conflict test that elicits competing motivations: the drive to eat and the fear of venturing into

the center of a brightly lit arena. The latency to begin eating is used as an index of anxiety/depression-like behavior, because classical anxiolytic drugs as well as chronic antidepressants decrease this measure. The NSF test is carried out during a 10-min period as previously described in René Hen's laboratory [15]. Briefly, the testing apparatus consisted of a plastic box (50x50x20 cm), the floor of which is covered with approximately 2 cm of wooden bedding. Twenty-four hours prior to behavioral testing, all food is removed from the home cage. At the time of testing, a single pellet of food (regular chow) is placed on a white paper platform positioned in the center of the box. Each animal is placed in a corner of the box, and a stopwatch is immediately started. The latency to eat (defined as the mouse sitting on its haunches and biting the pellet with the use of forepaws) is timed. Immediately afterwards, the animal is transferred to its home cage, and the amount of food consumed by the mouse in the subsequent 5 min is measured, serving as a control for change in appetite as a possible confounding factor.

3.1.4. Tail Suspension Test (TST)

The TST was initially described by Steru et al., (1985) [37] as an antidepressant activity screening test [37] often used to test compounds that are expected to affect depression related behaviors. Mice are suspended by their tails with tape, in such a position that they cannot escape or hold on to nearby surfaces. During this test, typically 6 minutes in duration, the resulting escape oriented behaviors are quantified using an automated tail suspension test apparatus (Bioseb, Vitrolles, France). A specific strain gauge linked to a computer quantifies the time spent by the animal trying to escape from this uncomfortable position.

3.1.5. Splash Test (ST)

This test was initially described by Surget and colleagues [6] and it consists of squirting 200 µl of a 10% sucrose solution on the mouse's snout. The grooming duration is

assessed at the end of the corticosterone regimen following (or not) by 4 weeks of drug treatment according to a protocol used elsewhere [15].

3.5. Emotionality Score

To address behavioral variability and obtain comprehensive and integrated measures in each group, emotionality-related data are normalized using a Z-score methodology previously described [31]. Z-scores for behavioral measures are first averaged within a test, then across tests to ensure equal weighting of the four tests comprising the final Z-score (Table 3). The TST as the FST, is excluded from the emotionality score analysis since it is primarily recognized as a screening test for antidepressant drug action. In the Z-score analysis, the vehicle/vehicle group is used as control. Increased emotionality in mice is revealed by decreased values of dependent variables in some tests (OF and EPM) and by increased values in other tests (NSF), and thus all measures indicative of increased emotionality should be reflected by positive numbers of standard deviations from the control group mean [31].

3.6. Collection of mouse blood and isolation of peripheral blood mononuclear cells

Candidate biomarkers that can accurately predict antidepressant-like responses must be identified. While there are currently no specific markers that are considered “gold standards”, a few candidates have emerged. Recently, we showed that PBMCs from anxious/depressed mice showed significantly reduced β -arrestin 1 levels [38]. These decreased β -arrestin 1 expression levels in PBMCs were restored to normal levels with chronic fluoxetine treatment. Blood is collected from anaesthetized mice as previously described [39,40]. In compliance with the laboratory animal care guidelines, approximately 0.4 ml of blood per mouse is collected in K₃EDTA tubes with a submandibular bleeding procedure [30]. 5 mm point size sterile lancets (MediPoint, Mineola, NY) are used to

puncture the location where the orbital vein and the submandibular vein join to form the jugular vein [40]. A light pressure with dry gauze is applied to the punctured area for hemostasis. Separation and extraction of peripheral blood mononuclear cells (PBMCs) are performed using an iodixanol mixer technique [41]. Mouse PBMCs are purified from whole blood by density centrifugation (300 g at 20°C for 30 min) using solution B of the OptiPrepTM gradient solution (Sigma-Aldrich Saint-Quentin Fallavier, France). Specifically, the OptiPrepTM gradient solution is used to separate blood into PBMC and plasma layers with centrifugation. The PBMC layers are then carefully removed from the tube and transferred to a new 50 ml conical tube. The PBMCs are then washed twice with solution B (1 min each). After another centrifugation (150 g at 20°C for 7 min) and 2 washing steps (1 min each), mouse PBMCs are recovered with a final centrifugation (1,000 g at 4°C for 5 min) and are stored at -80°C.

Equal amounts of proteins are separated by 10% sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF), (Amersham Biosciences, Les Ulis, France). The membranes are then incubated overnight with a primary mouse monoclonal anti- β -Arrestin 1 antibody (#610551, BD Bioscience Pharmingen, France; 1:100). In order to ensure that equal amounts of total protein (30 μ g) are loaded in each lane, β -actin protein levels were also assessed (β -actin (C4) HRP, Santa Cruz Biotechnology, Germany, 1:10,000). Immune complexes are detected using appropriate peroxide-conjugated secondary antibodies and a Chemiluminescent reagent kit (Pierce Biotechnology). Immunoblot quantifications are performed by densitometric scanning with Image lab software (Bio-Rad). Signals are in the linear range. The densitometry values are normalized against the β -actin values.

3.7. Statistical Analysis

Results from data analyses are classically expressed as mean \pm SEM. Data are analyzed using StatView 5.0 software (SAS Institute, Cary, NC). One-way ANOVAs is applied to the behavioral and neurochemical data as appropriate. Significant main effects and/or interactions are followed by Fisher's PLSD post hoc analysis. In the NSF test, the Kaplan-Meier survival analysis is used due to the lack of normal distribution of the data. Mantel-Cox log-rank test is used to evaluate differences between experimental groups. Statistical significance is set at $P < 0.05$.

4. Data Analysis and Anticipated Results

Behavioral data can be analyzed and presented separately using classical parameters such as time or entries in the center in the Open Field, time or entries in the opened arm in the Elevated Plus Maze, the latency to feed in the Novelty Suppressed Feeding, the grooming duration in the Splash test and the immobility duration in the Tail Suspension/Forced Swim Test (table 4). Excluding the TST/FST, chronic corticosterone treatment affects all the behavioral parameters by inducing an anxiety/depressive-like phenotype. For example, long-term corticosterone exposure does not change immobility duration in the mouse FST, which might imply that this test is more appropriate to identify antidepressant-like activity of a molecule than a depressive-like state, as originally described by Porsolt (1977) [42].

Classical monoaminergic antidepressants such as fluoxetine, reboxetine, imipramine or agomelatine, a novel antidepressant drug with melatonergic agonist and 5-HT_{2C} receptor antagonist properties, display antidepressant/anxiolytic-like properties in the CORT model [15,33], correcting behavioral deficits in the OF, EPM, NSF or ST. Applying z-normalization across complementary measures in different behavioral tests after chronic corticosterone increases emotionality score [31]. The emotionality score (z-score) is easy-to-apply and “generalizable”. Z-score methodology can increase the reliability and comprehensiveness of

behavioral testing from a variety of non-exclusive tasks, but along cohesive behavioral dimensions. An important feature of its application to behavioral data is to ensure conformity with the direction of effects. Thus, chronic corticosterone is producing a persistent anxiety/depressive-like phenotype in mice, as measured by an increase in emotionality score.

It is extremely interesting to note that, unlike fluoxetine, a 5-HT₄ receptor agonist, such as RS67333, is able to correct the increase in the emotionality score induced by chronic corticosterone after a week of treatment. A longer duration of treatment (28 days) is required for fluoxetine to exert anxiolytic-like effects comparable to 7 days of RS67333 treatment.

Data from pre-clinical [15,25][19] and clinical [25,28,27] studies performed by Avissar's group support the importance of measuring β -arrestin 1 levels as a peripheral biomarker of the pathophysiology of mood disorders and predicting the antidepressant-like response [30,25,27]. We recently found that β -arrestin 1 protein levels in leukocytes were reduced when mice were treated by chronic corticosterone [30,35]. The reduced β -arrestin 1 levels exposed to chronic corticosterone were alleviated by fluoxetine treatment after chronic, but not subchronic treatment (Fig 3). In contrast, RS67333 treatment was able to correct this decrease in β -arrestin 1 expression in CORT-treated mice as early as 7 days of treatment. The rapid onset of anxiolytic/antidepressant-like activity observed after a subchronic treatment with RS67333 is predicted by a change in β -arrestin 1 levels in PBMC isolated from anxious/depressive-like mice. The relationship between changes in β -arrestin 1 levels at the periphery, as well as in various brain tissues and the anxiolytic/antidepressant-like activity of 5-HT₄ receptor agonists is still unknown and requires further investigation.

5. Troubleshooting

Several practical recommendations, summarized here, may help the researchers to obtain more reliable and reproducible behavioral data.

1-The CORT model in mice has been developed in adult male C57BL/6Ntac mice. Strains differences may occur. The researchers may need to re-assess the strain's sensitivity to long-term corticosterone treatment for their experiment, e.g. re-examine the dose of corticosterone.

2-Experimenters should keep in mind that the emotionality score should be used only for screening purposes and not for mechanistic approaches. Indeed, antidepressant-like activity of monoaminergic drugs at least, required neurogenesis-dependent and independent action in the mouse CORT model [15]. For example, in the NSF test, both fluoxetine and a 5-HT₄ receptor agonist had an anxiolytic/antidepressant-like effect only after chronic treatment, suggesting that the neurobiological mechanisms involved in this paradigm are different from those underlying the other behavioral tests (OF, EPM, TST, ST). If after a subchronic treatment, the emotionality score for a 5-HT₄ receptor agonist can reverse/block the increase in z-score induced by chronic corticosterone treatment, it only reflects a part of its activity, the neurogenesis-independent one.

3- Measuring protein levels in mouse PBMCs at several time points is a powerful technique that can be used to reveal potential biomarkers for the pathophysiology of depression and the antidepressant response. Our study focused on β -arrestin 1 level, however, disease conditions are most often signified by the dysregulation of complex biological pathways involving multiple key factors. Experimenter should keep in mind that several factors may influence the outcome of PBMC separation. Factors such as addition of medium to whole blood before centrifugation, PBMC separation, medium temperature, separation media from different sources, centrifugation speed and frequency and addition of serum are likely to have an impact on cell quality and quantity recover and therefore a low yield in protein levels.

6. Conclusions

We believe the CORT model described in this chapter is ideally suited to address the basic molecular mechanisms of anxiety/depression and screening drugs with innovative targets. The present study is encouraging for the development of 5HT₄ receptor agonist or signaling molecules that interact with its receptor leading to a fast anxiolytic/antidepressant activity for the benefit of patients. Finally, we also demonstrated that PBMCs isolated from a small volume of whole blood in unanesthetized mice using a submandibular bleeding method might provide a useful biological tool to assess circulating proteins including β -arrestin 1. Likewise, we confirmed that measurements of β -arrestin 1 level in PBMCs may serve as a biochemical marker of depression in humans and can predict fast onset of action of drugs such as 5-HT₄ receptor agonist. Overall, with the CORT model associated with an emotionality score analysis and a β -arrestin 1 measure in PBMC, we developed powerful tools to predict the effectiveness of antidepressant drugs.

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Legend

Figure 1: Protocol to assess fast onset of action of new drugs in anxio/depressive mouse model

(A) In place of normal drinking water, grouped-housed male C57BL/6Ntac mice are presented with vehicle (0.45% hydroxypropyl- β -cyclodextrin) or corticosterone (35 μ g/ml) in the presence or absence of a 5-HT₄ agonist (RS67333, 1.5 mg/kg/day, Alzet® mini pump model 2006 implanted subcutaneously) or fluoxetine, 18 mg/kg/day.

(B) The anxiety/depressive-like phenotype of chronic corticosterone is assessed by comparing a chronic corticosterone/vehicle group *versus* a vehicle/vehicle group. The same animals are successively tested in the OF paradigm, the EPM, the NSF, the ST, and the TST after subchronic (days 7 to 11) or chronic (days 28 to 33) drug treatment.

Figure 2: Classical behavioral paradigms used to screen anxiolytic/antidepressant-like activity in mice

The Open Field (OF) and the Elevated plus maze (EPM) paradigms are used to screen anxiolytic-like activity. The Splash test (ST), the Forced Swim test (FST) and the Tail Suspension Test are useful paradigm to predict antidepressant-like activity.

Figure 3: Rapid onset of action of 5-HT₄ agonist treatment in a mouse model of anxiety/depression

Effects of a subchronic and a chronic 5-HT₄ ligand, RS67333, or fluoxetine treatment on anxiety- and depression-like behaviors on the Emotionality score after subchronic (A) or chronic treatment (B). Test z-values (Open-Field, Elevated Plus maze, Novelty Suppressed

Feeding and Splash Test) are then calculated by averaging individual z-scores and averaged to obtain emotionality z-score after subchronic (A) and chronic treatment (B). One-factor ANOVA with Fisher's PLSD post hoc analysis against the control group (vehicle/vehicle) revealed that only chronic RS67333 treatment reversed increase in emotionality score induced by corticosterone after subchronic treatment ($F(3,56)=4.9$, $p<0.01$) while it takes 28 days for fluoxetine treatment ($F(3,56)=9.25$, $p<0.01$). Values plotted are mean \pm SEM ($n=15$ per group). ** $p<0.01$, ## $p<0.01$, versus vehicle/vehicle group and corticosterone/vehicle group, respectively.

Figure 1

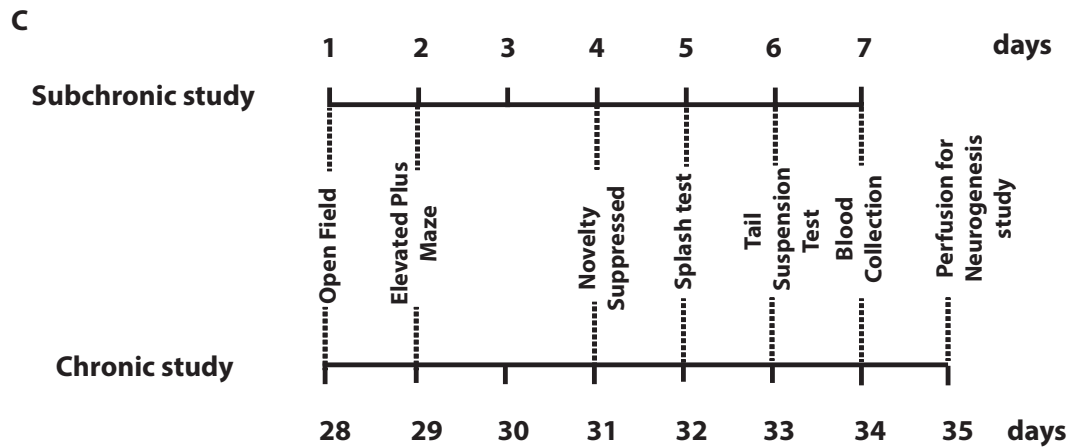
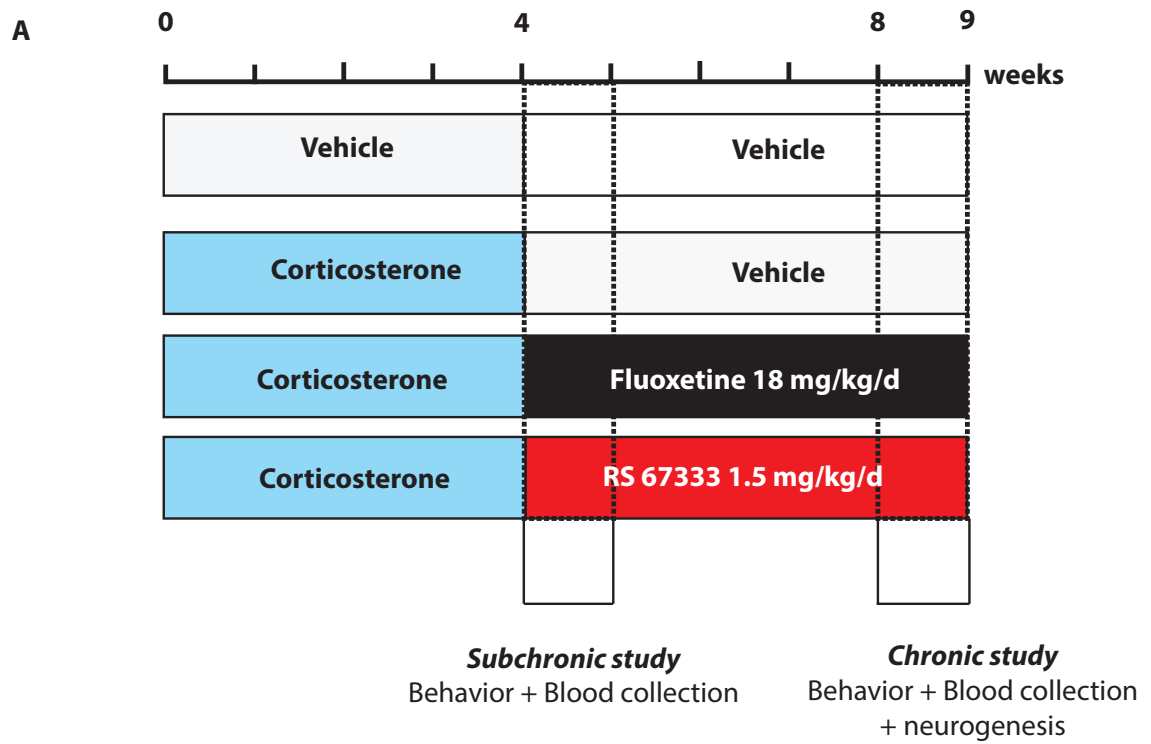


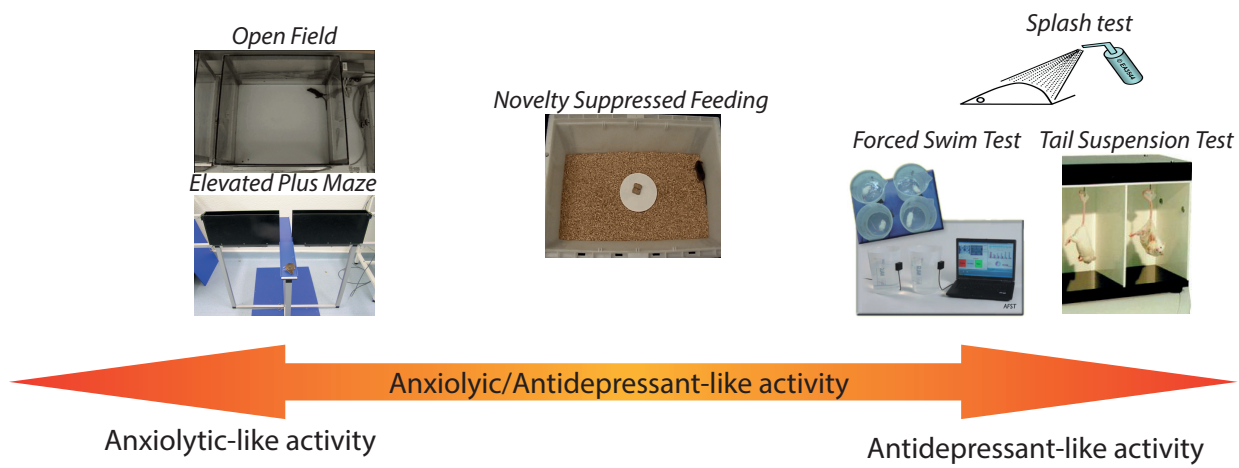
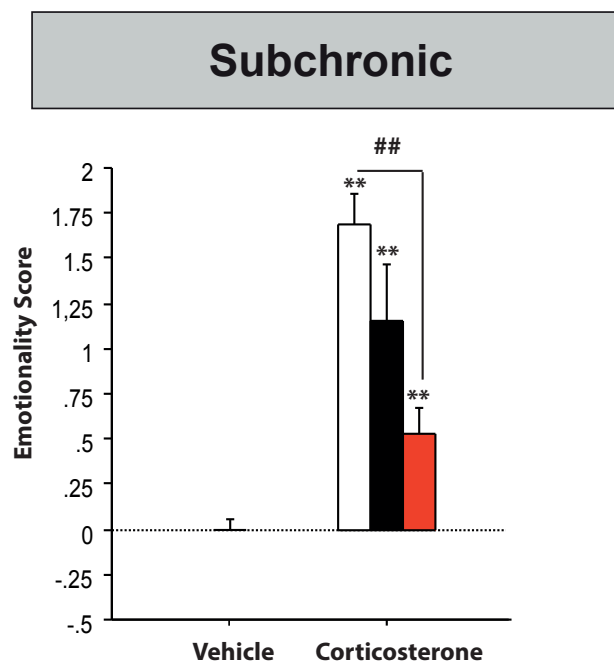
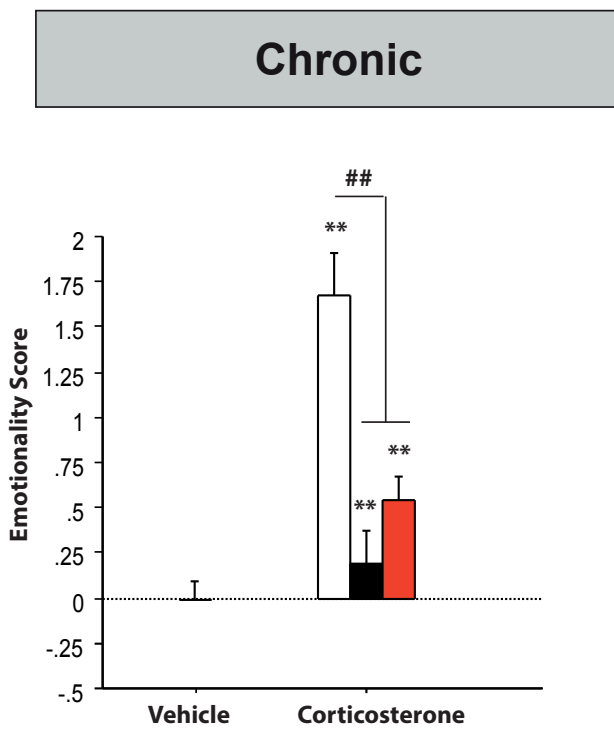
Figure 2

Figure 3

A



B



□ Vehicle

■ Fluoxetine 18mg/kg/day

■ RS 67333 1.5mg/kg/day

Table 1: Weekly procedure to assess rapid onset therapeutic activity in a model of anxiety/depression

Week	Days	Procedures
0	0	Reception of animals, housed by 5
1	7	Mouse Identification (<i>ear punch</i>)
2	14 (<i>day 0 of the treatment</i>)	Start of Corticosterone treatment Weigh animals
	17	Change Corticosterone
3 (<i>week 1 of the treatment</i>)	21 (<i>day 7 of the corticosterone treatment</i>)	Change Corticosterone Weigh animals
	24	Change Corticosterone
4 (<i>week 2 of the treatment</i>)	28 (<i>day 14 of the corticosterone treatment</i>)	Change Corticosterone Weigh animals
	31	Change Corticosterone
5 (<i>week 3 of the treatment</i>)	35 (<i>day 21 of the corticosterone treatment</i>)	Change Corticosterone Weigh animals
	38	Change Corticosterone
6 (<i>week 4 of the treatment</i>)	42 (<i>day 28 of the corticosterone treatment</i>)	Change Corticosterone Weigh animals
	45	Change Corticosterone
7 (<i>week 5 of the treatment</i>)	49 (<i>day 35 of the corticosterone treatment</i>)	Start fluoxetine and drug treatment (5-HT ₄ receptor agonist RS67333, mini-pump) Change Corticosterone Weigh animals
	52	Change Corticosterone
8 (<i>week 6 of the treatment</i>)	56 (<i>day 42 of the corticosterone treatment, day 7 of drugs treatment</i>)	Change Corticosterone and fluoxetine Weigh animals Open Field Paradigm
	57	Elevated Plus Maze
	58	Food Deprivation
	59	Change Corticosterone Novelty Suppressed Feeding
	60	Splash Test
	61	Tail Suspension Test
	62	Blood collection
9 (<i>week 7 of the treatment</i>)	63 (<i>day 49 of the corticosterone treatment, day 14 of drugs treatment</i>)	Change Corticosterone and fluoxetine Weigh animals
	66	Change Corticosterone
10 (<i>week 8 of the treatment</i>)	70 (<i>day 56 of the corticosterone treatment, day 21 of drugs treatment</i>)	Change Corticosterone and fluoxetine Weigh animals
	73	Change Corticosterone
11 (<i>week 9 of the treatment</i>)	77 (<i>day 63 of the corticosterone treatment, day 28 of drugs treatment</i>)	Change Corticosterone and fluoxetine Weigh animals Open Field Paradigm
	78	Elevated Plus Maze
	79	Food Deprivation
	80	Change Corticosterone Novelty Suppressed Feeding
	81	Splash Test
	82	Tail Suspension Test
	83	Blood collection
12 (<i>week 10 of the treatment</i>)	84	Sacrifice of animals (<i>perfusion</i>)

Table 2: Method to prepare chronic corticosterone

Chronic Corticosterone administration (mice) in the drinking water
<ul style="list-style-type: none"> ▪ Dissolving Corticosterone <ul style="list-style-type: none"> ▪ Dissolve corticosterone in 20% β-cyclodextrin ▪ Protect corticosterone from excessive light exposure. <p><i>Preparation of the solution of 100 ml of a 20% β-cyclodextrin solution (β-cyclodextrin can be prepare weekly and stored at 4°C)</i></p> <ul style="list-style-type: none"> ▪ <i>Weigh 20g of β-cyclodextrin</i> ▪ <i>Raise volume to 100 ml with water.</i> ▪ <i>Stir 30 min at room temperature</i> <p><i>Preparation of the solution of 35μg/ml/day of corticosterone</i></p> <ul style="list-style-type: none"> ▪ To 37.5 ml 20% β-cyclodextrin add 58.34 mg corticosterone ▪ Sonicate circa 30 min ▪ Raise volume to 1666 ml with water. <ul style="list-style-type: none"> ▪ Compounds used: <ul style="list-style-type: none"> ▪ Corticosterone: Sigma-C2505 ▪ β-cyclodextrin: Sigma H107

Table 3: Behavioral paradigms and their appropriated parameters recorded

Paradigms	Parameters recorded	Parameters used to analyze behavioral data	Parameters used to calculate Emotionality score
Open Field	-Time in the center and in the periphery, -Entries in the center and in the periphery, -Ambulatory distance in the center and in the periphery.	-Time in the center (in sec) -Entries in the center -Total Ambulatory Distance (in cm) -Ratio Ambulatory Distance in Center/Total Ambulatory Distance	-Time in the center (in sec) -Entries in the center -Ratio Ambulatory Distance in Center/Total Ambulatory Distance
Elevated Plus Maze	-Time in opened and closed arms, -Entries in opened and closed arms, -Ambulatory distance in opened and closed arms.	-Time in opened arms (in sec) -Entries in opened arms -Ambulatory distance in opened arms (Total entries could also be used -Total Distance	-Time in opened arms (in sec) -Entries in opened arms -Total Distance
Novelty Suppressed Feeding	-Latency to feed, -Food consumption in the home cage, -Mouse body weight before and after food deprivation.	- A univariate scattergram showing latency to feed (in sec) of individual mouse subjects -Food consumption in the home cage (in mg/g of mouse)	-Latency to Feed (in sec)
Tail Suspension Test or Forced Swim test	-Immobility duration	-Immobility duration (in sec)	Not applicable
Splash Test	-Grooming duration, -Number of grooming episodes, -Latency to groom.	-Groom ing duration (in sec) -Number of grooming episodes and latency to groom could also be added	-Grooming duration (in sec)

Table 4: Example of table used to calculate the emotionality score

Cage_#ID			Mouse_#ID	Treatment	RAW DATA OF	RAW DATA EPM	RAW DATA NSF	RAW DATA ST
			OF_Center/Total_Distance_Ratio	OF_Center Time OF_Center Entries	EPM_Open/Total arm entries ratio EPM_Open Arms Time EPM_Total Distance	NSF_Latency	Splash_TEST_Grooming duration	
1	1							
	2							
	3							
	4							
	5							
2	6							
	7							
	8							
	9							
	10							
3	11							
	12							
	13							
	14							
	15							
4	16							
	17							
	18							
	19							
	20							
5	21							
	22							
	23							
	24							
	25							
6	26							
	27							
	28							
	29							
	30							
7	31							
	32							
	33							
	34							
	35							
8	36							
	37							
	38							
	39							
	40							
9	41							
	42							
	43							
	44							
	45							
10	46							
	47							
	48							
	49							
	50							
11	51							
	52							
	53							
	54							
	55							
12	56							
	57							
	58							
	59							
	60							

Table 5: β -arrestin 1 levels in peripheral blood mononuclear cells after subchronic and chronic treatment with the 5-HT₄ agonist, RS67333

Treatment Duration	Vehicle/Vehicle	Corticosterone/Vehicle	Corticosterone/ Fluoxetine (18 mg/kg/day)	Corticosterone/ RS67333 (1.5 mg/kg/day)
7 days	100 \pm 2.1	72.5 \pm 10.8*	81 \pm 39.8	185.6 \pm 33.04##
28 days	100 \pm 13.4	61.4 \pm 10.6**	91.1 \pm 6.1#	124.0 \pm 21.2##

Data are expressed in % of the Vehicle/Vehicle Group \pm SEM.

One-factor ANOVA with Fisher's PLSD post hoc analysis against the control group (vehicle/vehicle) revealed that only chronic RS67333 treatment reversed decrease in β -arrestin 1 levels in peripheral blood mononuclear cells induced by corticosterone after subchronic treatment while it takes 28 days for fluoxetine treatment to reverse this effect [$F(6,45)=3.62$, $p<0.01$]. Values plotted are mean \pm SEM ($n=6-7$ per group).

* $p<0.05$, ** $p<0.01$, # $p<0.05$, ## $p<0.01$, versus vehicle/vehicle group and corticosterone/vehicle group, respectively.

RÉSULTATS EXPÉRIMENTAUX

Résultats Expérimentaux

Article 4:

Rapid anxiolytic effects of a 5-HT₄ receptor agonist are mediated by a neurogenesis-independent mechanism. Accepted for publication in Neuropsychopharmacology, manuscript NPP-13-1008RR

Article 5:

A method for biomarker measurements in peripheral blood mononuclear cells isolated from anxious and depressed mice: β -arrestin 1 protein levels in depression and treatment. Frontiers in Pharmacology(4):124.

Résultats complémentaires:

A method for biomarker measurements in β -arrestin 1 protein levels in peripheral blood mononuclear cells isolated from anxious and depressed mice can predict fast onset of action of 5-HT₄ agonist.

Article 4: Rapid anxiolytic effects of a 5-HT₄ receptor agonist are mediated by a neurogenesis-independent mechanism.

Indira Mendez-David, Denis J David, Flavie Darcet, Melody V Wu, Saadia Kerdine-Römer S,
Alain M Gardier, René Hen

Accepted for publication in Neuropsychopharmacology, manuscript NPP-13-1008RR

Introduction of the study

Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed drugs for the treatment of depression and several anxiety disorders. Unfortunately, the onset of action of SSRIs is often delayed by 3 to 6 weeks (Artigas, 2013). The existence of this delayed action combined with the fact that one third of patients do not respond to treatment emphasizes the need for faster acting and more effective antidepressants (Samuels et al., 2011).

It has been proposed that 5-HT₄ receptor agonists such as RS67333 may bring new hope for treating depression (Lucas, 2009; Lucas et al., 2005; Lucas and Debonnel, 2002; Lucas et al., 2010; Lucas et al., 2007).

While a number of studies have assessed the antidepressant-like activity of 5-HT₄ receptor agonists, none have so far evaluated their anxiolytic-like profile. We also assessed whether chronic 5-HT₄ receptor stimulation can affect proliferation of newborn cells and maturation of newborn neurons. Finally, using our mouse model of anxiety/depression combined with ablation of hippocampal neurogenesis by X-irradiation, we assessed whether the anxiolytic/antidepressant action of RS67333 after 7-days and 28-days of treatment recruits a neurogenesis-dependent mechanism.

Rapid anxiolytic effects of a 5-HT₄ receptor agonist are mediated by a neurogenesis-independent mechanism

Running: 5-HT₄ receptor contribution to antidepressant response

Authors: Indira Mendez-David^{1*}, Denis J. David^{1*}, Flavie Darcet¹, Melody V. Wu³, Saadia Kerdine-Romer², Alain M. Gardier¹, René Hen^{3,§}

¹EA 3544, Lab. Neuropharmacologie, Faculté de Pharmacie, Université Paris-Sud, Châtenay-Malabry F-92296, France.

²INSERM UMR 996, Faculté de Pharmacie, Université Paris-Sud, Châtenay-Malabry F-92296, France.

³Departments of Neuroscience and Psychiatry, Columbia University, New York, NY, 10032, USA; Department of Integrative Neuroscience, New York State Psychiatric Institute, New York, NY 10032, USA

*These authors contributed equally to this work

§ To whom correspondence should be addressed.

Pr René Hen

Departments of Neuroscience and Psychiatry,

Columbia University,

New York, NY, 10032, USA

Tel: +1 212 543 5328

Fax: +1 212-543-5074

E-mail: rh95@columbia.edu

ABSTRACT

Selective serotonin reuptake inhibitors (SSRIs) display a delayed onset of action of several weeks. Past work in naïve rats showed that 5-HT₄ receptor agonists had rapid effects on depression-related behaviors and on hippocampal neurogenesis. We decided to investigate whether 5-HT₄ receptor stimulation was necessary for the effects of SSRIs in a mouse model of anxiety/depression and whether hippocampal neurogenesis contributed to these effects. Using the mouse corticosterone model of anxiety/depression, we assessed whether chronic treatment with a 5-HT₄ receptor agonist (RS67333, 1.5 mg/kg/day) had effects on anxiety and depression-related behaviors as well as on hippocampal neurogenesis in comparison to chronic fluoxetine treatment (18 mg/kg/day). Then, using our anxiety/depression model combined with ablation of hippocampal neurogenesis, we investigated whether neurogenesis was necessary for the behavioral effects of subchronic (7-days) or chronic (28-days) RS67333 treatment. We also assessed whether a 5-HT₄ receptor antagonist, (GR125487, 1 mg/kg/day) could prevent the behavioral and neurogenic effects of fluoxetine. Chronic treatment with RS67333, similar to fluoxetine, induced anxiolytic/antidepressant-like activity and stimulated adult hippocampal neurogenesis, specifically facilitating maturation of newborn neurons. However, unlike fluoxetine, anxiolytic effects of RS67333 were already present after 7 days and did not require hippocampal neurogenesis. Chronic treatment with GR125487 prevented both anxiolytic/antidepressant-like and neurogenic effects of fluoxetine, indicating that 5-HT₄ receptor activation is necessary for these effects of SSRIs. 5-HT₄ receptor stimulation could represent an innovative and rapid onset therapeutic approach to treat depression with comorbid anxiety.

Key words: 5-HT₄ receptor, anxiolytic, neurogenesis, fast onset

INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed drugs for the treatment of depression and several anxiety disorders. Unfortunately, the onset of action of SSRIs is often delayed by 3 to 6 weeks (Artigas, 2013). The existence of this delayed action combined with the fact that one third of patients do not respond to treatment emphasizes the need for faster acting and more effective antidepressants (Samuels *et al*, 2011).

It has been proposed that 5-HT₄ receptor agonists such as RS67333 may bring new hope for treating depression (Lucas, 2009; Lucas *et al*, 2005; Lucas and Debonnel, 2002; Lucas *et al*, 2010; Lucas *et al*, 2007). Indeed, administration of 5-HT₄ agonists induced similar molecular and behavioral changes as common antidepressants in rodents (Bockaert *et al*, 2008; Lucas *et al*, 2007; Pascual-Brazo *et al*, 2012). Depressed-like state in the olfactory bulbectomy or chronic mild stress model was completely abolished after 10-14 days of RS67333 treatment in rats, suggesting a more rapid response mechanism in comparison to classical antidepressants (Lucas *et al*, 2007). A positive behavioral response in the novelty suppressed feeding (NSF) test in rat, a complete reversion of anhedonic-like state (sucrose consumption), and an increase in swimming behavior in defeated mice in the forced swim test (FST) were also observed after a short period of RS67333 treatment (Gomez-Lazaro *et al*, 2012; Pascual-Brazo *et al*, 2012). In addition to behavioral data, and in agreement with a previous report from Lucas and colleagues, a recent study performed in naïve rats confirmed that a short period of treatment with RS67333 increased the number of newborn cells in the dentate gyrus (DG) (Pascual-Brazo *et al*, 2012). These results are interesting because hippocampal neurogenesis has been implicated in some of the behavioral effects of antidepressants in adult rodents (David *et al*, 2009; Santarelli *et al*, 2003). However, no direct evidence has yet linked the antidepressant-like effects of 5-HT₄ receptor activation and its neurogenic effects. Lastly, it has been suggested that SSRIs and 5-HT₄ receptor agonists share common mechanisms of action. Indeed, the 5-HT₄ receptor agonist,

RS67333, augmented the acute effect of paroxetine on extracellular 5-HT levels in rat ventral hippocampus, and after only 3 days of administration increased basal hippocampal 5-HT levels (Licht *et al*, 2010). The coadministration of the SSRI citalopram and RS67333 strongly potentiated the antidepressant-like properties of the latter in several electrophysiological, molecular, and behavioral paradigms (Lucas *et al*, 2010).

While a number of studies have assessed the antidepressant-like activity of 5-HT₄ receptor agonists, none have so far evaluated their anxiolytic-like profile. It is noteworthy that some SSRIs are often prescribed for the treatment of anxiety disorders (Burghardt and Bauer, 2013). Anxiety disorders have a lifetime prevalence of over 25% making them the most common psychiatric disorders (Kheirbek *et al*, 2012). Moreover, a co-morbidity between depression and anxiety disorders is commonly observed. Thus, this study aimed to investigate both antidepressant and anxiolytic-like effects of either subchronic or chronic administration of a 5-HT₄ receptor agonist in a model of anxiety/depression based on the elevation of glucocorticoids in mice (CORT model) (David *et al*, 2009). “Standard models of depression that rely on environmental stress manipulations, such as learned helplessness or the chronic mild stress (CMS) are hampered by protocol variability and reported difficulties in replication, thus highlighted the need for a reliable, easily replicable depression model (Nestler *et al*, 2002). The corticosterone model is a chronic exposure method optimized for use in modeling the persistent anxiety/depression-like state in rodents, allowing for multiple behavioral tests in the same animals using an etiologically relevant model of depression that is easily replicable between and within laboratories (David *et al*, 2009; David *et al*, 2010; Gould, 2011; Mendez-David *et al*, 2013).

We also assessed whether chronic 5-HT₄ receptor stimulation can affect proliferation of newborn cells and maturation of newborn neurons. Finally, using our mouse model of anxiety/depression combined with ablation of hippocampal neurogenesis by X-irradiation, we

assessed whether the anxiolytic/antidepressant action of RS67333 after 7-days and 28-days of treatment recruits a neurogenesis-dependent mechanism.

MATERIALS and METHODS:

The supplemental data for this article include supplemental experimental procedures, six tables, and four figures.

Subjects

Adult male C57BL/6Ntac mice were purchased from Taconic Farms (Lille Skensved, Denmark and Germantown, NY, USA for the pharmacological and the X irradiation studies respectively). All mice were 7-8 weeks old, weighed 23-25g at the beginning of the treatment, and were maintained on a 12L:12 D schedule (lights on at 0600). They were housed in groups of five. Food and water were provided *ad libitum*. All testing was conducted in compliance with the laboratory animal care guidelines and with protocols approved by the Institutional Animal Care and Use Committee (Council directive # 87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions # 92-256B to D.J.D.).

Drugs

Corticosterone (4-pregnen-11b-DIOL-3 20-DIONE 21-hemisuccinate (35µg/mL) from Sigma Aldrich (Saint-Quentin Fallavier, France) was dissolved in vehicle (0.45% hydroxypropyl-β-cyclodextrin, (β-CD), Sigma-Aldrich Saint-Quentin Fallavier, France). Fluoxetine hydrochloride (160 mg/mL, equivalent to 18mg/kg/day) was purchased from Anawa Trading, (Zurich, Switzerland) and dissolved in 0.45% β-CD/corticosterone solution. Hydrochloride(1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-butyl-4piperidinyl)-1-propanone hydrochloride) (RS67333), and (5-Fluoro-2-methoxy-[1-[2-[(methylsulfonyl)amino]ethyl]-4-

piperidinyl]-1H-indole-3-methylcarboxylate sulfamate) (GR125487) were purchased from Tocris Bioscience (Bristol, United Kingdom) and dissolved in 0.9% saline solution. RS67333 and GR125487 were chosen based on previous work (Cachard-Chastel *et al*, 2007; Lucas *et al*, 2007).

RS67333 shows high binding affinity for the 5-HT₄ receptor with a pKi of 8.7 (Bockaert *et al*, 2004; Eglen *et al*, 1995). Except for the sigma receptors, which are bound at affinities comparable to 5-HT₄ (sigma 1: pKi = 8.9; and sigma 2: pKi = 8.0), RS67333 has a pKi of less than 6.7 for other neurotransmitter receptors including 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C}, dopamine D₁, D₂ and muscarinic M₁-M₃ receptors. However little is known about the function of sigma receptors". However little is known about the function of sigma receptors.

GR125487 is the most selective 5-HT₄ receptor antagonist with a pKi = 10.6 (Schiavi *et al*, 1994) presenting a selectivity more than 1000 fold over other 5-HT receptor (Gale *et al*, 1994). The dose of RS67333 and GR125487 used in this study were chosen based on previous works (Cachard-Chastel *et al*, 2007; Lucas *et al*, 2007).

Corticosterone model and treatment

Our model of elevated glucocorticoids (also named CORT model) is able to blunt the response of the hypothalamic-pituitary-adrenal axis (HPA) as shown by the markedly attenuated stress-induced corticosterone levels observed in these mice (David *et al*, 2009). This is probably a consequence of the negative feedback exerted by corticosterone on the HPA axis. This model displays hallmark characteristics of anxiety and depression.

The dose and duration of corticosterone treatment was selected based on previous studies (David *et al*, 2009; Rainer *et al*, 2011). Corticosterone (35 µg/ml, equivalent to about

5 mg/kg/d) or vehicle (0.45 % β -cyclodextrin, β -CD) was available *ad libitum* in the drinking water in opaque bottles to protect it from light. Corticosterone-treated water was changed every 3 d to prevent any possible degradation. Thereafter, while administration with β -CD or corticosterone continued, mice were treated with vehicle (0.45% β -CD), fluoxetine, RS67333, GR125487 alone, or GR125487 in the presence of fluoxetine (Supplementary Fig. 1). Both RS67333 and GR125487 were delivered by osmotic mini-pumps at a dose of 1.5 mg/kg/day and 1mg/kg/day respectively (Lucas *et al*, 2005). Fluoxetine (18 mg/kg per day) was delivered in the drinking water as previously described (David *et al*, 2009). Osmotic minipumps (42 days mini-pumps, 2006 model, Alzet, Cupertino, CA) were implanted subcutaneously under light anesthesia (ketamine/xylazine; (75/20 mg/kg) from Sigma Aldrich (Sigma-Aldrich Saint-Quentin Fallavier, France). Controls animals (Vehicle/Vehicle or Corticosterone/Vehicle groups) were also implanted with a mini-pump containing 0.9% saline (2006 model, Alzet, Cupertino, CA). Treatment was always maintained until the end of the experiments. Corticosterone and fluoxetine dosages were calculated assuming an average fluid intake of about 5mL per day (David *et al*, 2009).

Behavioral tests

The same cohort of animals was tested in five different behavioral models of anxiety and depression. Each animal, over a week, was successively tested in the Open Field (OF), Elevated Plus Maze (EPM), Novelty Suppressed Feeding (NSF), Splash Test (ST) and Tail Suspension Test (see supplemental material). Behavioral testing occurred during the light phase between 0700 and 1900. Behavioral paradigms occurred after 7 or 28 days of drug treatment depending on the study (Figure S1A, S1B).

Immunohistochemistry

The effects of chronic RS67333 treatment on cell proliferation or maturation of newborn neurons was assessed in corticosterone-treated animals. After anesthesia with ketamine and xylazine (100 mg/ml ketamine; 20mg/ml xylazine), mice were perfused transcardially (cold saline for 2 min, followed by 4% cold paraformaldehyde at 4°C). The brains were then removed and cryoprotected in 30% sucrose and stored at 4°C. Serial sections (35 µm) were cryosectioned through the entire hippocampus and stored in PBS with 0.1% NaN₃.

Proliferation of newborn cells

We first looked at proliferation of newborn cells using Ki-67 immunohistochemistry as described previously (Xia *et al*, 2012). Sections were washed in PBS, blocked (PBS containing 0.3% triton and 10% NDS) and incubated with primary antibody overnight at 4° C (Ki67 rabbit, 1:100, Vector, Burlingame, CA). Following washes in PBS, sections were incubated with fluorescence coupled rabbit secondary antibody (Jackson ImmunoResearch, Beckman, France). Stereological quantification of Ki-67 labeling was performed using an Olympus BX51 microscope (Germany).

Maturation of newborn neurons

For doublecortin staining, the procedure consisted of the following steps: 1 hr incubation in 0.1M TBS with 0.5% Triton X-100 and 10% normal donkey serum (NDS), followed by goat anti-doublecortin primary antibody (1:100) in TBS/Tx/NDS for 24 hrs at 4°C. The secondary antibody was biotinylated donkey anti-goat (1:500) in TBS/NDS for 1 hr

at room temperature, followed by a 1hr amplification step using an avidin-biotin complex (Vector, USA). The immunohistochemistry protocol was adapted from David et al. (David *et al*, 2009). DCX-positive (DCX^+) cells were subcategorized according to their dendritic morphology: DCX^+ cells without and DCX^+ cells with tertiary (or higher order) dendrites. The maturation index was defined as the ratio of DCX^+ cells possessing tertiary dendrites to the total number of DCX^+ cells.

Sholl analysis

Sholl analysis was performed as described elsewhere (Guilloux *et al*, 2013). DCX^+ cells with tertiary or higher order dendrites were traced using Neurolucida software (MicroBrightField, Williston, VT) on an Olympus BX51 microscope equipped with a motorized stage device and x100 immersion oil objective. Sholl analysis for dendritic complexity was performed using the accompanying software (NeuroExplorer; MicroBrightField, version 10), to determine dendritic length and number of intersections (branch points). One DCX^+ cell was traced for each 35 μm hippocampal slice; $n = 6$ cells per brain for DAB-stained sections).

X-irradiation

A separate batch of mice were anesthetized with ketamine and xylazine (75/20 mg/kg), placed in a stereotaxic frame, and exposed to cranial irradiation using a PXI X-RAD 320 X-ray system operated at 300 kV and 12 mA with a 2 mm Al filter. Animals were protected with a lead shield that covered the entire body, but left unshielded a 3.22 X 11-mm treatment field above the hippocampus (interaural 3.00 to 0.00) exposed to X-ray, thus

effectively preventing irradiation from targeting the rest of the brain (Santarelli *et al*, 2003). The corrected dose rate was approximately 0.95 Gy per min at a source to skin distance of 36 cm. The procedure lasted 2 min and 39 sec, delivering a total of 2.5 Gy. Three 2.5 Gy doses were delivered on days 1, 4, and 7 as previously described (Quesseveur *et al*, 2013). This 7.5 Gy cumulative dose was determined from prior pilot experiments to be the minimum dosage necessary to result in permanent ablation of adult-born neurons in the dentate gyrus as assessed by expression of the immature neuronal marker doublecortin. The reason for using a fractionated paradigm rather than a single high dose of 7.5Gy is that the ablation is not permanent after a single high dose. Histological staining for CD68 as a marker of inflammation throughout the brain revealed that irradiated mice were indistinguishable from sham animals eight weeks post-irradiation, indicating minimal non-specific side effects of irradiation at time of behavioral testing (Meshi et al, 2006). Immunohistochemistry confirmed the ablation of adult hippocampal neurogenesis (Supplementary Fig. 4).

Data Analysis and Statistics

Results from data analyses were expressed as mean \pm SEM. Data were analyzed using StatView 5.0 software (SAS Institute, Cary, NC). For all experiments, one-way or two-way ANOVAs with repeated-measures were applied to the data as appropriate. Significant main effects and/or interactions were followed by Fisher's PLSD post hoc analysis, unpaired t tests. In the NSF test, we used the Kaplan-Meier survival analysis due to the lack of normal distribution of the data. Mantel-Cox log-rank test was used to evaluate differences between experimental groups. Statistical significance was set at $P < 0.05$. A summary of statistical measures is included in Tables S1-S6, available online.

RESULTS

5-HT₄ receptor stimulation produces anxiolytic-like and antidepressant-like effects in a model of anxiety/depression.

To induce an anxious/depressed-like state in C57BL/6Ntac mice we administered a low dose of corticosterone (35 µg/ml) for 4 weeks as described in (David *et al*, 2009) (“CORT model”). After chronic corticosterone, we tested the effects of a 4-week treatment with the 5-HT₄ agonist RS67333 (1.5 mg/kg/day) in comparison to fluoxetine (18 mg/kg/day). To assess the selectivity of these effects, we also tested whether the 5-HT₄ antagonist GR125487 (1mg/kg/day), alone or in combination with fluoxetine, affected the behavioral phenotype (see experimental design, Figure S1). In the open field (OF), the anxiety-like phenotype induced by chronic corticosterone was reversed by chronic fluoxetine and by the 5-HT₄ agonist RS67333 (One-way ANOVA, * $p < 0.05$, Figure 1A). Indeed, chronic fluoxetine and RS67333 treatment increased time spent in the center (Figure 1A). A trend for an increase in the number of entries in the center was also observed with both compounds (Figure S2A-B). It is unlikely that this effect was the consequence of a change in locomotor activity since the total ambulatory distance was not affected and the ratio of ambulatory distance in the center divided by total distance was increased for both treatments (One-way ANOVA, * $p < 0.05$, Figure 1B). Interestingly, while the 5-HT₄ antagonist GR125487 by itself did not affect any anxiety parameters, it prevented fluoxetine induced-anxiolytic-like effects. Indeed, the fluoxetine-induced increase in time spent in the center was prevented by chronic GR125487 administration. These data indicate that 5-HT₄ stimulation induces an anxiolytic-like effect, and is necessary for the anxiolytic effect of chronic fluoxetine treatment.

To further validate these results, we next tested the effects of RS67333 and fluoxetine alone or in the presence of GR125487 in the same animals in another anxiety-related test, the

elevated plus maze (EPM). We found that chronic RS67333 and fluoxetine induced a trend for an increase in time spent in and number of entries into the open arms, (Figure 1C and 1D). This anxiolytic-like effect of fluoxetine was completely abolished by treatment with the 5-HT₄ antagonist, GR125487.

We next assessed whether chronic treatment with the 5-HT₄ agonist RS67333 could also produce antidepressant-like effects. Thus the same mice were tested in the splash test and the tail suspension test. We observed that after squirting a 10% sucrose solution on the mouse's snout, increased grooming duration was observed in both the fluoxetine and the RS67333 groups (One-way ANOVA, $**p < 0.01$, Figure 1E). Chronic treatment with the 5-HT₄ antagonist GR125487 prevented the antidepressant-like activity of chronic fluoxetine. Similarly, in the tail suspension test both fluoxetine and RS67333 had antidepressant-like effects and these effects of fluoxetine were blocked by GR125487 (One-way ANOVA, $**p < 0.01$, Figure 1F).

Finally, we tested these mice in the NSF test, which is sensitive to both acute anxiolytics and chronic antidepressants (Guilloux *et al*, 2013) (Figure 1G, 1H). Like chronic fluoxetine, chronic RS67333 decreased the latency to feed (Kaplan-Meier survival analysis, Mantel-Cox log-rank test, $**p < 0.01$) without affecting the home-cage food consumption (Figure S2C). Chronic treatment with the 5-HT₄ antagonist (GR125487) prevented the effect of fluoxetine. Altogether, these data indicate that 5-HT₄ receptor activation produces both anxiolytic-like and antidepressant-like effects comparable to those of fluoxetine in the chronic corticosterone model of anxiety/depression. Furthermore we show that 5-HT₄ activation is necessary for the anxiolytic and antidepressant effects of fluoxetine in this model.

5-HT₄ receptor activation facilitates the maturation of newborn neurons in the adult hippocampus.

To investigate the potential cellular mechanisms underlying the behavioral effects of the 5-HT₄ agonist RS67333, we next evaluated changes in adult hippocampal neurogenesis that may be relevant to antidepressant action (Surget *et al*, 2011).

In agreement with previous observations (David *et al*, 2009; Rainer *et al*, 2011), chronic fluoxetine exposure resulted in an increase in the number of dividing neural precursors as assessed by the number of Ki67-positive cells in the subgranular zone of the dentate gyrus (One-way ANOVA, * $p < 0.05$, Figure 2A). The 5-HT₄ agonist, RS67333 also increased the number of neural precursors, but to a lesser extent than fluoxetine (+51% versus +170%). The 5-HT₄ antagonist partially blocked the effect of chronic fluoxetine. These results suggest that 5-HT₄ receptors contribute to the effects of fluoxetine on proliferation, but that other 5-HT receptors are likely to be also involved.

We next assessed the number of young adult-born neurons in the dentate gyrus that express doublecortin (DCX), a protein that is expressed for about a month after the birth of adult-born neurons (Couillard-Despres *et al*, 2005) (Figure S3). We also subcategorized the DCX⁺ cells according to their dendritic morphology: total number of DCX⁺ cells and DCX⁺ cells with complex, tertiary dendrites (Figure 2B-2G). As previously described, chronic fluoxetine increased the number of doublecortin-positive cells with tertiary dendrites and the maturation index, defined as the ratio of DCX⁺ cells possessing tertiary dendrites over the total DCX⁺ cells in control animals (David *et al*, 2009) (One-way ANOVA, ** $p < 0.01$, Figure 2B, 2C and 2D). Chronic treatment with RS67333 affected modestly both the total number of DCX positive cells and also the number of DCX cells with tertiary dendrites.

The 5-HT₄ antagonist, GR125487, partially blocked the neurogenic effects of fluoxetine. However, while the effects of chronic fluoxetine on the number of doublecortin-positive cells with tertiary dendrites are larger than those of chronic 5-HT₄ receptor activation, the effect of these compounds on the maturation index is similar (+51% and 44% for fluoxetine and RS67333, respectively).

The dendrites of adult-born granule cells become progressively more complex during the first 4 weeks after their birth, a stage when the cells express DCX (Couillard-Despres *et al*, 2005). To further examine the effect of 5-HT₄ receptor activation on the dendritic morphology of newborn cells, we performed Sholl analyses on DCX+ cells with tertiary dendrites. DCX+ cells in chronic fluoxetine-treated and RS67333-treated animals displayed an increase in dendritic length (One-way ANOVA, ** $p < 0.01$, Figure 2E and 2F) and in number of dendritic intersections (One-way ANOVA, ** $p < 0.01$, Figure 2E and 2G). Fluoxetine-induced increase in dendritic complexity was abolished by a chronic treatment with the 5-HT₄ antagonist, GR125487.

Overall, these results suggest that 5-HT₄ receptor activation facilitates the maturation of newborn neurons in the adult hippocampus.

An assessment of causality between the neurogenic and behavioral effects of short and long term treatments with the 5-HT₄ agonist in the chronic CORT model.

Since we have shown that long-term 5-HT₄ activation induced anxiolytic/antidepressant-like effects and facilitated maturation of newborn neurons, we decided to test the requirement of hippocampal neurogenesis for the emergence of behavioral changes after 5-HT₄ receptor activation in our CORT model. Moreover, a recent study in rats reported that the behavioral and neurogenic (proliferation of newborn cells) effects of the 5-

HT₄ receptor agonist RS67333 occur after short-term administration (3 to 7 days depending on the paradigm) (Pascual-Brazo *et al*, 2012). Thus, we also investigated whether subchronic RS67333 treatment induced a rapid onset of behavioral effects. To address these points, mice were submitted to focal hippocampal X-irradiation before the start of chronic corticosterone treatment alone or in combination with the 5-HT₄ agonist RS67333 (1.5 mg/kg/day) or fluoxetine (18 mg/kg/day) (Figure S1B). These animals were tested in anxiety and depression related tests first after 7 days of treatment and again after 28 days of treatment.

As previously described (David *et al*, 2009; Rainer *et al*, 2011), the chronic CORT paradigm resulted in an anxious/depressed-like phenotype. The efficacy of corticosterone model was assessed by comparing the behavioral phenotype of controls to corticosterone-treated mice (Figure 3). In anxiety-related tests, chronic corticosterone treatment had a marked effect on all anxiety parameters, resulting in decreased time spent in center and ratio of distance in center divided by total distance in the OF (One-way ANOVA, * $p < 0.05$ or ** $p < 0.01$, Figure 3A, 3I, 3J), and in decreased time and entries in the open arms in the EPM (One-way ANOVA, ** $p < 0.01$, Figure 3C, 3K, 3L). In the Splash test, which is a depression-related test, chronic CORT resulted in a decrease in grooming (One-way ANOVA, ** $p < 0.01$, Figure 3O), and in the NSF test, which is related to both anxiety and depression, chronic CORT increased the latency to feed (Kaplan-Meier survival analysis, Mantel-Cox log-rank test, ** $p < 0.01$, Figure 3E, 3F, 3M, 3N). As previously observed in a similar paradigm, the FST (David *et al*, 2009; Rainer *et al*, 2011), chronic corticosterone treatment did not affect the immobility duration in the TST (Figure 3H, 3P), suggesting distinct underlying mechanisms between these tests and the OF, EPM, NSF or ST.

The rapid anxiolytic and antidepressant-like effects of a subchronic 5-HT₄ agonist treatment do not require hippocampal neurogenesis.

A 7-day treatment with RS67333 produced anxiolytic and antidepressant-like effects in a battery of behavioral tests (Figure 3A to 3H). In the OF and EPM paradigms, all anxiety-related parameters were impacted. The time spent in the center (Figure 3A), the number of entries in the center (Figure S5A), the ratio of center distance/total distance traveled (Figure 3B), the time spent in open arms (Figure 3C) and the number of entries in the open arms (Figure 3D) were increased after subchronic treatment with RS67333, regardless of whether the mice were exposed to X-irradiation or not (two-way ANOVA with significant treatment factor, $**p<0.01$, Figures 3A-C). In contrast, subchronic treatment with fluoxetine did not have an anxiolytic effect in the OF and EPM paradigms (Figures 3A-B and 3C-D). These results indicate that the anxiolytic effects of RS67333 have a faster onset than those of fluoxetine and that these effects do not require adult hippocampal neurogenesis.

Interestingly, in the NSF test, neither subchronic RS67333 nor subchronic fluoxetine had an effect on latency to feed in both sham and X-irradiated groups (Figures 3E-F) indicating that in this test, the anxiolytic/antidepressant activity of RS67333 and fluoxetine require a longer treatment.

In the ST and TST (Figures 3G-H), after 11 and 12 days of administration, both RS67333 and fluoxetine increased grooming duration and decreased immobility duration, respectively (two-way ANOVA with significant treatment factor, $**p<0.01$). These antidepressant-like effects were not affected by focal hippocampal X-irradiation.

Altogether, these results demonstrate that, unlike fluoxetine, the 5-HT₄ agonist RS67333 elicits a rapid anxiolytic and antidepressant-like effect in all the paradigms tested (OF, EPM, ST and TST) except the NSF. However, hippocampal neurogenesis is not required for these effects of RS67333.

In this study we assessed the behavioral activity of RS67333 after both subchronic and chronic treatments. Thus, the same animals were tested after 7 and 28 days of treatment. It is therefore not surprising to observe changes in the basal values in control animals since they have been exposed twice to behavioral tests. We have seen these effects of repeated testing routinely. For example, in the Novelty Suppressed Feeding paradigm, the latency to feed was decreased after a second exposure to the test (Wang et al., 2008). In the present study, we observed a decrease in the time spent in the center of the arena, due to the re-exposure to the test in all treated groups. However, the size of the anxiolytic-like effect of RS67333, remains the same between the first and the second exposure to the open field.

The behavioral effects of long term 5-HT₄ agonist treatment are mediated by both neurogenesis-dependent and -independent mechanisms.

Since we previously demonstrated that chronic 5-HT₄ activation produced anxiolytic/antidepressant-like activity in the CORT model, we proceeded to investigate whether these behavioral effects require adult hippocampal neurogenesis.

The same battery of behavioral tests was performed again after 28 days of treatment with fluoxetine or RS67333 (Figures 3I-P). In the OF (Figures 3I-J) and the EPM (Figures 3K-L) paradigms, chronic RS67333 maintained the anxiolytic-like effect observed subchronically (two-way ANOVA with significant treatment factor, * $p < 0.05$, Figures 3I-3L). Chronic fluoxetine also elicited an anxiolytic-like effect while it had no effect after subchronic treatment (two-way ANOVA with significant treatment factor, * $p < 0.05$, Figures 3I-3J). Moreover, these anxiolytic-like effects of RS67333 and fluoxetine were not affected by the ablation of adult hippocampal neurogenesis by X-irradiation.

In contrast, the anxiolytic/antidepressant-like effects of RS67333 and fluoxetine in the NSF paradigm were completely abolished by hippocampal X-irradiation (Figures 3M and 3N;

Kaplan-Meier survival analysis, Mantel-Cox log-rank test, $**p<0.01$, two-way ANOVA with significant interaction between irradiation and treatment $**p<0.01$) indicating that these effects require adult hippocampal neurogenesis. Home cage food consumption was not affected by drug treatment or irradiation (Figure S5D).

In the splash test and TST, long-term administration of RS67333 and fluoxetine induced an increase in grooming duration and a decrease in immobility duration that were not affected by focal X-irradiation (two-way ANOVA with significant treatment factor, $**p<0.01$ for both tests, Figures 3O-3P).

Altogether these results indicate that the effects of chronic treatment with RS67333 and fluoxetine in the OF, EPM, ST, and TST are independent of hippocampal neurogenesis. In contrast, the anxiolytic/antidepressant-like effects of these compounds in the NSF test require neurogenesis.

DISCUSSION

Fast anxiolytic action of a 5-HT₄ agonist.

Most current antidepressant treatments are limited by a significant degree of non responsiveness among patients (Trivedi *et al*, 2006), delayed onset of therapeutic efficacy, and a number of side effects (Kato and Serretti, 2010). The development of new antidepressants is therefore of considerable importance (Wong *et al*, 2010), and understanding the mechanisms underlying the delayed onset should offer insights into new approaches. Recent studies as well as our present results indicate that 5-HT₄ receptor agonists are faster acting than SSRIs (Lucas *et al*, 2007; Pascual-Brazo *et al*, 2012; Tamburella *et al*, 2009).

While a 7-day treatment with fluoxetine or RS67333 induced antidepressant-like activity in the TST and ST, only the 5-HT₄ agonist RS67333 resulted in anxiolytic-like activity in the

OF and the EPM. A longer duration of treatment (28 days) is required for fluoxetine to exert anxiolytic-like effects comparable to 7 days of RS67333 treatment. While recent evidence indicates that 5-HT₄ receptors may represent a new target for antidepressant drugs (Bockaert *et al*, 2004; Lucas *et al*, 2007; Pascual-Brazo *et al*, 2012; Tamburella *et al*, 2009), the role of 5-HT₄ receptor ligands in anxiety is unclear. Discrepancies in results observed with 5-HT₄ receptor antagonists have been observed. In one study, the 5-HT₄ receptor antagonists, SDZ205-557, GR113808, and SB204070, administered acutely, failed to induce anxiolytic-like behavior in the light/dark choice test in mice (Costall and Naylor, 1997), while in two other studies, acute administration of the 5-HT₄ receptor antagonists, SB204070, GR113808 (Silvestre *et al*, 1996), and SB207266A (Kennett *et al*, 1997; Silvestre *et al*, 1996), had an anxiolytic-like effect in rats in the elevated plus maze. 5-HT₄ receptor knock-out mice do not display an anxious or depressed-like phenotype, though an attenuated response to novelty may be relevant to mood disorders (Compan *et al*, 2004). In our hands, chronic treatment with GR125487 did not affect the anxiety-like phenotype induced by chronic corticosterone treatment. 5-HT₄ receptor agonists have mainly been tested in behavioral tests of antidepressant-like activity (see (Lucas, 2009) for review). Only one study investigated the effects of RS67333 in the OF paradigm over a 5-min period (Lucas *et al*, 2007). The authors showed that hyperlocomotion induced by olfactory bulbectomy was totally abolished after 14 days of RS67333 treatment in rats. To our knowledge, the present study is the first to clearly demonstrate fast anxiolytic-like activity of a 5-HT₄ receptor agonist in a mouse model of anxiety/depression. Our data in regards to the literature support a direct effect of RS67333 on the 5-HT₄ receptor. There is considerable evidence that RS67333 is a specific agonist of the 5-HT₄ receptor. Indeed, three studies have evaluated the effects of RS67333 in the presence of the selective 5-HT₄ antagonist GR125487, and shown that the effects of RS67333 are blocked by GR125487. Lucas and colleagues showed that the increase in 5-HT firing induced by RS67333 (1.5 mg/kg, acutely, during 3 days or 21

days) is prevented by GR125487 administration (Lucas *et al*, 2005). This effect of RS67333 on the firing of 5-HT neurons is likely to contribute to the phenotype we observe in the current study. Enhanced cognition induced by RS67333 was blocked by the 5-HT₄ receptor antagonist GR 125487 (1mg/kg, intravenous [i.v.]) (Freret *et al*, 2012; Lamirault and Simon, 2001). Both studies tested the specificity of the effects of RS67333 in the Novel Object Recognition test by using the highly potent and selective 5-HT₄ receptor antagonist, GR125487. They found that 1 mg/kg of GR125487, which had no effect per se on the discrimination index, totally reversed the beneficial effects of RS67333, arguing thus that these effects of RS67333 are mediated by 5-HT₄ receptor.

Interestingly, in the NSF test, both fluoxetine and RS67333 had an anxiolytic/antidepressant-like effect only after chronic treatment, suggesting that the neurobiological mechanisms involved in this paradigm are different from those underlying the other tests (OF, EPM, TST, ST). Indeed, we show that the effects of RS67333 and fluoxetine in the OF, EPM, TST, and ST are independent of hippocampal neurogenesis, while the effects of these compounds in the NSF test require neurogenesis. This is in agreement with our previous study showing both neurogenesis-dependent (NSF) and independent (OF, FST) effects of fluoxetine in the CORT model (David *et al*, 2009). It is also noteworthy that the only test (NSF) that requires neurogenesis is also the one that requires a chronic administration. This observation is likely related to the fact that young adult-born neurons take several weeks to mature and that the critical period during which adult-born neurons contribute to behavior extends from 4 to 6 weeks after their birth (Denny *et al*, 2012). Interestingly, the effect of the 5-HT₄ agonist RS67333 on proliferation of neural precursors are weaker than those of fluoxetine, while the effects of RS67333 on the maturation of young neurons are similar to those of fluoxetine. Newborn neurons undergo an accelerated maturation after chronic fluoxetine treatment (Wang *et al*, 2008) and possibly also after 5-HT₄

receptor activation. These results suggest that the neurogenesis-dependent effect of RS67333 and fluoxetine in the NSF test is more likely to result from increased maturation than from increased proliferation.

The fast onset of action of the 5-HT₄ receptor agonist could be a consequence of an increase in serotonergic output to projection areas (Lucas *et al*, 2005; Lucas *et al*, 2002). Indeed, by measuring spontaneous electrical activity in mice lacking 5-HT₄ receptors, Conductier *et al* demonstrated that 5-HT₄ receptors exert a tonic positive influence on the firing activity of DRN (dorsal raphe nucleus) 5-HT neurons (Conductier *et al*, 2006) and previous studies have shown that 5-HT₄ receptor activation by selective agonists modulates central 5-HT neurotransmission, increasing the firing of DRN 5-HT neurons (Lucas *et al*, 2002). There is also accumulating evidence that cortical regions are involved in 5-HT₄-induced anxiolytic/antidepressant-like activities (Lucas *et al*, 2005) (for review see also (Lucas, 2009)). 5-HT₄ receptors in the prefrontal cortex control the firing rate of midbrain serotonergic neurons via descending inputs (Lucas *et al*, 2005) and their activation leads to increases in serotonin release in projection sites including the hippocampus (Ge and Barnes, 1996).

Requirement of 5-HT₄ receptors for the behavioral and neurogenic effects of SSRIs.

A blockade of 5-HT₄ receptors with the antagonist GR125487 prevented the anxiolytic and antidepressant-like effects of fluoxetine. These results show that 5-HT₄ receptor activation is necessary for the behavioral effects of SSRIs. Our results are consistent with a previous study showing a specific induction of 5-HT₄ expression after SSRI treatment (Schmidt *et al*, 2012). Moreover, using the NIMH Psychoactive Drug Screening Program database, we did not find any study looking at the binding affinity of fluoxetine at the mouse

5-HT₄ receptor. The only study looking at binding affinities of fluoxetine for serotonergic receptor demonstrated negligible binding of fluoxetine to the 5-HT₄ receptor in pig striatal membranes (Lucchelli *et al*, 1995). In addition, quantitative autoradiography revealed that the binding of the 5-HT₄ receptor ligand [³H]GR113808 was not significantly changed in fluoxetine-treated mice (Kobayashi *et al*, 2012). Thus, in the present study, the anxiolytic/antidepressant-like effects of fluoxetine likely resulted from an indirect activation of the 5-HT₄ receptor through an increase in endogenous 5-HT levels in the synaptic cleft following the blockade of the selective serotonin transporter. However, it is unlikely that 5-HT₄ receptor activation alone can be responsible for all SSRIs-mediated anxiolytic/antidepressant-like activity. Among the 14 known 5-HT receptor subtypes, the 5-HT_{1A} receptor has been prominently implicated in the modulation of mood and anxiety-related behaviors (Santarelli *et al*, 2003). 5-HT_{1A} receptor knockout (KO) mice were insensitive to the behavioral effects of chronic fluoxetine, suggesting that activation of 5-HT_{1A} receptors is also a critical component in the mechanism of action of SSRIs. Recent data also suggest a potential non-cell autonomous mechanism by which serotonin regulates neurogenesis and the response to antidepressants through 5-HT_{1A} receptor (Samuels, personal communication). However, we cannot rule out adaptive changes in the serotonergic system, including variations in 5-HT₄ receptor levels, which could explain the absence of behavioral effects of fluoxetine in 5-HT_{1A} receptor knock-out mice. Indeed, decreases in the density of the serotonin transporter (5-HTT) were measured in several brain regions of these 5-HT_{1A} mutant mice (Ase *et al*, 2001), and a recent study described that variation in serotonin transporter expression could cause adaptive changes in 5-HT₄ receptor levels in serotonin transporter overexpressing mice (Jennings *et al*, 2012).

SSRIs are potent stimulators of adult hippocampal neurogenesis (Klempin *et al*, 2010; Santarelli *et al*, 2003). However, the role of each serotonergic receptor in the neurogenic

effects of SSRIs is still a matter of investigation. We have showed that the 5-HT₄ agonist, RS67333, increased neurogenesis (proliferation and maturation) to a lesser extent than fluoxetine and that the 5-HT₄ antagonist, GR125487, partially blocked neurogenic effects of chronic fluoxetine. These results suggest that 5-HT₄ receptors contribute to the effects of fluoxetine on proliferation and maturation of newborn neurons, but that other 5-HT receptors are likely to be involved. Pharmacological manipulations suggested that 5-HT_{1A} receptors are involved in proliferation of precursor cells, whereas 5-HT₂ receptors affect both proliferation and promote neuronal differentiation (Klempin *et al*, 2010). Moreover, fluoxetine had no effect on neurogenesis (proliferation and survival) in 5-HT_{1A} KO mice (Santarelli *et al*, 2003).

These results suggest that both 5-HT₄ and 5-HT_{1A} receptors contribute to the effects of SSRIs on behavior and neurogenesis. Interestingly, both receptors are expressed in the dentate gyrus, which may be the site responsible for their effects on neurogenesis. Recently, it has been suggested that 5-HT₄ receptor activation may also be involved in antidepressant-induced dematuration of mature dentate granule cells (Kobayashi *et al*, 2010). The exact mechanisms underlying in this phenomenon still needs further investigations. However, our results also show that most effects of SSRIs and 5-HT₄ agonists do not require hippocampal neurogenesis. Examining effects of tissue-specific manipulations of these receptors will be important to identify the circuits responsible for their fast acting anxiolytic and antidepressant actions.

Conclusions

Taken together, our results show, for the first time, in a mouse model of anxiety/depression, that a 5-HT₄ receptor agonist may be a fast-acting anxiolytic agent, and that 5-HT₄ stimulation is necessary for the behavioral and neurogenic effects (proliferation and maturation) of fluoxetine, a classic SSRI antidepressant. Furthermore, we showed that, with the exception of the NSF test, the anxiolytic and antidepressant-like effects of the 5-HT₄

agonist were independent of hippocampal neurogenesis (Table 1). The present study is encouraging for the development of RS-67333 as an anxiolytic/antidepressant compound for use in patients. However, the use of the 5-HT₄ receptor as a novel antidepressant target may be hampered by the fact that it also plays important roles outside the central nervous system, e.g., in the heart, gastrointestinal tract, adrenal gland, and urinary bladder (Tonini and Pace, 2006), which may prevent its development as an effective anxiolytic/antidepressant drug (Bockaert *et al*, 2004, 2008). Thus, signaling molecules that interact with the 5-HT₄ receptor such as P11 (Egeland *et al*, 2011; Warner-Schmidt *et al*, 2009) may represent novel targets for fast-acting anxiolytic/antidepressant treatments. There is indeed recent evidence that cortical neurons that express both P11 and 5-HT₄ receptors are involved in the behavioral effects of SSRIs (Schmidt *et al*, 2012) and that chronic treatment with fluoxetine results in an increase in 5-HT₄ receptor expressions in cortical neurons (Schmidt *et al*, 2012).

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Legend

Figure 1: Effects of chronic 5-HT₄ receptor stimulation (28-days) on the anxious/depressed-like phenotype induced by chronic corticosterone exposure.

(A and B) Effects of chronic treatment with 5-HT₄ ligands or fluoxetine, starting after 4 weeks of corticosterone (35 µg/ml), on anxiety behaviors in the open field test. Anxiety is measured as mean time spent in the center in seconds (A) or the ratio of ambulatory distance in the center/total ambulatory distance (B). Values plotted are mean ± SEM (n= 10–15 per group). *p<0.05, **p<0.01, versus corticosterone/vehicle group.

(C and D) Effects of chronic treatment with 5-HT₄ ligands or fluoxetine, starting after 4 weeks of corticosterone (35 µg/ml), on anxiety behaviors in the elevated plus maze. Anxiety is expressed as mean time in (C) or entries into (D) the open arms. Values plotted are mean ± SEM (n= 10–15 per group). #p<0.05 versus corticosterone/fluoxetine group.

(E) Effect of chronic treatment with 5-HT₄ ligands or fluoxetine on corticosterone-induced depression-related behaviors in the splash test. Results are expressed as mean duration of grooming after receiving a 10% sucrose solution on the snout. Values plotted are mean ± SEM (n = 10–15 per group). **p<0.01, ###p<0.01, versus corticosterone/vehicle group and corticosterone/fluoxetine group, respectively.

(F) Effect of chronic treatment with 5-HT₄ ligands or fluoxetine in the tail suspension test following chronic corticosterone. Results are expressed as mean of immobility duration in seconds. Values plotted are mean ± SEM (n= 10–15 per group). **p<0.01, ###p<0.01, versus corticosterone/vehicle group and corticosterone/fluoxetine group, respectively.

(G and H) Effects of chronic treatment with 5-HT₄ ligands or fluoxetine on anxiety- and depression-like behaviors in the novelty suppressed feeding paradigm after chronic

corticosterone. Values plotted are cumulative survival of animals that have not eaten over 10 min (n= 10–15 per group) (G) or mean of latency to feed in seconds \pm SEM (H). $**p<0.01$, $##p<0.01$, versus corticosterone/vehicle group and corticosterone/fluoxetine group, respectively.

Figure 2: Effects of chronic 5-HT₄ receptor stimulation (28 days) on proliferation and dendritic maturation of young neurons in the dentate gyrus of the adult hippocampus.

(A) Effect of chronic treatment with 5-HT₄ ligands or fluoxetine, starting after 4 weeks of corticosterone (35 μ g/ml), on cell proliferation. Cell proliferation is measured as mean number of Ki-67 positive cells (A). Values plotted are mean \pm SEM (n= 3–5 per group). $*p<0.05$, $**p<0.01$, $##p<0.01$, versus corticosterone/vehicle group and corticosterone/fluoxetine group, respectively.

(B) Effect of chronic treatment with 5-HT₄ ligands or fluoxetine on total number of doublecortin-positive cells (mean \pm SEM; n= 4-5 per group) was measured after chronic corticosterone. $**p<0.01$, $##p<0.01$ versus corticosterone/vehicle group, and corticosterone/fluoxetine group, respectively.

(C and D) Doublecortin-positive cells were categorized as to whether they exhibited tertiary dendrites. Effects of fluoxetine treatment on the doublecortin-positive cells with tertiary dendrites (C) and maturation (D) of newborn granule cells were measured after chronic corticosterone. Values are mean \pm SEM (n= 4-5 per group). $**p<0.01$, $\#p<0.05$, $##p<0.01$, versus corticosterone/vehicle group, and corticosterone/fluoxetine group, respectively.

(E) Representative image and traces from Sholl analyses of doublecortin-positive cells with tertiary branches after vehicle, chronic fluoxetine, chronic RS67333, GR125487 in presence

or not of fluoxetine in corticosterone-treated animals ($n = 3-4$ mice per group, 6 cells per mouse).

(F and G) Effects of chronic treatment with the 5-HT₄ ligands RS67333 or fluoxetine on the dendritic length (F) or the number of intersections (G) following a Sholl analysis. Values are mean \pm SEM ($n = 4-5$ per group). ** $p < 0.01$ versus corticosterone/vehicle group.

Figure 3. Neurogenesis-dependent and -independent effects of subchronic (7-days) or chronic (28-days) 5-HT₄ agonist treatment on corticosterone-induced behavioral changes in mice.

(A–B/I–J) Effects of subchronic (A–B) or chronic (I–J) treatment with RS67333, a 5-HT₄ agonist, after focal X-irradiation of the mouse hippocampus on corticosterone-induced anxiety-like behaviors in the open field test. Anxiety is expressed as mean time spent in the center, in seconds, for the entire session (A or I), and also as the mean of percentage ambulatory distance in the center over total ambulatory distance traveled (B or J). Values are mean \pm SEM ($n = 9-15$ per group for corticosterone-treated animals and $n=5$ for vehicle/vehicle). ** $p < 0.01$, # $p < 0.05$, ## $p < 0.01$, versus control vehicle/vehicle group and corticosterone/RS67333 or corticosterone/vehicle group, respectively.

(C–D/K–L) Effects of subchronic (C–D) or chronic (K–L) treatment with RS67333, a 5-HT₄ agonist, after focal X-irradiation of the mouse hippocampus on corticosterone-induced anxiety-like behaviors in the elevated plus maze paradigm. Anxiety is expressed as mean time in the open arms (C or K), and also as the mean entries in the open arms (D or L). Values are mean \pm SEM ($n = 9-15$ per group for corticosterone-treated animals and $n=5$ for vehicle/vehicle). ** $p < 0.01$, # $p < 0.05$, versus control vehicle/vehicle group and corticosterone/RS67333 or corticosterone/vehicle group, respectively.

(E–F/M–N) Effects of subchronic (E–F) or chronic (M–N) treatment with RS67333, a 5-HT₄ agonist, after focal X-irradiation on corticosterone-induced anxiety- and depression-related behaviors in the novelty suppressed feeding paradigm. Results are cumulative survival of animals that have not eaten over 10 min (E or M) or mean \pm SEM of latency to feed in seconds (F or N) (n = 9–15 per group for corticosterone-treated animals and n=5 for vehicle/vehicle). **p<0.01, ###p<0.01, versus control vehicle/vehicle group and corticosterone/RS67333 or corticosterone/vehicle group, respectively.

(G–O) Effects of subchronic (G) or chronic (O) treatment with RS67333, a 5-HT₄ agonist, after X-irradiation on behavior in the splash test. Results are expressed as mean \pm SEM duration of grooming after receiving a 10% sucrose solution on the snout (n = 9–15 per group for corticosterone-treated animals and n=5 for vehicle/vehicle). *p<0.05, #p<0.05, versus control vehicle/vehicle group and corticosterone/vehicle group, respectively.

(H–P) Effects of subchronic (H) or chronic (P) treatment with RS67333, a 5-HT₄ agonist, after X-irradiation on behavior in the tail suspension test. Results are expressed as mean \pm SEM immobility duration (in seconds) (n = 9–15 per group for corticosterone-treated animals and n=5 for vehicle/vehicle). **p<0.01, #p<0.05, versus control vehicle/vehicle group and corticosterone/vehicle group, respectively.

Figure 1

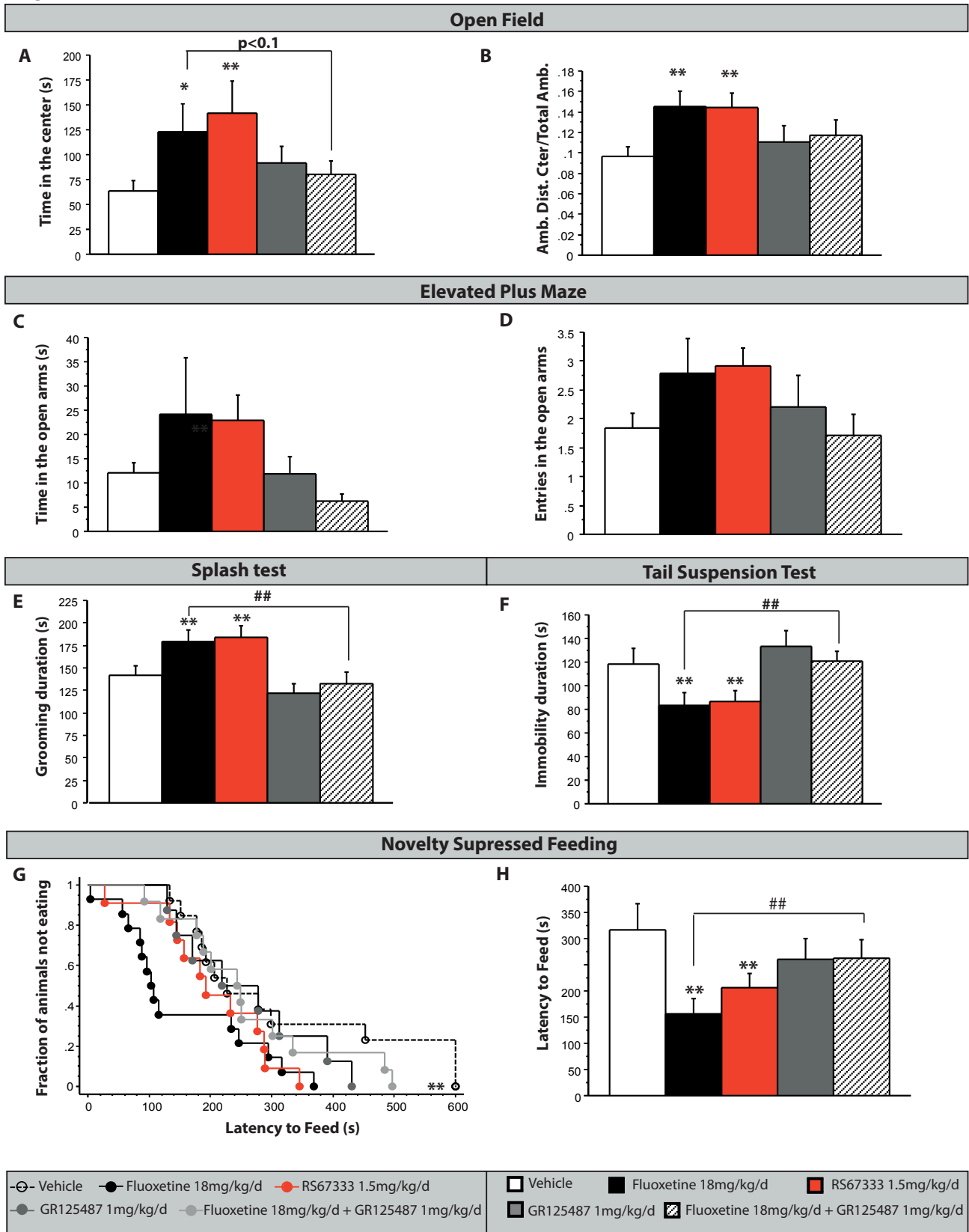


Figure 2

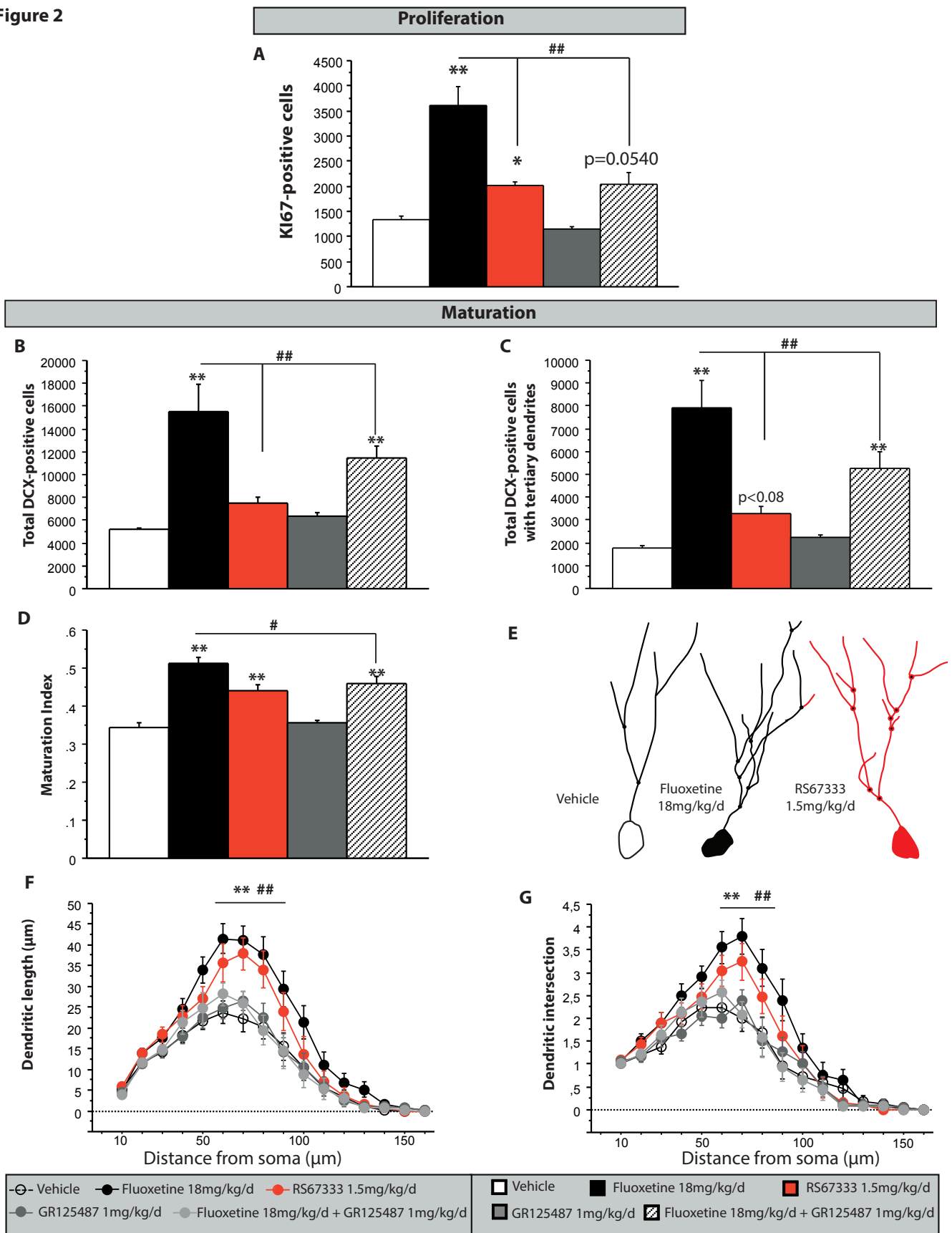


Figure 3

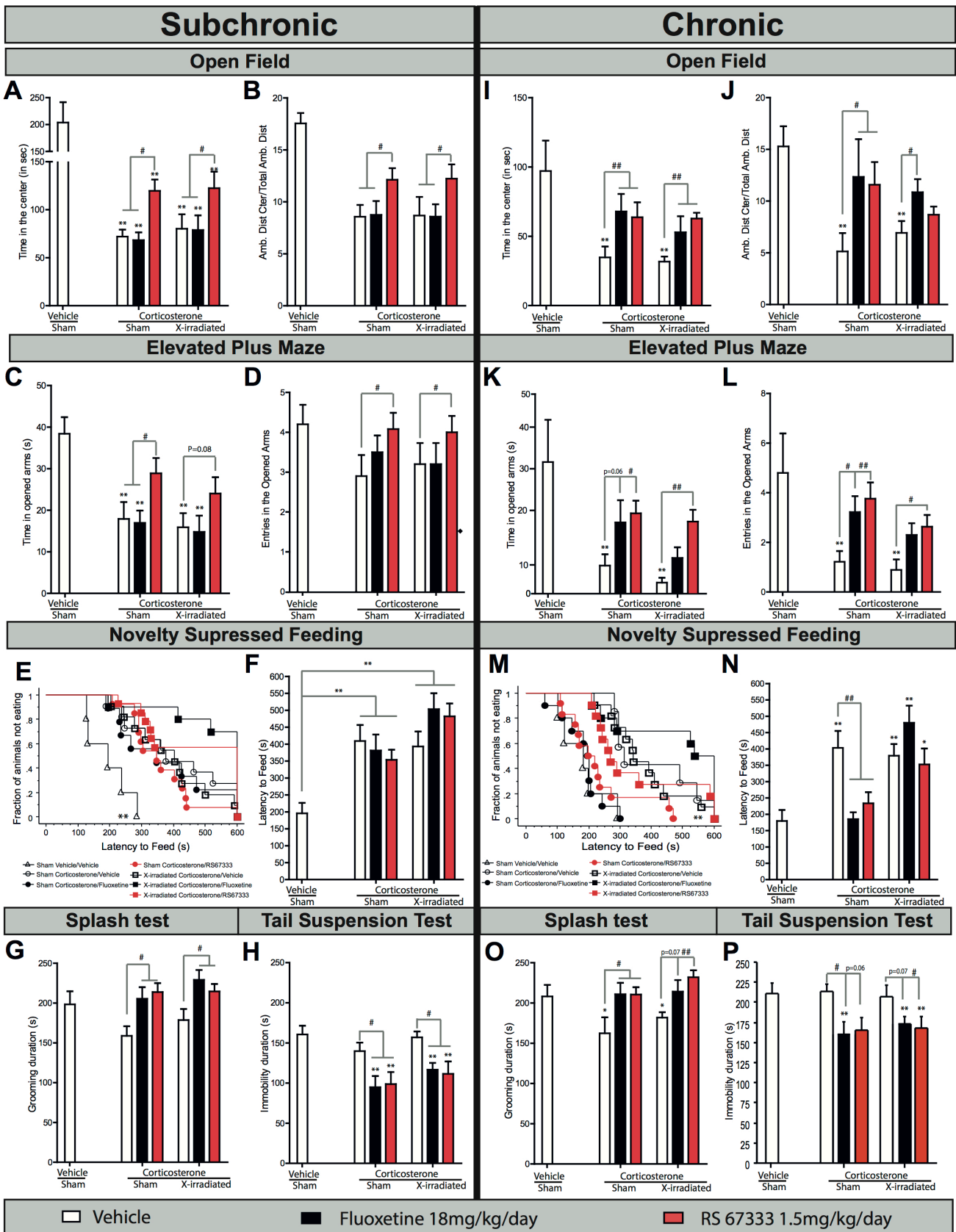


Table 1: Neurogenesis-dependent and independent mechanism involved in the behavioral effects of subchronic and chronic 5-HT₄ agonist treatment

	Fluoxetine (18 mg/kg/d)		RS67333 (1.5 mg/kg/d)	
	Subchronic	Chronic	Subchronic	Chronic
Open Field	Ø	+	+	+
	/	Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent
Elevated Plus Maze	Ø	+	+	+
	/	Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent
Novelty Suppressed Feeding	Ø	+	Ø	+
	/	Neurogenesis-dependent	/	Neurogenesis-dependent
Tail Suspension Test	+	+	+	+
	Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent
Splash Test	+	+	+	+
	Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent

SUPPLEMENTAL DATA

Rapid anxiolytic effects of a 5-HT₄ receptor agonist are mediated by a neurogenesis-independent mechanism

Authors: Indira Mendez-David^{1*}, Denis J. David^{1*}, Flavie Darcet¹, Melody V. Wu³, Saadia Kerdine-Romer², Alain M. Gardier¹, René Hen^{3,§}

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Behavioral tests

Open Field (OF)

This test was performed as described by David and colleagues (David *et al*, 2009). Motor activity was quantified in four 43 x 43 cm² Plexiglas open field boxes (MED Associates, Georgia, VT). Two sets of 16 pulse-modulated infrared photobeams were placed on opposite walls 2.5-cm apart to record x–y ambulatory movements. Activity chambers were computer interfaced for data sampling at 100-ms resolution. The computer defined grid lines that divided each open field into center and surround regions, with each of four lines being 11 cm from each wall. Dependent measures in the center were the total time and the number of entries over a 30-min test period. The activity in the center was quantified as distance traveled in the center divided by total distance traveled.

Elevated Plus Maze (EPM)

This test was performed as described by David and colleagues (David *et al*, 2009). The maze is a plus-cross-shaped apparatus, with two open arms and two arms closed by walls linked by

a central platform 50 cm above the floor. Mice were individually put in the center of the maze facing an open arm and were allowed to explore the maze during 5 min. The time spent in and the number of entries into the open arms were used as an anxiety index. All parameters were measured using a videotracker (EPM3C, Bioseb, Vitrolles, France).

Novelty-Suppressed-Feeding (NSF)

The NSF is a conflict test that elicits competing motivations: the drive to eat and the fear of venturing into the center of a brightly lit arena. The latency to begin eating is used as an index of anxiety/depression-like behavior, because classical anxiolytic drugs as well as chronic antidepressants decrease this measure. The NSF test was carried out during a 10-min period as previously described (David *et al*, 2009). Briefly, the testing apparatus consisted of a plastic box (50x50x20 cm), the floor of which was covered with approximately 2 cm of wooden bedding. Twenty-four hours prior to behavioral testing, all food was removed from the home cage. At the time of testing, a single pellet of food (regular chow) was placed on a white paper platform positioned in the center of the box. Each animal was placed in a corner of the box, and a stopwatch was immediately started. The latency to eat (defined as the mouse sitting on its haunches and biting the pellet with the use of forepaws) was timed. Immediately afterwards, the animal was transferred to its home cage, and the amount of food consumed by the mouse in the subsequent 5 min was measured, serving as a control for change in appetite as a possible confounding factor.

Tail Suspension Test (TST)

The TST is an antidepressant activity screening test (Steru *et al*, 1985) often used to test compounds that are expected to affect depression related behaviors. Mice are suspended by

their tails with tape, in such a position that they cannot escape or hold on to nearby surfaces. During this test, typically 6 minutes in duration, the resulting escape oriented behaviors are quantified using an automated tail suspension test apparatus (Bioseb, Vitrolles, France). A specific strain gauge linked to a computer quantifies the time spent by the animal trying to escape.

Splash Test

This test consisted of squirting 200 µl of a 10% sucrose solution on the mouse's snout. The grooming duration was assessed at the end of the corticosterone regimen in the presence or absence of 4-weeks of drug treatment according to a protocol used elsewhere (David *et al*, 2009).

Immunohistochemistry

Doublecortin-immunostaining consisted of the following steps: slices were washed with 3 times with PBST (PBS 1X + 0.3% triton) for 15 min, blocked with 10%NDS (normal donkey serum) + PBST during 2hrs and incubated with by goat anti-doublecortin primary antibody (1:100) in PBS/Triton/NDS for 24 hrs at 4°C. After secondary antibody incubation, sections were developed using CY2 (Donkey anti-rabbit) 1/250 diluted (dilute in PBS) for 2hrs.

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SUPPLEMENTAL FIGURES

Supplemental figure 1: Experimental timeline.

(A) In a first set of experiments, in place of normal drinking water, grouped-housed male C57BL/6Ntac mice were presented with vehicle (0.45% hydroxypropyl- β -cyclodextrin) or corticosterone (35 μ g/ml) in the presence or absence of a 5-HT₄ agonist (RS67333, 1.5 mg/kg/day, Alzet® mini pump model 2006 implanted subcutaneously), fluoxetine, 18 mg/kg/day, or a 5-HT₄ antagonist alone (GR125487, 1 mg/kg/day, Alzet® mini pump model 2006 implanted subcutaneously) or in combination with fluoxetine. We investigated whether the behavioral changes induced by chronic corticosterone were reversed by chronic 5-HT₄ ligands, fluoxetine alone, or fluoxetine in combination with 5-HT₄ receptor antagonist treatment. The same animal was successively tested in the OF paradigm, the EPM, the NSF, the ST, and the TST and then sacrificed for neurogenesis

(B) In another set of experiments, focal X-irradiation of the hippocampus was employed to assess whether the mechanisms underlying RS67333 mediated rescue of corticosterone-induced anxiety/depressive-like behavior were neurogenesis-dependent. X-irradiation (2.5 Gy) was delivered five weeks before the start of corticosterone treatment. All animals (Sham or X-irradiated) received corticosterone (35 μ g /ml) in the presence of vehicle, RS67333 (1.5 mg/kg/day), or fluoxetine (18 mg/kg/day). The anxiety/depressive-like phenotype of chronic corticosterone was assessed by comparing a chronic corticosterone/vehicle group *versus* a vehicle/vehicle group. The same animals were successively tested in the OF paradigm, the EPM, the NSF, the ST, and the TST after subchronic (days 7 to 11) or chronic (days 28 to 33) drug treatment.

Supplemental figure 2: Effects of chronic 5-HT₄ receptor stimulation on entries in the center in the open field paradigm and home food consumption in the NSF test in anxious/depressive mice.

(A-B) Effect of chronic 5-HT₄ ligands or fluoxetine treatment, started after 4 weeks of corticosterone (35 µg/ml), on the number of entries in the center (A) and on total ambulatory distance in the open field test (B). Values plotted are mean ± SEM (n= 10–15 per group).

(C) Effect of chronic 5-HT₄ ligands or fluoxetine treatment, started after 4 weeks of corticosterone (35 µg/ml), on home food consumption in the NSF test. Values plotted are mean ± SEM (n= 10–15 per group).

Supplemental figure 3: Images of doublecortin staining following corticosterone for 8 weeks ± chronic RS67333 (1.5 mg/kg/day), GR125487 (1 mg/kg/day), or fluoxetine (18 mg/kg/day) alone or in combination with GR125487 treatment.

Images of coronal sections of mouse hippocampal dentate gyrus stained for doublecortin (10x magnification).

Supplemental figure 4: Images of doublecortin staining following corticosterone for 8 weeks in Sham/vehicle or X-irradiated/vehicle animals.

Images of coronal sections of mouse hippocampal dentate gyrus stained for doublecortin (10x magnification) in sham/vehicle (A) or in Xray/vehicle-treated animals (B).

Supplemental figure 5: Effects of subchronic or chronic 5-HT₄ receptor stimulation on entries in the center in the open field paradigm and home food consumption in the NSF test in anxious/depressive X-irradiated mice.

(A) Effect of subchronic 5-HT₄ agonist or fluoxetine treatment, started after 4 weeks of corticosterone (35 µg/ml), on entries in the center in the open field test in X-irradiated mice. Values plotted are mean ± SEM (n = 9–15 per group for corticosterone-treated animals and n=5 for vehicle/vehicle). **p<0.01, #p<0.05, versus control vehicle/vehicle group and corticosterone/vehicle group, respectively.

(B) Effect of chronic 5-HT₄ agonist or fluoxetine treatment, started after 4 weeks of corticosterone (35 µg/ml), on home food consumption in the NSF test in X-irradiated mice. Values plotted are mean ± SEM (n = 9–15 per group for corticosterone-treated animals and n=5 for vehicle/vehicle). **p<0.01, #p<0.05, versus control vehicle/vehicle group and corticosterone/vehicle group, respectively.

(C) Effect of subchronic 5-HT₄ agonist or fluoxetine treatment, started after 4 weeks of corticosterone (35 µg/ml), on food consumption in the novelty suppressed feeding test in X-irradiated mice. Values plotted are mean ± SEM (n = 9–15 per group for corticosterone-treated animals and n=5 for vehicle/vehicle).

(D) Effect of chronic 5-HT₄ agonist or fluoxetine treatment, started after 4 weeks of corticosterone (35 µg/ml), on food consumption in the novelty suppressed feeding test in X-irradiated mice. Values plotted are mean ± SEM (n = 9–15 per group for corticosterone-treated animals and n=5 for vehicle/vehicle).

SUPPLEMENTAL DATA

Supplemental Table 1: complete statistical summary analysis for behavioral data after chronic treatment

Behavioral paradigm	Measurement	Statistical Test	Comparison	Statistics	Degrees of freedom	p	Fig.
Open Field	Total Time in the center	One-way ANOVA	Factor treatment	F=2.48	4,59	<0.05*	1A
		PLSD	Cort/Veh vs Cort/Flx			<0.05*	
		Post-hoc test	Cort/Veh vs Cort/RS			<0.01**	
	Ratio Amb. Dist Cter/Total Amb Dist.	One-way ANOVA	Factor treatment	F=2.70	4,59	<0.05*	1B
		PLSD	Cort/Veh vs Cort/Flx			<0.01**	
		Post-hoc test	Cort/Veh vs Cort/RS			<0.01**	
	Entries in the center	One-way ANOVA	Factor treatment	F=0.70	4,59	>0.5	Supp 2A
	Total Am	One-way ANOVA	Factor treatment	F=0.66	4,59	>0.5	Supp 2B
Elevated Plus Maze	Total Time in the open arms	One-way ANOVA	Factor treatment	F=1.6	4,59	>0.05	1C
		PLSD	Cort/Flx vs Cort/FLX+GR			<0.05#	
	Entries in the open arms	One-way ANOVA	Factor treatment	F=1.78	4,59	>0.05	1D
Splash Test	Grooming duration	One-way ANOVA	Factor treatment	F=4.95	4,59	<0.01**	1E
		PLSD	Cort/Veh vs Cort/Flx			<0.01**	
			Cort/Veh vs Cort/RS			<0.01**	
			Cort/Flx vs Cort/FLX+GR			<0.01##	
Tail Suspension Test	Immobility duration	One-way ANOVA	Factor treatment	F=3.76	4,59	<0.01**	1F
		PLSD	Cort/Veh vs Cort/Flx			<0.01**	
			Cort/Veh vs Cort/RS			<0.01**	
			Cort/Flx vs Cort/FLX+GR			<0.01##	
Novelty Suppressed Feeding	Latency to Feed	Kaplan-Meier Survival analysis				<0.01**	1G
		One-way ANOVA	Factor treatment	F=2.83	4,59	<0.05*	1H
		PLSD	Cort/Veh vs Cort/Flx			<0.01**	
			Cort/Veh vs Cort/RS			<0.01**	
			Cort/Flx vs Cort/FLX+GR			<0.01##	
	Food Cons.	One-way ANOVA	Factor treatment	F=1.18	4,59	>0.05	Supp 2B

Legend: CORT:corticosterone; Flx:fluoxetine; Veh:Vehicle; RS:RS67333; GR:GR125487

Supplemental table 2: complete statistical summary analysis for neurogenic data after chronic treatment

Behavioral paradigm	Measurement	Statistical Test	Comparison	Statistics	Degrees of freedom	p	Fig.
Proliferation	KI-67 positive cells	One-way ANOVA	Factor treatment	F=20.07	4,13	<0.05*	2A
		PLSD Post-hoc test	CORT/Veh vs CORT/Flx			<0.01**	
			CORT/Veh vs CORT/RS			<0.05*	
			CORT/Flx vs CORT/RS			<0.01##	
Maturation	DCX-positive cells	One-way ANOVA	Factor treatment	F=9.36	4,13	<0.01**	2B
		PLSD Post-hoc test	CORT/Veh vs CORT/Flx			<0.01**	
			CORT/Veh vs CORT/FLX+GR			<0.01**	
			CORT/Flx vs CORT/FLX+GR			<0.01##	
			CORT/Flx vs CORT/RS			<0.01##	
	DCX-positive cells with tertiary dendrites	One-way ANOVA	Factor treatment	F=12.90	4,13	<0.01**	2C
		PLSD Post-hoc test	CORT/Veh vs CORT/Flx			<0.01**	
			CORT/Veh vs CORT/FLX+GR			<0.01**	
			Cort/Flx vs Cort/FLX+GR			<0.01##	
			CORT/Flx vs CORT/RS			<0.08	
	Maturation Index	One-way ANOVA	Factor treatment	F=34.95	4,13	<0.01**	2D
		PLSD Post-hoc test	CORT/Veh vs CORT/Flx			<0.01**	
			CORT/Veh vs CORT/RS			<0.01**	
			CORT/Veh vs CORT/FLX+GR			<0.01**	
			CORT/Flx vs CORT/FLX+GR			<0.01#	
	Dendritic length	One-way ANOVA	Factor treatment	F=8.94	4,91	<0.01**	2F
		PLSD Post-hoc test	CORT/Veh vs CORT FLX (50, 60,70, 80, 90, 100 µm)			<0.01**	
			CORT/Veh vs CORT/RS (70 µm)			<0.01**	
			CORT/Flx vs CORT Flx+GR (50, 60,70, 80, 90, 100 µm)			<0.01##	
	Dendritic intersection	One-way ANOVA	Factor treatment	F=7.49	4,91	<0.01**	2G
		PLSD Post-hoc test	CORT/Veh vs CORT Flx (60,70, 80, 90 µm)			<0.01**	
			CORT/Veh vs CORT/RS (70, 80µm)			<0.01**	
			CORT/Flx vs CORT Flx+GR (60,70, 80, 90, 100 µm)			<0.01##	

Legend: CORT:corticosterone; Flx:fluoxetine; Veh:Vehicle; RS:RS67333; GR:GR125487

Supplemental Table 3: complete statistical summary analysis for behavioral data after subchronic treatment in sham or X-irradiated mice

Behavioral paradigm	Measurement	Statistical Test	Comparison	Statistics	Degrees of freedom	p	Fig.
Open Field	Total Time in the center	One-way ANOVA	Factor treatment	F=6.33	6,65	<0.01**	3A
		PLSD Post-hoc test	SHAM/Veh/Veh vs SHAM/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs SHAM/CORT/Flx			<0.01**	
			SHAM/Veh/Veh vs SHAM/CORT/RS			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Flx			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/RS			<0.01**	
	Entries in the center	One-way ANOVA	Factor treatment	F=3.90	6,65	<0.01**	Supp 4A
		PLSD Post-hoc test	SHAM/Veh/Veh vs SHAM/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs SHAM/CORT/Flx			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Flx			<0.01**	
Elevated Plus Maze	Total Time in the open arms	One-way ANOVA	Factor treatment	F=3.35	6,65	<0.01**	3C
		PLSD Post-hoc test	SHAM/Veh/Veh vs SHAM/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs SHAM/CORT/Flx			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Flx			<0.01**	
Novelty Suppressed Feeding	Latency to feed	Kaplan-Meier Survival analysis				<0.01**	3E
		One-way ANOVA	Factor treatment	F=3.79	6,65	<0.01**	3F
		PLSD Post-hoc test	SHAM/Veh/Veh vs SHAM/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs SHAM/CORT/Flx			<0.01**	
			SHAM/Veh/Veh vs SHAM/CORT/RS			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Flx			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/RS			<0.01**	
	Food cons.	One-way ANOVA	Factor treatment	F=2.20		<0.06	Supp 4C
Tail Suspension Test	Immobility duration	One-way ANOVA	Factor treatment	F=2.38	6,65	<0.05*	3H
		PLSD Post-hoc test	SHAM/Veh/Veh vs SHAM/CORT/Flx			<0.01**	
			SHAM/Veh/Veh vs SHAM/CORT/RS			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Flx			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/RS			<0.01**	
						<0.01**	

Legend : CORT:corticosterone; Flx:fluoxetine; Veh:Vehicle; RS:RS67333; XRAY:X-irradiated

Supplemental table 4: complete statistical summary analysis for behavioral data after subchronic treatment in sham or X-irradiated mice (two-way ANOVA)

Behavioral paradigm	Measurement	Statistical Test	Comparison	Statistics	Degrees of freedom	p	Fig.
Open Field	Total Time in the center	2-way ANOVA	Factor 1 Pre-treatment	F=0.38	1,61	>0.5	3A
			Factor 2 Treatment	F=8.0	2,61	<0.01**	
			Interaction (F1 x F2)	F=0.039	2,61	>0.5	
		PLSD Post-hoc test	SHAM/CORT/RS vs SHAM/CORT/Veh			<0.05#	
			SHAM/CORT/RS vs SHAM/CORT/Flx			<0.05#	
			XRAY/CORT/RS vs XRAY/CORT/Veh			<0.05#	
			XRAY/CORT/RS vs XRAY/CORT/Flx			<0.05#	
	Ratio Amb. Dist Cter/Total Amb Dist.	2-way ANOVA	Factor 1 Pre-treatment	F< 0.0001	1,61	>0.5	3B
			Factor 2 Treatment	F=4.91	2,61	<0.05*	
			Interaction (F1 x F2)	F=0.005	2,61	>0.5	
		PLSD Post-hoc test	SHAM/CORT/RS vs SHAM/CORT/Veh			<0.05#	
			SHAM/CORT/RS vs SHAM/CORT/Flx			<0.05#	
			XRAY/CORT/RS vs XRAY/CORT/Veh			<0.05#	
			XRAY/CORT/RS vs XRAY/CORT/Flx			<0.05#	
	Entries in the center	2-way ANOVA	Factor 1 Pre-treatment	F=0.27	1,61	>0.5	Supp 4A
			Factor 2 Treatment	F=10.41	2,61	<0.01**	
			Interaction (F1 x F2)	F=0.120	2,61	>0.5	
		PLSD Post-hoc test	SHAM/CORT/RS vs SHAM/CORT/Veh			<0.05#	
			SHAM/CORT/RS vs SHAM/CORT/Flx			<0.05#	
			XRAY/CORT/RS vs XRAY/CORT/Veh			<0.13	
			XRAY/CORT/RS vs XRAY/CORT/Flx			<0.13	
Elevated Plus Maze	Total Time in the open arms	2-way ANOVA	Factor 1 Pre-treatment	F=0.86	1,61	>0.3	3C
			Factor 2 Treatment	F=4.96	2,61	<0.01**	
			Interaction (F1 x F2)	F=0.28	2,61	>0.5	
		PLSD Post-hoc test	SHAM/CORT/RS vs SHAM/CORT/Veh			<0.05#	

			SHAM/CORT/RS vs SHAM/CORT/Veh			<0.05#	3D
			XRAY/CORT/RS vs XRAY/CORT/Veh			<0.09	
	Entries in the open arms	2-way ANOVA	Factor 1 Pre-treatment	F=0.14	1,61	>0.5	
			Factor 2 Treatment	F=3.09	2,61	<0.053	
			Interaction (F1 x F2)	F=0.92	2,61	<0.41	
		PLSD Post-hoc test	SHAM/CORT/RS vs SHAM/CORT/Veh			<0.05#	
			XRAY/CORT/RS vs XRAY/CORT/Veh			<0.05#	
Splash test	Grooming duration	2-way ANOVA	Factor 1 Pre-treatment	F=2.28	1,61	>0.5	3G
			Factor 2 Treatment	F=7.16	2,61	<0.01**	
			Interaction (F1 x F2)	F=0.022	2,61	>0.5	
		PLSD Post-hoc test	SHAM/CORT/Flx vs SHAM/CORT/Veh			<0.05#	
			SHAM/CORT/RS vs SHAM/CORT/Veh			<0.05#	
			XRAY/CORT/Flx vs XRAY/CORT/Veh			<0.05#	
			XRAY/CORT/RS vs XRAY/CORT/Veh			<0.05#	
Tail Suspension Test	Immobility duration	2-way ANOVA	Factor 1 Pre-treatment	F=2.28	1,61	<0.14	3H
			Factor 2 Treatment	F=7.16	2,61	<0.01**	
			Interaction (F1 x F2)	F=0.02	2,61	>0.5	
		PLSD Post-hoc test	SHAM/CORT/Veh vs SHAM/CORT/Flx			<0.05#	
			SHAM/CORT/Veh vs SHAM/CORT/RS			<0.05#	
			XRAY/CORT/Veh vs XRAY/CORT/Flx			<0.05#	
			XRAY/CORT/Veh vs XRAY/CORT/RS			<0.01##	

Factor 1 Pre-treatment: Sham or X-irradiation; Factor 2 Treatment: Vehicle or Corticosterone; CORT:corticosterone; Flx:fluoxetine; Veh:Vehicle; RS:RS67333; XRAY:X-irradiated

Supplemental Table 5: complete statistical summary analysis for behavioral data after chronic treatment

Behavioral paradigm	Measurement	Statistical Test	Comparison	Statistics	Degrees of freedom	p	Fig.
Open Field	Total Time in the center	One-way ANOVA	Factor treatment	F=3.94	6,59	<0.01**	3I
		PLSD Post-hoc test	SHAM/Veh/Veh vs SHAM/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Veh			<0.01**	
	Amb. Dist Center/Total Amb. Dist	One-way ANOVA	Factor treatment	F=2.48	6,59	<0.05*	3J
		PLSD Post-hoc test	SHAM/Veh/Veh vs SHAM/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Veh			<0.01**	
	Entries in the center	One-way ANOVA	Factor treatment	F=2.94	6,59	<0.05*	Supp 4B
		PLSD Post-hoc test	SHAM/Veh/Veh vs SHAM/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Veh			<0.01**	
Elevated Plus Maze	Total Time in the open arms	One-way ANOVA	Factor treatment	F=5.32	6,59	<0.01**	3K
		PLSD Post-hoc test	SHAM/Veh/Veh vs SHAM/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Veh			<0.01**	
	Entries in the open arms	One-way ANOVA	Factor treatment	F=3.91	6,50	<0.01**	3L
		PLSD Post-hoc test	SHAM/Veh/Veh vs SHAM/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Veh			<0.01**	
Novelty Suppressed Feeding	Latency to feed	Kaplan-Meier Survival analysis				<0.01**	3M
		One-way ANOVA	Factor treatment	F=7.31	6,59	<0.01**	3N
		PLSD Post-hoc test	SHAM/Veh/Veh vs SHAM/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Veh			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/Flx			<0.01**	
			SHAM/Veh/Veh vs XRAY/CORT/RS			<0.05*	
	Food cons.	One-way ANOVA	Factor treatment	F=1.28	6,59	<0.3	Supp 4D

Splash Test	Grooming duration	One-way ANOVA	Factor treatment	F=3.40	6,59	<0.01**	3O
		PLSD Post-hoc test	SHAM/Veh/Veh vs SHAM/CORT/Veh			<0.05*	
			SHAM/Veh/Veh vs XRAY/CORT/Veh			<0.26	
Tail Suspension Test	Immobility duration	One-way ANOVA	Factor treatment	F=2.36	6,59	<0.05*	3P
		PLSD Post-hoc test	SHAM/Veh/Veh vs SHAM/CORT/Flx			<0.05*	
			SHAM/Veh/Veh vs XRAY/CORT/Flx			<0.05*	
			SHAM/Veh/Veh vs SHAM/CORT/RS			<0.06	
			SHAM/Veh/Veh vs XRAY/CORT/RS			<0.07	

Legend: Factor 1 Pre-treatment: Sham or X-irradiation; Factor 2 Treatment: Vehicle or Corticosterone; CORT:corticosterone; Flx:fluoxetine; Veh:Vehicle; RS:RS67333; XRAY:X-irradiated

Supplemental Table 6: complete statistical summary analysis for behavioral data after chronic treatment in sham or X-irradiated mice (two-way ANOVA)

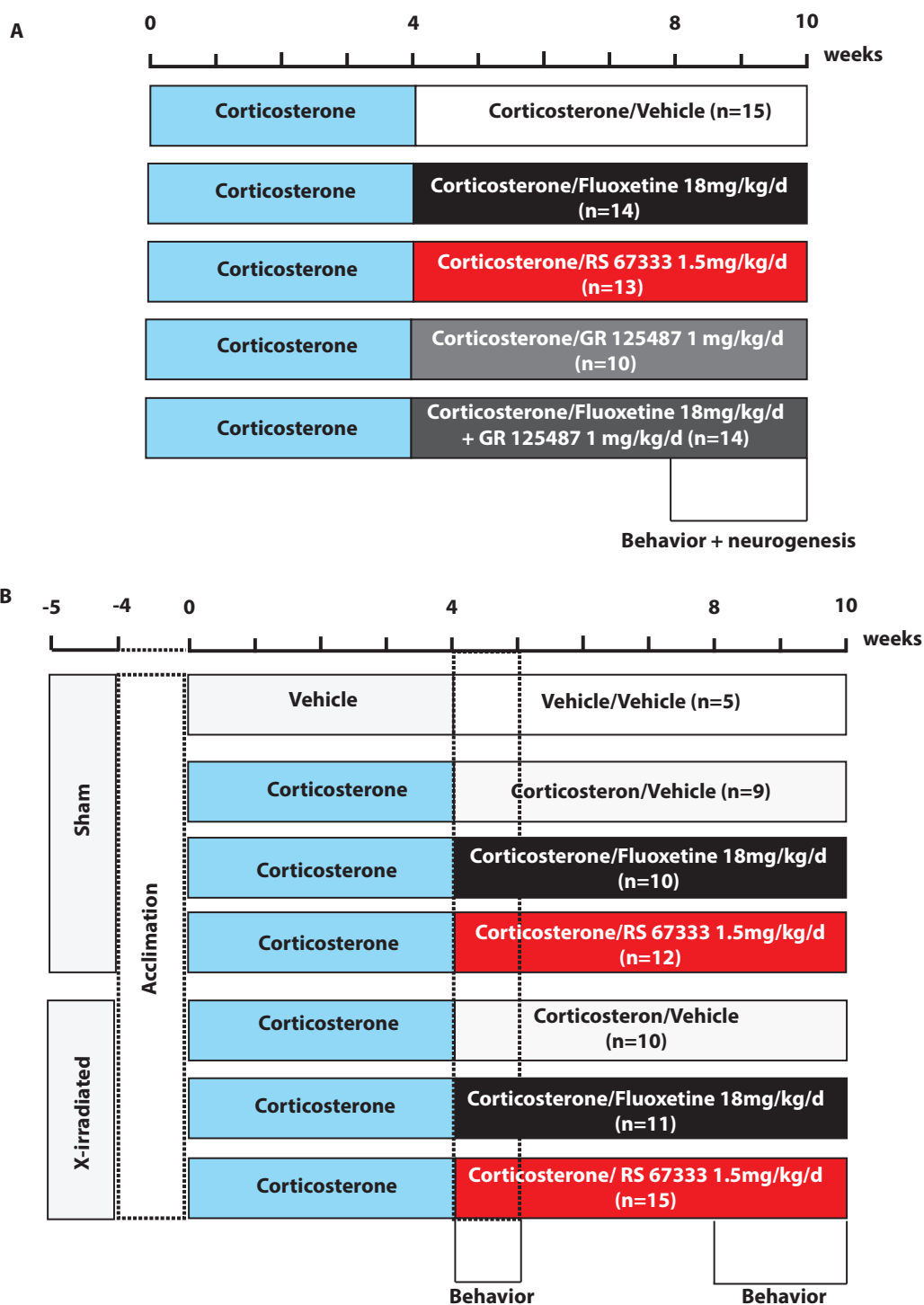
Behavioral paradigm	Measurement	Statistical Test	Comparison	Statistics	Degrees of freedom	p	Fig.
Open Field	Total Time in the center	2-way ANOVA	Factor 1 Pre-treatment	F=0.64	1,55	>0.4	3I
			Factor 2 Treatment	F=5.37	2,55	<0.01**	
			Interaction (F1 x F2)	F=0.32	2,55	>0.5	
		PLSD Post-hoc test	SHAM/CORT/Veh vs SHAM/CORT/Flx			<0.01##	
			SHAM/CORT/Veh vs SHAM/CORT/RS			<0.01##	
			XRAY/CORT/Veh vs XRAY/CORT/Flx			<0.1	
			XRAY/CORT/Veh vs XRAY/CORT/RS			<0.05#	
	Ratio Amb. Dist Cter/Total Amb Dist.	2-way ANOVA	Factor 1 Pre-treatment	F=0.24	1,55	>0.5	3J
			Factor 2 Treatment	F=3.17	2,55	<0.05*	
			Interaction (F1 x F2)	F=0.60	2,55	>0.5	
		PLSD Post-hoc test	SHAM/CORT/Veh vs SHAM/CORT/Flx			<0.01##	
			SHAM/CORT/Veh vs SHAM/CORT/RS			<0.05#	
			XRAY/CORT/Veh vs XRAY/CORT/Flx			<0.2	
	Entries in the center	2-way ANOVA	Factor 1 Pre-treatment	F=0.052	1,55	>0.5	Supp 4B
			Factor 2 Treatment	F=5.47	2,55	<0.01**	
			Interaction (F1 x F2)	F=0.40	2,55	>0.5	
		PLSD Post-hoc test	SHAM/CORT/Veh vs SHAM/CORT/Flx			<0.05#	
			SHAM/CORT/Veh vs SHAM/CORT/RS			<0.05#	
			XRAY/CORT/Veh vs XRAY/CORT/Flx			<0.07	
			XRAY/CORT/Veh vs XRAY/CORT/RS			<0.01##	
Elevated Plus Maze	Total Time in the open arms	2-way ANOVA	Factor 1 Pre-treatment	F=3.45	1,55	<0.07	3K
			Factor 2 Treatment	F=9.28	2,55	<0.01**	
			Interaction (F1 x F2)	F=0.56	2,55	>0.5	

		PLSD Post-hoc test	SHAM/CORT/Veh vs SHAM/CORT/Flx			<0.06	
			SHAM/CORT/Veh vs SHAM/CORT/RS			<0.05#	
			XRAY/CORT/Veh vs XRAY/CORT/Flx			<0.09	
			XRAY/CORT/Veh vs XRAY/CORT/RS			<0.01##	
	Entries in the open arms	2-way ANOVA	Factor 1 Pre- treatment	F=2.98	1,55	<0.09	3L
			Factor 2 Treatment	F=7.91	2,55	<0.001***	
			Interaction (F1 x F2)	F=0.27	2,55	>0.5	
		PLSD Post-hoc test	SHAM/CORT/Veh vs SHAM/CORT/Flx			<0.05#	
			SHAM/CORT/Veh vs SHAM/CORT/RS			<0.01##	
			XRAY/CORT/Veh vs XRAY/CORT/Flx			<0.13	
			XRAY/CORT/Veh vs XRAY/CORT/RS			<0.042#	
Novelty Suppressed Feeding	Latency to feed	2-way ANOVA	Factor 1 Pre- treatment	F=14.42	1,55	<0.01**	3N
			Factor 2 Treatment	F=2.73	2,55	<0.08	
			Interaction (F1 x F2)	F=6.86	2,55	<0.01**	
		PLSD Post-hoc test	SHAM/CORT/Veh vs SHAM/CORT/Flx			<0.05#	
			SHAM/CORT/Veh vs SHAM/CORT/RS			<0.01##	
	Food Cons.	2-way ANOVA	Factor 1 Pre- treatment	F=1.48	1,55	>0.2	Supp 4D
			Factor 2 Treatment	F=0.67	2,55	>0.5	
			Interaction (F1 x F2)	F=1.42	2,55	>0.2	
Splash test	Grooming duration	2-way ANOVA	Factor 1 Pre- treatment	F=2.22	1,55	>0.1	3O
			Factor 2 treatment	F=8.85	2,55	<0.01**	
			Interaction (F1 x F2)	F=0.35	2,55	>0.5	
		PLSD Post-hoc test	SHAM/CORT/Veh vs SHAM/CORT/Flx			<0.05#	
			SHAM/CORT/Veh vs SHAM/CORT/RS			<0.01##	
			XRAY/CORT/Veh vs XRAY/CORT/Flx			<0.07	
			XRAY/CORT/Veh vs XRAY/CORT/RS			<0.01##	
Tail Suspension	Immobility duration	2-way ANOVA	Factor 1 Pre- treatment	F=0.055	1,55	>0.5	3P
			Factor 2 Treatment	F=5.43	2,55	<0.01**	

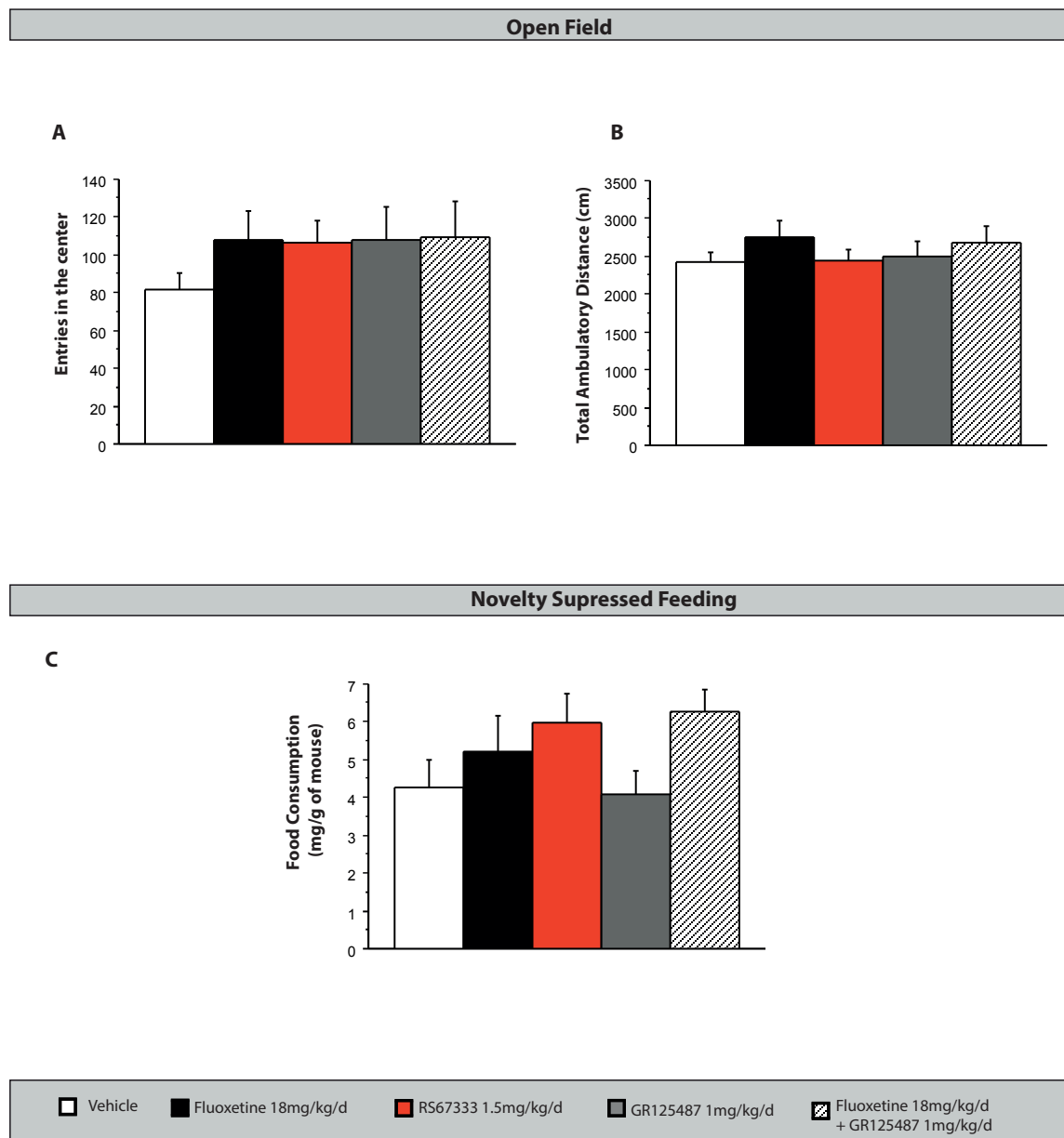
Test			Interaction (F1 x F2)	F=0.18	2,55	>0.5
			SHAM/CORT/Veh vs SHAM/CORT/Flx			<0.05#
			SHAM/CORT/Veh vs SHAM/CORT/RS			<0.06
			XRAY/CORT/Veh vs XRAY/CORT/Flx			<0.07
			XRAY/CORT/Veh vs XRAY/CORT/RS			<0.05#

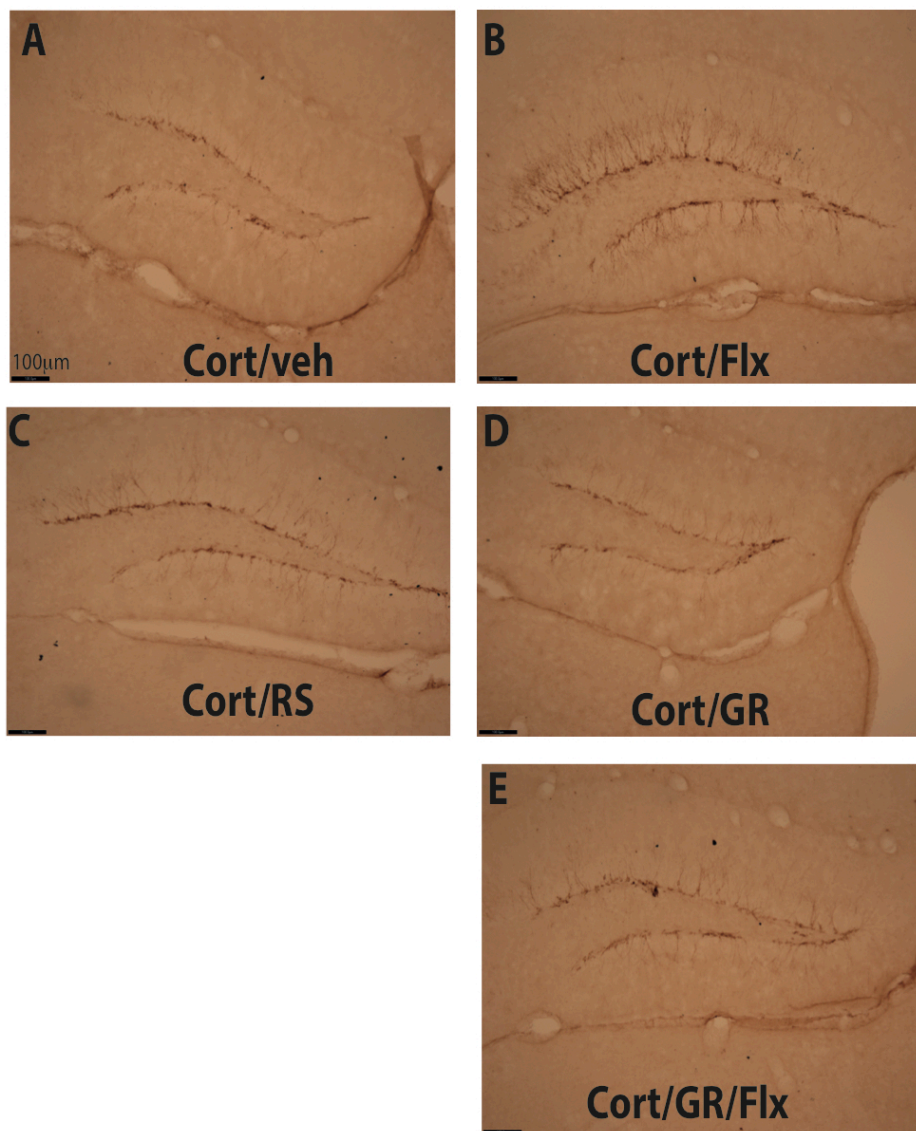
Legend: Factor 1 Pre-treatment: Sham or X-irradiation; Factor 2 Treatment: Vehicle or Corticosterone; CORT:corticosterone; Flx=fluoxetine; Veh:Vehicle; RS:RS67333; XRAY:X-irradiated

Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3**Maturation**

Supplemental Figure 4

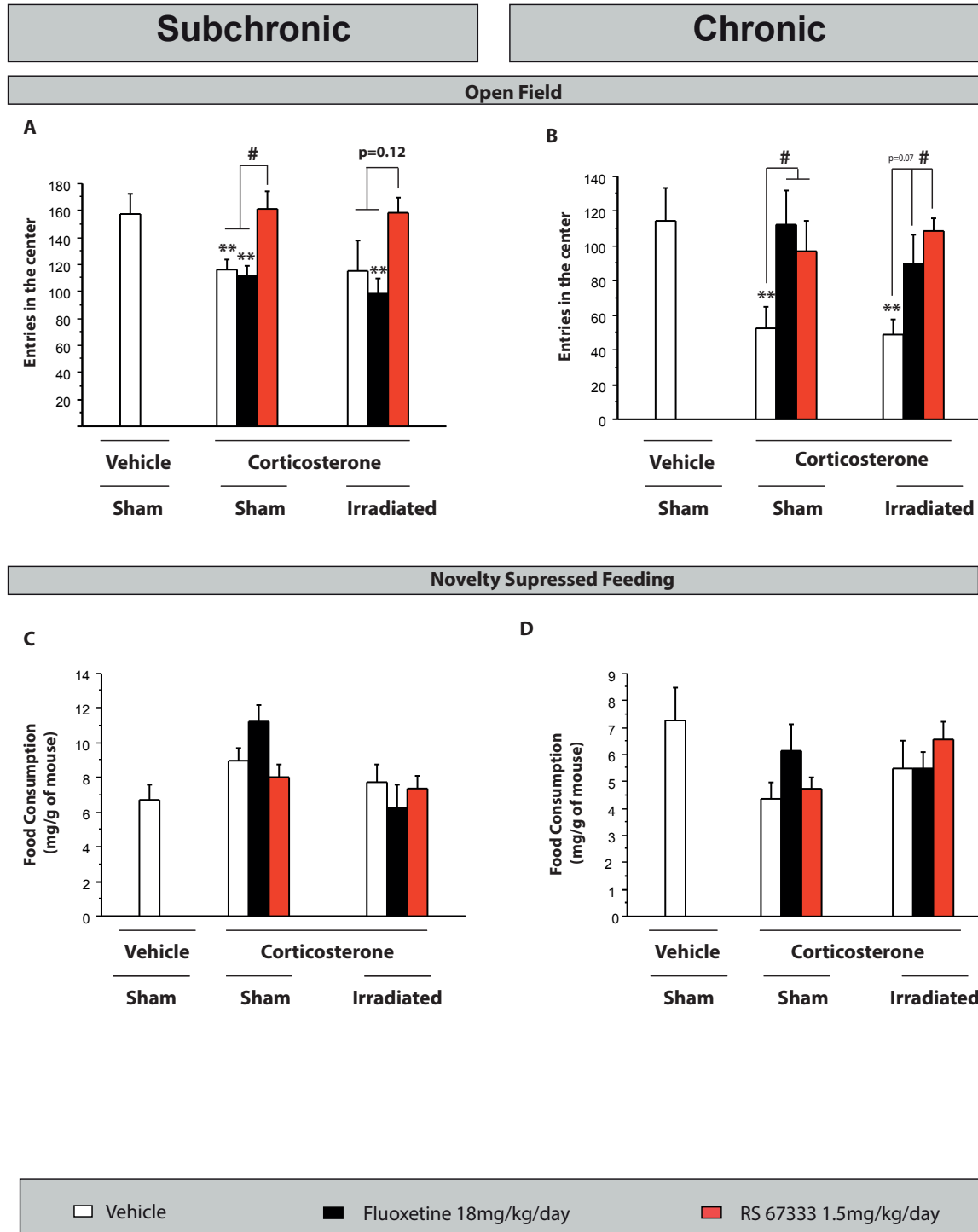
A



B



Supplemental Figure 5



Conclusion of the study

Our study shows, for the first time, in a mouse model of anxiety/depression, that a 5-HT₄ receptor agonist may be a fast-acting anxiolytic agent, and that 5-HT₄ stimulation is necessary for the behavioral and neurogenic effects (proliferation and maturation) of fluoxetine, a classic SSRI antidepressant. Furthermore, we showed that, with the exception of the NSF test, the anxiolytic and antidepressant-like effects of the 5-HT₄ agonist were independent of hippocampal neurogenesis.

The present study is encouraging for the development of RS-67333 as an anxiolytic/antidepressant compound for use in patients. However, the use of the 5-HT₄ receptor as a novel antidepressant target may be hampered by the fact that it also plays important roles outside the central nervous system (Tonini and Pace, 2006), which may prevent its development as an effective anxiolytic/antidepressant drug (Bockaert et al., 2004, 2008). Thus, signaling molecules that interact with the 5-HT₄ receptor such as P11 (Egeland et al., 2011; Warner-Schmidt et al., 2009) may represent novel targets for fast-acting anxiolytic/antidepressant treatments.

We next wanted to investigate whether the fast onset of anxiolytic/antidepressant action of 5-HT₄ receptor agonist treatment can be predicted by measuring in Peripheral Blood Mononuclear Cells, β -arrestin 1 levels, a protein known to play a role in the pathophysiology of depression and in antidepressant mechanism of action (Avissar et al., 2004; Schreiber and Avissar, 2004; Matuzany-Ruban et al., 2005; Beaulieu et al., 2008; David et al., 2009; Schreiber et al., 2009; Golan et al., 2010).

Article 5: A method for biomarker measurements in peripheral blood mononuclear cells isolated from anxious and depressed mice: β -arrestin 1 protein levels in depression and treatment.

Indira Mendez-David, Zeina El Ali, René Hen, Alain M Gardier, Saadia Kerdine-Römer S,
Denis J David,

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Introduction of the study

A recent review from Duman's group highlighted the need to develop a biomarker panel for depression. This biomarker panel should profile diverse peripheral factors that together will provide a biological signature of depressive disorders subtypes and predict treatment response (Schmidt et al., 2011). Assessing peripheral protein levels in PBMCs is an attractive method because PBMCs are circulating cells that can be easily collected and monitored. Previous studies demonstrated that PBMCs can be isolated from mouse blood to assess immunological responses (Fuss et al., 2009).

In this study we developed a method to screen putative biomarkers of the pathophysiology of mood disorders and the response to Ads, measuring and assessing circulating proteins, especially β -arrestin 1 in PBMCs. If β -arrestin 1 is known as a protein to mediate receptor desensitization, endocytosis and G protein-independent signaling, it also recognized as a protein involved in the pathophysiology of depression and in antidepressant mechanism of action. Furthermore, we examined whether changes in β -arrestin 1 levels in mouse PBMCs were observed in a model of anxiety/depression (David et al., 2009; Guilloux et al., 2011; Rainer et al., 2011) and whether these levels could be corrected by chronic treatment with the SSRI fluoxetine.



A method for biomarker measurements in peripheral blood mononuclear cells isolated from anxious and depressed mice: β -arrestin 1 protein levels in depression and treatment

Indira Mendez-David¹, Zeina El-Ali², René Hen^{3,4}, Bruno Falissard⁵, Emmanuelle Corruble⁵, Alain M. Gardier¹, Saadia Kerdine-Römer^{2†} and Denis J. David^{1*†}

¹ EA3544, Faculté de Pharmacie, Université Paris-Sud, Châtenay-Malabry, France

² INSERM UMR 996, Faculté de Pharmacie, Université Paris-Sud, Châtenay-Malabry, France

³ Department of Neuroscience, Columbia University, New York, NY, USA

⁴ Department of Psychiatry, Columbia University, New York, NY, USA

⁵ INSERM U669, Département de Psychiatrie, Hôpital Bicêtre, Assistance Publique Hôpitaux de Paris, Université Paris-Sud, Le Kremlin-Bicêtre, France

Edited by:

Thibault Renou, Florey Institute of Neuroscience and Mental Health, Australia

Reviewed by:

Ciaran J. Faherty, Cadence

Pharmaceuticals, USA

El Chérif Ibrahim, Aix-Marseille

UNiversité - Centre National de la Recherche Scientifique, France

*Correspondence:

Denis J. David, EA3544, Faculté de Pharmacie, Université Paris-Sud, 5, rue J-B Clement, Tour D1, 2e étage, F-92296 Châtenay-Malabry Cedex, France

e-mail: denis.david@u-psud.fr

[†] Saadia Kerdine-Römer and Denis J. David are joint last authors.

A limited number of biomarkers in the central and peripheral systems which are known may be useful for diagnosing major depressive disorders and predicting the effectiveness of antidepressant (AD) treatments. Since 60% of depressed patients do not respond adequately to medication or are resistant to ADs, it is imperative to delineate more accurate biomarkers. Recent clinical studies suggest that β -arrestin 1 levels in human mononuclear leukocytes may be an efficient biomarker. If potential biomarkers such as β -arrestin 1 could be assessed from a source such as peripheral blood cells, then they could be easily monitored and used to predict therapeutic responses. However, no previous studies have measured β -arrestin 1 levels in peripheral blood mononuclear cells (PBMCs) in anxious/depressive rodents. This study aimed to develop a method to detect β -arrestin protein levels through immunoblot analyses of mouse PBMCs isolated from whole blood. In order to validate the approach, β -arrestin levels were then compared in naïve, anxious/depressed mice, and anxious/depressed mice treated with a selective serotonin reuptake inhibitor (fluoxetine, 18 mg/kg/day in the drinking water). The results demonstrated that mouse whole blood collected by submandibular bleeding permitted isolation of enough PBMCs to assess circulating proteins such as β -arrestin 1. β -Arrestin 1 levels were successfully measured in healthy human subject and naïve mouse PBMCs. Interestingly, PBMCs from anxious/depressed mice showed significantly reduced β -arrestin 1 levels. These decreased β -arrestin 1 expression levels were restored to normal levels with chronic fluoxetine treatment. The results suggest that isolation of PBMCs from mice by submandibular bleeding is a useful technique to screen putative biomarkers of the pathophysiology of mood disorders and the response to ADs. In addition, these results confirm that β -arrestin 1 is a potential biomarker for depression.

Keywords: peripheral blood mononuclear cells, β -arrestin 1, anxiety, depression, mouse models, fluoxetine, biomarkers

INTRODUCTION

Elucidation of the neurobiological bases of depression and anxiety are significant challenges for today's society. Mood disorders impact 7% of the world's population and rank among the top 10 causes of disability (Kessler et al., 2005). Selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs) are the most commonly prescribed antidepressant (AD) drugs for major depressive disorders (MDD; Samuels et al., 2011). However, key questions about the molecular and cellular mechanisms underlying the effects of ADs remain unanswered. Approximately 60% of depression patients do not respond adequately or are resistant to these drugs (Samuels et al., 2011). Therefore, there are clear benefits of having valid, reliable, selective, and feasible biomarkers for MDD. Several studies have reported genome-wide expression changes

associated with AD responses in MDD (Iga et al., 2007a,b; Belzeaux et al., 2010; Lakhan et al., 2010; Mamdani et al., 2011). However, candidate biomarkers that can accurately predict AD responses must be identified. While there are currently no specific markers that are considered "gold standards," a few candidates have emerged. Peripheral/serum brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), and cytokines may serve as biomarkers of MDD and treatment response (for review, see Schmidt et al., 2011).

Recently, a substantial body of evidence indicates that β -arrestins (β -arrestin 1 and 2), proteins that regulate G protein receptor coupling, play major roles in the pathophysiology of mood disorders and in the mechanisms underlying AD actions (Avissar et al., 2004; Schreiber and Avissar, 2004; Matuzany-Ruban et al., 2005; Beaulieu et al., 2008; David et al., 2009; Schreiber

et al., 2009; Golan et al., 2010). The β -arrestin-signaling cascade has recently gained attention as a potential pre-clinical/clinical bridging biomarker for depressive states and treatment effects. In naïve rats, SSRI, SNRI, and non-selective reuptake inhibitor ADs significantly elevate β -arrestin 1 levels in the cortex and the hippocampus (Avissar et al., 2004; Beaulieu and Caron, 2008; Beaulieu et al., 2008; David et al., 2009). Similarly, β -arrestin 1 expression is decreased in the hypothalamus and hippocampus in anxious/depressed mice exposed to glucocorticoid elevation, and is restored by chronic fluoxetine treatment (David et al., 2009). Moreover, β -arrestin 1 and 2 signaling is involved in mediating the response to fluoxetine and lithium (Beaulieu et al., 2008; David et al., 2009).

Clinical data from Avissar et al. (2004) suggest that β -arrestin 1 mRNA and protein levels are highest in peripheral blood leukocytes of MDD patients. Therefore, β -arrestin 1 may be a putative candidate biochemical marker in clinical practice for depressive pathophysiology and the response to ADs (for review, see Schreiber et al., 2009). β -Arrestin mRNA levels and β -arrestin 1 protein levels in mononuclear leukocytes of untreated patients with MDD are lower than the levels found in healthy subjects. Furthermore, reduced levels of β -arrestin 1 protein and mRNA are significantly correlated with the severity of depressive symptoms (Avissar et al., 2004; Schreiber et al., 2009). However, the low β -arrestin 1 protein and mRNA levels are alleviated by AD treatment. Therefore, these low levels can predict clinical improvement (Avissar et al., 2004; Golan et al., 2010).

These clinical data suggest that assessment of β -arrestin 1 levels may prove useful for diagnosing depression with high sensitivity and specificity (Golan et al., 2013). This hypothesis must first be validated in animal models of anxiety–depression. Most of the current understandings of mood disorders and AD activities are based on studies performed on animal models of anxiety–depression (Belzung and Lemoine, 2011). No animal studies have investigated whether β -arrestin 1 protein levels in peripheral blood mononuclear cells (PBMCs) are a marker of the pathophysiology of depression and the AD response. However, if PBMCs can be successfully used to define biomarkers, they provide a system of circulating cells that can be easily collected from patients and monitored to predict therapeutic responses.

In this study, we developed a method to measure and assess circulating proteins (such as β -arrestin 1 in PBMCs) that are collected through submandibular bleeding from unanesthetized animals. Furthermore, we examined whether changes in β -arrestin 1 levels in mouse PBMCs were observed in a model of anxiety/depression (David et al., 2009; Guilloux et al., 2011; Rainer et al., 2012b), and whether these levels could be corrected by chronic treatment with the SSRI fluoxetine.

EXPERIMENTAL PROCEDURES

SUBJECTS

Adult male C57BL/6Ntac mice were purchased from Taconic Farms (Lille Skensved, Denmark). All mice were 7–8 weeks old, weighed 23–25 g at the beginning of the treatment and were maintained on a 12L:12D schedule (lights on at 0600 hours). The mice were group-housed with each cage containing five animals. Food and water were provided *ad libitum*. All testing were conducted

in compliance with the laboratory animal care guidelines and with protocols approved by the Institutional Animal Care and Use Committee (Council directive # 87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions # 92-256B to Denis J. David).

DRUGS

Corticosterone (4-pregnen-11 β -DIOL-3 20-DIONE 21-hemisuccinate from Sigma (Sigma-Aldrich, Saint-Quentin-Fallavier, France) was dissolved in 0.45% hydroxypropyl- β -cyclodextrin (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Fluoxetine hydrochloride (18 mg/kg/day in the drinking water) was purchased from Anawa Trading (Zurich, Switzerland).

ISOLATION OF HUMAN AND MOUSE PERIPHERAL BLOOD MONONUCLEAR CELLS

Collection of human blood and isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were purified from 7.5 ml of human whole circulating blood obtained from Etablissement Français du Sang (Ivry-Sur-Seine, France) through density centrifugation (850 g at 20°C for 20 min) using a Ficoll gradient (PAA Laboratories GmbH, Pashing, Austria; **Figure 1A**). This centrifugation separated lymphocytes, monocytes, and plasma. The PBMC layers were carefully removed from the tube and transferred to a new 50 ml conical tube. The PBMCs were then washed twice (1 min each) with 1 \times phosphate-buffered saline (PBS)/fetal calf serum (FCS, 2%). After centrifugations (150 g at 20°C for 7 min), the cells were resuspended in the appropriate volume of 1 \times PBS. The human PBMCs were then recovered with a final centrifugation (1,000 g at 4°C for 5 min) and were stored at -80°C .

Collection of mouse blood and isolation of peripheral blood mononuclear cells

Blood was collected from unanesthetized mice as previously described (Golde et al., 2005; Joslin, 2009). In compliance with the laboratory animal care guidelines, approximately 0.4 ml of blood per mouse was collected in K₃EDTA tubes with a submandibular bleeding procedure. Five millimeters point size sterile lancets (MediPoint, Mineola, NY, USA; **Figure 1B**) were used to puncture the location where the orbital vein and the submandibular vein join to form the jugular vein (Joslin, 2009). A light pressure with dry gauze was applied to the punctured area for hemostasis. Separation and extraction of PBMCs were performed using an iodixanol mixer technique (Ford and Rickwood, 1990). Mouse PBMCs were purified from whole blood by density centrifugation (300 g at 20°C for 30 min) using solution B (see **Table 1** for preparation) of the OptiPrepTM gradient solution (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Specifically, the OptiPrepTM gradient solution was used to separate blood into PBMC and plasma layers with centrifugation. The PBMC layers were then carefully removed from the tube and transferred to a new 50 ml conical tube. The PBMCs were then washed twice with solution B (1 min each). After another centrifugation (150 g at 20°C for 7 min) and two washing steps (1 min each), mouse

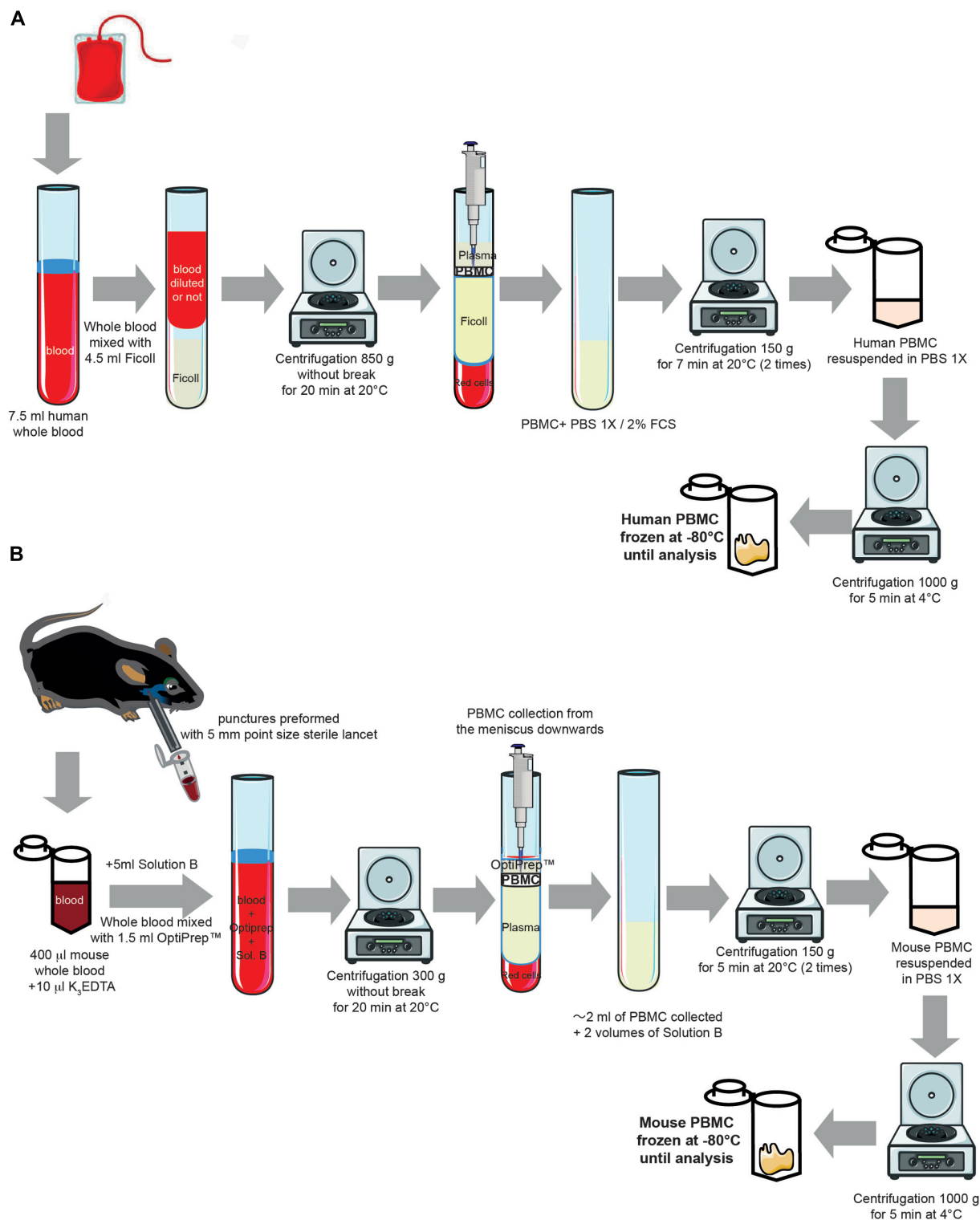


FIGURE 1 | Experimental protocol for isolating human and mouse peripheral blood mononuclear cells from whole blood. (A) Cartoon representing the different steps for isolating human PBMC from whole circulating blood (for full details of the method, see *Blood collection and Peripheral blood mononuclear cells Isolation in human* from the Section “Experimental Procedures”). Some elements of this figure were produced

using Servier Medical Art image bank (www.servier.com). **(B)** Cartoon representing the different steps for isolating mouse PBMC from whole circulating blood (for full details of the method, see *Blood collection and Peripheral blood mononuclear cells Isolation in mouse* from the Section “Experimental Procedures”). Some elements of this figure were produced using Servier Medical Art image bank (www.servier.com).

Table 1 | Solution used to prepare peripheral blood mononuclear cells from mouse whole blood.

	OptiPrep™ density gradient medium	Tricine-buffered saline (TBS)	Solution B
Solutions	D1556-250ML (Sigma-Aldrich, France)	0.85% NaCl, 10 mM; Tricine-NaOH, pH 7.4 (Tricine as 100 mM stock solution at 4° C; 1.79 g/100 ml water)	Dissolve 0.85 g NaCl in 50 ml water; add 10 ml of Tricine stock; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml

PBMCs were recovered with a final centrifugation (1,000 g at 4°C for 5 min) and were stored at −80°C.

β -ARRESTIN 1 LEVELS IN HUMAN AND MOUSE PERIPHERAL BLOOD MONONUCLEAR CELLS

Protein extraction from peripheral blood mononuclear cells and immunoblots

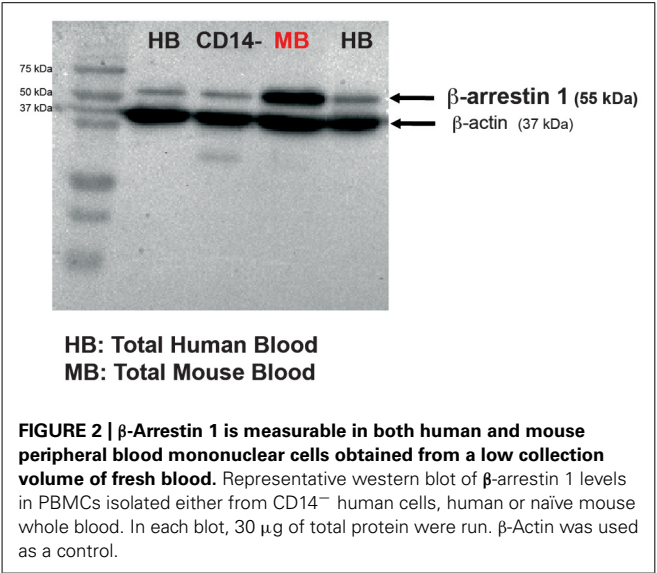
Peripheral blood mononuclear cells were thawed and homogenized with cell lysis buffer containing [20 mM Tris pH 7.4, 137 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA) pH 7.4, 1% Triton X-100, 25 mM β -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin and 100 mM orthovanadate], were incubated on ice for 20 min, were then subjected to centrifugation at 21,130 g at 4°C for 20 min. Protein concentrations were quantified using a BCA Protein Assay Kit (Pierce Biotechnology).

β -Arrestin 1 level measurements with immunoblot analyses

Equal amounts of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Les Ulis, France). The membranes were then incubated overnight with a primary mouse monoclonal anti- β -arrestin 1 antibody (#610551, BD Biosciences Pharmingen, France; 1:100). In order to ensure that equal amounts of total protein (30 μ g) were loaded in each lane, β -actin protein levels were also assessed [β -actin (C4) horseradish peroxidase (HRP), Santa Cruz Biotechnology, Germany, 1:10,000]. Immune complexes were detected using appropriate peroxide-conjugated secondary antibodies and a chemiluminescent reagent kit (Pierce Biotechnology). Immunoblot quantifications were performed by densitometric scanning with Image Lab Software (Bio-Rad). Signals were in the linear range. The densitometry values were normalized against the β -actin values (Figures 2 and 3).

CORTICOSTERONE MODEL AND TREATMENT

The dose and duration of corticosterone treatment (CORT model) were selected based on previous studies (David et al., 2009; Guilloux et al., 2011; Hache et al., 2012; Rainer et al., 2012a,b). Exposure to chronic corticosterone results in a phenotype that is similar to a chronic stress phenotype, including a deterioration of the coat state and anxiety/depression-related behaviors. At the end, a higher emotionality score is observed (Guilloux et al., 2011). Corticosterone (35 μ g/ml/day, equivalent to about 5 mg/kg/day) or vehicle (0.45% β -cyclodextrin, β -CD) were available to mice *ad libitum* in the drinking water in



opaque bottles. Corticosterone-treated water was changed every 3 days to prevent degradation. Group-housed mice were also treated with the SSRI fluoxetine (18 mg/kg/day) for the last 4 weeks of the experiment (see the experimental protocol on Figure 3A).

STATISTICAL ANALYSIS

β -Arrestin 1 levels were quantified and then expressed with a scatterplot or as mean \pm SEM normalized to vehicle levels. Data were analyzed using Prism 6.0 software (GraphPad, La Jolla, USA). One-way ANOVAs were used to evaluate data when appropriate. Significant main effects were further analyzed by Fisher’s *post hoc* test. Statistical significance was set at $p < 0.05$.

RESULTS

β -ARRESTIN 1 IS DETECTED IN HUMAN AND MOUSE PBMC

We first collected blood in order to assess whether β -arrestin 1 could be detected. Single-use lancets were used for submandibular bleeding and permitted drawing of ~0.4 ml of blood without the use of anesthesia (Golde et al., 2005). The mouse PBMCs were lysed and subjected to immunoblotting. A ~55 kDa band that corresponded to the molecular weight of β -arrestin 1 protein was detected with a monoclonal antibody against mouse anti- β -arrestin 1 that is known to detect human β -arrestin 1 (Avissar et al., 2004; Matuzany-Ruban et al., 2005; Golan et al., 2013; Figure 2). Therefore, this method of PBMC isolation from fresh mouse

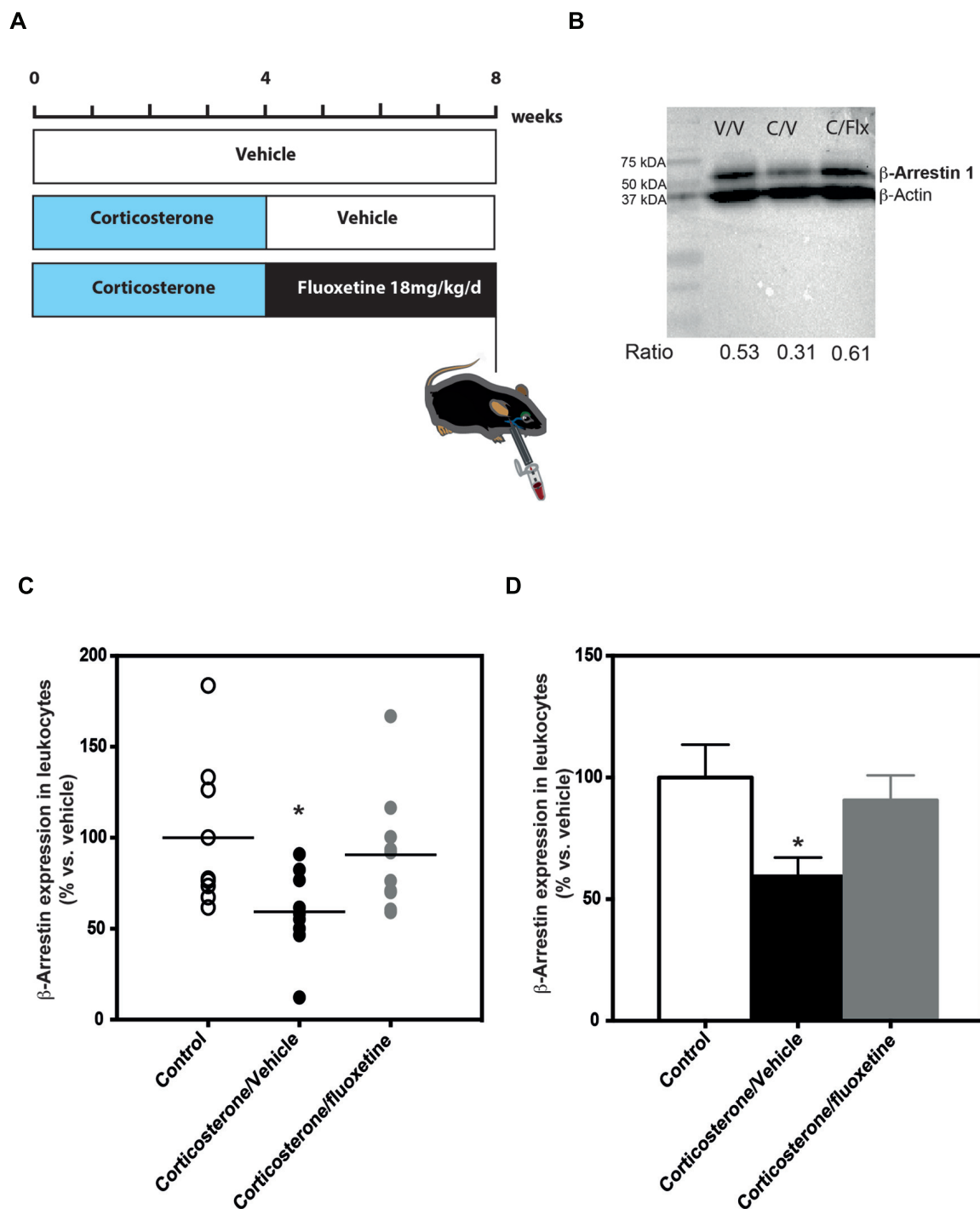


FIGURE 3 | Chronic fluoxetine treatment (18 mg/kg/day for 28 days) restored β -arrestin 1 levels in the peripheral blood mononuclear cells from anxi/depressive mice treated with chronic corticosterone treatment. (A) Experimental timeline used to measure β -arrestin 1 levels in peripheral blood mononuclear cells from naïve or anxi-depressive mouse submandibular bleeding chronically treated or not with fluoxetine during 28 days (18 mg/kg/day in the drinking water). **(B)** Representative western blot of β -arrestin 1 levels in peripheral blood mononuclear cells isolated from naïve (vehicle/vehicle, V/V), corticosterone (35 μ g/ml/day; corticosterone/vehicle, C/V) or corticosterone/fluoxetine (18 mg/kg/day; corticosterone/

fluoxetine, C/F) mouse whole blood. In each blot, 30 μ g of protein from mouse PBMC were run. The densitometry values for each band allowed the calculation of a ratio: optical density for β -arrestin 1/optical density β -actin value. **(C,D)** Scatterplot of the individual effects **(C)** or bar charts of mean \pm SEM of the effects **(D)** of a chronic administration (28 days) treatment with fluoxetine on β -arrestin 1 levels in the peripheral blood mononuclear cells from mice treated with chronic corticosterone in comparison to untreated animals. Data are expressed in percentage normalized to vehicle/vehicle expression; $n = 9-10$. * $p < 0.05$, versus control vehicle/vehicle group.

blood successfully permitted measurements of β -arrestin 1 levels. This method can potentially be used to investigate levels of other proteins as well. Lysates of human total PBMCs and CD14 negative PBMC fraction cells (CD14[−]) were used as positive controls. In addition, we were also able to detect β -arrestin 1 in human PBMCs isolated from low fresh circulating blood volume (7.5 ml) of healthy adult donors obtained from Etablissement Français du Sang (Figure 2). To our knowledge, this is the first study to detect β -arrestin 1 in this fashion.

Next, we decided to quantify β -arrestin 1 levels in PBMCs isolated from C57BL/6Ntac mice exposed to chronic corticosterone (David et al., 2009; Rainer et al., 2012b) that was given either alone or in combination with the SSRI fluoxetine (18 mg/kg/day; Figure 3A).

CHRONIC FLUOXETINE TREATMENT NORMALIZES β -ARRESTIN 1 EXPRESSION IN PBMC ISOLATED FROM ANXIOUS/DEPRESSIVE-LIKE MICE

In mouse PBMCs isolated from blood of mice treated chronically with corticosterone (35 μ g/ml/day), we found that β -arrestin 1 levels were significantly lower (−41%; 59% of expression compared to 100% in the control group) than the levels in naïve animals [one-way ANOVA, $F(2,25) = 3.81$; $*p < 0.05$; Figures 3C,D]. Interestingly, a 4-week treatment with the SSRI fluoxetine normalized these β -arrestin 1 expression levels so that they were not significantly different than the levels observed in naïve animals (Figures 3C,D).

DISCUSSION

We developed a new method to assess circulating proteins such as β -arrestin 1 through immunoblot analyses of mouse PBMCs isolated from whole blood. We showed significantly reduced β -arrestin 1 levels in PBMCs from anxious/depressed mice. These decreased β -arrestin 1 expression levels were restored to normal levels with chronic fluoxetine treatment.

PBMCs WERE ISOLATED FROM UNANESTHETIZED MICE

A recent review from Duman's group highlighted the need to develop a biomarker panel for depression. This biomarker panel should profile diverse peripheral factors that together will provide a biological signature of MDD subtypes and predict treatment response (Schmidt et al., 2011). Assessing peripheral protein levels in PBMCs is an attractive method because PBMCs are circulating cells that can be easily collected and monitored. Previous studies demonstrated that PBMCs can be isolated from mouse blood to assess immunological responses (Fuss et al., 2009). However, to our knowledge this is the first study to collect PBMCs from circulating blood of unanesthetized animals. Single-use lancets were used for submandibular bleeding. This method permitted PBMCs to be collected from peripheral blood circulation in living and unanesthetized mice. Thus, submandibular bleeding is a useful method to screen putative biomarkers of the pathophysiology of mood disorders and the response to ADs. This technique can be easily performed multiple times in the same animals and can be used with other rodent species such as rats.

β -ARRESTIN 1 PROTEIN LEVELS CAN BE MEASURED IN MOUSE AND HUMAN PBMCs

We measured β -arrestin 1 protein levels to determine whether mouse PBMCs are useful biological materials to screen biomarkers for MDD pathophysiology and the AD response. Over the last decade, several G protein receptor-related genes such as β -arrestins were found to be involved in the pathophysiology of mood disorders (Schreiber and Avissar, 2004; Beaulieu et al., 2008; David et al., 2009). Numerous data from clinical studies support the importance of measuring β -arrestin 1 levels as a peripheral biomarker of the pathophysiology of mood disorders and predicting the AD response (Avissar et al., 2004; Schreiber et al., 2009; Golan et al., 2013). However, no previous study demonstrated *ex vivo* measurements of β -arrestin 1 levels in leukocytes isolated from whole blood to compare levels between naïve and anxious/depressed rodents. In addition, this is the first study to assess β -arrestin 1 by immunoblot in human and in mouse leukocytes simultaneously by using the same monoclonal antibody.

In the human experiments, we were able to recover PBMCs from 7.5 ml of whole circulating blood from healthy volunteers. Previous studies showed that larger amounts of blood were needed for the detection of β -arrestin 1 in human leukocytes (Avissar et al., 2004; Matuzany-Ruban et al., 2005; Golan et al., 2013). Here, 7.5 ml was sufficient to acquire 30 μ g of PBMC lysate for immunoblotting (Figure 2).

Avissar et al. (2004) demonstrated that β -arrestin 1 levels were elevated by chronic ADs in rat cortex and hippocampus. However, by contrast with their human study, they did not provide data showing that β -arrestin 1 levels in rat PBMCs are affected by chronic AD treatment (Avissar et al., 2004). Therefore, we also compared β -arrestin 1 levels in PBMCs of anxious/depressed mice before and after chronic AD treatment (Figure 3).

β -ARRESTIN 1 IS A PREDICTIVE MARKER OF THE PATHOPHYSIOLOGY OF DEPRESSION AND THE ANTIDEPRESSANT RESPONSE

To induce an anxious/depression-related phenotype, we utilized a chronic corticosterone treatment that results in hallmark characteristics of anxiety and depression (for review, see David et al., 2009; Mendez-David et al., 2013). In order to delineate a panel of biomarkers of the pathophysiology and the treatment of depression, it is first essential to screen putative candidates in a model of anxiety/depression. β -Arrestin 1 protein levels in leukocytes were reduced when mice were exposed to chronic corticosterone. As found in previous human studies (Matuzany-Ruban et al., 2005; Golan et al., 2013), these reduced β -arrestin 1 levels were alleviated by AD treatment.

LIMITATIONS OF THE STUDY

Measuring protein levels in mouse PBMCs at several time points is a powerful technique that can be used to reveal potential biomarkers for the pathophysiology of depression and the AD response. However, this study has some limitations that must be considered when interpreting the current findings. For example, it is important to distinguish diagnostic biomarkers from treatment biomarkers (Schmidt et al., 2011). This study does not address this difference. Further studies are required to assess whether β -arrestin 1 is a reasonable biomarker for diagnostic and/or drug

treatments. A study that compares peripheral levels of β -arrestin 1 in stressed animals before and after AD treatment could definitively address this question. It also may be interesting to study whether there is a correlation between β -arrestin 1 levels and the severity of the anxiety/depressive state (Guilloux et al., 2011). Moreover, disease conditions are most often signified by the dysregulation of complex biological pathways involving multiple key factors (Dudley and Butte, 2009). Thus, it is unlikely that β -arrestin 1 alone will be a sufficient diagnostic and treatment biomarker. However, mouse PBMCs might provide useful material to screen a panel of biomarkers and to provide biological signatures of MDD and AD treatments. Finally, in our study, β -arrestin 1 levels were measured using western blots, which is a semi-quantitative method of evaluating protein levels. The development of an enzyme-linked immunosorbent assay (ELISA) to assess β -arrestin 1 levels would provide a more quantitative method.

CONCLUSION

In this study, we demonstrated that PBMCs isolated from a small volume of whole blood in unanesthetized mice using a submandibular bleeding method may provide a useful biological tool to assess circulating proteins. This method will permit future studies to screen potential biomarkers for the pathophysiology of depression and AD responses. We also confirmed that measurements of β -arrestin 1 levels in PBMCs may serve as a biochemical marker of depression in humans (Avisar et al., 2004). Overall, we developed a powerful tool for translational studies that can

easily be used to assess proteins measurements and to provide a biological signature of treatment response. Identification of a biological signature could predict the effectiveness of ADs (Fuss et al., 2009).

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AUTHOR CONTRIBUTIONS

Indira Mendez-David, Alain M. Gardier, René Hen, Saadia Kerdine-Römer, and Denis J. David designed research; Indira Mendez-David and Zeina El-Ali performed research and draw Figure 2; Indira Mendez-David analyzed data; Indira Mendez-David, Saadia Kerdine-Römer, and Denis J. David wrote the manuscript. Indira Mendez-David, Zeina El-Ali, René Hen, Emmanuelle Corruble, Bruno Falissard, Alain M. Gardier, Saadia Kerdine-Römer, and Denis J. David contributed to the preparation of the manuscript.

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Conclusion of the study

In this study, we demonstrated that PBMCs isolated from a small volume of whole blood in unanesthetized mice using a submandibular bleeding method may provide a useful biological tool to assess circulating proteins. This method will permit future studies to screen potential biomarkers for the pathophysiology of depression and AD responses.

We also confirmed that measurements of β -arrestin 1 levels in PBMCs may serve as a biochemical marker of depression in humans (Avissar et al., 2004). Overall, we developed a powerful tool for translational studies that can easily be used to assess proteins measurements and to provide a biological signature of treatment response. Identification of a biological signature could predict the effectiveness of ADs (Fuss et al., 2009).

Thus, we used this method to assess the fast anxiolytic-like activity induced by 5-HT₄ receptor stimulation with RS67333 could be predicted by measuring β -arrestin 1 levels in PBMCs.

Résultats complémentaires: A method for biomarker measurements in β -arrestin

1 protein levels in peripheral blood mononuclear cells isolated from anxious and depressed mice can predict fast onset of action of 5-HT₄ agonist

Indira Mendez-David, Denis J David, René Hen, Saadia Kerdine-Römer S, Alain M Gardier

Introduction of the study

The β -arrestin-signaling cascade has recently gained attention as a potential pre-clinical/clinical bridging biomarker for depressive states and treatment effects (Golan et al., 2013). Previously (article 5), we demonstrated that β -arrestin 1 measurement in PBMCs could serve as a biological marker in response to fluoxetine in our model of anxiety/depression (Mendez-David et al., 2013). We also corroborated previous evidences that agonist activation of 5-HT₄ receptors mediate fast-acting antidepressant-like effects since the behavioral activity that we observed occurs at 7 days after the start of the treatment. Thus, in this study, we wanted to investigate whether, the fast anxiolytic-like activity induced by 5-HT₄ receptor stimulation with RS67333 could be predicted by measuring β -arrestin 1 levels in PBMCs.

It has been demonstrated that GRK5 as well as a post-translational modification of β -arrestin 1 determine the nature of the signaling pathway, which is predominantly engaged on activation of the 5-HT₄ receptor. Overall, β -arrestin 1 has been involved in 5-HT₄ receptor desensitization (Barthet et al., 2009) and 5-HT₄ receptor internalization (Mnie-Filali et al., 2010). Such a control in signaling pathways could be critical for 5-HT₄ receptor in behavior (Barthet et al., 2009). We also explored the consequences of a chronic treatment with RS67333 on β -arrestin 1 expression in two brain areas involved in anxiolytic/antidepressant behavioral activity, the cortex and the hippocampus in corticosterone-treated mice.

EXPERIMENTAL PROCEDURES:

Corticosterone treatment

Our designed protocol consists in chronic administration of corticosterone in the drinking water. For this purpose, corticosterone (4-pregnen-11 β -DIOL-3 20-DIONE 21-hemisuccinate (Sigma-Aldrich Saint-Quentin Fallavier, France) is dissolved in vehicle (0.45% hydroxypropyl- β -cyclodextrin, Sigma-Aldrich Saint-Quentin Fallavier, France). The dose and duration of corticosterone treatment are selected based on previous studies (David et al., 2009). For a cohort of 60 animals corticosterone (35 μ g/ml, equivalent to about 5 mg/kg/d) is available *ad libitum* in the drinking water in. Corticosterone powder begins to degrade once in solution. Thus, to protect corticosterone from light, opaque bottles should be used. Control animals (vehicle group) should receive the vehicle (0.45 % β -cyclodextrin, β -CD).

Drugs treatment

While administration with β -CD or corticosterone continued, mice are treated with vehicle (0.45% β -CD), fluoxetine hydrochloride (Anawa Trading, Zurich, Switzerland), RS67333 hydrochloride (1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-butyl-4piperidinyl)-1-propanone hydrochloride) (Tocris Bioscience, Bristol, United Kingdom). RS67333 is delivered by osmotic mini-pumps at a dose of 1.5 mg/kg/day. Fluoxetine (18 mg/kg per day) is delivered in the drinking water as previously described. Osmotic minipumps (42 days mini-pumps, 2006 model, Alzet, Cupertino, CA) is implanted subcutaneously under light anesthesia (ketamine/xylazine; (75/20 mg/kg) from Sigma Aldrich (Sigma-Aldrich Saint-Quentin Fallavier, France). Treatment is always maintained until the end of the experiments.

β-arrestine expression

Blood collection and Peripheral blood mononuclear cells (PBMC) Isolation

The procedure is done on unanesthetized mice as previously described elsewhere²². About 0.4 ml of blood per mice was collected in K₃EDTA tubes using the submandibular bleeding method after 7 or 28 days of treatment. The punctures were performed with 5 mm point size sterile lancets (MediPoint, Mineola, NY). Briefly, blood was mixed with the OptiPrep™ gradient solution and extractions of Peripheral blood mononuclear cells (PBMC) were done using the iodixanol mixer technique. After centrifugation and several washing steps the PBMC's were recovered and stored at -80°C.

Protein extraction from PBMC's and Immunoblots

PBMC's were thawed and homogenized with cell lysates buffer containing (20mM Tris PH :7.4, 137mM NaCl, 2mM ethylenediaminetetraacetic acid (EDTA) Ph7.4, 1% triton X 100, 25mM β -glycerophosphate, 1mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin and 100mM Orthovanadate), incubated on ice for 20 min and followed by centrifugation at 15,000 rpm at 4°C for 20 min. Protein concentration was quantified using BCA Protein Assay Kit (Pierce Biotechnology). Equal amount of proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF), (Amersham Biosciences, Les Ulis, France). Membranes were incubated overnight in the following antibodies: a primary monoclonal antibody Mouse Anti- β -Arrestin 1 (BD Bioscience Pharmigen) and β-Actin (Santa Cruz Biotechnology, CA). Immune complexes were detected using appropriate peroxide-conjugated secondary antibodies along with a Chemiluminescent reagent kit (Pierce Biotechnology). Proteins bands were quantified with Image lab software (Bio-Rad).

Western blotting analysis

For Western Blot analysis, mice were killed by cervical dislocation, cortex and hippocampi were rapidly dissected and the proteins were extracted in 20 mM Tris-HCl pH7,6, 137 mM NaCl, 1% NP-40, 10% glycerol, 1% protease and phosphatase inhibitor cocktails (Sigma). Cell lysates were centrifuged 10,000g for 10 min at 4°C. Electrophoresis was performed in 8% (or 15% for BDNF detection) SDS-polyacrylamide gels and the proteins were transferred to Protran nitrocellulose membranes (Whatman). After blocking in 10 mM Tris-HCl, pH:7,6, 200 mM NaCl, 0,1% Tween-20 and 5% BSA, membranes were incubated with primary monoclonal antibody Mouse Anti- β -Arrestin 1 (BD Bioscience Pharmigen) and β -Actin (Santa Cruz Biotechnology, CA). Primary antibodies were detected with appropriate HRP-coupled secondary antibodies and ECL signals (SuperSignal West Pico Chemiluminescent Substrate (Pierce, Erembodegem, Belgium) were quantitated with Image lab software (Bio-Rad).

Data Analysis and Statistics

Results from data analyses were expressed as mean \pm SEM. Data were analyzed using StatView 5.0 software (SAS Institute, Cary, NC). For all experiments, one-way ANOVAs was applied to the data as appropriate. Significant main effects and were followed by Fisher's PLSD post hoc analysis.

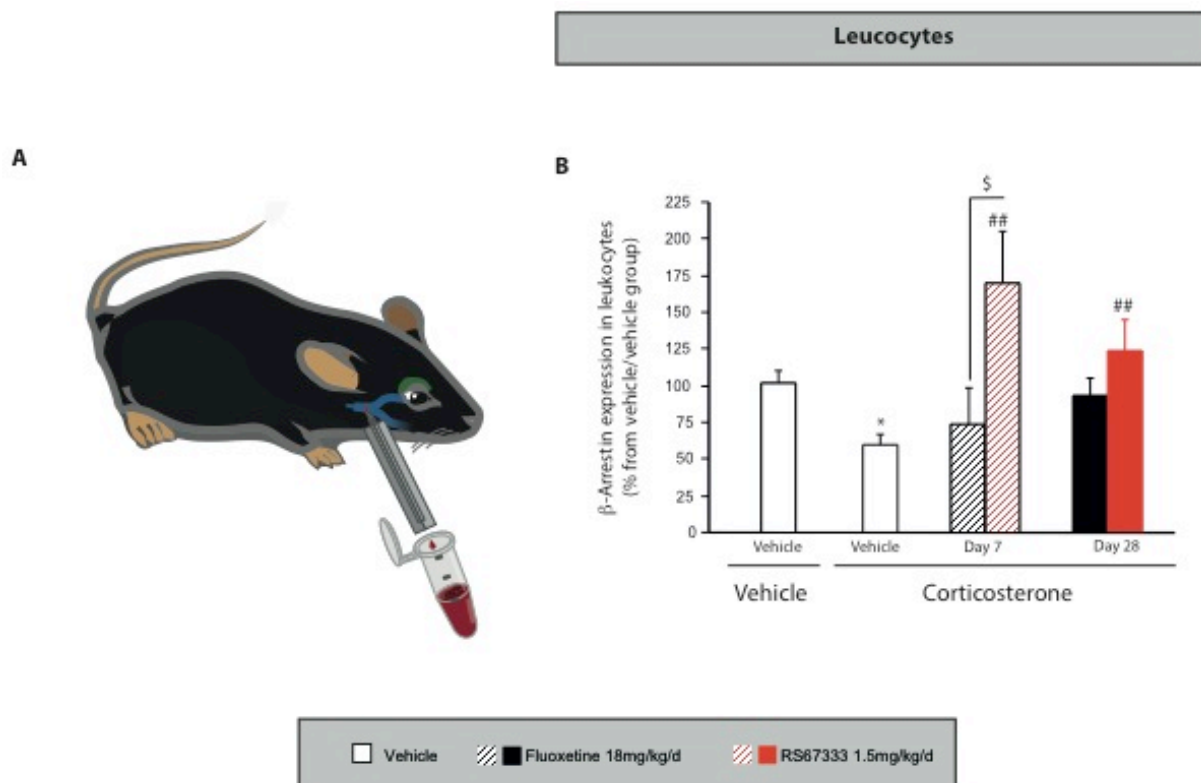


Figure 12 : β -arrestin 1 levels in peripheral blood mononuclear cells after subchronic (7 days) and chronic treatment (28 days) with the 5-HT₄ agonist, RS67333 (1.5 mg/kg/day) or the Selective Serotonin Reuptake Inhibitor, fluoxetine (18 mg/kg/day) in corticosterone-treated mice.

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from small volume of whole blood in unanesthetized mice using a submandibular bleeding method (A). Data are expressed in % β -arrestin 1 of the Vehicle/Vehicle Group \pm SEM in mouse PBMCs. One-factor ANOVA with Fisher's PLSD post hoc analysis against the control group (vehicle/vehicle) revealed that only chronic RS67333 treatment reversed decrease in β -arrestin 1 levels in peripheral blood mononuclear cells induced by corticosterone after subchronic treatment while it takes 28 days for fluoxetine treatment to reverse this effect ($F(6,45)=3.62$, $p<0.01$; $p<0.05^*$ versus vehicle/vehicle group, $p<0.01^{##}$ versus Corticosterone/vehicle group; $p<0.05^{\$}$ versus corticosterone/fluoxetine group).

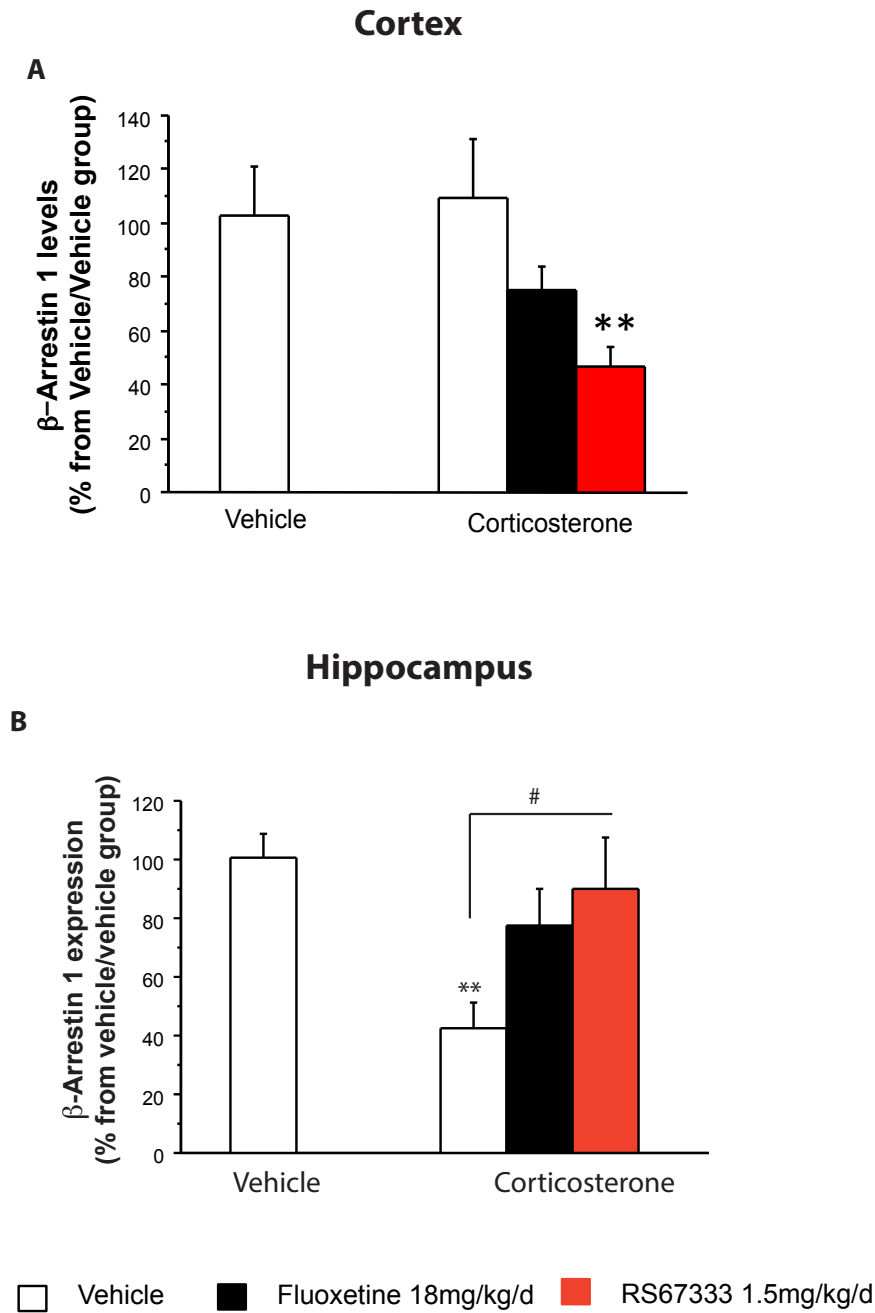


Figure 13 : β -arrestin 1 levels in cortex after chronic treatment (28 days) with the 5-HT₄ agonist, RS67333 (1.5 mg/kg/day) or the Selective Serotonin Reuptake Inhibitor, fluoxetine (18 mg/kg/day) in corticosterone-treated mice.

Data are expressed in % β -arrestin 1 protein levels of the Vehicle/Vehicle Group \pm SEM in mouse cortex (A) or in mouse hippocampus. One-factor ANOVA with Fisher's PLSD post hoc analysis against the control group (vehicle/vehicle) revealed that chronic RS67333 treatment decreased in β -arrestin 1 levels in mouse cortex treated by corticosterone ($F(3, 20)=3.41$, $p<0.05$), while, as fluoxetine, it reversed decreased in β -arrestin 1 levels in the mouse hippocampus ($F(3, 20)=3.22$, $p<0.05$; $p<0.01^{**}$ versus vehicle/vehicle group, $p<0.05\#$ versus Corticosterone/vehicle group).

Conclusion

In this study, we wanted to further explore whether the fast onset of action of a 5-HT₄ receptor activation by RS67333 could be predicted by a peripheral biomarker expression. To this end, we used β -arrestin 1 expression in leukocyte as a candidate since previously preclinical and clinical studies linked this protein to mood disorders (Golan et al., 2013). Thus, peripheral blood mononuclear cells were isolated from non-stressed and stressed-treated animals with vehicle, RS67333 (1.5 mg/kg/d) or fluoxetine (18 mg/kg/d) (Figure A). Interestingly, β -Arrestin 1 level, measured by immunoblot, were lowered in our cort model of Stress-Related (Anxiety/Depression), as previously shown (article #5) (Mendez-David et al., 2013). This results goes in line with recent clinical data that point out the role of β -arrestin 1 level for biochemical diagnostic purposes.

Previous behavioral data (article #4) showed that, unlike fluoxetine, a 5-HT₄ receptor stimulation with RS67333, induced rapid anxiolytic/antidepressant-like activity in a stress-related model of Anxiety/Depression. If we did not detect any change in β -arrestin 1 mouse leukocytes under fluoxetine in corticosterone-treated animals after 7 days of treatment, a short term exposure with RS67333, restored the level of this protein. This effect with RS67333 lasted after 28 days of treatment. In regard to the fluoxetine-treated animals, a restoration was only observed in the corticosterone model after a longer exposure.

The effects of a chronic treatment with RS67333 on β -arrestin 1 expression levels in the mouse brain treated with chronic corticosterone gave us opposite effect depending on the brain region. We observed that, in contrast to the hippocampus, a chronic treatment with the 5-HT₄ agonist reduced the expression of β -arrestin 1 protein in the cortex. These results point out region-specific consequences of a chronic treatment with RS67333 on β -arrestin 1 protein expression.

Recent reports showed that chronic treatment with various classes of antidepressants (SSRIs fluoxetine and paroxetine or dual antidepressant venlafaxine) induced desensitization and/or downregulation of the 5-HT₄ receptors in cerebral regions implicated in depression including basal ganglia and hippocampus (Licht et al., 2009; Vidal et al., 2009; Vidal et al., 2010), probably reflecting a desensitization process, due to a less efficient coupling to Gs proteins. Another study, demonstrated that short-term administration (7 days) of RS67333 is required to fully desensitize the post-receptor signaling pathway associated to 5-HT₄ receptors (Pascual-Brazo et al., 2012). The fact that chronic SSRI treatments led to 5-HT₄ receptor stimulation is also suggested by the observation that such treatments induced a downregulation of those receptors in hippocampus (particularly CA1 region), striatum, and substantia nigra but, interestingly, not in medial Prefrontal Cortex (mPFC) (for review, Bockaert et al., 2011). These results are in line with ours demonstrating a restoration of β -arrestin 1 expression in the hippocampus under RS67333 or fluoxetine chronic treatment. Indeed, β -arrestin proteins are involved in desensitization and internalization of 5-HT₄ receptor (for review, Mnie-Filali and Pineyro, 2012). In contrast, the decrease in β -arrestin proteins expression in the cortex after RS67333 or fluoxetine treatment is in favor to an absence of downregulation in the cortex. Further studies investigating the difference of β -arrestin 1 expression, using local application of antidepressant or RS6733 are required to understand the link of β -arrestin1 and 5-HT₄ receptors and behavioral activity.

DISCUSSION

Discussion

Most current antidepressant treatments are limited by a significant degree of non responsiveness among patients (Trivedi et al, 2006), delayed onset of therapeutic efficacy, and a number of side effects (Kato and Serretti, 2010). The development of new antidepressants is therefore of considerable importance (Wong et al, 2010), and understanding the mechanisms underlying the delayed onset should offer insights into new approaches.

Selective Serotonin Reuptake Inhibitors (SSRI) act as indirect agonists of monoamine receptors. Serotonin is released throughout the forebrain by axons emanating from cell bodies located in the midbrain raphe (Barnes and Sharp, 1999). The largely neuromodulatory effects of 5-HT are mediated through 14 receptor subtypes, whereas 5-HT levels are limited by two inhibitory autoreceptors expressed on 5-HT raphe neurons (Barnes and Sharp, 1999). The 5-HT_{1A} receptor, a major inhibitory receptor subtype, is expressed on 5-HT raphe neurons as an autoreceptor, limiting release of 5-HT at nerve terminals, and in diverse target regions as a heteroreceptor mediating cellular responses to release 5-HT. A diversity of evidence has demonstrated that 5-HT_{1A} receptors contribute to the establishment of anxiety-related behaviors and to antidepressant response (Richardson-Jones et al., 2011). It is believed that overactivation and/or overexpression of 5-HT_{1A} autoreceptor would delay the onset of antidepressant effect, whereas the functional desensitization of this receptor after sustained administration of SSRIs is an adaptive change relevant to their therapeutic activity (Gardier et al., 1996). At the postsynaptic levels, the absence of the 5-HT_{1A} receptor prevents the behavioral effects of fluoxetine (Santarelli et al., 2003, Richardson et al., 2011). SSRIs are also potent stimulators of adult hippocampal neurogenesis (for review Mendez-David et al., 2013; Santarelli et al., 2003) and the role of each serotonergic receptor in the neurogenic effects of SSRIs is still a matter of investigation.

The recent findings raising the possibility that 5-HT₄ receptor activation may constitute a new way to treat anxiety/depression triggered my interest for further investigation of this 5-HT receptor. 5-HT₄ receptors are localized in the limbic system suggesting a role in emotion control. These receptors mediate intracellular signaling through cAMP increase, PKA activation, phosphorylation of CREB, and transcription of BDNF (Bockaert et al., 2004). This same signal transduction machinery is activated by chronic antidepressants as well (Nibuya et al., 1996). Due to these same mechanisms, direct activation of 5-HT₄ receptors may also yield antidepressant-like effects (Lucas et al., 2007; Pascual-Brazo et al., 2012; Tamburella et al., 2009). However, the current literature in regard to the antidepressant-like activity of 5-HT₄ receptor is limited. Indeed, almost all related studies have been performed in non-stressed rats (except one study measuring the locomotor activity in olfactory bulbectomized rats, and another study measuring sucrose consumption after 2 weeks of chronic mild stress in rats) (Lucas et al., 2007; Pascual-Brazo et al., 2012) and none have so far evaluated the anxiolytic-like profile of 5-HT₄ agonists. Moreover, only one step of the adult neurogenesis process was investigated, *i.e.* proliferation. Thus, the 5-HT₄ hypothesis is far from being validated and more studies are required to evaluate the rapid anxiolytic/antidepressant-like effect of 5-HT₄ receptor agonists.

The first aim of my thesis was to focus on whether 5-HT₄ receptor stimulation was necessary for the effects of SSRIs in a mouse model of anxiety/depression and whether this effect occurs rapidly. Thus, using the mouse corticosterone model of anxiety/depression, we assessed whether chronic treatment with a 5-HT₄ receptor agonist (RS67333, 1.5 mg/kg/day) had effects on anxiety and depression-related behaviors as well as on hippocampal neurogenesis in comparison to chronic fluoxetine treatment (18 mg/kg/day).

We showed that the 5-HT₄ receptor agonist, RS67333, elicits, like SSRIs, both anxiolytic and antidepressant effects, as well as an increase in neurogenesis in the dentate

gyrus (**article 4**). We also showed, using a brain penetrant 5-HT₄ receptor antagonist, GR 125487, that activation of 5-HT₄ receptors by endogenous 5-HT is necessary to both anxiolytic/antidepressant-like and neurogenic effects of the indirect 5-HT receptor agonist, fluoxetine. Serotonin has a high affinity with a pK_d = 8.4 and 7.7 for the human and the rat 5-HT₄ receptors respectively (Bender et al., 2000; Adham et al., 1996). Thus, the anxiolytic/antidepressant-like effects of fluoxetine likely resulted from an indirect activation of the 5-HT₄ receptor through an increase in endogenous 5-HT levels in the synaptic cleft, which is a consequence of the blockade of the selective serotonin transporter (SSRI).

In addition to behavioral data, and in agreement with a previous report from Lucas and colleagues (Lucas et al., 2007), a recent study performed in naïve rats confirmed that a short period of treatment with RS67333 increased the number of newborn cells in the dentate gyrus (Pascual-Brazo et al, 2012). However, our data showed that maturation of newborn neurons seems to be the most important neurogenesis step. These results are interesting because hippocampal neurogenesis has been implicated in some of the behavioral effects of antidepressants in adult rodents (David et al, 2009; Santarelli et al, 2003). While no direct evidence has yet linked the antidepressant-like effects of 5-HT₄ receptor activation and its neurogenic effects, we demonstrated that the 5-HT₄ agonist acts faster than SSRIs and that its anxiolytic effects do not require hippocampal neurogenesis (**table 10**).

We then explored whether the fast onset of action of 5-HT₄ receptors activation by RS67333 could be predicted by a peripheral biomarker expression. There are clear benefits of having valid, reliable, selective, and feasible biomarkers for MDD. The β -arrestin-signaling cascade has recently gained attention as a potential pre-clinical/clinical bridging biomarker for depressive states and treatment effects (Golan et al., 2013). Moreover, β -arrestin 1 has been involved in 5-HT₄ receptors desensitization (Barthet et al., 2009), and 5-HT₄ receptors

internalization process (Mnie-Filali et al., 2010). Thus, β -arrestin 1 could be a good candidate to predict fast onset of antidepressant activity.

However, no animal studies have investigated whether β -arrestin 1 protein levels in peripheral blood mononuclear cells (PBMCs) can be used as a biomarker tool for the pathophysiology of depression and the antidepressant response. Thus, we developed a new method to assess circulating proteins through immunoblot analyses of mouse PBMCs isolated from whole blood in anesthetized animals. Measuring protein levels in mouse PBMCs at several time points is a powerful technique that can be used to reveal potential biomarkers for the pathophysiology of depression and the antidepressant response. We showed significantly reduced β -arrestin 1 levels in PBMCs from anxious/depressed mice. These decreased β -arrestin 1 expression levels were restored to normal levels with chronic fluoxetine treatment.

We next apply this strategy to corticosterone-treated animals receiving a subchronic or a chronic treatment of the 5-HT₄ agonist, RS67333, in comparison to chronic fluoxetine. Interestingly, β -Arrestin 1 levels, measured by immunoblot, were lowered in our CORT model, as previously shown (**article # 5**) (Mendez-David et al., 2013). This result goes in line with recent clinical data that suggest that β -arrestin 1 level may be used for biochemical diagnostic purposes. Previous behavioral data (**article #4**) showed that, unlike fluoxetine, a 5-HT₄ receptor stimulation with RS67333, induced rapid anxiolytic/antidepressant-like activity in a stress-related model of anxiety/depression. While we did not detect any change in β -arrestin 1 levels in mouse leukocytes after 7 days of treatment with fluoxetine in corticosterone-treated animals, a short term exposure with RS67333, restored the level of this protein to control levels. This effect of RS67333 lasted after 28 days of treatment. In fluoxetine-treated animals, a restoration was only observed in the corticosterone model after a longer exposure.

Tableau 10: Neurogenesis-dependent and independent mechanism involved in the behavioral effects of subchronic and chronic 5-HT₄ agonist treatment.

	Fluoxetine (18 mg/kg/d)		RS67333 (1.5 mg/kg/d)	
	Subchronic	Chronic	Subchronic	Chronic
Anxiety tests	Ø	+	+	+
	/	Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent
Novelty Suppressed Feeding	Ø	+	Ø	+
	/	Neurogenesis-dependent	/	Neurogenesis-dependent
Depressive test	+	+	+	+
	Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent

The effects of a chronic treatment with RS67333 on β -arrestin 1 expression levels in the mouse brain treated with chronic corticosterone gave us opposite effect depending on the brain region. We observed that, in contrast to the hippocampus, a chronic treatment with a 5-HT₄ agonist, reduced the expression of β -arrestin 1 protein in the cortex. These results point out region-specific consequences of a chronic treatment with RS67333 on β -arrestin 1 protein expression.

Tableau 11: β -arrestin 1 levels in peripheral blood mononuclear cells after subchronic and chronic treatment with the SSRI, fluoxetine and with the 5-HT₄ agonist, RS67333.

Treatment Duration	Corticosterone/ Vehicle	Corticosterone/ Fluoxetine (18 mg/kg/day)	Corticosterone/ RS67333 (1.5 mg/kg/day)
7 days	↘↘	↘	=
28 days	↘↘	=	=

↘ ; ↘↘ : Decrease in β -arrestin expression ; = no change in comparison to vehicle/vehicle group

Finally, some important findings of our investigation such as (fast onset of action of a 5-HT₄ receptor stimulation, neurogenesis-dependent/independent effects, detection of a putative biomarker of response) come from methodological choices made during the study. Indeed, to demonstrate a fast onset of action, a neurogenesis dependent/independent mechanism and a biomarker of response, some critical steps have to be respected:

- **Need for chronic treatment:** *depression is a chronic, recurrent disease and antidepressant efficacy takes time.*
- **Comparison with a reference molecule:** *we have chosen to use fluoxetine, since its behavioral effects in our CORT model have been well characterized.*

- **The mouse CORT model:** *we previously showed that in stressed-animals, the neurogenic response is potentiated (for review: David et al., 2010; Mendez-David et al., 2013, article 2).*
- **Role of the genetic background of mice in response to the tests:** *strains with a high number of basal proliferating cells in the dentate gyrus of the hippocampus should be used only under stressed conditions to detect the effects of antidepressants on adult neurogenesis (for review David et al., 2010).*
- **Characteristics of the protocol to highlight a rapid effect of onset:** *behavioral studies occurred at different time points of the treatment, 7 days versus 28 days.*
- **Different steps of adult neurogenesis, with various markers (BrdU, Ki67, DCX), have been studied in the dentate gyrus:** *it allowed us to unravel the effects of 5-HT₄ receptor agonist on specific stages of the neurogenic process.*
- **Need to use a large battery of behavioral tests to assess both anxiolytic and antidepressive-like responses of a molecule:** *Anxiety disorders have a lifetime prevalence of over 25% making them the most common psychiatric disorders (Hirschfeld, 2001). Moreover, a co-morbidity between depression and anxiety disorders is commonly observed.*
- **X-irradiation of a restricted region of mouse brain containing the hippocampus:** *it allows the classification of these behavioral tests as neurogenesis-dependent and neurogenesis-independent tests, demonstrating in our study the neurogenic and behavioral effects of RS 67333 and fluoxetine.*
- **Develop a simple and repeatable method to collect blood samples and isolate PBMC in mice at different period of time:** *it will allow to search for biomarkers of this fast action of the drugs and make some correlation between changes in levels of these biomarkers in the CNS and the periphery system (article 5).*

Conclusions & Perspectives

We demonstrated that 5-HT₄ receptors act on at least two distinct brain circuits:

- one that is independent of hippocampal neurogenesis and that results in rapid anxiolytic effects.
- one that requires hippocampal neurogenesis and that results in delayed antidepressant-like effects.

It is now important to dissect the mechanism of action involved in the fast onset of action of the 5-HT₄ receptor agonists. Lucas et al. suggested that 5-HT₄ receptors exert an excitatory control on the firing activity of rat DRN 5-HT neurons. Nonetheless, several studies performed in rodent brain have shown that the DRN is lacking of 5-HT₄ receptor mRNA and protein (Waeber et al., 1994). Perhaps, an indirect control may involve 5-HT₄ receptors located in the medial prefrontal cortex? A new study has used optogenetic methods to stimulate prefrontal-brainstem neuromodulatory pathways while animals face environmental stressors; these results provided further compelling evidence that prefrontal control of neuromodulatory function can have a dramatic effect on motivated behavior (Warden et al., 2012). Indeed, they were able to show that the amount of time the rat spends engaging in motivated behavior is specifically influenced by these projections from mPFC to DRN. Complementary experiments, with intracerebral injection of RS67333 directly in the mPFC or in the hippocampus followed by behavioral testing should validate our current hypothesis: perhaps, fast onset of antidepressant action results in mPFC/DRN activation and will lead to a neurogenesis-independent response. Someone can argue that the selectivity of the 5-HT₄ receptor agonist tested, RS67333, is not optimal. However, except for the sigma receptors, which are bound to affinities comparable to 5-HT₄ receptor (5-HT₄: pK_i = 8.7; sigma 1: pK_i = 8.9; and sigma 2: pK_i = 8.0), RS67333 shows low binding affinity for other neurotransmitter receptors including 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C}, dopamine D₁, D₂ and

muscarinic M_1 - M_3 receptors ($pK_i < 6.7$) (Bockaert et al, 2004; Eglen et al, 1995). Ultimately, the development of a new strategy to manipulate 5-HT_4 receptors in the mPFC without affecting the dentate gyrus and *vice versa* and generating inducible tissue specific 5-HT_4 KO mice, might resolve this question (for instance breeding flox/flox 5-HT_4 receptors KO mice with Calcium/calmodulin-dependent protein kinase II alpha CreERT2 or NestinCre ERT2 mice).

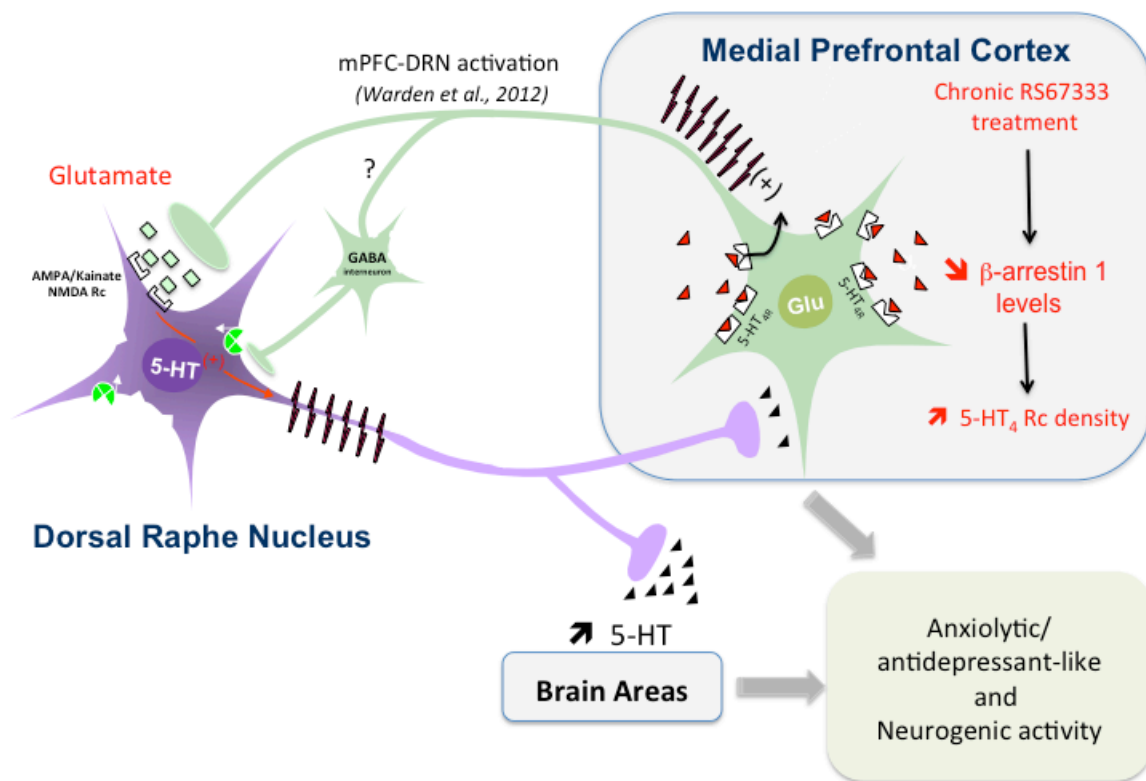


Figure 14 : A hypothesis on the physiological role of the 5-HT_4 -mediated cortico-raphe positive long-loop and its impact on serotonin levels and behavior under stress (adapted from Lucas et al., 2009).

Prefrontal Cortex projections to the dorsal raphe nucleus specifically induce antidepressant-like activity, activating behavioral responses with an increase in swimming behavior in the Forced Swim test (Warden et al., 2012). When mPFC pyramidal neurons become activated, the 5-HT_4 -dependent control also becomes more excitatory, therefore modifying the equilibrium of the loop in favor of its 5-HT_4 component. Consequently, DR extracellular concentrations of glutamate are increased, which results in a stronger excitation of 5-HT cell bodies. The impulse flow of 5-HT projections is enhanced, allowing an elevation of 5-HT release in the different brain areas innervated by the 5-HT neuron. The role of DR GABA interneurons, which also receive glutamatergic projections from the mPFC is still unclear (Shenhav and Botvinick, 2013).

Another important result of our study concern the neurogenesis process. We

concluded that maturation of newborn neurons seems to be the most important neurogenesis step linked to 5-HT₄ receptors activation. Our results suggested that 5-HT₄ receptors contribute to the effects of fluoxetine on proliferation and maturation of newborn neurons, but that other 5-HT receptors are likely to be involved, including the 5-HT_{1A} or the 5-HT₂ receptor (Santarelli et al., 2003; Klempin et al., 2010). Interestingly, both receptors are expressed in the dentate gyrus, which may be the site responsible for their effects on neurogenesis. Recently, it has been suggested that 5-HT₄ receptors activation may also be involved in antidepressant-induced dematuration process of mature dentate granule cells (Kobayashi *et al.*, 2010). However, the exact mechanisms underlying this phenomenon still need further investigations.

Finally, the activation of 5-HT₄ receptors in the brain could represent an innovative and rapid onset therapeutic approach to treat depression with comorbid anxiety (new classification of MDD in the DSM 5.0). However, the use of the 5-HT₄ receptor as a novel antidepressant target may be hampered by the fact that it also plays important roles outside the central nervous system. For instance, its cardiac and intestinal distribution makes it delicate the use of 5-HT₄ receptor agonists with the development of side effects such as arrhythmia (Tonini and Pace, 2006; Bockaert et al., 2004, 2008). Thus, signaling molecules that interact with the 5-HT₄ receptor such as P11 (Egeland et al., 2011; Warner-Schmidt et al., 2009) may represent novel targets for fast-acting anxiolytic/antidepressant treatments. There is indeed recent evidence that cortical neurons that express both P11 and 5-HT₄ receptors are involved in the behavioral effects of SSRIs (Schmidt et al., 2012).

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TITRE : Effets anxiolytiques/antidépresseurs et neurogéniques des ligands du récepteurs 5-HT₄ chez la souris : rôle de la protéine β -arrestine 1

RESUME : Les inhibiteurs sélectifs de recapture de la sérotonine (ISRS), agonistes indirects des récepteurs de la sérotonine (5-HT), ont un début d'effet antidépresseur retardé de plusieurs semaines. Des travaux antérieurs suggèrent que le récepteur 5-HT₄ de la sérotonine serait une cible directe pour traiter la dépression et un nouvel espoir pour traiter plus rapidement ces pathologies anxio-dépressives. Toutefois, l'hypothèse « 5-HT₄ » doit encore être validée dans des modèles animaux d'anxiété/dépression. Les questions posées étaient : la stimulation des récepteurs 5-HT₄ centraux est-elle nécessaire aux effets comportementaux des ISRS ? la neurogenèse hippocampique adulte contribue-t-elle à ces effets ? En utilisant le modèle de stress chronique à la corticostérone (CORT) chez la souris, nous avons évalué les effets sur ces paramètres d'un traitement chronique avec un agoniste du récepteur 5-HT₄ (RS67333, 1,5 mg/kg/jour pendant 4 semaines) comparé à un traitement à la fluoxétine (18 mg/kg/jour). Nous avons ensuite utilisé ce modèle murin combiné à l'ablation de la neurogenèse hippocampique par rayons-X afin d'examiner si la neurogenèse est nécessaire aux effets comportementaux d'un traitement subchronique (7 jours) ou chronique (28 jours) avec le RS67333. Nous avons également évalué le blocage des effets de la fluoxétine par un antagoniste du récepteur 5-HT₄ (GR125487, 1 mg/kg/jour). Le traitement chronique avec RS67333, comme celui de la fluoxétine, induit une activité anxiolytique/antidépresseive et stimule la neurogenèse hippocampique adulte. Cependant, contrairement à la fluoxétine, les effets anxiolytiques du RS67333 sont déjà présents après 7 jours de traitement, sans nécessité l'activation de la neurogenèse. Le traitement chronique avec le GR125487 empêche les deux effets anxiolytique/antidépresseur et neurogénique de la fluoxétine, indiquant que l'activation du récepteur 5-HT₄ est nécessaire à ces effets de l'ISRS. Nous avons ensuite cherché à savoir si le court délai d'action antidépresseur du RS67333 peut être prédit par l'expression d'un biomarqueur périphérique. Des données de la littérature indiquent que la cascade de signalisation de β -arrestine 1 (impliquée dans la désensibilisation et l'internalisation du récepteur 5-HT₄) serait un biomarqueur potentiel pré-clinique/clinique des états dépressifs et des effets d'un traitement antidépresseur. À cette fin, nous avons développé une nouvelle méthode d'évaluation des taux de protéines circulantes grâce à une analyse par immunoblot des leucocytes (PBMC) isolés à partir du sang total de souris. Les taux de β -arrestine 1 sont diminués dans les leucocytes des souris pré-traitées à la CORT. Il faut 7 jours de traitement avec le RS67333, mais 28 jours avec la fluoxétine chez ces animaux pour restaurer un taux de β -arrestine 1 comparable à celui des animaux contrôles. Ces résultats suggèrent que le taux sanguin de β -arrestine 1 est un biomarqueur de la rapidité de la réponse antidépresseive. Enfin, l'activation du récepteur 5-HT₄ dans le cerveau peut représenter une approche thérapeutique innovante d'apparition pour traiter plus rapidement des symptômes dépressifs associés à l'anxiété.

MOTS CLES : Récepteur 5-HT₄, anxiété, dépression, neurogenèse, protéine β -arrestine 1

LABORATOIRE DE RATTACHEMENT :

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Laboratoire de Pharmacologie

Faculté de Pharmacie

5, Rue J.B. Clément

92296 – CHATENAY MALABRY CEDEX