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Etude des mécanismes d'action de l'ocytocine sur la modulation des circuits astro-neuronaux de régulation de la douleur

Jérôme Wahis

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ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ
Institut des Neurosciences Cellulaires et Intégratives

THÈSE présentée par :

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**Etude des mécanismes d'action de l'ocytocine
sur la modulation des circuits astro-neuronaux
de régulation de la douleur.**

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C. RESUME ETENDU EN FRANCAIS

Introduction du sujet de thèse

La douleur est un phénomène complexe, partant de la nociception jusqu'à l'affect douloureux, décrit de la molécule jusqu'au comportement. La douleur n'est pas une simple sensation mais bien une perception qui est ressentie et possède une composante émotionnelle. L'association internationale d'étude de la douleur la définit en ces termes : une sensation et une expérience émotionnelle désagréable en réponse à une atteinte tissulaire réelle ou potentielle ou décrites en ces termes. Au sein du système nerveux central (SNC), de nombreuses zones cérébrales sont impliquées dans la régulation du message douloureux, de la moelle épinière au cortex cérébral. On nomme l'ensemble de ces dernières « matrice de la douleur » au niveau du SNC, on décrit en général deux voies principales de la douleur : les voies ascendantes, qui apportent l'information nociceptive, et les voies descendantes qui modulent l'activité de la première, en augmentant ou en inhibant l'intensité du message nociceptif. De nombreuses molécules sont impliquées tout au long de cette matrice de la douleur, et atténuent ou accentuent différents aspects de la douleur. Parmi elles se trouve l'ocytocine (OT). Ma thèse vise à éclaircir les mécanismes d'action de cette molécule au sein du système nerveux.

L'OT est une hormone vitale aux rôles nombreux. Elle fut d'abord identifiée par Sir Henry Dale (Dale, 1906) qui remarqua les propriétés utérotoniques d'extrait de glande pituitaire humaine sur des chattes gestantes. Cette substance fut purifiée vers la fin des années 20 (Kamm et al., 1928), et nommée ocytocine pour « ocy » du grec *ὄκυσ*, *ôkus* : rapide et de « tocine » *τόκος* : accouchement. Il faut aussi noter que ce fut le premier polypeptide à être séquencé puis synthétisé, ce qui valut le prix Nobel de chimie 1955 à Vincent Du Vigneaud (Du Vigneaud, 1956). Ce nonapeptide (nona- pour ses 9 acides aminés) est synthétisé par des neurones de l'hypothalamus situés dans les noyaux paraventriculaires, supraoptiques et accessoires. Il existe deux types de neurones produisant de l'ocytocine, les neurones parvocellulaires et magnocellulaires. Ces derniers sont des neurones neuroendocrines : Ils projettent des axones à la glande pituitaire postérieure et sécrètent l'OT dans la circulation sanguine, étant ainsi responsables de l'effet endocrine de l'ocytocine, qui ne peut pas passer la barrière hémato-encéphalique. Les neurones parvocellulaires, eux, ne communiquent pas au-delà de la barrière hémato-encéphalique, mais projettent des axones dans le tronc cérébral et la moelle épinière (Gimpl et al., 2001). L'OT est une molécule clé modulant de nombreuses fonctions touchant à la propagation de l'espèce : la formation des liens sociaux, la régulation de la copulation, le contrôle des contractions utérines lors de l'accouchement et des montées de lait, et les comportements de protection des petits. A lire cette liste, non exhaustive, on comprend pourquoi

Lee et collègues ont intitulé leur revue « oxytocine, le grand facilitateur de la vie » (Lee et al., 2009b). Mais ce ne sont pas les seules fonctions de l'OT, elle régule aussi des fonctions biologiques comme la régulation osmotique ou encore le contrôle des seuils douloureux. C'est cette dernière fonction qui concerne mes travaux. Plus précisément, ma thèse est focalisée sur l'étude des mécanismes d'actions de l'oxytocine sur certains réseaux neuronaux et aussi astro-neuronaux, entre neurones et astrocytes. En effet, depuis le début des années 2000, la neurobiologie connaît un regain d'intérêt pour un type cellulaire primordial et pourtant peu étudié du SNC : les astrocytes. Ces cellules, qui regroupées avec la microglie et les oligodendrocytes (et d'autres) forment la glie, ont longtemps été considérées, à tort, comme négligeables dans le traitement de l'information par le SNC. Or, de nombreuses publications récentes démontrent un rôle important et primordial de la glie, qui se trouve être partie prenante du traitement de l'information, de concert avec les neurones, et dès lors essentielle au fonctionnement du SNC (Sofroniew and Vinters, 2010).

Avant d'étudier un rôle des astrocytes dans l'effet analgésique de l'OT, ma thèse au sein du laboratoire des déterminants moléculaires de la douleur fut d'abord consacrée à l'exploration de certaines connexions entre neurones oxytocinergiques de l'hypothalamus et leurs rôles dans la modulation de la douleur au niveau central et périphérique. En effet, les neurones OT magnocellulaires ont une fonction neuroendocrine bien décrite, mais ils régulent également les circuits du SNC. Des travaux récents ont en effet montré qu'ils pouvaient à la fois projeter à la glande pituitaire et dans de nombreuses régions du cerveau, ce qui était auparavant une fonction purement attribuée aux neurones OT parvocellulaires (Knobloch et al., 2012; Swanson and Sawchenko, 1983). Mes travaux ont donc d'abord porté sur l'étude d'une sous-population de 30 neurones parvocellulaires du noyau paraventriculaire, qui projettent à la fois à la moelle épinière et sur des neurones magnocellulaires des noyaux supraoptiques voisins et permettent ainsi une modulation de la douleur.

Dans une deuxième partie, j'ai étudié le rôle de l'OT dans la modification de l'activité des circuits de l'amygdale centrale (CeA). Des travaux ont montré précédemment que des axones transportant de l'OT projetaient dans cette zone, où ils font des synapses avec des interneurons GABAergiques de l'amygdale centrale latérale (CeL). Le relâchement d'oxytocine dans cette zone a été démontré comme activant ces neurones GABAergiques, qui ce faisant inhibent une population de neurones de projection situés dans l'amygdale centrale médiale (CeM) (Huber et al., 2005; Knobloch et al., 2012; Viviani et al., 2011). En partant de ces travaux, j'ai testé l'hypothèse que les astrocytes contribuaient à l'effet de l'OT dans la régulation des micro-circuits de la CeA. Enfin, la CeA étant une zone clé dans le contrôle endogène des seuils de douleur, j'ai mis à l'épreuve la contribution supposée des astrocytes dans l'effet analgésique de l'OT dans la CeA sur un modèle animal de douleur chronique.

Résultats scientifiques

A) Etude d'une sous-population de neurones ocytocinergiques

Via une collaboration avec le laboratoire du Prof. Valery Grinevich, du DKFZ à Heidelberg, nous avons décrypté les connexions entre neurones ocytocinergiques de différents noyaux. En utilisant des techniques de traçage viral rétrograde, nous avons pu montrer qu'une sous-population de seulement 30 neurones parvocellulaires du noyau paraventriculaire (NPV) du rat projetait à la fois sur d'autres neurones OT magnocellulaires, situés dans le noyau supra-optique (NSO), et en parallèle sur des neurones dit « wide dynamic range » (WDR) de la moelle épinière. Ces neurones compilent des entrées sensorielles nociceptives et tactiles, et sont capables d'une première amplification ou diminution de l'intensité du message nociceptif, qu'ils transportent au niveau de l'encéphale.

J'ai pu démontrer, en collaboration directe avec Meggane Melchior, doctorante au laboratoire, que la stimulation spécifique par des techniques d'optogénétique des 30 neurones parvocellulaires était suffisante pour produire une inhibition du codage en fréquence du message douloureux par les neurones WDR de la moelle. En utilisant du patch-clamp en cellule entière sur des neurones WDR de la moelle, j'ai pu montrer qu'en effet l'OT inhibe les potentiels d'action évoqués de ces cellules. J'ai ensuite démontré que la stimulation des axones de ces mêmes 30 neurones du NPV qui projettent sur des neurones magnocellulaires du NSO activait ces derniers, via le relâchement d'OT et de glutamate. Nous avons par la suite démontré que de cette activation résultait une augmentation de la concentration plasmatique d'OT. Nous avons ensuite testé le rôle de ce système sur les seuils douloureux d'animaux souffrant d'une douleur inflammatoire, et montré un effet analgésique de la stimulation optogénétique de notre population de 30 neurones parvocellulaires.

Cet effet analgésique est donc double. Il résulte d'abord une baisse rapide de la transmission du message nociceptif par les neurones WDR de la moelle épinière. Ensuite, la sous-population de neurones parvocellulaires du NPV induisent le relâchement d'OT dans le sang par la stimulation des neurones du NSO. Cela produit une analgésie par action périphérique, probablement au niveau des ganglions rachidiens. Ces travaux ont fait l'objet d'une publication présentée ici (ARTICLE 1).

B) Régulation des réseaux astro-neuronaux de l'amygdale par l'ocytocine.

Tout d'abord, j'ai montré que les astrocytes de l'amygdale centrale latérale répondait à la stimulation spécifique des fibres ocytocinergiques (via optogénétique) innervant la CeL. En effet, la stimulation de ces fibres induit, sur de longues durées, des augmentations oscillantes de la concentration

intracellulaire en calcium des astrocytes, mesurées par imagerie calcique. J'ai ensuite démontré que ces réponses astrocytaires étaient nécessaires à l'effet d'agonistes de l'OT-R sur les neurones. En effet, l'inactivation des astrocytes de la CeA provoque la disparition de tout effet de l'OT sur l'activité synaptique dans la CeA. J'ai ensuite cherché à explorer les mécanismes intracellulaires de la réponse des astrocytes à l'activation de l'OTR, par des moyens pharmacologiques. J'ai identifié les sources de calcium de la réponse astrocytaire comme étant majoritairement extracellulaire et partiellement intracellulaire. Plus précisément, en réponse à une application de TGOT (agoniste spécifique de l'OT-R), le calcium du milieu extracellulaire pénètre par des canaux calciques voltage-dépendant, les canaux Ca_v3 . En effet, le blocage de ceux-ci inhibe la partie oscillante de la réponse calcique. La purge des réserves calciques du reticulum endoplasmique diminue aussi, dans une moindre mesure, la réponse astrocytaire. Ces résultats révèlent des mécanismes complexes, impliquant plusieurs voies classiques d'augmentation du calcium cytosolique dans l'astrocyte. En parallèle, j'ai cherché à identifier le sous type de la protéine G_α lié à l'OT-R. Mes données utilisant des agonistes de l'OT-R biaisés pour un sous type de protéine G_α ou des inhibiteurs des voies qui y sont liées, pointent vers deux sous type de G_α , G_i et G_q . Ces résultats démontrent de façon originale un couplage complexe et atypique de l'OT-R astrocytaire à deux voies de signalisation, laissant néanmoins de nombreuses questions en suspens quant aux mécanismes précis en jeu (voir la DISCUSSION en anglais).

Ensuite, pour mieux comprendre le rôle de l'activation astrocytaire dans l'effet connu du TGOT sur les réseaux neuronaux de la CeA, j'ai cherché à identifier les moyens de communication entre astrocytes et neurones. En utilisant le patch clamp sur neurones, mes résultats montrent la participation de la signalisation liée au récepteur NMDA, son blocage inhibant la réponse neuronale au TGOT et non la réponse astrocytaire. Une hypothèse proposée par de nombreux chercheurs est que les astrocytes ont la capacité de relâcher, après stimulation, de la D-Serine qui agit alors comme co-agoniste au niveau du récepteur NMDA neuronal (Oliet and Mothet, 2009). J'ai prouvé que c'était également le cas lors de la stimulation des astrocytes par le TGOT. En effet la destruction préalable de la D-serine via une enzyme spécifique annihile toute réponse au TGOT, réponse restaurée sur les mêmes cellules lorsqu'est incubée par après de la D-Serine exogène.

Enfin j'ai testé *in vivo* le bien-fondé de ces résultats. J'ai utilisé un modèle de douleur chronique, le modèle d'épargne du nerf sural (Decosterd and Woolf, 2000), et testé le rôle des astrocytes dans l'effet analgésique et anxiolytique du TGOT au sein de la CeA (Han and Yu, 2009). En effet, les animaux (et les humains) souffrant de douleurs chroniques développent entre autres des troubles anxieux (Asmundson and Katz, 2009; Nicholson and Verma, 2004). L'inactivation des astrocytes de la CeA par l'utilisation de fluorocitrate, un poison métabolique des astrocytes (Fonnum et al., 1997), bloque tout effet analgésique du TGOT infusé ensuite dans la CeA. J'ai confirmé ces résultats en

patch-clamp sur tranches aiguës en montrant que le TGOT n'avait plus d'effet sur l'activité neuronale de la CeA si les astrocytes étaient au préalable inactivés par le fluorocitrate, comme mentionné plus haut.

Ces résultats démontrent donc la nécessité d'une fonction astrocytaire préservée dans les mécanismes de l'analgésie induite par le TGOT au sein de la CeA. Ensuite, en utilisant des construits viraux permettant l'expression astrocytaire de canaux cationiques photosensibles (C1V1), j'ai pu stimuler l'entrée de calcium spécifiquement dans les astrocytes. J'ai montré que cette activation suffisait à induire une augmentation de l'activité synaptique de la CeA similaire à celle induite par le TGOT. Ils semblent donc que les astrocytes soient nécessaires et suffisants (au moins *ex vivo*) dans l'effet analgésique et anxiolytique de l'ocytocine au sein de la CeA.

En résumé, mon travail de thèse a permis de révéler différents mécanismes par lesquels l'ocytocine agit au sein du SNC pour moduler différents circuits cérébraux de la matrice de la douleur, en impliquant notamment plusieurs types cellulaires. Il reste évidemment de nombreuses zones d'ombre à éclaircir, comme je le détaille dans la DISCUSSION. Pour finir, ce travail ouvre de nouvelles pistes de recherche, en détaillant d'inédits mécanismes de régulation de la douleur et, ce faisant, ouvre de potentielles pistes thérapeutiques.

Liste des publications scientifiques

Articles scientifiques :

1. En qualité de premier auteur :

Titre : **A New Population of Parvocellular Oxytocin Neurons Controlling Magnocellular Neuron Activity and Inflammatory Pain Processing.**

Auteurs : Eliava M, Melchior M, Knobloch-Bollmann HS, **Wahis J**, da Silva Gouveia M, Tang Y, Ciobanu AC, Triana del Rio R, Roth LC, Althammer F, Chavant V, Gourmon Y, Gruber T, Petit-Demoulière N, Busnelli M, Chini B, Tan LL, Mitre M, Froemke RC, Chao MV, Giese G, Sprengel R, Kuner R, Poisbeau P, Seeburg PH, Stoop R, Charlet A, Grinevich V.

Journal : Neuron 89, 1291–1304 (2016). doi: 10.1016/j.neuron.2016.01.041.

2. En qualité de deuxième auteur :

Titre : **Favoring inhibitory synaptic drive mediated by GABA A receptors in the basolateral nucleus of the amygdala efficiently reduces pain symptoms in neuropathic mice.**

Auteurs : Zeitler A, Kamoun N, Goyon S, **Wahis J**, Charlet A, Poisbeau P, Darbon P.

Journal : Eur J Neurosci. 2016 Apr;43(8):1082-8. doi:10.1111/ejn.13217

3. En cours de réécriture, en qualité de premier auteur (déjà soumis, reviewé puis refusé, en cours de nouvelle soumission) :

Titre : **A key role for amygdala astrocytes in regulation of negative affective processing by oxytocin.**

Auteurs : **J. Wahis**, M. da Silva Gouveia, B. Bellanger, M. Eliava, M. Abatis, B. Boury-Jamot, H. S. Knobloch-Bollmann, M. Pertin, B. Boutrel, C. M. Lamy, I. Décosterd, J. Y. Chatton, R. Stoop, P. Poisbeau, V. Grinevich, A. Charlet.

Chapitre de livre :

1. **Optogenetics: From Neuronal Function to Mapping and Disease Biology.** Publication prévue en mai 2017, isbn: 9781107053014.

Editeur : Krishnarao Appasani, GeneExpression Systems, Inc., Massachusetts

Auteurs : Gero Miesenböck, Georg Nagel, Krishnarao Appasani, Raghu K. Appasani, Boris V. Zemelman, Koutarou D. Kimura, Karl Emanuel Busch, Jatin Nagpal, Dany Adams Ai-Sun Tseng, Michael Levin, Vitaly Shevchenko, Ivan Gushchin, Vitaly Polovinkin, Kirill Kovalev, Taras Balandin, Valentin Borshchevskiy, Valentin Gordeliy, Yonatan Katz, Ilan Lampl, Olga Malyshevskaya, Nobuhiko Yamamoto, Christian Renicke, Christof Taxis, Marc Folcher, Ute Hochgeschwender, William F. Kaemmerer, Anja G. Teschemacher, Sergey Kasparov, Yan Tang, **Jérôme Wahis**, Meggane Melchior, Valery Grinevich, Alexandre Charlet, Alexander M. Herman, Jay M. Patel, Benjamin R. Arenkiel, Michael Kohl, Dennis Kaetzel, Andrea L. Gutman, Ryan T. LaLumiere, Barbara Juarez, Allyson K. Friedman, Ming-Hu Han, Adam C. G. Crego, Stephen E. Chang, William N. Butler, Kyle S. Smith, Jan Tonnensen, Marco Ledri, Merab Kokaia, J. Barney Bryson, Linda Greensmith, Dasha Nelidova, Federico Esposti, Sonja Kleinlogel, Grégory Gauvain, Antoine Chaffiol, Jens Duebel, Serge A. Picaud, Adi Schejter Bar-Noam, Inna Gefen, Shy Shoham, Hiroshi Tomita, Eriko Sugano, Priyamvada Rajasethupathy, Charles-Francois Vincent Latchoumane, Hee-Sup Shin, Jenny X. Chen, Elliott Kozin, Christian Brown, Daniel J. Lee, Victor Hugo Hernandez Gonzalez, Tobias Moser, Joan Fallon, Sabine Müller, Henrik Walter.

Communication orale :

- **Relevance of neuron-glia interactions to the action of oxytocin in pain processing (2015).** J. Wahis, A. Charlet. *Présenté au 11ème symposium du réseau français d'étude de la douleur, à Strasbourg.*

Communication par affiche :

- **Relevance of the astrocyte-neuron interaction to the action of oxytocin in the central amygdala and its effect on pain processing (2016).** J. Wahis, V. Grinevich, P. Poisbeau, A. Charlet. *Présenté au 3ème « Bordeaux Neurocampus Conference » à Bordeaux.*
- **Relevance of the astrocyte-neuron interaction to the action of oxytocin in the central amygdala and its effect on pain processing (2016).** J. Wahis, T. Maduna, V. Lelièvre, V. Grinevich, P. Poisbeau, A. Charlet. *Présenté au 10^{ème} forum de la FENS à Copenhague.*

- **Relevance of neuron-glia interactions to the action of oxytocin in pain processing (2015)**
J. Wahis, T. Maduna, V. Lelièvre, P. Poisbeau, A. Charlet. *Présenté au congrès annuel de la « Society for Neurosciences » à Chicago.*
- **Relevance of astrocyte-neuron interactions in modulatory action of oxytocin in the amygdala (2015)** J. Wahis, P. Poisbeau, A. Charlet. *Présenté au congrès annuel de la société française des Neurosciences, à Montpellier.*

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- ANNEXE 1: List of different OTR agonists and antagonist
- ANNEXE 2: An overview of the OT-OTR signaling network
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G. LISTE DES ABBREVIATIONS

[Ca²⁺]_i - intracellular calcium concentration

ACTH - adrenocorticotropin hormone

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ANP - atrial natriuretic peptide

ASICs - acid sensing ion channels

AVP – Arginine vasopressin

BBB - blood-brain barrier

BNST - bed nucleus of stria terminalis

BRET - bioluminescence resonance energy transfer

CCK - cholecystokinin

CeA - central amygdala

CeL - lateral part of the central amygdala

CeM - medial part of the central amygdala

CGRP - calcitonin gene-related peptide

CNS - Central nervous system

CRH - Corticotropin releasing hormone

CSF - Cerebro-spinal fluid

DRG - dorsal root ganglion

EPM - elevated plus maze

EPSC - excitatory post-synaptic current

ERE - Estrogen response elements

GABA - γ-Aminobutyric acid

GECI - genetically encoded calcium indicators

GFAP - glial fibrillary acidic protein

GPCR - G-protein coupled receptor

I.C.V - Intra-cerebro-ventricular

LDCV - large dense core vesicles

LTP- long term potentiation

mGluR - metabotropic glutamate receptor

NMDAR - N-methyl-D-aspartate receptor

nNOS - neuronal nitric oxide synthase

OT – Oxytocin

OTR – Oxytocin receptor

OXT- Oxytocin gene

P2 - purinergic receptors

PAG - periaqueductal gray

P-LAP - placental leucine aminopeptidase

PLC - phospholipase C

SC – Spinal cord

TGOT - [Thr4,Gly7] oxytocin

TRPA1 - Transient receptor potential cation channel, member A1

TTX - tetrodotoxin

VGCC - voltage gated calcium channel

WT – Wild type

H. INTRODUCTION

1. The neuropeptide oxytocin and its receptor

1.1. History of oxytocin discovery

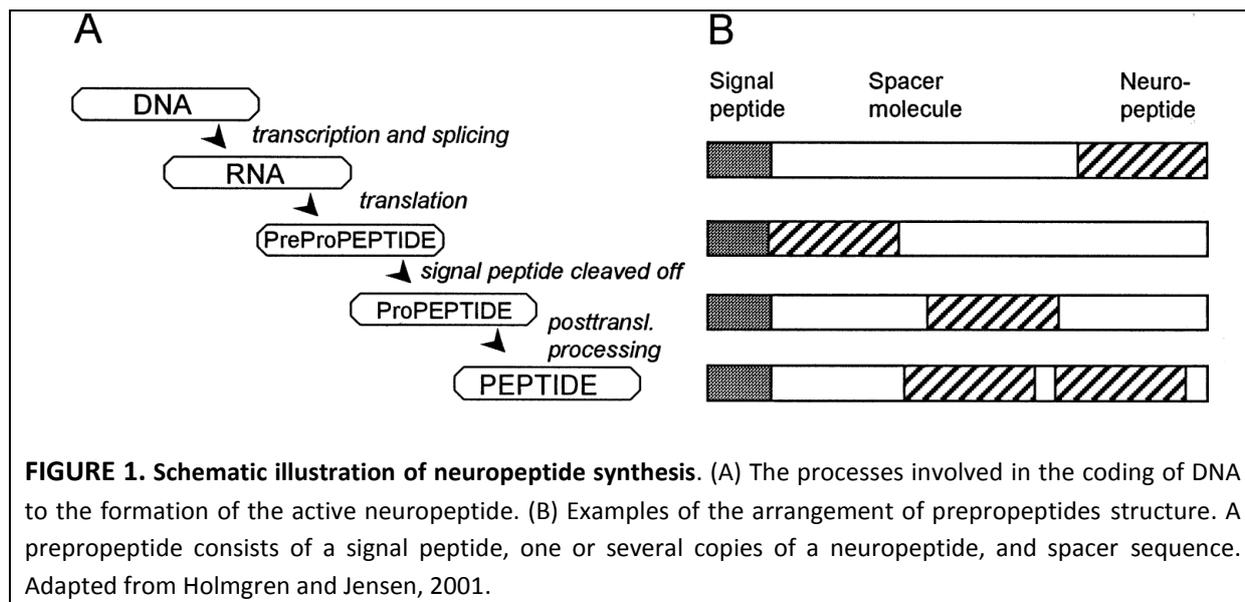
The neuropeptide oxytocin (OT) discovery dates back to 1906, when Sir Henry H. Dale, pharmacologist and physiologist, discovered that human posterior pituitary gland extracts were able to induce uterine contractions in pregnant cats (Dale, 1906). Later this uterotonic compound was isolated from pituitary extracts (Kamm et al., 1928) and named oxytocin, from the greek $\omega\chi\nu\xi$, $\tau\omicron\chi\omicron\xi$, for "swift birth". The next big step for OT was its sequence determination and artificial synthesis, achieved in the 50s by Vincent du Vigneaud (Du Vigneaud, 1956; Du Vigneaud et al., 1954; Du Vigneaud et al., 1953) and for which he was awarded the 1955 Nobel prize in chemistry for "his work on biochemically important Sulphur compounds, especially for the first synthesis of a polypeptide hormone", namely OT.

1.2. OT and its receptor: from genes to molecules

1.2.1. OT : From gene to polypeptide

The oxytocin gene (*OXT*) is 850 base pairs long. It is located on chromosome 20 in the human genome, chromosome 3 in the rat and 2 in the mouse. The mammalian *OXT* gene is on the same chromosomal locus and closely related to the vasopressin (*AVP*) gene, with an intergenic region varying in length across species (11 kb in rat and human, 3.6 kb in the mouse) (Hara et al., 1990; Ivell and Richter, 1984; Mohr et al., 1988; Rao et al., 1992; Sausville et al., 1985). Both genes share the same structure, containing three exons and two introns, but are transcribed in opposite directions. The first exon encodes for a translocator signal, the hormone itself, the tripeptide processing signal and part of the associated neurophysin. The second exon encodes for the other residues of the neurophysin, and the third one for the carboxy-terminal region of neurophysin (Gimpl et al., 2001).

OT (and AVP) is not different from other neuropeptides with regard to its biosynthesis. In all cases, the initial transcript translates into a pre-pro-peptide, later cleaved into the pro-peptide and then into the peptide, as summarized in FIGURE 1. The mature product of *OXT* biosynthesis consists of a dimer, OT-neurophysin. The dimer is processed and transported as one protein in the cell. The dissociation of OT from neurophysin is facilitated by its release from acidic vesicles, namely the neuro-secretory granules, into the more basic plasma or cerebrospinal fluid (CSF), after which it can bind to its receptor (Blumenstein et al., 1979; Breslow and Burman, 1990).



1.2.1.1. *OXT* gene regulation

Upstream regulatory elements: example of the composite hormone response element

The expression of the *OXT* gene is highly regulated, especially at the transcriptional level. For instance, ~160 base pair upstream of the transcription initiation site, a highly conserved sequence closely related to the estrogen response element motifs (ERE) is present in the proximal 5' flanking region of the rat and human *OXT* gene. Due to the high but not perfect resemblance with an ERE, this motif is named composite or multiple hormone response element (Stedronsky et al., 2002). The presence of such a motif in addition to the presence of a ERE in rat indicates the possibility for many true and orphan nuclear receptor family members to regulate *OXT* gene expression (Burbach, 2002). In the case of the human *OXT* gene, estradiol was indeed able to induce expression of OT in cell cultures (Richard and Zingg, 1990) and a single dose of estrogen increases the plasma levels of OT in women (Chiodera et al., 1991). Although there is no expression of estrogen receptor α in oxytocinergic neurons (Axelson and Leeuwen, 1990; Shughrue et al., 1997) it is not the case for the estrogen receptor β , which is found in some hypothalamic oxytocinergic neurons (Alves et al., 1998; Hrabovszky et al., 2004). Studies suggest that estradiol acting on estrogen receptor β is either an up or down-regulator of *OXT* gene in the hypothalamus (Nomura et al., 2002; Patisaul et al., 2003; Shughrue et al., 2002). The thyroid hormone was reported as being able to slightly upregulate the *OXT* gene transcription in the rat hypothalamus (Adan et al., 1992), whereas retinoic acid seems to either down or up regulate its expression in non-cerebral cells (Larcher et al., 1995; Lipkin et al., 1992). Regarding the regulation of the *OXT* gene by nuclear orphan receptors, several studies highlighted a dual role of such elements: a direct translational control, and an indirect translational

control through modulation of hormonal activation of the *OXT* gene (Burbach, 2002; Koohi et al., 2005; Stedronsky et al., 2002).

Other regulatory elements are present upstream of the *OXT* gene, notably a POU-Homeodomain. (FIGURE 2). These cis-regulatory upstream elements highlight the complex regulations of *OXT* transcription, notably in a cell and tissue specific manner (Burbach, 2002; Gimpl et al., 2001).

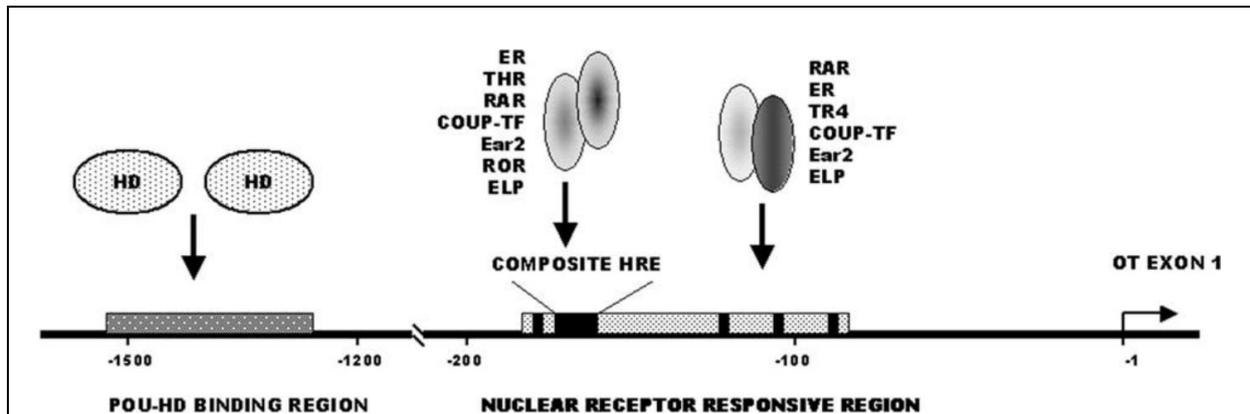


FIGURE 2. Regulatory elements in the 5'-flanking region of the *OXT* gene. The schematic representation of the main known regulatory regions includes a complex domain which confers activities of nuclear hormone receptors, and a distal region binding POU-homeodomain proteins (**POU-HD**). The nuclear receptor responsive region contains multiple half site motifs of the AGGTCA-type in different orientations and with different spacing. The strongest regulatory element is composed of three such motifs, the composite hormone response element (**HRE**) and allows binding of hormone receptors, the estrogen receptor **ER**, thyroid hormone receptors (**THR**s), and retinoic acid receptors (**RAR**), as well as a number of orphan receptors, as indicated. Competition between receptors for binding and protein-protein interactions result in positively as well as negatively modulating actions on promoter activity. For other abbreviations, see Burbach, 2002

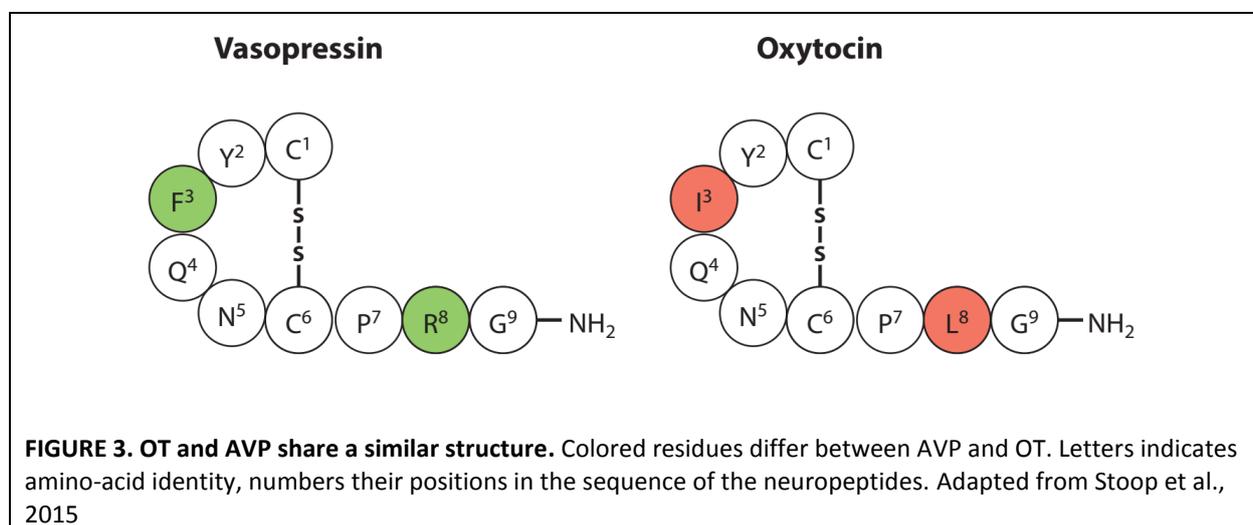
Downstream regulatory elements and cell type specificity

The use of transgenic mice to study the influence of deletion of cis-regulatory elements of the *OXT* gene revealed the importance of downstream sequences for the cell specific expression of OT in sub populations of magnocellular hypothalamic neurons (Murphy and Wells, 2003; Young and Gainer, 2003). Further studies using viral transfection of magnocellular neurons from hypothalamic organotypic cultures revealed that the two downstream enhancers of OT and AVP were quite similar in sequence (Fields et al., 2003), and Gainer reports that switching those two enhancers did not affect expression levels of OT and AVP. This highlight the possibility that the regulatory elements for cell type specific expression of OT might actually all reside in the upstream 5' region of *OXT* gene. The use of viral vector to identify key regulatory elements is a novel state of the art tool, and studies are pursued to tackle the precise sequences needed for a proper OT (and AVP) expression in different tissues (Gainer, 2012). One should note that the design of such specific viral vectors allowed specific

expression of a protein of interest in OT neurons, and such tools were used in my first publication, among others.

1.2.2. OT and its analogs throughout evolution

As mentioned above, the genes coding for OT and AVP are found on the same chromosome in mammals, and separated by a 12 kb or less intergenic region. Both nonapeptides share a very similar sequence, differing by only two amino-acids (FIGURE 3). One difference regarding the amino acid composition resides in that vasopressin (and its family members) contains a basic amino acid in position 8 when OT possesses a neutral one. Regarding the position 3, it is the polarity of amino acids that differs the most, and is believed to confer the specificity of interaction with their respective receptors (Barberis et al., 1998; Gimpl and Fahrenholz, 2001).



1.2.2.1. Oxytocin, vasopressin and related peptides: a common ancestor

The ancestral peptide from which the OT/AVP family evolved is estimated to have emerged before the separation between protostomian and deuterostomian animals (Feldman et al., 2016). The similar sequences and chromosomal locations of OT and AVP indicate that they arose from a common ancestor of jawed vertebrates following tandem duplication of the ancestral gene encoding vasotocin, more than 500 million years ago (Yamashita and Kitano, 2013). With some exceptions, invertebrates possess only one homolog to OT/AVP (Beets et al., 2013). Members of the OT/AVP family are represented along with their amino acid sequences in TABLE 1. Overall, since the duplication of the ancestral gene, the OT/AVP family stayed highly conserved (Banerjee et al., 2017), especially among eutherian mammals (Wallis, 2012). Some striking experimental proof of the conservation of those genes came from the observation that genomic integration of the isotocin

gene of the teleost fish *Fugu* in rat and mouse resulted in its correct expression in OT neurons. Furthermore, the OT gene response to an osmotic stimulus was also mimicked (Gilligan et al., 2003; Venkatesh et al., 1997). This indicates that the regulatory regions of OT and istococin genes are virtually unchanged in their function, at least since the tetrapode / fish divergence.

	1	2	3	4	5	6	7	8	9	
Oxytocin	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Leu	Gly(NH ₂)	Placentals, some marsupials, ratfish (<i>Hydrolagus collieti</i>)
Mesotocin	*	*	*	*	*	*	*	Ile	*	Marsupials, nonmammalian tetrapods, lungfishes
Isotocin	*	*	*	Ser	*	*	*	Ile	*	Osteichthyes
Glumitocin	*	*	*	Ser	*	*	*	Gln	*	Skates (Chondrichthyes)
Valitocin	*	*	*	*	*	*	*	Val	*	Sharks (Chondrichthyes)
Aspartocin	*	*	*	Asn	*	*	*	*	*	Sharks (Chondrichthyes)
Asvatocin	*	*	*	Asn	*	*	*	Val	*	Sharks (Chondrichthyes)
Phasvatocin	*	*	Phe	Asn	*	*	*	Val	*	Sharks (Chondrichthyes)
Cephalotocin	*	*	Phe	Arg	*	*	*	Ile	*	<i>Octopus vulgaris</i> (Molluscs)
Annetocin	*	Phe	Val	Arg	*	*	*	Thr	*	<i>Eisenia foetida</i> (Annelids)
Vasotocin	*	*	*	*	*	*	*	Arg	*	Nonmammalian vertebrates, cyclostomes
Vasopressin	*	*	Phe	*	*	*	*	Arg	*	Mammals
Lysipressin	*	*	Phe	*	*	*	*	Lys	*	Pig, some marsupials
Phenypressin	*	Phe	Phe	*	*	*	*	Arg	*	Macropodids (Marsupials)
Locupressin	*	Leu	*	Thr	*	*	*	Arg	*	<i>Locusta migratoria</i> (Insects)
Arg-conopressin	*	Ile	*	Arg	*	*	*	Arg	*	<i>Conus geographicus</i> (Molluscs)
Lys-conopressin	*	Phe	*	Arg	*	*	*	Lys	*	<i>Lymnaea stagnalis</i> (Molluscs)

TABLE 1. OT compared to related peptides and associated species. Asterisks indicate residues identical to those of OT sequence. (Gimpl and Fahrenholz, 2001)

1.2.2.2. Conservation and adaptation of OT functions

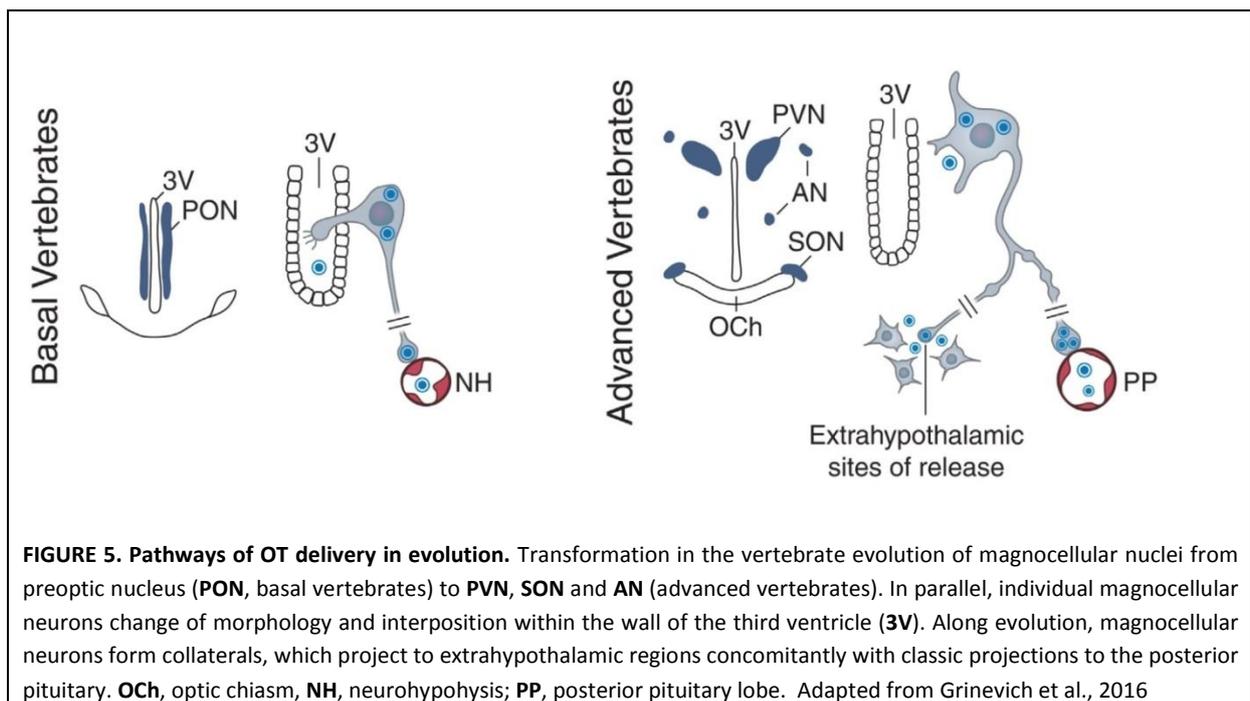
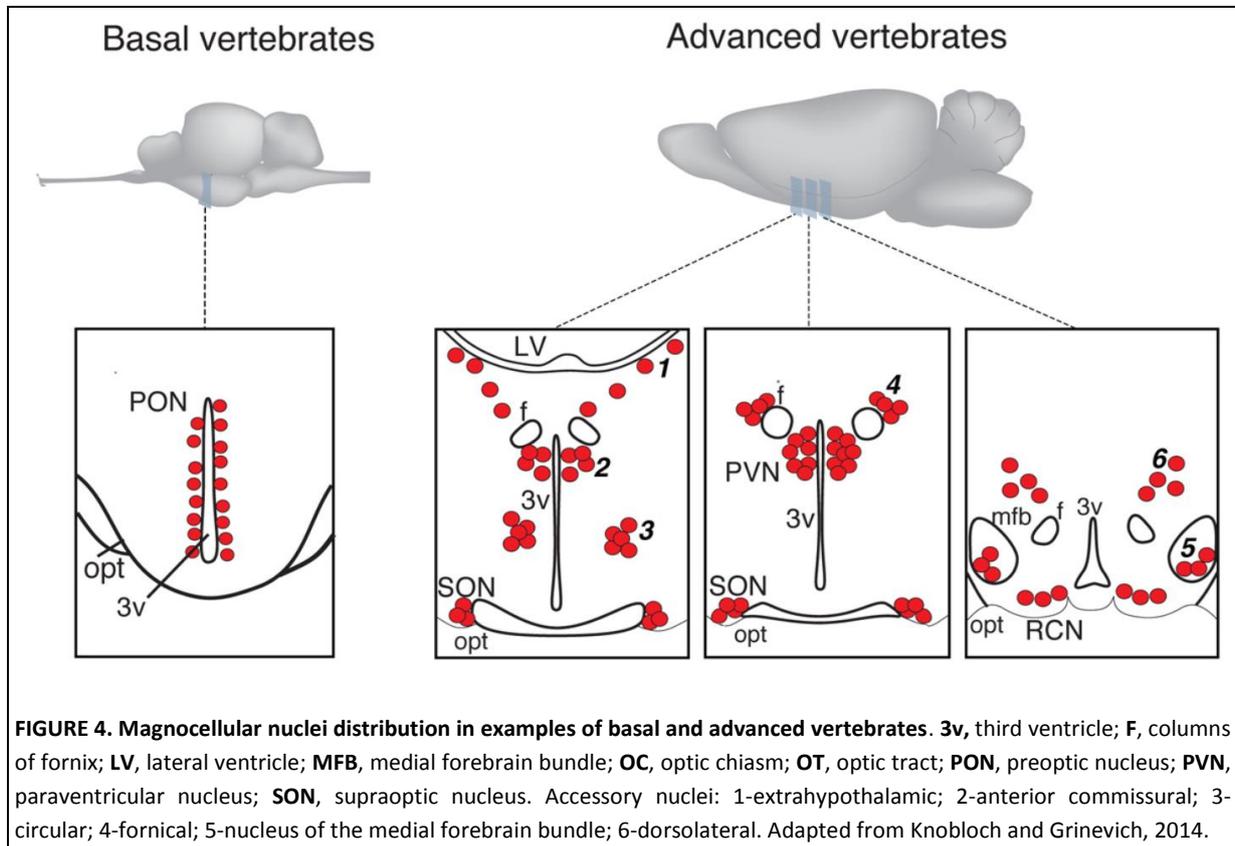
A striking feature of OT and its analogues is that in all species, it conserves a fundamental role in regulating life sustaining functions, ranging from body homeostasis to reproduction. For instance, a basic function of OT, its myoactive effects, is observed from mammals (e.g. uterine contraction) to leeches, in which the OT analog produces twitching, a stereotyped behavior linked to reproduction (Wagenaar et al., 2010). Another example is found in *C.elegans*, in which OT/AVP related peptides also have a role in reproductive behavior (Garrison et al., 2012). But in mammals, OT also regulates other complex functions, like social behaviors (Donaldson and Young, 2008). Chang and colleagues provide an interesting general hypothesis regarding the attribution of new functions to evolutionary old systems, such as the OT/AVP one. They propose that ancestral mechanisms are duplicated and repurposed to support the more complex social behaviors (Chang et al., 2013). For instance in the case of OT in mammals, the functions of OT on reproductive physiology, e.g. the initiation of parturition and lactation, are conserved. But OT also induces maternal behavior in rats (Pedersen et al., 1982) and modulate complex social behavior between humans (Heinrichs et al., 2009), which are necessary for a successful education of the offspring (Feldman et al., 2016). This highlights the idea that the role of OT (and its analogues) in regulation of reproductive functions is conserved along

evolution, and repurposed to modulate species specific behaviors surrounding the reproduction (Lee et al., 2009a). A similar discussion about the conserved function of OT peptide family in the regulation of animal reproductive behavior is given by Beets and colleagues, by comparing AVP and OT systems and their functions between invertebrates such as nematodes and vertebrates such as mammals (Beets et al., 2013).

1.2.2.3. Conservation and adaptation of OT brain centers and OT cells morphology

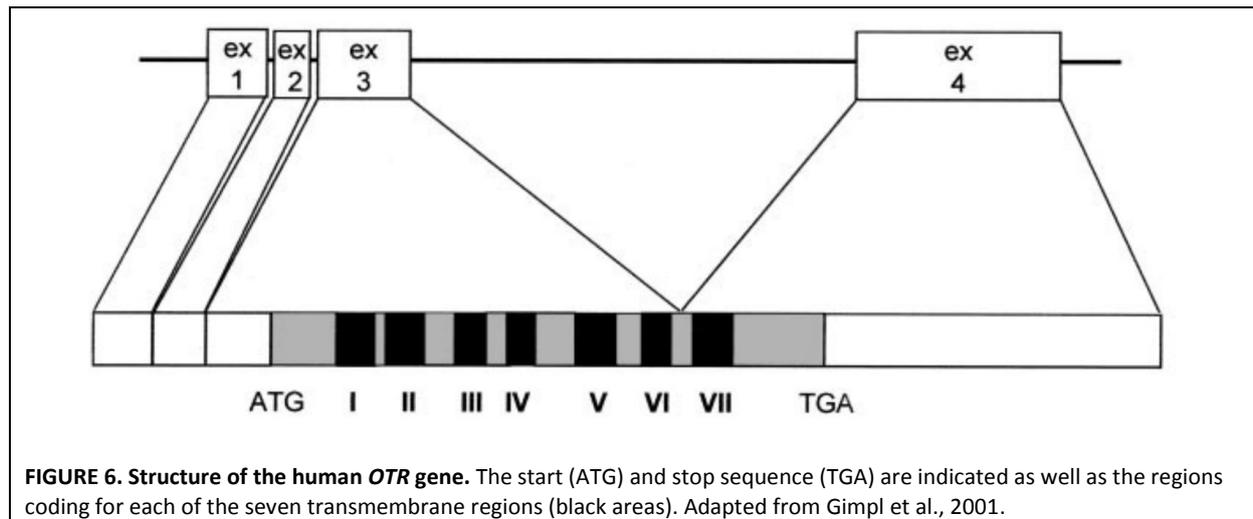
In line with the conservations of sequences and functions among the OT/AVP analogues, a certain degree of conservation is present in the brain centers and cell types producing and releasing those neuropeptides. In mammals, OT and AVP are synthesized in the paraventricular, supraoptic and accessory nuclei (PVN, SON, AN, respectively) of the hypothalamus. They contain, among others, magnocellular neurosecretory neurons that project to the posterior pituitary to release OT and AVP into the blood circulation (McEwen, 2004). In invertebrates, OT and AVP analogs are synthesized by neurons whose cell bodies reside in cerebral or peripheral ganglia (Beets et al., 2013). In basal vertebrates (*Anamnia*), magnocellular neurons expressing OT analogs reside in the ancestral preoptic area (Herget et al., 2014), adjacent to the third ventricle. The magnocellular neurons are distributed randomly in the preoptic area, among other cell types. In advanced vertebrates (*Amniota*), the distribution of magnocellular neurons is more defined, limited between the PVN, SON and AN. A precise description and discussion about the evolution of anatomical distributions of magnocellular OT neurons among basal and advanced vertebrates can be found in the review from Knobloch and Grinevich, 2014, from which FIGURE 4 is extracted. Another interesting feature is the evolution of the morphology of cells expressing OT and its analogs. In *C.elegans*, nematocin expressing neurons have ciliated processes, from which they can release nematocin containing granules in the pseudocoelomic fluid (Beets et al., 2013). In basal vertebrates, cilia are also present in the dendrites of magnocellular neurons and protrude into the third ventricle, allowing the release of neuropeptides in the CSF. This feature is still present in the mammalian brain, although sparsely (Knobloch and Grinevich, 2014). A feature of magnocellular cells, conserved throughout the phylogenetic tree of vertebrates, is the endocrine neurosecretion through axo-vasal contacts within the posterior pituitary. This feature remains constant in all vertebrates, down to the ray-finned fish (Egorova et al., 2003). Along the evolution, it seems that a neuronalisation of OT cells occurred, meaning an evolution from a primitive uni- or bipolar glandule-like neuron into a multipolar one, with a richer dendritic tree. Those features enable magnocellular neurons to release neuropeptides from dendrites through exocytosis (Pow and Morris, 1989), allowing a paracrine communication inside hypothalamic nuclei and in all likelihood diffusion of OT into the third ventricle, as in basal vertebrates. A unique trait of OT neurons from advanced vertebrates is the presence of long-range

axonal projections (Grinevich et al., 2016). Those projections might stand for the precise regulation of complex functions by OT and AVP, such as the regulation of maternal behavior and social recognition (Marlin et al., 2015; Oettl et al., 2016). A schematic summary of the positional and morphological changes of magnocellular neurons throughout evolution of vertebrates can be found in FIGURE 5.



1.2.3. Oxytocin receptor : from gene to protein

The oxytocin receptor (OTR) is a member of the class I G-protein coupled receptors (GPCRs) family, composed of the prototypical seven transmembrane domains. The gene encoding OTR has been isolated as one copy on chromosome 3 in human (Kimura et al., 1992), 4 in the rat (Rozen et al., 1995) and 6 in mice (Kubota et al., 1996). The 17 kb long *OTR* gene contains three introns and four exons. The exons one and two contain the 5' non coding region, exons three and four the 389 amino acids sequence of OTR (FIGURE 6).



1.2.3.1. *OTR* gene regulation

Several regulatory sequences can be found in the 5' flanking region of the *OTR* gene. In the rat gene, an ERE, a palindromic ERE, a half steroid response element and a cAMP responsive element are present, among others (Gimpl and Fahrenholz, 2001). These indicate a role for estrogen and protein kinase A and C in regulation of *OTR* gene expression, among others (Bale and Dorsa, 1997). Indeed, forskolin treatment in cultured rabbit or human cells promoted OTR upregulation, indicating PKA/PKC linked pathways as potential inducers of OTR expression (Bale and Dorsa, 1998; Hinko and Soloff, 1993; Jeng et al., 1998).

OTR gene regulation by sex steroids is peculiar. Estrogen is able to increase the amount of OTRs binding sites and its mRNAs, whereas progesterone had an effect only on binding site availability, indicating that it acts on the OTR itself (Grazzini et al., 1998; Patchev et al., 1993; Zingg et al., 1998). But results concerning progesterone regulation of OTR are contradictory (Ivell et al., 2001). Although estrogen receptor α , (and not β) is as an inducer of OTR binding in the brain, OTR are still present in estrogen receptor α KO mice (Patisaul et al., 2003; Young et al., 1998), suggesting the existence of other mechanisms regulating OTR expression (Gimpl et al., 2001; Ivell and Walther, 1999). Furthermore, transfection studies using fusion proteins containing the promoter region of the *OTR*

gene followed by a reporter gene failed to express the construct upon application of estrogen (Ivell et al., 2001; Kimura et al., 2003). Taken together, studies about the regulation of OTR expression by sex steroids point towards a clear but indirect role of those hormones (Fleming et al., 2006; Ivell and Walther, 1999; Ivell et al., 2001). It seems that the *OTR* gene might be constitutively active (Ivell et al., 1998) thereby suggesting that the repression of the promoter might be a key player of OTR expression control. Indeed, some reports show different levels of methylation around intron 1 and in intron 3 in different tissues, thus suppression of OTR transcription by epigenetic mechanisms might be the key element of tissue specific expression of OTR (Kimura et al., 2003; Kusui et al., 2001). An element of proof is the fact that various levels of methylation of the *OTR* gene promoter do indeed control the different levels of OTR mRNA in different brain regions in mice (Harony-Nicolas et al., 2014). Finally, in humans, different levels of methylation of the *OTR* gene are associated with different patterns of brain activity in emotional processing and social perception (Puglia et al., 2015) and linked to mental disorders such as autism (Kumsta et al., 2013).

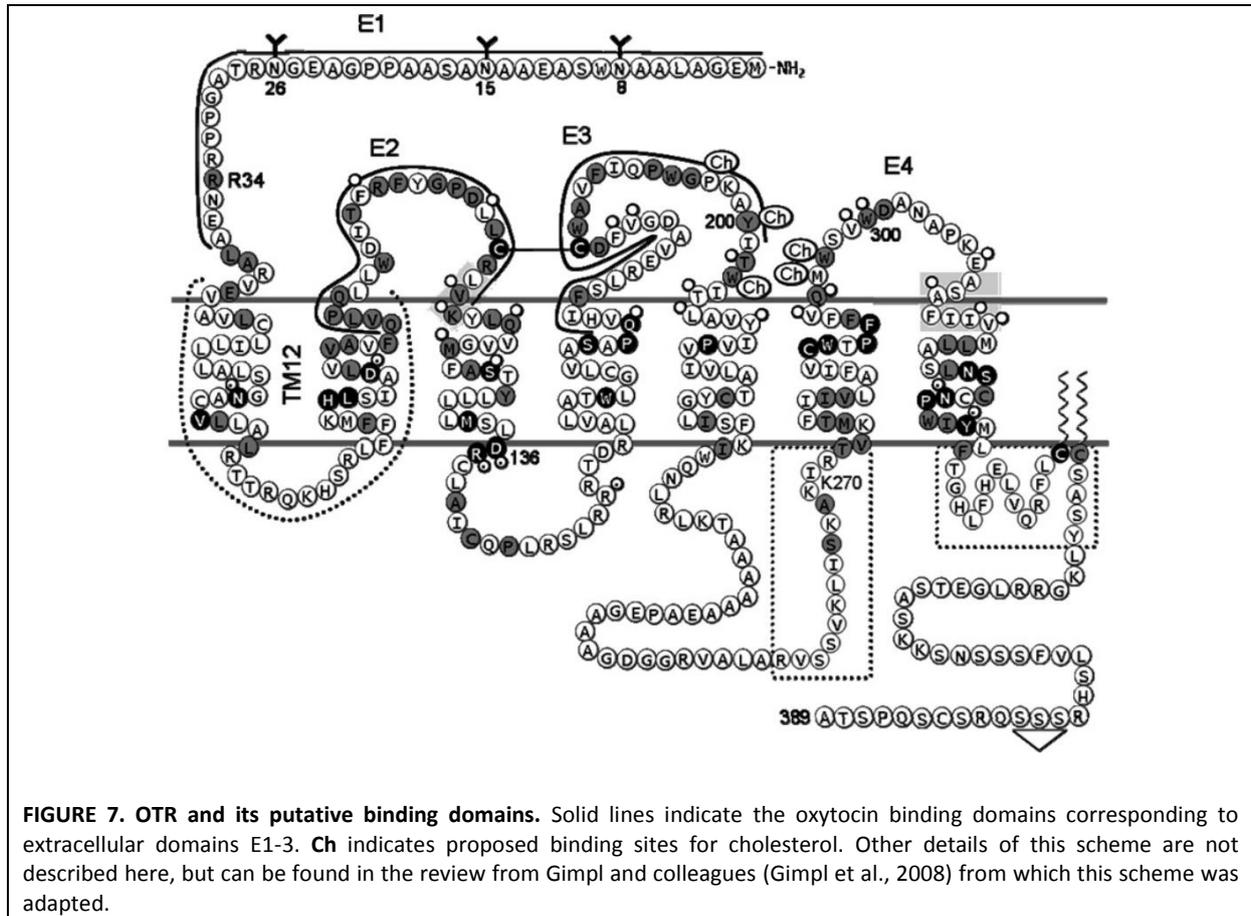
1.2.4. OTR characteristics

1.2.4.1. *Ligand binding*

The ligand-receptor interaction for OTR has been extensively studied using several technical approaches. Conclusions drawn from those studies identify the extracellular N terminus and the first two extracellular loops of the transmembrane domain as the sites of specific interactions between OTR and OT. It seems that the N-terminus of the OTR binds a wide variety of OTR agonists, and therefore do not discriminate between them (Wesley et al., 2002). The OT specific binding site resides in the region E2 and E3, depicted in FIGURE 7 below (Fanelli et al., 1999).

As mentioned earlier, OT amino acid sequence is very close to the AVP one. Indeed, AVP is a ligand of OTR, for which it possesses an affinity similar than OT (Akerlund et al., 1999) and acts as a partial agonist (Chini et al., 1996). This peculiar selectivity profile led to the intense development of selective agonists and antagonists of OTR (and AVP receptors). In this regard, Busnelli and colleagues reports that in the last decades, at least a thousand synthetic peptides have been developed and tested for their ability to bind and activate the different OT/AVP receptors (Busnelli et al., 2013). A non-exhaustive list of different available agonists and antagonists of OT and AVP receptors can be found in the following review (Manning et al., 2012a) as well as in ANNEXE 1. It should be noted here that OT also binds the AVP receptors ($K_i = 120$ nM for V_{1A} , > 1000 nM for other receptors), but with a much lower affinity than AVP for OTR ($K_i = 1.7$ nM). Nevertheless, in researches aiming at deciphering OTR linked phenomenon in tissues or cells also expressing AVP receptors, the use of synthetic

agonists with a high selectivity profile for OTR is to be preferred, as for instance [Thr4,Gly7]OT in rats, thereafter referred as TGOT (Elands et al., 1988a).



1.2.4.2. OTR, cholesterol and divalent ions

Another peculiar property of OTR is its functional dependence on cholesterol and divalent cations. There is a clear relationship between the availability of cholesterol and the affinity of OT for OTR, that switches from a ~ 1 nM K_d to a ~ 100 nM K_d in absence of cholesterol (Klein et al., 1995). This property of cholesterol is specific and not linked to change in membrane fluidity (Gimpl et al., 1997). Further work was done to identify specific binding domains of cholesterol in the OTR protein, those are shown in FIGURE 7, indicated by the letter "Ch". Gimpl and colleagues propose that cholesterol may act as a stabilizer of a high affinity state of OTR and facilitates efficient receptor expression. Indeed addition of cholesterol in the culture medium of insect cells (that have naturally low cholesterol content) was more efficient to gain high affinity OTRs expression than addition of cholesterol to the plasma membrane (Gimpl et al., 1995). The authors also propose that cholesterol protects OTR against thermal or proteolytical degradation (Gimpl and Fahrenholz, 2002). Furthermore, OTRs are more stable in cholesterol rich microdomains of plasma membrane in HEK cells, as revealed by the delay in inactivation time in those region, especially at physiological

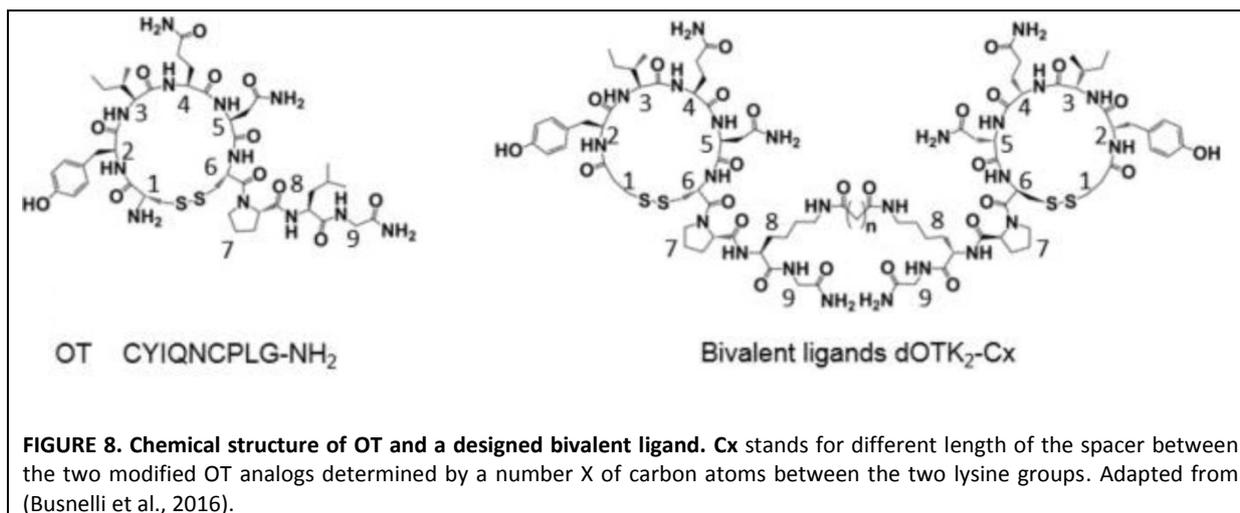
temperatures (Gimpl and Fahrenholz, 2000). The addressing of OTR to lipid rafts is even able to change the cellular effects of OTR activation. When such addressing was conducted using a fusion protein of OTR with calveolin, OTR activation effects on cell growth switched from inhibitory to proliferative (Guzzi et al., 2002). Those data might explain the findings of high and low affinity OTR populations in uterine cells (Crankshaw et al., 1990; Plieika et al., 1986) as being OTRs in portions of plasma membrane with different amounts of cholesterol. Divalent ions like magnesium have a similar effect on OTR. They increase the binding affinity of OT to OTR as well as the affinity state of the OTR (Gimpl and Fahrenholz, 2001).

1.2.4.3. OTR dimers

The capacity of GPCRs to associate together to form homo-/hetero-dimers or even oligomers has been well demonstrated by biochemical and biophysical techniques such as co-immunoprecipitation and bioluminescence/fluorescence resonance energy transfer (B/F-RET) (Bouvier, 2001; Ferre et al., 2014). The OTR is no exception and can form either homo- or hetero-dimers (Cottet et al., 2010). Few studies tested the existence of such association of GPCRs for OTR:

- *In vitro*: during biosynthesis, OTR and AVP receptors form homo and hetero dimers (Terrillon et al., 2003).
- In tissue: in mammary glands of rats, OTR homodimers are present on cells surface (Albizu et al., 2010)
- In tissue: Dopamine receptor (D2) and OTR heterodimers (Romero-Fernandez et al., 2012)
- *In vitro*: β 2-adrenergic receptor and OTR (Wrzal et al., 2012a, 2012b)

The physiological relevance of such complex of OTRs has been elegantly tested by Busnelli and colleagues. They designed homobivalent ligands, shown in FIGURE 8. They first showed the ability of such homobivalent ligands to bind OTR homodimers by BRET. They report that, as mentioned previously, OTR exists in two affinity states, low and high. The low affinity OTRs were not differently affected by the new homobivalent ligand compared to OT, whereas the high affinity population showed superagonistic responses upon binding of the bivalent ligand of appropriate length (8 carbons). They next tested the effect of the more potent homobivalent ligand they designed *in vivo*. Behavioral assays where the effect of OT has been well characterized, the three chamber test in mice and the shoaling behavior in zebrafish, were used to test the effect of the homobivalent ligand. Those two tests measure the sociability of animals. It has been previously reported that OT was able to favor sociability in those two tests. Intra-cerebro-ventricular (i.c.v) administration of the bivalent ligand could also favor sociability in those two tests, with a potency increased between forty and a hundred folds compare to OT (and Isotocin in fish).



This result is consistent with the existence of OTR dimers in the central nervous system (CNS) of at least two very different vertebrates (Busnelli et al., 2016). The role of such dimers in modifying different cellular responses to OTR activation remains to be further explored.

1.2.4.4. Intracellular signaling

OTR: A GPCR with promiscuous G-protein coupling

The intracellular loops of the transmembrane domain and the intracellular C-terminal domain of GPCRs are linked to heterotrimeric G proteins complexes. Each complex is composed of three G proteins: G_α, G_β, G_γ. Upon binding of their ligands, conformational changes of the receptor leads to the exchange of the guanine diphosphate bound to the G_α to a guanine tri-phosphate form. G_α then dissociate from G_{βγ}. The G_{βγ} dimer then diffuses laterally in the plasma membrane and activates various effectors, whereas G_α diffuses away from them to target other effectors, depending on the subtype of G_α. Next, different second messengers of GPCR signaling are activated, resulting in an amplification of the signal, which modulates numerous cellular functions. The GPCR is next recycled to the membrane or degraded through various cellular pathways. The coupling of a given GPCRs to different G_α subtypes is not fixed as previously thought, and depends on many factors as for instance the cell type it is expressed in. The OTR has been described as being functionally coupled to various G_α subtypes, such as G_{q/11}, G_{i/o}, G_h and possibly G_s (Gimpl et al., 2001; Reversi et al., 2005a). Different G-protein coupling of OTRs is even possible in the same cell type (Gravati et al., 2010) and might be favored by OTRs localization in different microdomains of the plasma membrane (Rimoldi et al., 2003). That being known, an interesting aspect is that different agonists of OTR can specifically activate OTRs linked to a given G-protein subtype, a property named biased agonism (Luttrell et al., 2015).

G-protein coupling specific ligand of OTRs

The original function associated to OT and that gave it its name, is uterine contraction. At the cellular level, the activation of OTR from myometrial cells by OT leads to the activation of $G_{q/11}$, which activate the phospholipase C (PLC) leading to inositol phosphate and diacylglycerol production. The inositol phosphate allows opening of the calcium channels from the endoplasmic reticulum, which finally leads to cellular and *in fine* myometrial contraction. To prevent too early contractions of the uterus during pregnancy, leading to pre-term labor, an antagonist of the OTR called Atosiban has been developed (Thornton et al., 2001). Atosiban is an antagonist of OTR, but only if the GPCR is linked to a $G_{q/11}$ subunit. In the case of an OTR coupled with a G_i , Atosiban acts as an agonist (Reversi et al., 2005b).

The other way around, a biased agonist of OTR toward the G_q pathway also exist. Carbetocin was initially developed as an analog of OT with a longer half-life in the peripheral circulation (Barth et al., 1974). It has prolonged effect on post-partum uterine activity in women compared to OT (Amsalem et al., 2014). But given in rats, Carbetocin and oxytocin have different, even opposite effects (Klenerova et al., 2009a, 2009b). Those differential effects might be explained by the fact that Carbetocin is in fact a biased agonist for OTR linked to the G_q pathways (Passoni et al., 2016a).

The multiple signaling pathways activated by differential coupling can either act synergistically, for instance by stimulation of the G_q and small G protein of the rho family, or the other way around, for instance by stimulation of the G_q and G_i pathways (Gravati et al., 2010). The use of those biased agonists allows a fine dissection of the signaling cascade following GPCRs activation in research, and their use in pharmacology is promising, since targeting only one function of a GPCR could allow more specific effects (Busnelli et al., 2012). For instance, Atosiban, being selective for G_i -coupled OTR, was able to inhibit the proliferation of mammary carcinomas in rat and mouse (Cassoni et al., 1996; Reversi et al., 2005b).

$G_{\beta\gamma}$ of OTR:

The role of the $G_{\beta\gamma}$ subunit upon OTR activation has not been extensively studied. Nevertheless, it may convey important functions of OTR signaling pathways, for instance it was shown to modulate the release of calcium from intracellular stores in G_i -coupled OTR (Hoare et al., 1999). G_q -coupled OTR signaling also relies partly on $G_{\beta\gamma}$, at least in myometrium (Zhong et al., 2003). Finally, the $G_{\beta\gamma}$ subunit of OTRs coupled to G_q alone is crucially involved in oxytocin-evoked burst firing of OT cells from lactating rats (Wang and Hatton, 2007a).

Downstream effectors of OTR linked G-proteins:

The intracellular signaling pathways activated by OTR signaling are various and numerous. The following diagram of FIGURE 9 has been proposed by Viero and colleagues, and summarizes most of the proven intracellular pathways that OTR activates (Viero et al., 2010). For a more complete overview of all the known downstream effectors of OTR, please refer to ANNEXE 2 and Chatterjee et al., 2016.

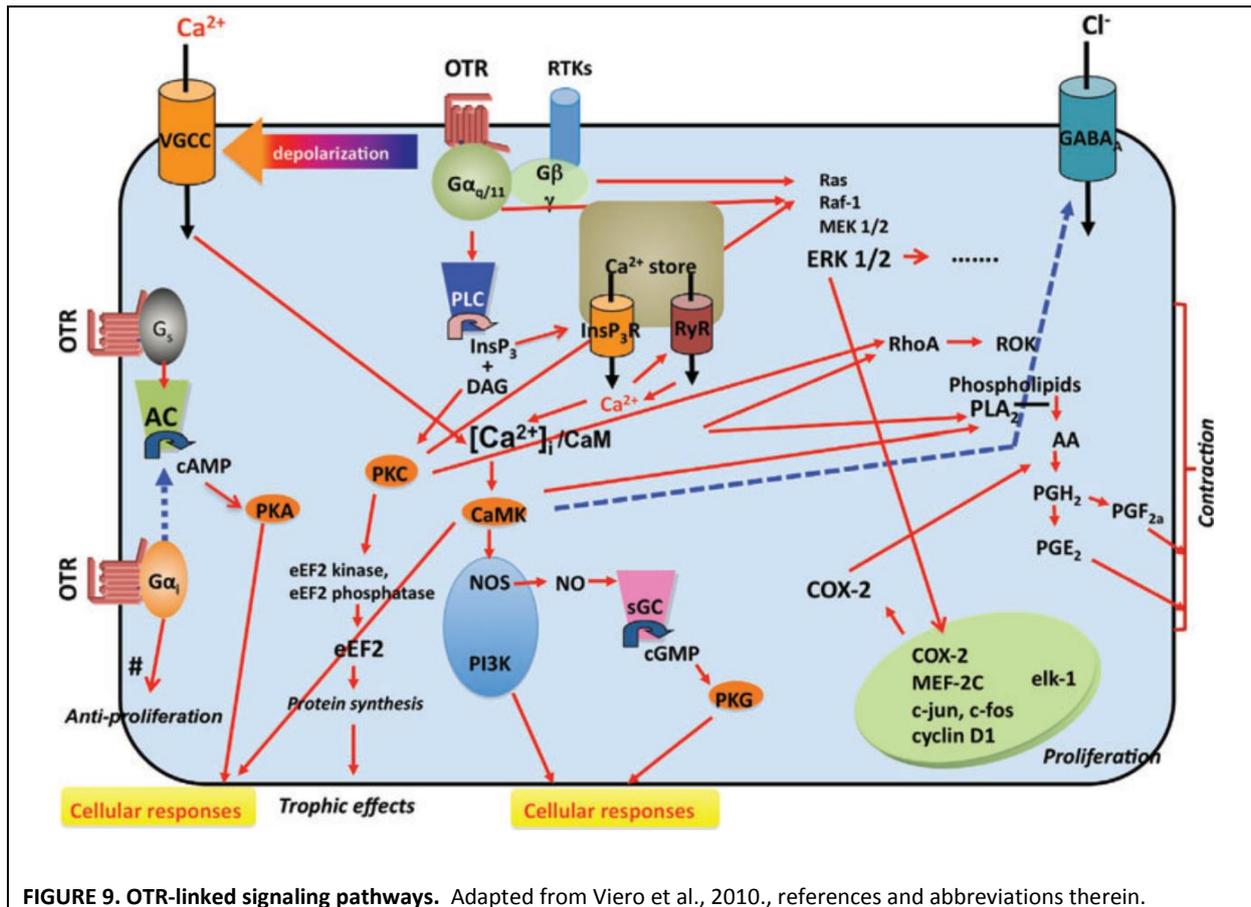


FIGURE 9. OTR-linked signaling pathways. Adapted from Viero et al., 2010., references and abbreviations therein.

Receptor internalization and inactivation

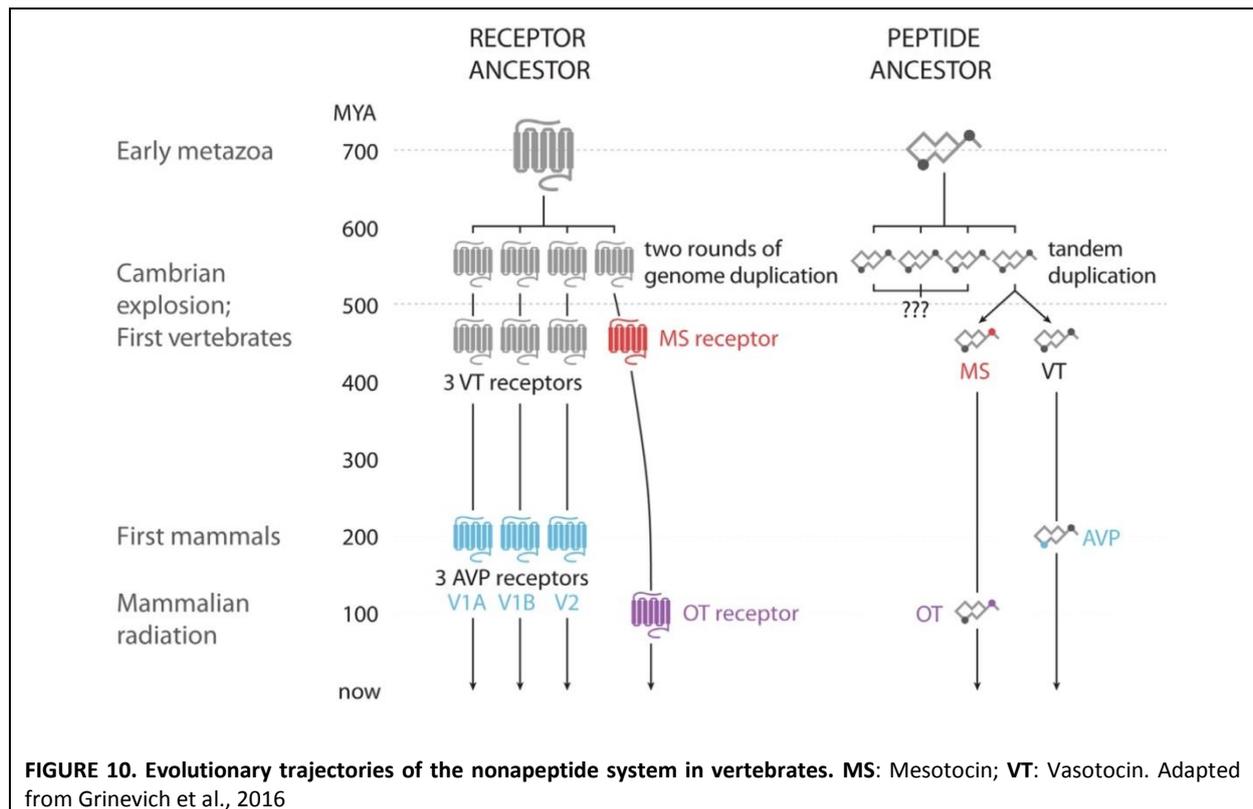
After persistent stimulations, a GPCR may desensitize. This phenomenon is thought to be mediated by GPCR phosphorylation, which inactivates the receptor, followed by β-arrestin binding (Wolfe and Trejo, 2007). This leads to receptor endocytosis, internalization or sequestration (Moore et al., 2007). From there, receptors can be either degraded in lysosomes or recycled to the membrane (Drake et al., 2006). OTR is no exception in this regard. OTR expressing myometrial cells exposed for 20 hours to OT show a tenfold reduction in OT binding and a down regulation of OTR mRNA production (Phaneuf et al., 1993; Plested and Bernal, 2001). Furthermore, myometrium biopsies of women

showed a decrease responsiveness to OT in culture, inferred by the decrease in intracellular calcium elevation upon OT exposition (Robinson et al., 2003). Those desensitization are probably due to internalization of receptors, that seems to happen within thirty minutes of agonist application (Guzzi et al., 2002). OTR desensitization occurs through protein kinase GRK2 (G-protein coupled receptor kinase 2) action, which promotes β -arrestin binding and endocytosis through clathrin-coated pits (Hasbi et al., 2004; Smith et al., 2006). It has been reported that the OTR belong to the class B receptor regarding their stable interaction with β -arrestin (Oakley et al., 2001). This would indicate that OTR are not rapidly recycled to the membrane, but are either degraded in lysosomes or slowly recycled to the plasma membrane. Conti and colleagues demonstrate that this is not the case, and that OTR in HEK cells are recycled to the plasma membrane after their internalization through a so called “short cycle” (~ four hours) (Conti et al., 2009). Interestingly, different agonists of OTR induce different mechanisms of desensitization. The previously described selective agonist Atosiban does not induce the association of OTR with β -arrestin, and the endocytosis of OTR is not observed even after sustained atosiban exposition (Busnelli et al., 2012). Carbetocin does promote OTR internalization, but through a β -arrestin independent pathway, and recycling of the OTR is not observed after exposition to this biased agonist (Passoni et al., 2016b).

1.2.5.OTR throughout evolution

As for the OT and AVP nonapeptides, the OT/AVP receptors originate from a common ancestral vasotocin receptor. Around the same time as the duplication of the ancestral vasotocin gene, the sequence for its receptor also underwent duplications, giving rise to four vasotocin receptors. Those genes evolved in the four receptors found in mammals, the three AVP receptors V1a, V1b, V2 and OTR, as shown in FIGURE 10 (Grinevich et al., 2016; Yamashita and Kitano, 2013).

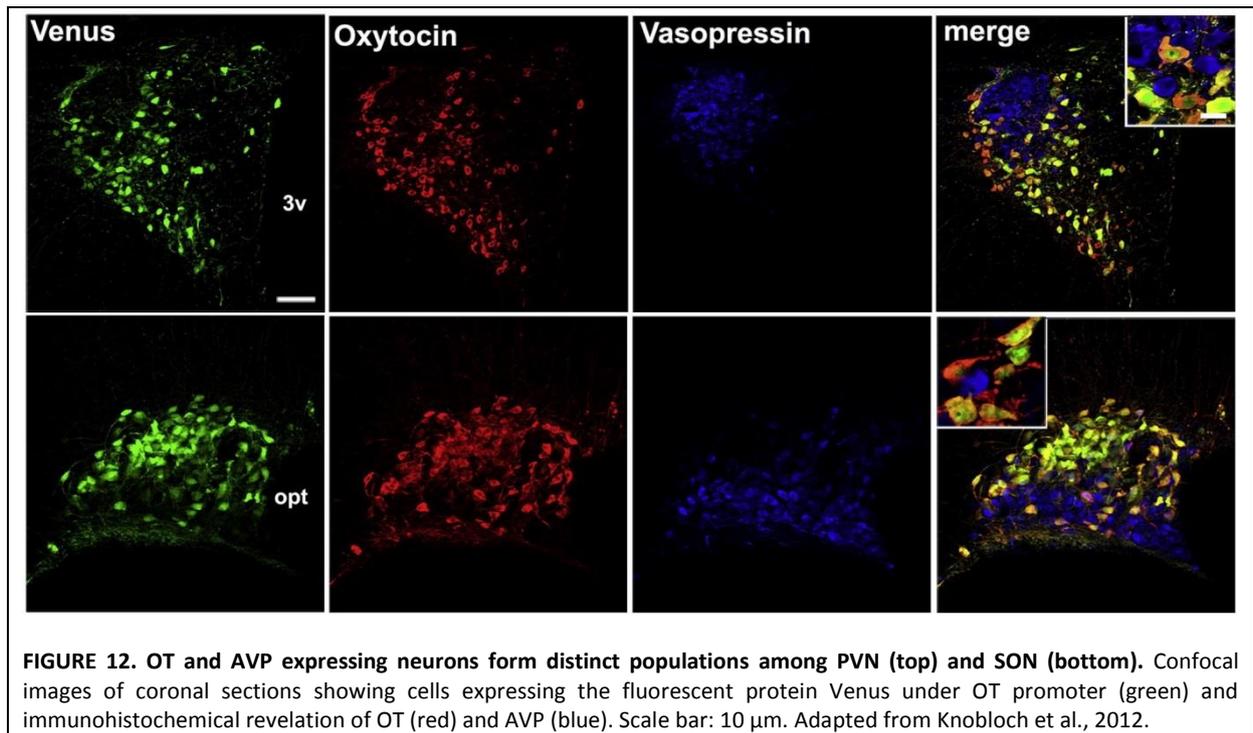
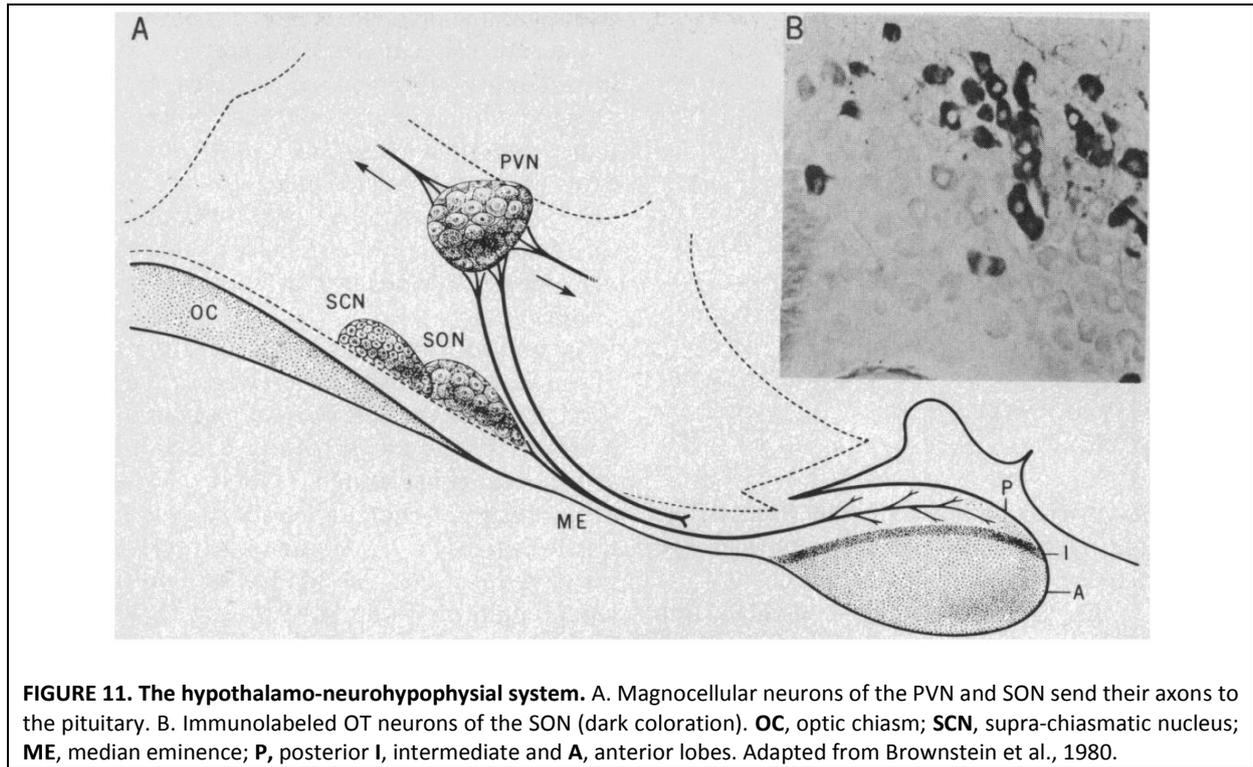
It appears that in placental mammals, the *OTR* gene is under evolutionary constraint compared to AVP receptors (Paré et al., 2016). An exception seems to be present in new world monkeys, in which new forms of the *OXT* gene and *OTR* gene have been detected and proposed to have co-evolved, and seem correlated to changes in male parental care (Vargas-Pinilla et al., 2015). Some evolutionary aspects of the pattern of OTR expression in the central nervous system will be discussed in 3.4.3.1.



2. OT, an hypothalamic neurohormone.

2.1. OT, a secreted neurohormone

The neuroendocrine functions of OT are mediated by the neurosecretion of OT from PVN, AN and SON magnocellular neurons of the hypothalamus (Burbach et al., 2001; Sofroniew, 1983; Swanson and Sawchenko, 1983). It is estimated that there are over 100,000 magnocellular neurons in the human brain (Manaye et al., 2005) and ~10,000 in the rat one (Rhodes et al., 1981). Those cells project their axons within the posterior pituitary lobe, in which they release OT in the bloodstream through axo-vasal contacts (see FIGURE 11 on the next page) (Brownstein et al., 1980). The magnocellular neurons of PVN and SON can be separated into two distinct populations, OT expressing and AVP expressing neurons (see FIGURE 12 on the next page) (van Leeuwen and Swaab, 1977; Vandesande and Dierickx, 1975) with distinct electrophysiological properties (Armstrong, 1995; Renaud and Bourque, 1991). Nevertheless, a small population of neurons do express both (1-5%), and some studies suggest that both populations synthesize both nonapeptides, but with a huge bias in quantities (Glasgow et al., 1999; Kiyama and Emson, 1990; Xi et al., 1999).



In magnocellular neurons, the first step of neurosecretion is the synthesis of OT prepropeptide in the rough endoplasmic reticulum. After cleavage of the signal sequence and disulfide bonds formation, the propeptide is transported into the golgi apparatus. It is then sequestered in membrane-bound vesicles, the secretory granules also named large dense core vesicles (LDCVs). Additional post-translational processing occurs while the granules are transported to the neuronal terminals in the

pituitary. Upon sufficient stimulation of neurons and consequent calcium entry at the level of the terminals, the neurophysin-oxytocin protein is secreted through exocytosis into the bloodstream, where further dissociation of neurophysin from OT is required for OT to act on its receptor (Burbach et al., 2001; McEwen, 2004).

2.1.1. Pharmacology

2.1.1.1. *Half- life and degradation*

OT half-life in the blood circulation is estimated to range between 3-8 minutes in humans and rats (Morin et al., 2008; Rydén and Sjöholm, 1969), compared to 19-28 minutes in the CSF of rats and guinea pigs (Jones and Robinson, 1982; Mens et al., 1983). OT is cleared from blood through chemical degradation in the liver and kidney (Claybaugh and Uyehara, 1993). Exo- and Endo-peptidases hydrolyze and cleave OT, notably the oxytocinase (full name: placental leucine aminopeptidase, P-LAP). The expression of P-LAP is regulated, for instance it increases during mid to late pregnancy and is important to maintain pregnancy at term, as its decrease in late pregnancy is linked to preterm labor (Nomura et al., 2005; Yamahara et al., 2000). Indeed, P-LAP deficient mice present an oxytocin hypersensitivity, resulting in pre-term labor (Ishii et al., 2009). Of importance, P-LAP also degrades AVP, angiotensin and endorphins, so its action is not specific to OT (Tsujiimoto and Hattori, 2005). Nevertheless, P-LAP is expressed by OT cells and is under regulation during lactation in rats, in which blocking P-LAP mimicked the reflex milk ejection induced by i.c.v OT injection (Tobin et al., 2014). Finally, changes in oxytocinase levels might also be differentially regulated in various central site of action of OT. Indeed, it seems to have a particular role in the modulation of OT actions in stress responses in the amygdala (Hernández et al., 2009). Even if a metabolite of AVP was shown to be active centrally (Burbach et al., 1983), no conclusive data are yet available regarding potential effects of OT metabolites.

2.1.2. Non central synthesis

OT is also synthesized by peripheral tissues and cells, such as:

- Ovary (Wathes and Swann, 1982; Wathes et al., 1982)
- Corpus luteum and placenta (Fields et al., 1983)
- Uterus and amnion (Arrowsmith and Wray, 2014)
- Male genital tract (Gimpl et al., 2001; Guldenaar and Pickering, 1985)
- Cardiovascular tissue (Gutkowska et al., 2014)
- Adrenal medulla (Ang and Jenkins, 1984)
- Thymus (Geenen et al., 1986)

- Pancreas (Amico et al., 1988)
- Bone (Colaiani et al., 2014)
- Skin (Denda et al., 2012)
- Adipocytes (Chaves et al., 2013)
- Macrophages & monocytes (Szeto et al., 2008)

Nevertheless, the reported concentration of OT are usually 100 to 1000 times lower in those peripheral organs compared to the hypothalamic synthesis sites, which may indicate a paracrine or autocrine role, as opposed to the endocrine one mediated by neurosecreted OT (Banerjee et al., 2017).

2.2. Peripheral targets of OT

2.2.1. OT targeted organs and corresponding physiological functions

2.2.1.1. OT role in uterus and parturition

As mentioned earlier, OT was named after its uterotonic action. Its effect on myometrium cells has been the focus of numerous researches. The consensus is that the OTR expressed in myometrium cells is coupled to a G_q protein. Myometrium OTR activation leads to an increase in cytoplasmic calcium concentration. Calcium binds Calmodulin, which induces actomyosin complex formation resulting in cellular/tissue contraction (Arrowsmith and Wray, 2014). The importance of OT in parturition seems clear when one looks at the pattern of OTR expression in female reproductive tissues during pregnancy. In all mammalian species studied so far, there is a dramatic increase of OTR expression towards the term of pregnancy (e.g. in humans: Fuchs et al., 1984). OTR activation in myometrium results in a direct uterotonic action. But in surrounding tissues, like decidua or uterine epithelium, OTR signaling is different. It increases the production of prostaglandins through interactions with cyclo-oxygenases pathways, that are necessary for a proper delivery, as exemplified by the failure of labor in prostaglandin receptor F2-alpha KO mice and their lack of response to OT (Sugimoto et al., 1997).

The role of OT neurosecretion in the contractions of the uterus, the expulsive phase of labor, is not disputed (Blanks and Thornton, 2003). On the contrary, the importance of endocrine OT in inducing parturition is not so clear. Even if a slight increase in plasma OT is detected before the expulsive phase of parturition, it does not parallel the huge increase in OTR expression. Some authors argue that a paracrine production of OT in intra-uterine tissue is also involved (Blanks and Thornton, 2003). In human, OT mRNA increases in uterine tissues during labor, and is probably regulated by estrogen (Chibbar et al., 1995). Furthermore, OT seems not necessary for the onset of delivery *per se*. Indeed, Mice in which the OT gene has been deleted still deliver viable pups, but cannot give milk (Nishimori

et al., 1996). In humans, women with pituitary dysfunctions can still deliver (Chard, 1989), and there is no correlated change in plasma OT concentration before or during labor (Leake et al., 1981). It seems that the role of OT during pregnancy is closely linked and complementary to the one of prostaglandin F₂ α and AVP receptors pathways. Therefore, OT role in labor induction seems to be to focus the timing of the onset of labor rather than initiate delivery itself (Arrowsmith and Wray, 2014; Blanks and Thornton, 2003).

Nevertheless, OTR agonists and antagonists are in wide use in obstetrics. OT itself is often prescribed for labor induction and augmentation, and carbetocin is prescribed to prevent uterine atony and hemorrhage following caesarian section. 20 to 30% of women receive labor induction treatments, but OT alone is not recommended, and is efficient only with a concomitant prostaglandin treatment. G_q-coupled OTR antagonist atosiban is often prescribed to prevent pre-term labor, among other drugs, but its efficiency is questioned (Flenady et al., 2014). The development of new and more specific antagonists for the prevention of preterm labor is difficult. So far, the tested drugs have a poor efficacy and/or bioavailability, topped with unwanted side effects and have then been rejected for human use. One exception is retosiban, which entered phase III of clinical trials in 2015 (Arrowsmith and Wray, 2014).

2.2.1.2. *OT and male reproductive tissues*

OT presence in the male reproductive tract is variable among species. For instance, no OT mRNAs are detectable in the testes of mice (Ang et al., 1991), whereas in human, OT mRNA and OTR protein are present in the testes, epididymis and prostate (Frayne and Nicholson, 1998). OT might regulate the seminiferous contractility and modulate steroidogenesis. OT can increase the resting tone of prostate tissue in several species, including human. Furthermore, a pulse of systemic OT, probably of hypothalamic origin, has been associated with ejaculation (Gimpl and Fahrenholz, 2001; Ivell et al., 1997).

2.2.1.3. *OT, mammary tissue and milk ejection*

OT has crucial role in the milk let down in mammals, as demonstrated by the absence of milk ejection in OT KO mice (Nishimori et al., 1996). Upon stimulation by suckling of the nipples, sensory information reaches the OT neurons of hypothalamus. This induces a synchronized high-frequency bursting of those neurons lasting for several seconds. Those bursts lead to a massive release of OT in the bloodstream, which causes contraction of the myoepithelial cells in the lactiferous ducts and other mammary tissue resulting in milk let down. In lactating rats, a huge increase in OTR is observed in mammary tissue, as well as in pigs (Lundin-Schiller et al., 1996; Soloff and Wieder, 1983). Surprisingly, in human no OTR mRNA elevation is observed during lactation, and OT receptors seem

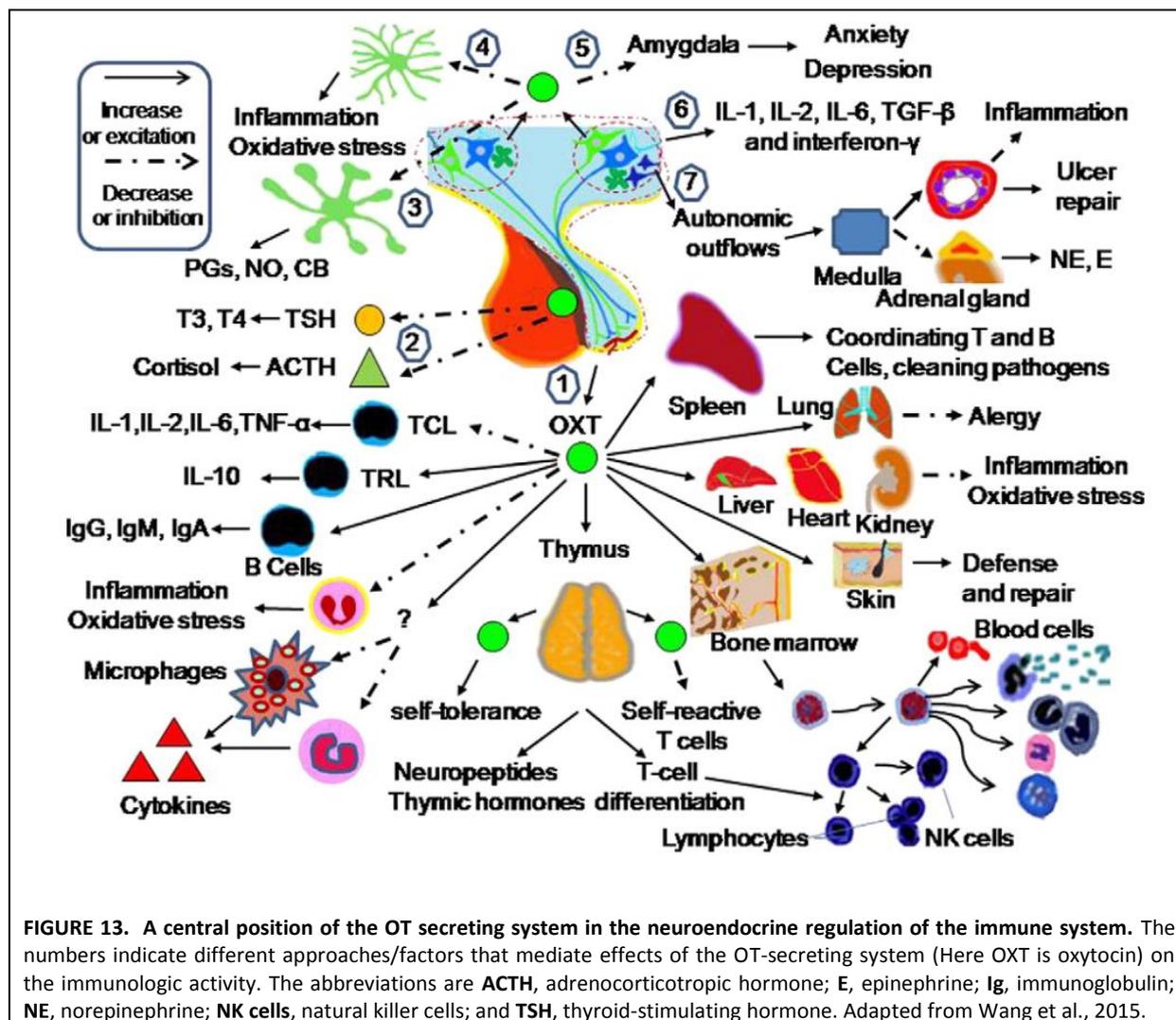
to be located more on the ductal and glandular epithelium as opposed to myometrial cells (Kimura et al., 1998). The mechanisms of bursts synchronization in OT neurons has been extensively studied, and will be discussed in part 3.2.2.

2.2.1.4. Regulation of renal functions

Intra-peritoneal injection of OT in rats causes dose-dependent increases in osmolality, natriuresis and kaliuresis (Haanwinckel et al., 1995). OTRs are expressed in the kidneys (Bichet, 1994) , and OT (and AVP) is secreted upon hypovolemia or hyperosmolarity. The level of circulating nonapetides increases as an exponential function of plasma sodium concentration (Verbalis and Dohanics, 1991). OT possesses a natriuretic effect, mainly due to a reduction in tubular sodium reabsorption (Conrad et al., 1993). Of importance, it seems that those effects of OT are not found in primates, including humans (Conrad et al., 1993).

2.2.1.5. Regulation of the immune system

OT was detected in the human thymus with a 1:1 ratio to its neurophysin and at concentrations consistent with a local production (Geenen et al., 1986). The high concentration of OT and its localization in the cytokeratin network of thymic epithelial cells led to the hypothesis that OT is used as the self-antigen of the neurohypophysial peptides family (Geenen et al., 1999). The thymic OT behaves like a cryptocrine signal in the epithelium membrane, where it can interact with pre-T cells, notably with the CD8+ cytotoxic family which express OTR (Martens et al., 1998). Moreover, the blocking of OTR receptors signaling can inhibit the differentiation of mouse thymic T-Cells (Hansenne, 2005). The OT secretion system is considered as pivotal in the neuroendocrine regulations of immune functions (Wang et al., 2015a). For instance, in case of sepsis in rats, the magnocellular OT neurons of SON linked to the neurosecretory pathway were activated and presented depleted contents of OT (Sendemir et al., 2013). In healthy men, intravenous OT can alleviate neuroendocrine and cytokine responses to bacterial endotoxin (Clodi et al., 2008). More generally, the OT secreting system can be considered as an integrative organ of the immune defense (Li et al., 2017). Wang and colleagues propose a summary scheme of the numerous roles of the OT neurosecretory system in immune functions, which can be found in FIGURE 13 (Wang et al., 2015a).



2.2.1.6. Regulation of cardiac functions

A peripheral injection of OT reduces the mean arterial pressure in rats (Pettersson et al., 1997) and is able to reduce the heart rate and the force of atrial contractions in isolated atria of rats (Favaretto et al., 1997). It seems that OT acts in concert with atrial natriuretic peptide (ANP) to mediate those actions, and cardiac tissue expresses the OTR (Gutkowska et al., 1997). The heart not only responds to OT but also produces it. It is proposed that *in situ* generated OT acts in a paracrine or autocrine manner in the heart, since the quantity measured would not allow an endocrine effect (Jankowski et al., 1998). The local action of OT in the cardiac tissue might still contribute to its natriuretic and other functions on the circulating blood volume, through ANP release stimulation (Favaretto et al., 1997; Gutkowska et al., 2014). OT also exerts an action on cardio-vascular responses to stress, but they are linked to OT central effects (Wsol et al., 2008), discussed in part 3.5.1.

2.2.1.7. Regulation of Bone tissue

Osteoblasts and osteoclasts both express the OTR and its stimulation increases the bone mass. Mice deficient in OT or OTR show impaired bone formation (Colaïanni et al., 2015). Furthermore, OT is synthesized by the bone marrow osteoblasts, under estrogen control. The effect of estrogen on bone mass is totally dependent on OTR, since estrogen injection in OTR KO mice had no effect on it (Colaïanni et al., 2014).

2.2.1.8. Pancreas

Peripherally circulating OT causes a rise in both glucagon and glucose levels in rats (Björkstrand et al., 1996) and humans (Paolisso et al., 1988). OTR is expressed by A and B cells of the islets of Langerhans of the rat pancreas (Suzuki et al., 2013) and OT (and AVP) induces glucagon release in isolated rat pancreas through specific action on its receptor (Yibchok-Anun et al., 1999).

2.2.1.9. Energy metabolism

Centrally or peripherally injected OT is known to reduce food intake (Arletti et al., 1989), and chronic peripheral OT administrations reduce weight in rats (Morton et al., 2012). Moreover in humans, anorexic subjects present higher levels of circulating OT after a meal compared to healthy subjects (Lawson et al., 2012). Adipocytes express the OTR, and OT can induce lipolysis in those cells (Yi et al., 2015) as shown *in vivo*, *ex vivo* and *in vitro* (Deblon et al., 2011). The observation that OT and OTR deficient mice develop obesity (Camerino, 2009; Takayanagi et al., 2008) adds to the numerous proofs that OT regulates energy metabolism, via both central and peripheral actions. In human, decreased levels of circulating OT have been observed in obese and type two diabetes mellitus patients (Qian et al., 2014) which makes the peripheral (and central) OT system a new target for therapeutic development against obesity and some forms of metabolic diseases (Altirriba et al., 2015; Chaves et al., 2013; Deblon et al., 2011).

2.2.2. OT action on the peripheral somatosensory system: focus on nociception

As detailed below in 2.2.3, OT does not cross the blood-brain barrier (BBB). Therefore, one can attribute effects of peripherally administered OT on the peripheral somatosensory system to non-central effects. Nociception is one of the fundamental roles of the somatosensory system. Nociceptors are the sensory cells that detect painful stimuli. Their cell bodies are located in the dorsal root or trigeminal ganglia.

OT has clear anti nociceptive and analgesic effects through various actions in the CNS, which will be discussed in part 3.5.9. But when injected intraperitoneally or intravenously in rats and mice, OT also possesses OTR mediated anti-nociceptive effects (Kang and Park, 2000; Lundeberg et al., 1994). Some studies indicate that OT acts at the level of the nociceptors cell bodies. Indeed, OTR is not expressed in the skin nociceptive terminals, but is restricted to the cell body of non-peptidergic C-fiber neurons, located in the dorsal root ganglion (DRG) (Moreno-López et al., 2013). Acutely dissociated DRG cells from rats show an inhibition of the depolarization induced increase in intracellular calcium upon application of OT. Those cells were also capsaicin sensitive, which is indicative of their nociceptive nature, and OT effect was dependent on OTR activation (Hobo et al., 2012). Yang and colleagues provided proof that OT could inhibit ATP evoked currents in freshly isolated DRG neurons, and that this effect involved the activation of the cAMP-PKA and Ca^{2+} pathways through OTR activation (Yang et al., 2002). Another study in cultured DRG neurons from adult rats showed that OT decreases membrane excitability and increases outward current and evoked membrane hyperpolarization in small DRG neurons (putative nociceptors). They further showed that OT produces an increase in intracellular calcium in those capsaicin responsive neurons and that the electrophysiological effects of OT were mediated by the Ca^{2+} /nNOS/NO/ K_{ATP} pathway. Immunohistochemistry indeed showed colocalization of OTR and neuronal nitric oxide synthase (nNOS) in cultured DRG cells classified as nociceptors (Gong et al., 2015).

In trigeminal nociceptive neurons of newborn rat, OT causes analgesia by reducing the GABA depolarizing action and evoked calcium transients (GABA being depolarizing in newborn) (Mazucca et al., 2011). Another study shows that OTR is also present in neuron of the trigeminal ganglion in adults, and colocalized with calcitonin gene-related peptide (CGRP), a marker of putative nociceptive neurons. OTR expression was upregulated when rats underwent either electrocutaneous stimulation or adjuvant induced inflammation. OT application reduced the capsaicin induced CGRP release from the dural nociceptors, an effect likely to induce analgesia *in vivo* (Tzabazis et al., 2016).

Similar results were obtained in mesenteric afferents, the nociceptors of the intestine. In *ex vivo* segments of jejunum, the discharges of mesenteric afferents were recorded. Nociceptive stimuli were evoked using a rise in intraluminal gut pressure, provoking distention of the intestine, or through pharmacological means using bradykinin applications. OT applications reduced the bradykinin induced increase in firing in identified mesenteric nociceptors. The same inhibitory effect of OT was observed on the increased firing in recorded units due to high pressure “painful” distension. Further pharmacological experiments revealed that OT effect was mediated by activation of the nNOS/NO/ K_{ATP} pathway in mesenteric afferent (Li et al., 2015). *In vivo*, visceromotor response to urinary bladder distension is decreased in lactating rats. The same effect is observed when OT is

injected intraperitoneally in non-lactating female rats, and not when it is administered with an OTR antagonist (Black et al., 2009). Another proof of the peripheral OT antinociceptive role in visceral pain is given by de Araujo and colleagues. They showed an up-regulation of OTR in nociceptors innervating the colon in mice suffering from chronic abdominal pain. Using a synthetic analog of OT administered directly in the colon, they demonstrated that OTR activation potently inhibited colonic nociceptors, both *in vitro* and *in vivo* (de Araujo et al., 2014).

Nevertheless, there is a strong (non-published) debate regarding the specificity of OTR antibodies used in those studies (and others), and some of the effects mentioned above could be in part mediated by V_{1A} AVP receptor. As mentioned above (see "ligand binding"), OT has a physiologically relevant affinity for this receptor. Furthermore, even if OTR was reported to be expressed in as high as 60% of rat DRG neurons (Ayar et al., 2014), another study found only weak expression of OTR mRNA in the mouse DRG (Schorscher-Petcu et al., 2010). Using OTR or V_{1A} R-KO mutant mice, a surprising result was observed when measuring the effect of systemic OT injection on pain thresholds using several tests. OT had the same analgesic effects in OTR-KO and wild type (WT) animals. On the other hand, this OT induced analgesia was totally absent in V_{1A} R-KO mice. This result was further confirmed by using V_{1A} R antagonist in WT animals, which fully prevented OT induced analgesia, as opposed to OTR antagonist. These results are strengthened by the presence of V_{1A} R and not OTR mRNAs in dorsal root ganglia (Schorscher-Petcu et al., 2010). Further studies corroborate those findings. In a model of orofacial mechanical hypersensitivity induced by infraorbital nerve injury, OT injected in the trigeminal ganglion did alleviate pain. OT also has "inhibitory" electrophysiological effects on cultured trigeminal ganglion neurons: Increased rheobase, hyperpolarized membrane and increased voltage gated potassium currents. As in the previously cited study, OT analgesic effect was blocked only when a V_{1A} R antagonist and not an OTR one was injected in the trigeminal ganglion (Kubo et al., 2016). A possible mechanistic explanation of the role of V_{1A} R activation by OT is that it decreases the amplitude of acid sensing ion channels (ASICs) currents. ASICs participate in nociception in peripheral sensory neuron and are key players in the pain evoked, for instance, by acetic acid injection in the skin. Qiu and colleagues demonstrate that, *in vitro*, OT diminishes the amplitude of ASICs currents and that this effect is, as previously shown, blocked only in presence of a V_{1A} R antagonist and not an OTR one. Furthermore, OT altered the acidosis-evoked increase in membrane excitability in patch-clamped DRG neurons of rats, thus inhibiting cell firing upon acidic stimuli. *In vivo*, both OT and AVP relieve the nociceptive response induced by acetic acid injections in rats paw, an effect also reported on WT mice, but not in V_{1A} R-KO strains (Qiu et al., 2014).

A possible mechanistic explanation resides in the various amount of peripheral OT. Juif and Poisbeau showed that while measuring nociceptive pain processing by recordings of spinal wide dynamic range

neurons, a low physiological intravenous dose of TGOT had an antinociceptive effect. The same antinociceptive effect was reached with AVP, but with a dose a hundred times higher. With much higher doses, pro-nociceptive effects were obtained for both peptides. When OTR or V_{1A}R antagonists were injected with the anti-nociceptive dose of TGOT or AVP, anti-nociceptive effects were abolished for both agonists only with the OTR antagonist. In contrast, at pro-nociceptive dose, only the V_{1A}R antagonist blocked the proalgesic effects of both TGOT and AVP. To test the physiological relevance of those mechanisms, the authors used a paradigm known to induce an endogenous release of OT producing an analgesic effect, the stress-induced analgesia by the forced swim test. Using von Frey tests after a forced swim stress, they assessed the mechanical threshold for pain. Through intra venous injection of OTR or V_{1A}R antagonist, they show that the analgesia induced by the forced swim was reduced only if the OTR antagonist was injected, and not the V_{1A}R one (Juif and Poisbeau, 2013). A mechanistic explanation could arise from the work of Tan and colleagues. They showed that low concentrations of OT enhanced GABA evoked currents in DRG neurons of rats, resulting in suppression of primary sensory transmission, whereas higher dose reduced the pre-synaptic GABA mediated inhibition (Tan et al., 2000). In all cases, OT acts as an analgesic when released or administered in the periphery in concentration close to physiological range, but the debate is still open regarding the receptor(s) it targets.

Human data corroborates animal ones regarding an analgesic role of OT on nociceptors in the periphery. Patients suffering from chronic pain present low endogenous OT. It is the case for children suffering from recurrent abdominal pain (Alfvén, 2004; Alfvén et al., 1994) and adults suffering from low back pain (Yang, 1994). A study in healthy women revealed that low levels of plasma OT are associated with a reduced pain threshold for noxious cold and ischemic stimuli (Grewen et al., 2008). But those observations cannot exclude a possible participation of central OT. A more convincing proof of peripheral OT role in nociception modulation is that when continuously infused intravenously, OT increased thresholds for colonic distention pain in adults with irritable bowel syndrome (Louvel et al., 1996).

In conclusion circulating OT clearly modulates nociception in the periphery, by acting on sensory neurons at the level of the cell bodies. Nevertheless, The exact role of the receptor(s) involved remains elusive (González-Hernández et al., 2014).

2.2.3. Modes of exogenous OT administration

There are several independent researches showing that OT do not easily cross the BBB (Ermisch et al., 1985; Kang and Park, 2000). Mens and colleagues estimate that 0.002% of intra-cutaneously

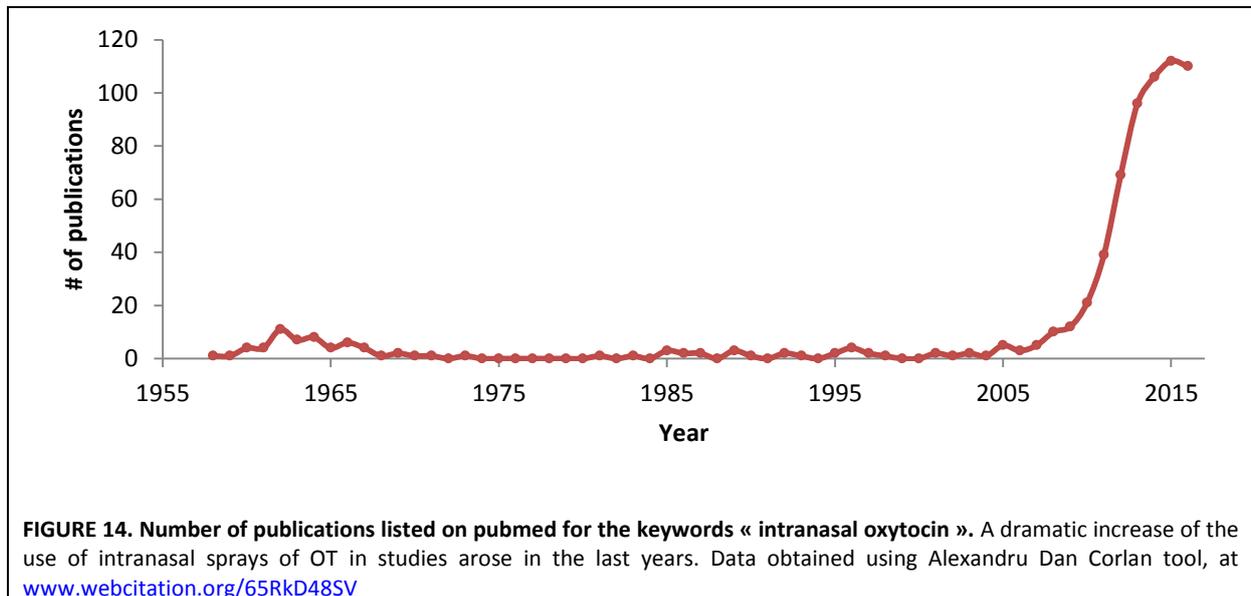
injected OT penetrates the brain (Mens et al., 1983). Levels of OT in human pituitary are estimated to be around 28 µg, whereas circulating levels of OT are estimated to be between 1 to 10 pg/mL (Leng and Ludwig, 2016). Nevertheless, another route of administration of OT can potentially reach the brain: intranasal application through sprays. Publications about the use of intranasal application of OT can be found as early as 1958, in which it was tested as a mean to facilitate milk let-down in women (Newton and Egli, 1958). Once administered intra-nasally, OT can reach the blood circulation through mucous membrane, but it is thought that it also penetrates the brain. The first postulate is that OT is internalized into olfactory or trigeminal neurons, transported through axons and released centrally through exocytosis (Leng and Ludwig, 2016). This postulate was rejected by Born and colleagues, since this phenomenon would require hours, which is in contradiction with the increased concentration of peptides found in CSF of healthy subjects 30 minutes after intranasal administration. They also argue that internalized peptides would get degraded (Born et al., 2002). Another, extracellular, route is more likely. Peptides, including OT, might pass through the intercellular clefts in the olfactory epithelium, and further diffuse in the subarachnoid space (Illum, 2000). One should note that Born and colleagues tested penetration in CSF of AVP and that OT was not part of their protocol. Another study measured CSF OT content in patients to which 24 IU of OT was given intranasally. No increase in OT was found after either 45 or 60 minutes, and only three out of eleven patients showed a higher than controls level of OT in CSF after 75 minutes (Striepens et al., 2013). Another study in macaques measured an increase in OT content in CSF after intranasally applied high dose of OT, but Leng and Ludwig highlight that this reflected the passage of only 0.002% of the applied dose, similarly as intra-cutaneous injections (Dal Monte et al., 2014). Overall, Leng and Ludwig argue that in seven studies they review, from rat to human, the content of OT applied intranasally was higher than the total content in pituitary but only 0.005% of it could reach the CSF, at most. In fact, intra-nasal OT administration induces a much more dramatic change in the OT blood levels (Leng and Ludwig, 2016). Nevertheless, since very high quantities are given in all human studies using intranasal OT delivery, the tiny amount that penetrates the CSF might still account for the various observed effects. Leng and Ludwig report that when OT is injected i.c.v in animal studies, 1 ng was the lowest dose that induced any behavioral effect, whereas 2 ng are needed to induce *c-fos* expression in areas known to express the OTR. In one study, 1 µg of OT applied intranasally in rats produced no activation in any regions of the forebrain, or in the olfactory bulb, one of the supposed primary targets of such a mode of administration. In line with this observation, no behavioral changes followed this intranasal OT administration (Ludwig et al., 2013).

Therefore, the most dramatic pharmacological effect of intranasal OT application is an increase in plasma concentration far above physiological levels, for instance a hundred fold increase in macaque

(Modi et al., 2014). At such high dosage, OT will induce many peripheral effects, for instance it modifies heart rate variability in humans (Kemp et al., 2012), and could possibly also act on the AVP receptor V_{1A} . Peripheral effects of intra-nasally administered OT might then have dramatic effects on several organs, as discussed above, which could provoke behavioral changes, a fact which is never or rarely considered in studies using intranasal OT, which usually infer that the observed effects are due to a central action of OT (Leng and Ludwig, 2016).

The number of studies using intranasal OT administration to tackle the role of OT in cognitive and behavioral processes in humans and other species has dramatically increased over the last years, as shown in FIGURE 14. But in view of the various critical points discussed in this part, the extent to which the observed effects might be due to a direct modulation of cerebral circuits by the intranasally administered OT have to be taken with caution (Leng and Ludwig, 2016).

In conclusion, endogenous OT released in the periphery by the pituitary, or synthesized locally in the periphery, acts only on its peripheral receptors, and does not reach the central nervous system. Therefore, one can consider that OT effects on various CNS regions are produced only by OT originating from inside the CNS, as will be discussed below.



3. OT as a neuromodulator

3.1. OT neurons of the hypothalamus: OT nuclei and OT neurons subtypes

Two distinct cell types in the hypothalamus produce and release OT: magnocellular and parvocellular neurons. The consensus states that magnocellular neurons mainly project to the posterior pituitary and underpin the neuroendocrine actions of OT, while parvocellular neurons project centrally to modulate the activity of other neuronal circuits, or release products either in the portal circulation in the anterior pituitary gland and directly onto blood vessels elsewhere in the brain (McEwen, 2004).

3.1.1. Cytoarchitecture of OT nuclei in rats

3.1.1.1. *PVN*

The PVN is subdivided into eight sub-regions, three containing mainly magnocellular neurons, five the parvocellular ones. Swanson and Kuypers describe in those words the organization of cell clusters in the PVN: “The PVN in the rat can be viewed most simply as three dense clusters of magnocellular neurons embedded in a larger shell of parvocellular neurons that consists of five components” (Swanson and Kuypers, 1980). Some authors further subdivide it to reach a total of eleven subdivisions (Simmons and Swanson, 2008). FIGURE 15 shows a 3D view from the surface of PVN, FIGURE 16 a scheme of coronal sections superimposed on Nissl stainings. Both figures are adapted from Simmons and Swanson, 2008.

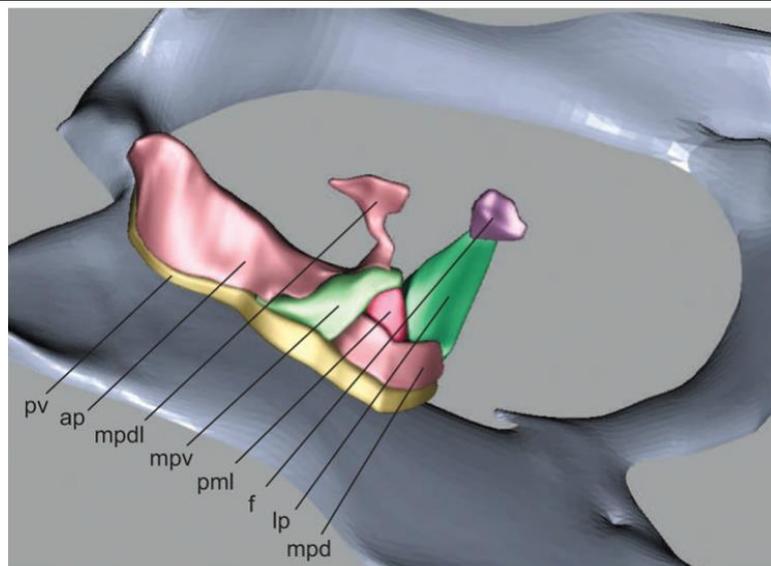


FIGURE 15. 3D model of the PVN surface. The bottom right corner points toward dorsal brain regions, upper right corner toward lateral brain regions. Abbreviations: **ap**, anterior parvocellular; **f**, forniceal; **lp**, lateral parvocellular; **mpd**, dorsal zone of medial parvocellular; **mpdl**, lateral wing of mpd; **mpv**, ventral zone of medial parvocellular; **pml**, lateral zone of posterior magnocellular; **pv** periventricular part. Adapted from Simmons and Swanson, 2008.

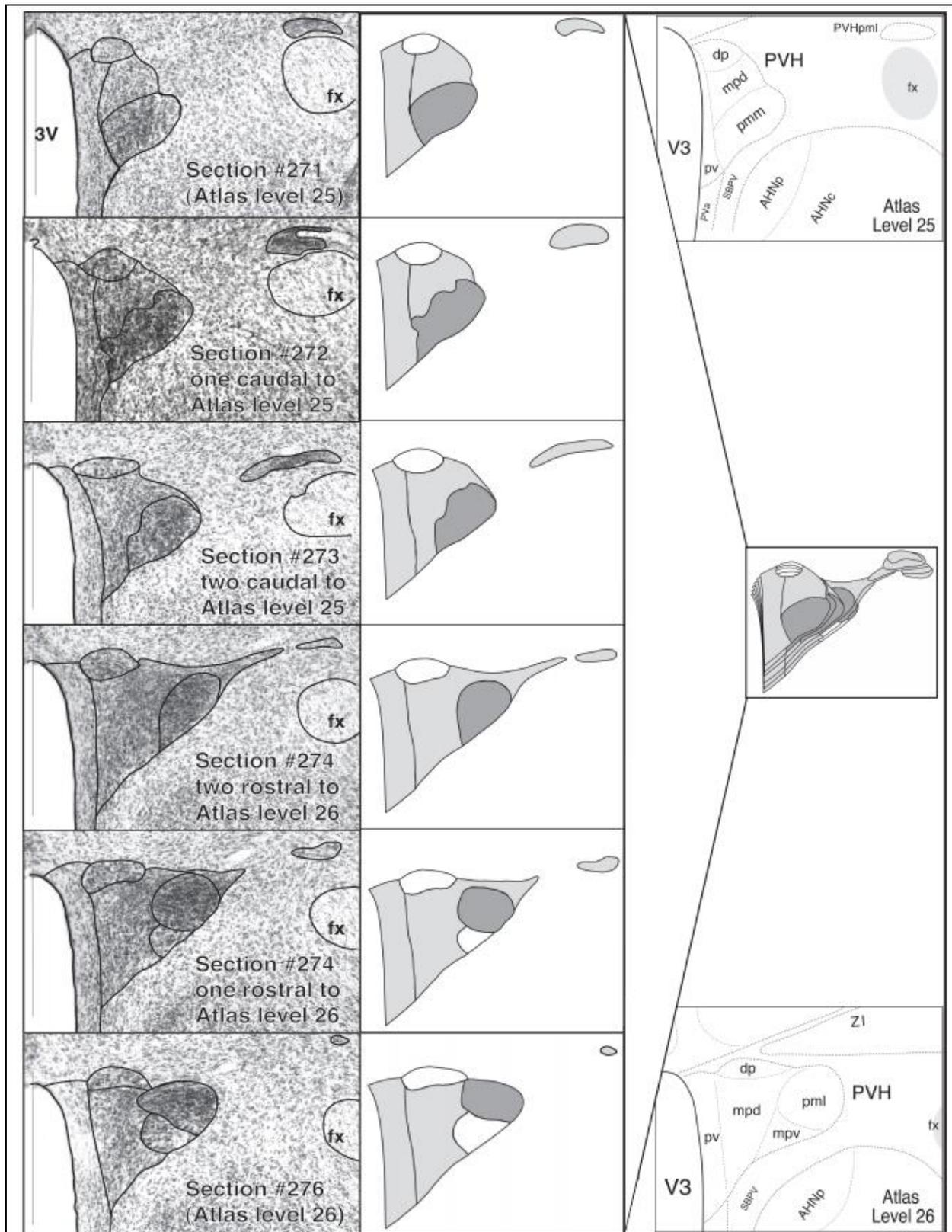


FIGURE 16. PVN subdivision outlines from Nissl coronal serial sections from « Brain Maps » atlas. Middle panels show in light gray the parvocellular neuroendocrine functional compartments, in dark gray, the magnocellular neuroendocrine compartment, in clear/white the parvocellular descending non endocrine functional compartment. On the middle right, a stack of coronal sections is proposed. Abbreviations: **dp**, dorsal parvocellular part; **mpd**, dorsal zone of medial parvocellular part; **mpdl**, lateral wing of mpd; **mpv**, ventral zone of medial parvocellular part; **pml**, lateral zone of posterior magnocellular part.; **pmm** medial zone of posterior magnocellular part; **pv** periventricular part. Adapted from Simmons and Swanson, 2008.

3.1.1.2. SON

A major difference between PVN and SON is the absence of parvocellular cells in the SON, which consists almost exclusively of magnocellular neurons. It was demonstrated that virtually every cell of the SON projects to the posterior pituitary. For instance, the uptake of retrograde tracers placed into the neurohypophysis resulted in the back-filling of every cell in the SON (Hatton, 1990).

3.1.1.3. AN

It is estimated that the accessory nuclei, located between the PVN and the SON, contain about 1/3 of all OT magnocellular neurons, and are an important source of forebrain projections from those cells (Knobloch et al., 2012; Rhodes et al., 1981). Six accessory nuclei have been described: the anterocommissural, circular, forniceal, dorsolateral, ventrolateral (or nucleus of the medial forebrain bundle) and extra-hypothalamic (see 3.1.4) (Grinevich and Polenov, 1998; Knobloch and Grinevich, 2014). A scheme summarizing the different groups of cells composing the AN can be found in the scheme for advanced vertebrates, in FIGURE 4.

3.1.1.4. OT vs AVP cells: distribution

As mentioned earlier, OT and AVP expressing cells of the hypothalamus are more or less segregated, as shown in FIGURE 12. For instance in the PVN, the anterior and medial magnocellular divisions are composed almost exclusively of OT cells, when more or less equal numbers of OT and AVP cells are concentrated in the different subdivisions of the posterior magnocellular division of PVN (Swanson and Sawchenko, 1983). AVP and OT neurons of the SON are not fully segregated, but AVP magnocellular neurons are more concentrated in the posteroventral portion of the SON, while the OT magnocellular neurons reside mostly in the anterodorsal portion (Swaab et al., 1975; Vandesande and Dierickx, 1975). An image depicting localization of OT and AVP cells in SON (and PVN) can be found in FIGURE 12.

3.1.2. Parvocellular neurons project into the CNS

Parvocellular neurons project to distinct brainstem nuclei and different regions of the spinal cord (SC) (Sawchenko and Swanson, 1982; Swanson and Sawchenko, 1983). They target: the preganglionic neurons of the parasympathetic centers in the caudal medulla, including the nucleus tractus solitarii, the dorsal motor nucleus of the vagus and the rostral ventrolateral medulla (Swanson, 1977; Swanson and Kuypers, 1980). At the level of the SC, they target the sympathetic centers at the thoracolumbar and sacral segments, in the intermediolateral cell column (Jójárt et al., 2009; Palkovits, 1999; Rinaman, 1998). Furthermore, some parvocellular neurons seem to target ventricular sites where they may release substances in the CNS, and onto blood vessels, where they

might influence vascular dynamics. Parvocellular neurons also project inside the hypothalamus. Projections can be found in the external zone of the medial eminence, where OT and AVP are secreted in the portal circulation where they further promote the release of pituitary hormones (Gimpl and Fahrenholz, 2001; McEwen, 2004). Finally, we found a small sub-population of parvocellular OT neurons projecting both to the SC and onto magnocellular OT neurons in the SON (see ARTICLE 1).

3.1.3. Magnocellular neurons are not just neurosecretory cells

The presence of OT fibers arising from magnocellular neurons in forebrain regions had already been described by the pioneering research on the neuroendocrine systems. OT containing axons have been observed in the amygdala, hippocampus, bed nucleus of stria terminalis (BNST) and septal nuclei of rats, among others (Buijs, 1978; Sofroniew, 1980). Those are not specific to the rat, as there are similar findings in human (Fliers et al., 1986) and non-human primates (Wang et al., 1997). Recent studies using fluorogold and viral based techniques to trace OT projections from magnocellular neurons revealed much more magnocellular OT fibers in brain tissue. Those studies demonstrated that magnocellular OT neurons from the PVN, AN and to a lesser extent SON innervate extensively forebrain regions, at least in voles, mice and rats (Knobloch et al., 2012; Mitre et al., 2016a; Ross et al., 2009).

3.1.4. Non-hypothalamic OT neurons?

As described above, OT neuron cell bodies are found in three nuclei of the hypothalamus: PVN, SON and AN. This might not be entirely true, as one study found OT mRNA in other brain regions such as the BNST, among others (Chung et al., 1991). Some cell bodies immunoreactive for OT or the OT neurophysin were found in the triangular nucleus of the septum and BNST in rats (Ingram and Moos, 1992; Sofroniew and Weind, 1978). However, the precise role of those cells is poorly understood nor studied (Kiss and Mikkelsen, 2005).

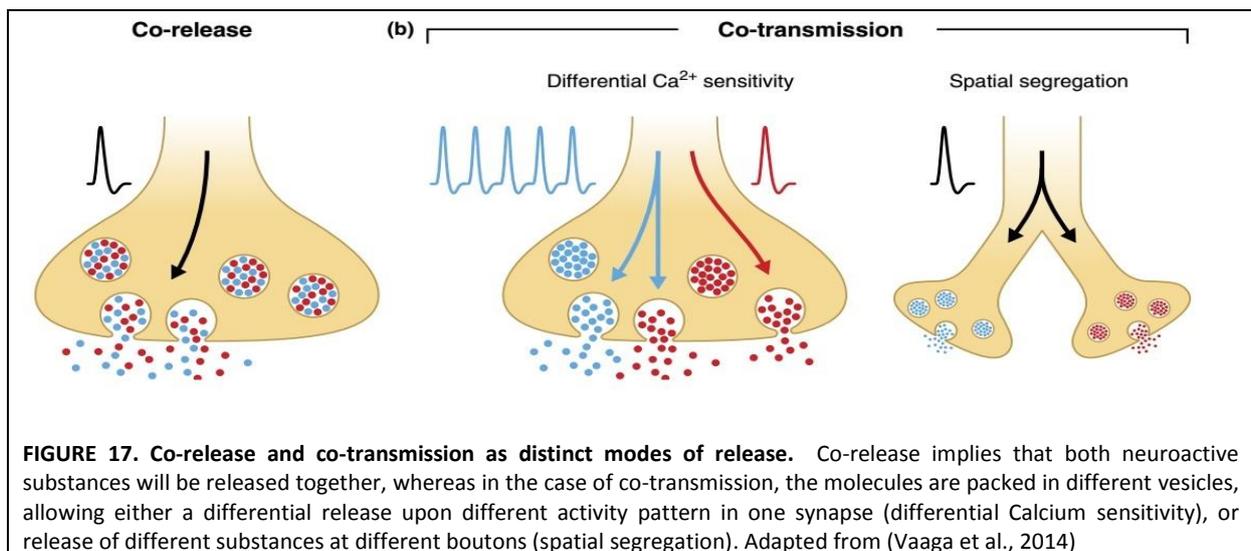
3.1.5. Do OT neurons only release OT?

No, they do not. OT magnocellular neurons also express various active substances, namely:

- Neuropeptide Y (Larsen et al., 1993).
- Tyrosine hydroxylase (Skutella et al., 1993).
- Dynorphin (Eriksson et al., 1996) and pro-enkephalin-A derived μ -opioid peptide fragments (Martin et al., 1983).
- Corticotropin releasing hormone (CRH) (Pretel and Piekut, 1990).
- Thyrotropin-releasing hormone (Tsuruo et al., 1988).

- Atrial natriuretic factor (Jirikowski et al., 1986).
- Galanin (Landry and Hökfelt, 1998; Landry et al., 1991).
- nNOS (Xiao et al., 2005).
- And others ...

OT neurons also express glutamate transporters (Hrabovszky and Liposits, 2008; Ponzio et al., 2006) and seem to release glutamate when stimulated (Knobloch et al., 2012). Furthermore, neurosecretory endings in the hypophysis include micro-vesicles which contain glutamate and are distinct from the LDCVs (Meeker et al., 1991; Navone and Di Gioia, 1988). It is then very likely that OT cells can release several bioactive molecules. The coexistence in one cell of neuropeptide(s) with more “classical” small neurotransmitters like GABA or glutamate is known since the 1980s, with neuropeptides and small neurotransmitters packed in different vesicles in terminals and which release termed “co-transmission”. An interesting hypothesis is that the release of neuropeptides from the LDCVs require a burst of actions potentials, as opposed to the small neurotransmitter vesicles that can undergo exocytosis with one action potential (Albers, 2015; Hökfelt, 1991). This means that depending on the cell activity, its effect on the post-synaptic (or nearby cells in case of non-synaptic release) neuron will differ, allowing a fine level of regulation of neural circuits in an activity dependent manner. Example of packaging of different neurotransmitters into the same vesicle has also been described and termed “co-release” as the exocytosis of the vesicle release both neuroactive substances together (Vaaga et al., 2014). A comparison of both modes of co-transmitters release is proposed in FIGURE 17. There are only very few data regarding the mechanism of co-release or co-transmission by OT neurons of OT and other neuroactive substances, peptidergic or not, so those mechanisms remain elusive.

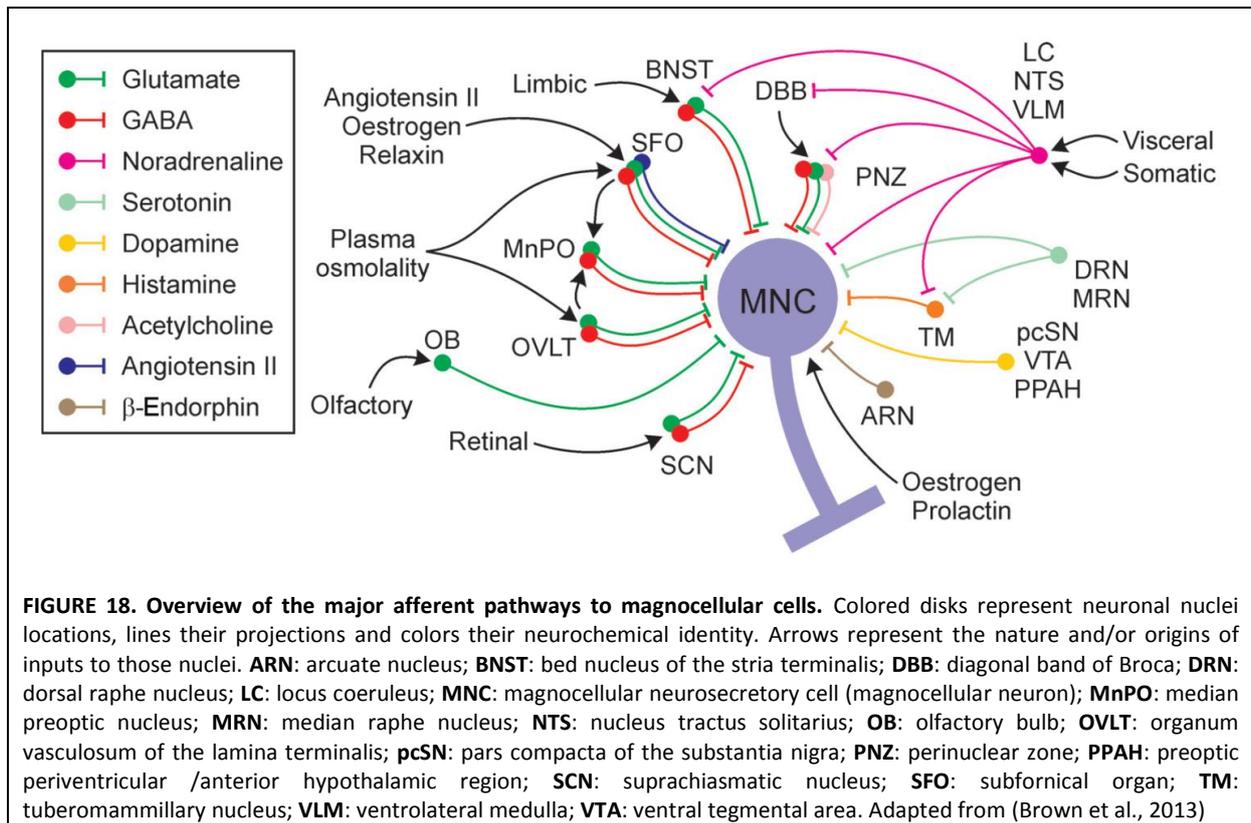


3.2. Afferents of OT nuclei and inter-/intra-nuclei connectivity

3.2.1. OT nuclei afferents

3.2.1.1. *Distal afferents to OT nuclei and their functions*

Magnocellular neurons receive numerous afferent inputs from various brain regions and the periphery, as depicted in FIGURE 18.



Next I will briefly explain some functions of those afferents and the physiological functions they modulate, through references found mostly in the reviews of Colin H. Brown (Brown, 2016; Brown et al., 2013).

Osmoregulation by extra hypothalamic structures (see FIGURE 18 for nuclei abbreviations)

The MnPO, OVLT and SFO relay information concerning the osmotic status of the organism to the magnocellular neurons. This was proven through lesions of those nuclei, which reduce osmotically-induced increase in firing of magnocellular neurons, leading to reduced OT and AVP release upon an hyper osmotic stimulation (Leng et al., 1989). By increasing neurosecretion of AVP (and OT in rats) those regions promote natriuresis and antidiuresis, but they also modulate thirst via projections to different cortical areas (Brown et al., 2013). Of importance here, magnocellular neurons are

themselves directly osmosensitive. The details of those mechanisms will not be discussed here, see Choe et al., 2016; Prager-Khoutorsky and Bourque, 2015 for more details.

Cardiac, reproductive and visceral inputs to OT cells are relayed by noradrenergic inputs.

Noradrenergic inputs to the magnocellular neurons can also relay information about the osmotic status of the body (Bourque, 2008), but noradrenergic inputs relay mostly information about the cardiovascular system through the NTS. Those inputs are also linked to the transmission of reproductive, somatosensory and gastrointestinal sensory information, which further modify magnocellular neuron activities (Leng et al., 1999). The NTS seems to project directly onto OT neurons (Raby and Renaud, 1989), and a further proof comes from the fact that *in vivo* OT neurons are under a noradrenergic tone, since they are inhibited by intra SON injection of α_1 receptor antagonist (Brown et al., 1998). One should note that various neuropeptides are co-expressed by NTS noradrenergic neurons, and some NTS neurons projecting to the magnocellular neurons do not express noradrenaline and are neuropeptidergic (Brown et al., 2013). Uterus and vagina afferents also terminate in the NTS (Ortega-Villalobos et al., 1990) and noradrenaline release in OT nuclei is increased during late pregnancy and lactation, where it promotes OT release (Bealer and Crowley, 1998; Lipschitz et al., 2004). This might be a necessary factor for the emergence of coordinated bursting activity of OT neurons during lactation and parturition through direct effect or through actions on other nuclei like BNST (Bealer et al., 2010; Brown et al., 2013). See part 3.2.2 for more details on the coordinated bursting activity during lactation. The gastrointestinal peptide cholecystokinin (CCK) can also directly activate OT cells (Chakfe and Bourque, 2000), but its action *in vivo* might arise from its effect on gastric vagal afferents that express CCK receptors. Indeed, peripheral CCK effect on OT cells is blocked via iontophoresis in SON of an α_1 receptor antagonist, and is mediated by the NTS, since its neurons present *c-fos* expression after peripheral CCK administration (Onaka et al., 1995a; Ueta et al., 1993; Zhu and Onaka, 2002).

Serotonergic inputs

Serotonergic inputs to OT neurons seem sparse and mostly indirect, targeting the surrounding regions of OT nuclei (Vertes et al., 1999), and their role seems to not be primordial. Nevertheless, as for noradrenergic inputs, CCK injections increase serotonin release in SON, which may then also act as a relay of visceral information (Onaka et al., 1995b).

Dopaminergic inputs

Magnocellular neurons receive dopaminergic inputs from the A9, A10, A14 and A15 cell groups (Cheung et al., 1998; van Vulpén et al., 1999). Dopamine depolarizes magnocellular neurons through

direct activation of D2 receptor expressed on magnocellular neurons and indirectly through pre-synaptic D4 receptors expressed in GABAergic and glutamatergic cells (Azdad et al., 2003; Price and Pittman, 2001; Yang et al., 1991). This indicates a fine regulation of circuits controlling magnocellular neurons by dopamine. Furthermore, dopamine might have a role in controlling OT cells during parturition, as its release is increased during late pregnancy (Herbison et al., 1997).

Histaminergic inputs

Histaminergic inputs arising from the tuberomammillary nucleus innervate magnocellular neurons and contribute to delivery at parturition (Luckman and Larsen, 1997). Histamine direct effect on OT cells seems inhibitory, whereas central histamine administration increase the release of OT in the circulation (Brown et al., 2013) and stimulation of central histamine receptors is necessary for suckling-induced OT secretion (Bealer et al., 2010).

Endogenous opioids

OT cells are innervated by opioidergic fibers originating in the arcuate nucleus. During pregnancy OT cells appear to be under opioid inhibition, probably to avoid premature labors due to OT release in case of stress like inflammation (Brunton and Russell, 2008; Brunton et al., 2006). Such inhibitory opioid actions are mediated both by a direct action on OT cells and an indirect one through inhibition of noradrenergic inputs (Onaka et al., 1995b; Ruan et al., 2011)

Suprachiasmatic inputs

Magnocellular neurons of the SON receive inputs from the suprachiasmatic nucleus, both by glutamatergic and GABAergic afferents (Cui et al., 1997). Indeed, both OT and AVP are secreted following a circadian rhythm (Forsling et al., 1998).

Olfactory inputs

Magnocellular neurons receive direct glutamatergic excitatory projections from the main and accessory olfactory bulb (Bader et al., 2012; Smithson et al., 1992) and might be of importance in the regulation of social and maternal behaviors, in rodents at least.

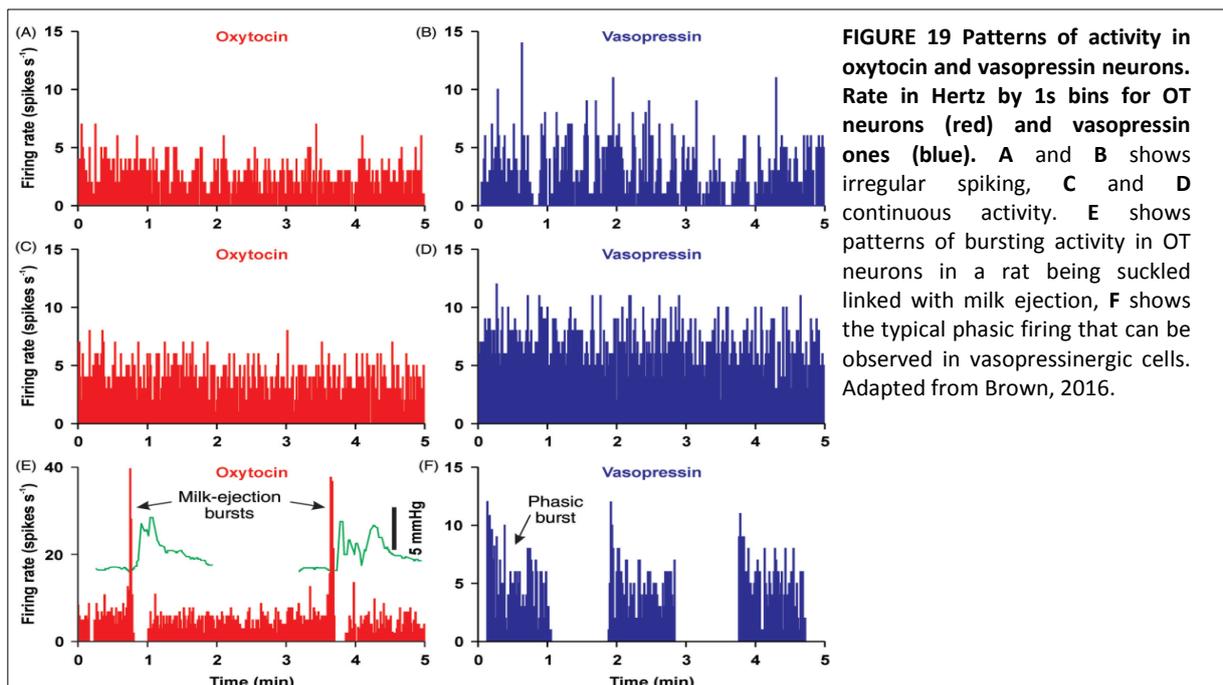
3.2.1.2. Local afferents and their functions

Neuroendocrine cells of the SON and PVN receive several local inputs from inside the hypothalamus. The perinuclear zone, dorsal to the SON, is a rich source of both glutamatergic and GABAergic innervations (Brown et al., 2013). Many axons from the perinuclear zone enter directly into SON, while some contact the PVN cells, both magno- and parvo-cellular (Armstrong and Stern, 1997;

Roland and Sawchenko, 1993), which might in part explain the inter OT nuclei activity coordination (see 3.2.2). The PVN also receive many glutamatergic and GABAergic projections from the anterior hypothalamus, BNST, medial preoptic area, perifornical nucleus and dorsomedial hypothalamus (Boudaba et al., 1996, 1997). The role of such local connections might be dual. The recorded patterns of synaptic potentials of those local inputs are stochastic in nature. A proposition is that they may generate a background noise against which extra hypothalamic inputs might be enhanced, through stochastic resonance (Brown et al., 2013). But inputs from “local” cells might also serve as a relay of distant inputs, as shown for noradrenergic inputs from the brainstem (see FIGURE 18) (Boudaba and Tasker, 2006; Daftary et al., 1998). Local excitatory relay of distant signals might also serve as an amplifier of those signals, for instance stimulation of perinuclear cells induces multiple excitatory post-synaptic currents (EPSCs) in SON cells (Boudaba et al., 1997). Local neuromodulation might also occur, since some cells in the perinuclear zone produce acetylcholine (Mason et al., 1983). Furthermore, magnocellular neurons of the SON express the nicotinic receptor and stimulation in the perinuclear network evokes synaptic potentials linked to its activation (Hatton and Yang, 2002; Zaninetti et al., 2000), as does exogenous application of a muscarinic receptor agonist (Luckman et al., 1994). PVN neurons are also directly stimulated by nicotinic receptor activation (Zaninetti et al., 2002).

3.2.2. Intra- and inter-OT nuclei connections: the case of synchronized bursting of OT cells

OT and AVP magnocellular neurons present various patterns of activity; they either fire regularly, irregularly or in bursts. The FIGURE 19 presents rate histograms of those different patterns of activities, measured in anesthetized rats.



Phasic bursting in AVP cells

The generation of phasic burst in AVP neuron has been the subject of many researches, but will not be discussed here. More details can be found in the review of Colin H. Brown (Brown, 2016). The overall physiological consequence of the patterns of activity of AVP cell is that even if the secretion at the level of individual cells is pulsatile, there is no apparent coordination of those bursts across the cell population, leading to the generally smooth, non-pulsatile, profile of AVP levels in the circulation (Leng et al., 2008)

Synchronized bursting of OT neurons during lactation

As mentioned earlier, circulating OT concentration increases gradually towards the end of pregnancy, but induce contraction of the uterus only when a sufficient number of OTR are expressed by myometrial cells (among others). Upon contraction of the uterus, a positive feedback is provided to OT neurons, leading to further increase in oxytocin secretion, named the “Ferguson reflex”. It seems that OT neurons respond to the uterine contraction itself and not its distension, since OT neurons firing rate is correlated with the increase in intra-uterine pressure and not uterine distension. As mentioned above, the sensory information about the pressure of the uterus is relayed mostly by noradrenergic afferents (Douglas et al., 2001). The bursts observed during birth and suckling last several seconds, in which the firing rate of OT cells reaches 100 Hz, compared to the 0-10 Hz basal frequency. Those bursts usually happen 5 to 10 minutes apart, and are immediately followed by a silent period. One primordial property of those bursts is that, as opposed to AVP neuron, the OT neurons bursts are coordinated across the all OT cells population, in both the PVN and SON, as proved more than 30 years ago by paired extracellular recordings during lactation (Belin et al., 1984). Those synchronized bursts lead to the release of a massive amount of OT in the systemic circulation. This triggers either rhythmic contractions of the uterus, or milk ejection from the breasts. The primordial role of OT cells synchronized bursts in the induction of contractions of the milk ducts or uterus is further proven by the fact that morphine, which silences OT neurons (Pumford et al., 1993; Ruan et al., 2011), can delay parturition (Luckman et al., 1993) and milk ejection reflex (Clarke et al., 1979). This effect can be rescued by repeated, not sustained, OT administrations that mimic the pulsatile secretion of OT (Luckman et al., 1993; Pumford et al., 1993).

OT neuron bursts are linked to sensory afferents. For instance, the burst firing in lactating dams occurs only during suckling *in vivo*, with a correlation between the number of nipples suckled and the number of action potentials in each burst (Lincoln and Wakerley, 1975). If the dependency to sensory afferents of burst generation is clear, this not the sole mechanism involved. Indeed, bursts can be generated in brain slices from male rats with a combination of a low calcium medium and an α_1

adreno-receptor agonist (Wang and Hatton, 2005). This implies that burst firing is an intrinsic property of OT neurons, gated by the appropriate afferent stimulus. We saw above that OT neurons receive synaptic inputs from GABA and glutamatergic perinuclear neurons. Both the inhibitory and excitatory drive might be necessary for burst generation, as spontaneous, synchronized bursts of EPSPs can drive OT neurons bursts *in vitro* (Israel et al., 2003) as does burst of mIPSPs, sometimes shared between magnocellular neurons, indicating a shared synapse (Popescu et al., 2010).

But how does the all population of OT neurons synchronize? The coordination of the bursts across the all population of OT cells, meaning also between OT nuclei, is essential to achieve a coordinated release of OT in the bloodstream from each individual neuron, leading to the pulsatile systemic release of OT. The synchronization of bursts between cells is not “spike for spike” and there are neither a given sequence of activation of one cell relative to the rest of the population nor spikes synchrony (Belin and Moos, 1986; Belin et al., 1984), highlighting that there is probably no strict pacemaker activity in one or a group of neurons leading the others, even if some coordinated bursts of IPSPs in pairs of OT neurons that has been observed might do so, as mentioned in the last paragraph.

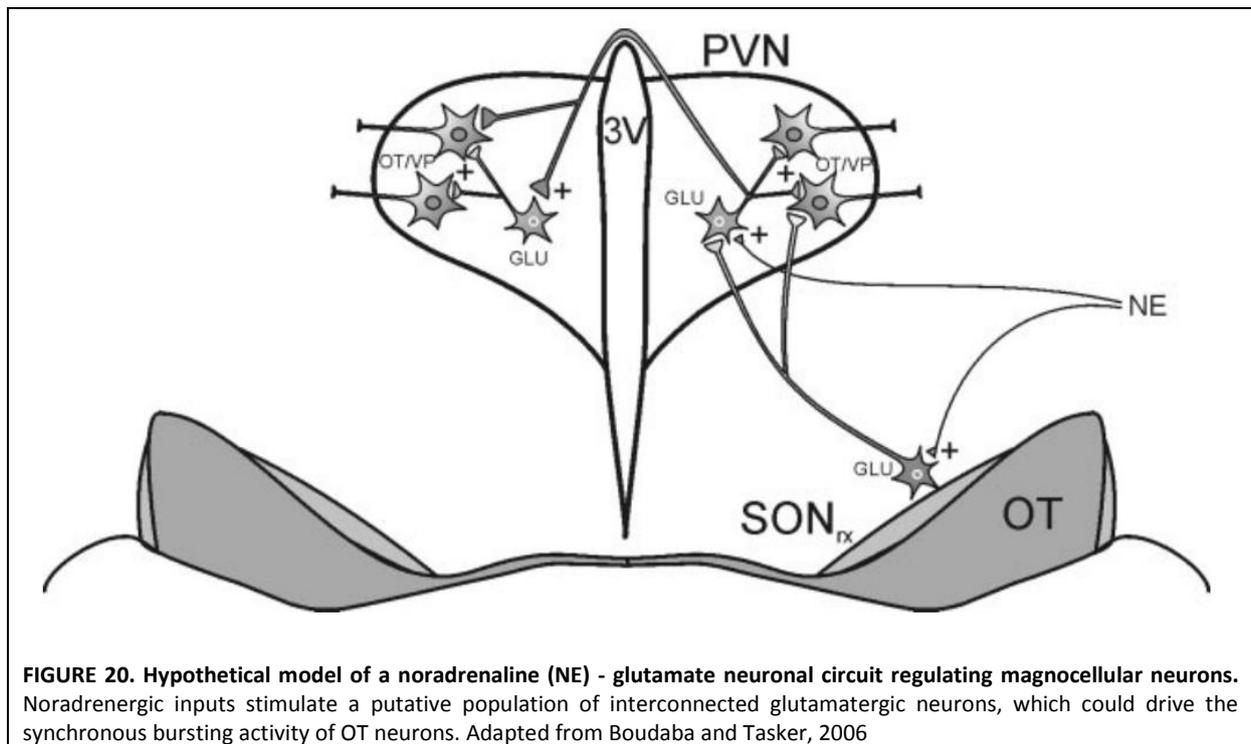
A proposed mechanism for synchronous bursting of OT cells is the somato-dendritic release of OT provoked by OT itself, acting on OTRs on the same cell and cells nearby (auto and paracrine action) (Meddle et al., 2007), while somato-dendritic release of AVP by AVP cells have an opposite, inhibitory, effect on AVP cells (Ludwig and Leng, 2006; Moos et al., 1984). This mechanism of OT release can be totally independent of axonal OT release, and even inhibit it (Sabatier et al., 2003). The somato-dendritic release of OT in OT nuclei is a prime suspect in the coordination of firing of OT cells, as it increases immediately before the onset of milk ejection bursts (Moos et al., 1989). Furthermore, exogenous OT administration in SON or PVN can enhanced milk ejection bursts in the same or contralateral nuclei (Moos and Richard, 1989). Nevertheless, somato-dendritic release of OT is not the trigger but more a priming or amplifying agent of milk-ejection bursts in OT cells, as their initiation require a suckling stimulus (Philippe Richard, Françoise Moos, 1991). The somato-dendritically released OT act in an autocrine and paracrine manner on neighboring dendrites of other OT cells, to promote further OT release. But it was also proposed that somato-dendritically released OT acts pre-synaptically on close by non-oxytocinergic cells or terminals contacting magnocellular neurons, and doing so induces a depression of evoked EPSPs in those magnocellular neurons (Kombian et al., 1997). Another proposed mechanism for such action is that the autocrine activation of OTR induces the release of endocannabinoid from magnocellular OT neurons that inhibit pre-synaptic excitatory synapses, which might be a way to terminate the milk ejections bursts and then underpin the post-burst silence observed in OT cells (Brown et al., 2013; Hirasawa et al., 2004).

Endocannabinoids also target pre-synaptic CB1 receptors on pre-synaptic GABA afferents of magnocellular dendrites (Ludwig et al., 2016; Oliet et al., 2007) thus inhibiting further GABA release onto OT neurons. This last result is proposed as a way to induce a uniform low probability of release in all GABA synapses, allowing a potent inhibition of firing of OT cells only upon a high frequency stimulation of GABA afferents. This mechanism gives the OT neurons a control on their own inhibition, which might be important in controlling the synchronous bursting activity. Moreover, such reduction of both excitatory and inhibitory synaptic transmission might act as a high pass filter that allow the modification of magnocellular neurons activity only by a strong activity of pre-synaptic afferents (like demonstrated for glutamatergic inputs by Israel et al., 2003).

These results do explain intra-nucleus synchronization, but is it enough for inter-nuclei synchronization? Even if OT could diffuse from the PVN to the SON and vice-versa and from one PVN to the contra-lateral PVN and in the process of diffusion, target peri-nuclear afferents, the slow rate of this process might not be sufficient for the observed synchronization between OT nuclei. The synchronization could then rely on external signals, such as the noradrenergic afferents coming from the NTS, which innervates both PVN and SON and have an increased effect during parturition (Herbison et al., 1997; Lipschitz et al., 2004; Swanson and Sawchenko, 1983) or from another population of neurons from the medulla oblongata (Moos et al., 2004). Those signals could increase the spiking of OT cells in both nuclei at the same time upon sensory stimuli, which would simultaneously prime the somato-dendritic vesicles for release and then drive synchronous bursting. Bealer and colleagues reports that the noradrenaline contents of the PVN was indeed increased during suckling, but was undetectable in the SON, even if the blockade of noradrenergic transmission in the SON and PVN did reduce OT release in both nuclei. Histaminergic afferents can also provoke intra-nuclear OT secretion and might at the same time increase the local release of noradrenaline and excitatory amino acids (Bealer and Crowley, 1998; Bealer et al., 2010).

Another potential actor of inter-nuclei synchronization, mentioned above and in 3.2.1.2, is the perinuclear zone from which cells innervate both OT nuclei (Boudaba et al., 1996, 1997; Roland and Sawchenko, 1993). Some of those cells could respond to external afferent like noradrenergic circuits and then drive simultaneously OT nuclei (Boudaba et al., 2003). Boudaba and Tasker revealed such a potential mechanism, implicating a circuit of glutamatergic neurons connecting the OT nuclei and responsive to noradrenaline (Boudaba and Tasker, 2006). They propose the scheme in FIGURE 20 as a summary of their findings. Other glutamatergic afferents arising from the dorsomedial hypothalamus and contacting magnocellular neurons might also be good candidates for driving bursting activities between OT nuclei (Honda and Higuchi, 2010; Takano et al., 1992). The mammillary body is also involved, as its lesion desynchronizes milk ejection bursts (Wang et al., 2013). Furthermore, a simple

proof of the need of “wired” connections between OT nuclei is the fact that hemi-section of the brain results in the loss of synchronized bursting *in vivo* (Moos and Richard, 1989).



Another, simple, hypothesis is an interconnection between OT cells from different nuclei. Indeed, magnocellular OT neurons can project collaterals in the brain (Knobloch et al., 2012), and the same goes for parvocellular OT neurons (see 3.1.2). Furthermore, afferents coming from the contralateral PVN and SON were indeed found in the PVN (Silverman et al., 1981) and one should keep in mind the results of Lambert and colleagues, showing that application of OT or OTR antagonist in one of the SON did modify OT neurons bursts properties in all the other nuclei (Lambert et al., 1993). The presence of synapses immuno-reactive for OT on both the pre- and post-synaptic elements was also reported in the SON (Theodosis, 1985). The proof of a functional connection between parvocellular OT neurons of the PVN and magnocellular OT neurons of the SON has been part of the study presented in the ARTICLE 1, but its role in the inter-nuclei synchronization of OT neurons bursts during lactation was not tested.

Another crucial actor of the various changes in activity of the OT nuclei, notably during lactation, is the astroglial cell. I will detail some of the novel functions attributed to astrocytes, with a focus on the modulation of OT neurons, in the following part.

3.3. Glial modulation of neuronal activities: OT nuclei as a model

Glial cells have long been considered as physical and metabolic support cells for neurons. They are defined as non-excitabile cells, and comprise in the CNS:

- Astrocytes
- Microglia
- Oligodendrocytes
- Ependymal cells
- the less known Polydendrocytes/NG2 cells

The classical roles attributed to glia are:

1. To surround neurons and hold them in place.
2. To supply nutrients and oxygen to neurons.
3. To insulate one neuron from another.
4. To destroy pathogens and remove dead cells and tissues.

Microglia support most of the immune functions in the CNS, ependymal cells line the ventricles and mediate the production of CSF and exchange with it. Oligodendrocytes ensheath axons of the CNS with myelin. Astrocytes have several functions, some fitting with the classical attributes of glial cells, some more recently attributed: maintenance of the BBB by interactions with endothelial cells, to store and provide nutrients to other cells, maintenance of the ions homeostasis, neurotransmitters uptake and release, a structural function, synapses isolation, a role in repairing neural tissue upon injury, vasomodulation and finally an active modulation of synaptic transmission. The last will be discussed in the following part.

3.3.1. An overlook of novel properties and functions of astrocytes

Astrocytes are estimated to represent at least 20 to 40% of the total number of cells in the mammalian brain, but the ratio of astroglia versus neurons is highly variable, across species, and across different brain areas (Herculano-Houzel, 2014). Research in neurosciences in the past decades has accumulated numerous proofs that the astrocytes are more than logistic managers of neurons, even if those supportive functions are still essential to the homeostasis of the CNS and should not be neglected. Nevertheless, not much is known about the functional diversity of astrocytes compared to the current knowledge about the neuronal one (Khakh and Sofroniew, 2015). Among the diverse phenotypes of astrocytes, an obvious distinction can be made based on morphology, the existence of protoplasmic and fibrous astrocytes residing in the gray and white matter, respectively (Miller and Raff, 1984). In a recent study, large scale transcriptomics analysis of sorted astrocytes from several

brain regions revealed clusters of at least five different sub-populations of astrocytes in terms of their transcriptomes and inferred putative functions linked to synaptogenesis (John Lin et al., 2017). One key property of astrocytes that is used to identify them is the fact they are non-electrically excitable cells. Some form of homogeneity in electrophysiological properties of astrocytes in different regions of the brain exists (Kuffler, 1967; Mishima and Hirase, 2010; Wang and Bordey, 2008): they are non-electrically excitable cells (no action potentials), with highly polarized resting membrane potential, governed mainly by the equilibrium potential of potassium (Orkand et al., 1966). Many advances in the study of neuronal functions came from electrophysiology techniques, and from this point of view, astrocytes seem indeed quite passive. Nevertheless, even from an electrophysiological point of view, astrocytes are actually not so homogenous and passive. Their resting potentials are various and their current profiles differ both within and between brain areas (Ambrosio et al., 1998; Chvátal et al., 1995; Graß et al., 2004; Guatteo et al., 1996; Zhou and Kimelberg, 2000), but some of those cells might actually be NG2 glial cells, a cell type thought as being a precursor for oligodendrocytes and other cell lineages. NG2 cells have “active” electrophysiological properties like a voltage dependency of some currents (Nishiyama et al., 2009, 2014). The electrophysiological recordings of astrocytes are nevertheless useful to study their ionic exchanges with the extracellular medium, notably the potassium flow and glutamate / GABA transporters currents, as detailed in Dallérac et al., 2013.

The quest for a molecular marker of astrocytes

At the molecular level, when asked for a molecular marker of the astrocytic nature of a cell, most students (and senior researchers) would respond GFAP, for glial fibrillary acidic protein. But GFAP has several caveats to be a proper identity marker of astroglial cells. It is a cytoskeletal protein that is upregulated upon injury, for instance. It is not expressed by all astrocytes in healthy tissue, especially in protoplasmic astrocytes, and is also expressed by non-astrocytic cells, such as neural progenitors (Zhang and Barres, 2010) making it neither required nor sufficient to characterize an astrocyte. Other proteins, such as the glutamine synthetase and the calcium binding protein S100 β suffer also from a lack of selectivity towards astrocyte (Wang and Bordey, 2008). Other common markers found in the literature are glutamate transporters GLT-1 and GLAST. They are commonly used and considered specific to astrocytes, but are also differentially expressed among brain regions, and in some cases reported in non astroglial cells. Furthermore GLT-1 provide only a punctuate staining (Lehre et al., 1995; Wang and Bordey, 2008). A more general marker identified through large scale genetic analysis of the astrocyte transcriptome in rodents and human is the aldehyde dehydrogenase 1 family, member L1 (ALDH1L1) (Cahoy et al., 2008; Yang et al., 2011d). But astrocytes analyzed in the study of Cahoy and colleagues were first sorted upon their expression of a reporter under the S100 β

promoter, probably letting some astrocytes out of the screening. Yang and colleagues found co-expression of GLT-1 and ALDH1L1 but a decreased expression of the latter in the spinal cord of mature mice. Nevertheless, the ALDH1L1 locus has been recently used by two different laboratories to express Cre/ERT2 in a transgenic mice line, allowing specific conditional expression of proteins of interest in mice astrocytes, with success (Srinivasan et al., 2016; Winchenbach et al., 2016). The hypothesis that ALDH1L1 is a pan-astrocytes marker was also recently confirmed through comparison of the transcriptome of ALDH1L1 sorted astrocytes for expression of other astrocytic genes signature (John Lin et al., 2017).

Calcium excitability of astrocytes

Despite the (fundamental) discussion about a proper molecular marker, The recent advances in imaging techniques, molecular genetics and transcriptomics among others, allowed to learn much more about the diversity in phenotypes and functions of astrocytes of the CNS (Khakh and Sofroniew, 2015; Oberheim et al., 2012; Zhang and Barres, 2010). One tool which allows to dynamically measure the activity of living astrocytes is calcium imaging. Indeed, the concentration of intracellular calcium ($[Ca^{2+}]_i$) in astrocytes is modified dynamically in response to numerous factors, for instance GPCRs activation by agonists released at synapses. Various signals generate a wide range of oscillations of $[Ca^{2+}]_i$ in astrocytes, and the idea that astrocyte can compute information through a fine regulation of the $[Ca^{2+}]_i$ gave rise to the term calcium excitability (Perea and Araque, 2005). The visualization of calcium concentrations through synthetic or genetically encoded calcium indicators (GECI) is now a common tool, which can be used to visualize astrocytic (and neuronal) calcium transients from cultured cells in petri dish to deep brain areas of freely moving animals (see e.g. Resendez et al., 2016). One should note here that calcium transients of astrocytes are complex and differs between the soma and the very fine processes, which can be independent from each other in terms of $[Ca^{2+}]_i$ variations. Those findings will be evoked in the DISCUSSION and are the subject of the following review and study (Bazargani and Attwell, 2016; Otsu et al., 2014).

The tripartite synapse and gliotransmission

From the numerous studies tackling the role of astrocytes in the CNS, the most striking (therefore debated) hypothesis is that astrocytes can directly affect neuronal activity by dynamic modulation of synapses. Astrocytes surround both the pre- and post-synaptic elements of synapses and interact with them. For instance, several studies have demonstrated the active role of astrocytes in the well-studied hippocampal long term potentiation (LTP) of CA3-CA1 synapses. One mechanism involves the neuronal release of acetylcholine on astrocytes, in which it activates muscarinic receptors, inducing astrocytic calcium transients originating from the intracellular stores. Those calcium transients lead

to astrocytic glutamate release on pre-synaptic metabotropic receptor which potentiates glutamate release in the synapse. Another study at the same synapse show that during LTP induction, extra-cellular calcium enters the astrocytes through TRPA1 (transient receptor potential cation channel, member A1) channels, leading to the release of the co-agonist D-Serine by astrocytes onto post synaptic NMDARs (N-methyl-D-aspartate receptors), leading again to synapse potentiation. This influence of astrocytes on synapses gave rise to the “tripartite synapse” model, and the concept of release of neuroactive substances by astrocytes is referred as “gliotransmission” (Volterra et al., 2014).

Active modulation of synapses by astrocytes have been established in other brain areas, with the striking observation that astrocytes can be circuit-specific, meaning that they modulate specific synapses belonging to a given circuit rather than all the synapses in their grasp. This was shown in the striatum while studying the role of astrocytes in modulation of the direct and indirect pathways of the basal ganglia. In the striatum, well characterized D1 or D2 dopamine receptors expressing medium spiny neurons convey information from each pathway separately, but are totally intermingled in the striatum. In this context, Martín and colleagues showed that specific subpopulations of astrocytes responded to the activation of one pathway and not the other, and vice-versa. The sole stimulation of one astrocyte potentiated the pathway-specific synapse it belonged to, and not the other (Martín et al., 2015).

In vivo, Poskanzer and colleagues demonstrated that specific activation of astrocytes in the cortex could trigger the switch of the cortical network to a slow-oscillation state, likely through release of glutamate in the vicinity of neurons (Poskanzer and Yuste, 2016).

Those studies and concepts have been discussed in several review and chapters (Araque et al., 2014; Khakh and Sofroniew, 2015; Perea and Araque, 2005; Perea et al., 2009; Sahlender et al., 2014; Santello et al., 2012; Volterra and Meldolesi, 2005). In the context of this manuscript, an interesting aspect of the modulation of neuronal circuits by astrocytes is their roles in the control of OT nuclei activity.

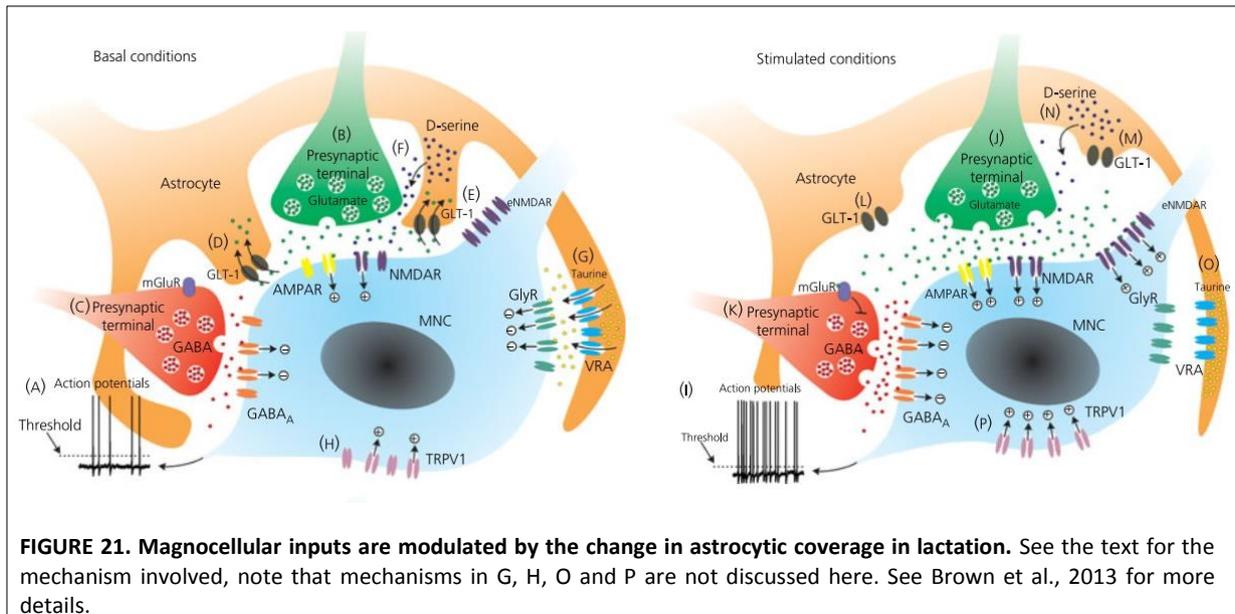
3.3.2. Astrocytes and magnocellular OT neurons during lactation

Direct synaptic modulation by astrocytes is also present in the OT nuclei. For instance, at glutamatergic synapses onto PVN magnocellular cells, afferent activity recruits astrocytes, which cause a fast and feed forward increase in amplitude of quantal synaptic currents. This is mediated by astrocytic metabotropic glutamate receptors (mGluRs) that respond to glutamate spillover from the synapse. Astrocytic mGluRs activation induces a rise in $[Ca^{2+}]_i$ followed by a release of ATP from the

astroglia, acting on post-synaptic purinergic receptors. Furthermore the authors demonstrate that this effect is actually extended to all the synapses of the recorded magnocellular neuron, and propose that such a mechanism could underlie the change in the excitability of magnocellular neurons leading to the facilitation of population bursts, as seen in lactation (Gordon et al., 2009). A similar mechanism was proposed, implicating this time astrocytic α 1-adrenoceptors, which stimulation by noradrenaline released close to the PVN induces calcium transients and further release of ATP, as in the study mentioned above. The activation of post-synaptic purinergic receptors is here proposed to induce the insertion of AMPA receptors in the synapse, potentiating it (Gordon et al., 2005).

A striking feature of astrocytes of the magnocellular nuclei is that they withdraw their processes from the magnocellular cells during lactation (and dehydration, not discussed here) (Theodosis et al., 1981). During lactation, only the OT magnocellular cells are physically released from astrocytes processes, indicating a specific and regulated mechanism (Theodosis et al., 1986). However, the blockade of astrocytes retraction does not alter milk ejection bursts in OT cells, indicating that the physical retraction of astrocytic processes *per se* is not essential to this phenomenon (Catheline et al., 2006). These authors propose that such retraction does not modulate burst firing *per se*, but might reduce responses of OT neuron to “perturbing” stimulations unrelated to lactation, a fact measured in other experiments (Higuchi et al., 1988). One of the functions attributed to astrocytic regulation of synapses is the clearance of secreted neurotransmitters like glutamate and GABA, through specific transporters. The retraction of astrocytic processes away from synapses down-regulates this uptake of neurotransmitters released at magnocellular neurons synapses. This leads to an increase in pre-synaptic mGluRs activation at glutamatergic and GABAergic terminals, inhibiting further synaptic release (Oliet et al., 2001; Park et al., 2006; Piet et al., 2004). Another mechanism implicating astrocytes might also explain the reduced synaptic efficacy in magnocellular neurons during lactation. One of the major gliotransmitter released by astrocytes onto synapses is the NMDAR co-agonist D-Serine. The release of D-Serine helps to potentiate synapses, therefore a retraction of astrocytes from magnocellular neurons synapses reduces synaptic efficacy (Panatier et al., 2006). Yet another mechanism implicating astrocytes retraction in lactating animals is the switch from a facilitating to an inhibitory kainate receptors subtype in GABAergic pre-synaptic neurons, due to the increase in ambient levels of glutamate (Bonfardin et al., 2010). Indeed, one of the key roles of astrocytes in maintaining a proper function of synapses relies in part on the uptake of neurotransmitters through transporters. Astroglia found at magnocellular neurons synapses express the glutamate transporter GLT-1. The blockade of this transporter induces an increased spillover of glutamate, resulting in an enhanced activation of extra-synaptic NMDARs in magnocellular neuron.

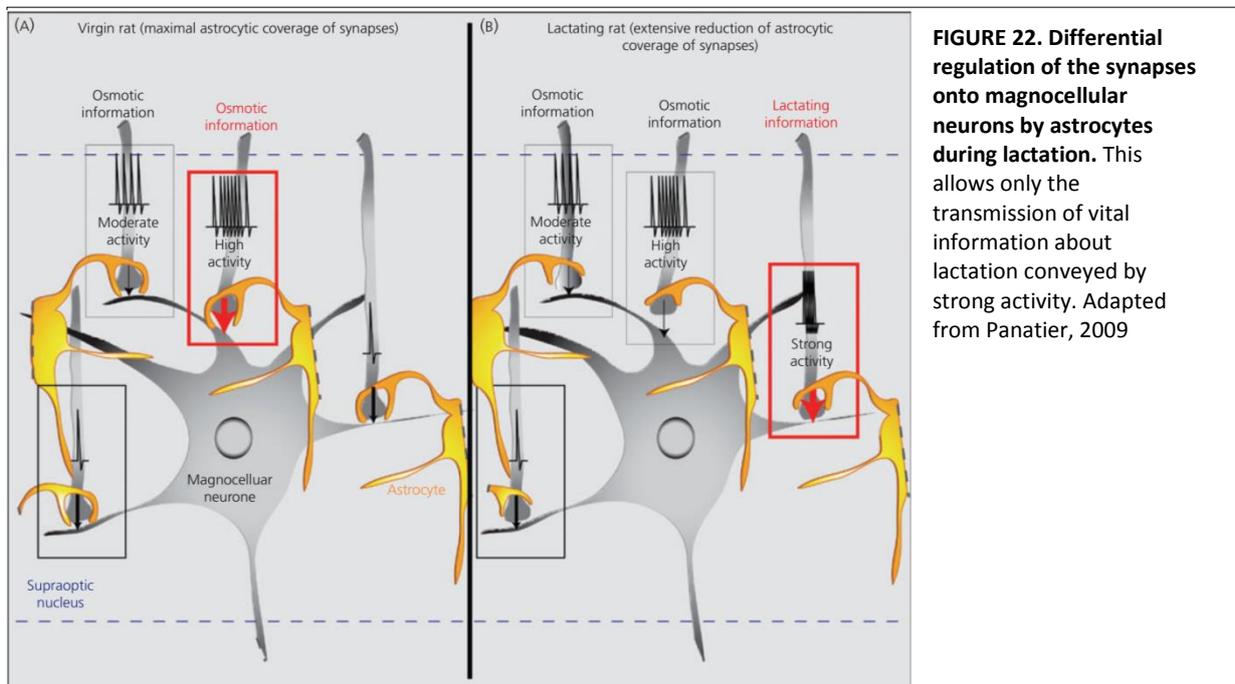
Such mechanism could participate in the increased excitability of magnocellular neurons during lactation (Fleming et al., 2011). This enhanced activity of extra-synaptic NMDAR also potentiates postsynaptic GABA_A receptors through calcium and phosphorylation dependent mechanisms, which might act as a compensatory mechanism for the increased glutamatergic (over-)excitation (Potapenko et al., 2013). A comprehensive scheme summarizing the role of astrocytes during normal and lactating conditions (or an osmotic challenge, not reviewed here) is proposed by Brown and colleagues and reproduced in FIGURE 21 (Brown et al., 2013).



Changes in the astrocytic covering of OT magnocellular neurons are linked to change in GFAP immunolabeling of SON astrocytes. Immediately after suckling there is a significant reduction in GFAP immunolabeling, which seems to be relocated to the somata of astrocytes (Wang and Hamilton, 2009). The retraction of astrocytes and relocation of GFAP in astrocytes and other cytoskeletal proteins in astrocytes and magnocellular neurons are initiated both by the noradrenergic inputs and the increased somato-dendritic release of OT upon suckling of the pups (Hatton et al., 1984; Wang and Hatton, 2007b). Further mechanisms underpinning the role of astrocytes retraction in facilitating the bursting activity of OT neurons during lactation and parturition are detailed in the following review (Wang and Hamilton, 2009).

Panatier summarizes the effects of astrocytes retraction during lactation as a way to create an high pass filter (mentioned in 3.2.2) letting only intense stimuli, coding for lactation related information (e.g. suckling stimulus), potentiate afferent synapses onto magnocellular OT neurons, and summarizes this hypothesis with the scheme proposed in FIGURE 22 (Panatier, 2009). Indeed OT neurons responses to osmotic challenge and hypovolemia is decreased during lactation (Summy-

Long and Kadekaro, 2001). This could also allow the preservation of the stores of OT for the lactation induction (Hartman et al., 1987).



In conclusion of this part, we saw that astrocytes have crucial roles in the functions and adaptations of the CNS to different states. Those functions have been extensively studied for the interaction of astrocytes of the hypothalamus covering synapses onto magnocellular OT cells. The second part of my work presented here aims to elucidate the role of astrocytes in the effects of OT released in the central amygdala from projections of magnocellular neurons, and its resulting analgesic and anxiolytic effects (see ARTICLE 2). Then, we will now focus on the OT projections in the CNS and their multiple functions.

3.4. OT projections and OTR maps

3.4.1. Volume transmission vs axonal release

The observations of the high quantity of OT that is released inside the hypothalamus by somato-dendritic release (Ludwig and Leng, 2006), the sparse numbers of

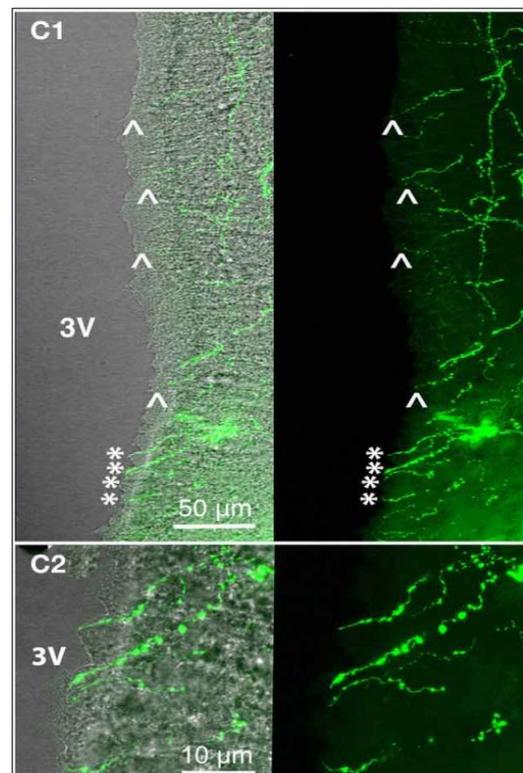
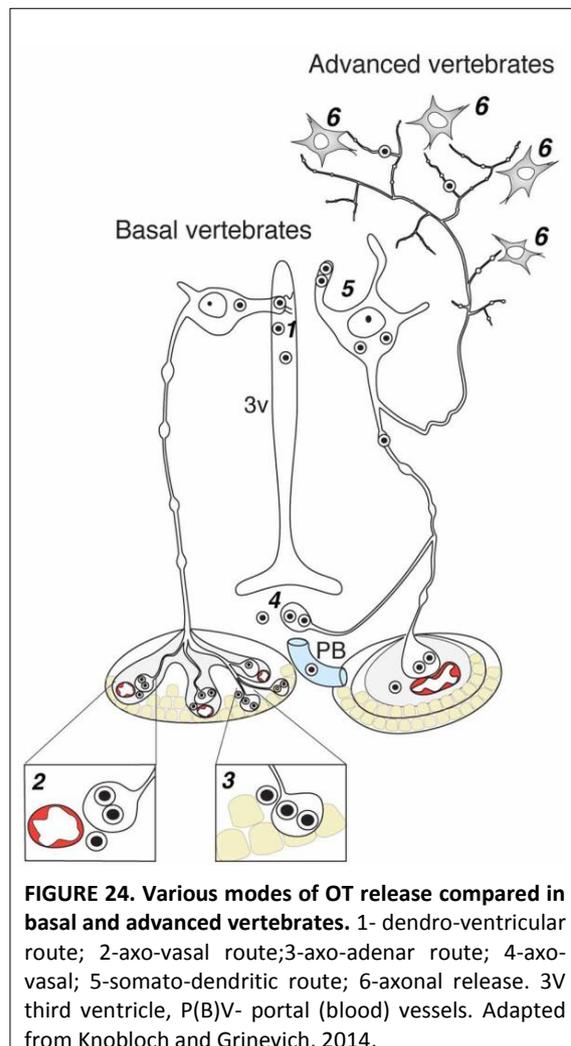


FIGURE 23. Venus expressing OT cells from the PVN . OT cells project their dendrites toward the third ventricle (*) and below the ependymal layer (arrow) or in the lumen. Adapted from Knobloch and Grinevich, 2014

OT axons found in the brain (Sofroniew, 1980, 1983) and the apparent mismatch of those projections with the patterns of OTR expression (see 3.4.3) has filled the hypothesis that OT could act by volume transmission, meaning by simple diffusion in brain tissue from OT nuclei or its release in the CSF at the level of the third ventricle. Various data are in line with such hypothesis. In basal conditions, micro-dialysis studies measured 4 pg of OT in SON after 30 minutes and 2 pg for PVN. In brain regions a few millimeters away, such as the amygdala, lateral septum and dorsal hippocampus, OT concentrations were only two to fourfold lower than in the SON (Wotjak et al., 2008). Actually those values correspond to an OT concentration in the extracellular fluid of ~1 nM (Leng and Ludwig, 2008), which corresponds to the binding affinity for OTR. Furthermore, several observations incline to think

that OT could act through its diffusion and transport in the CSF. 80% of OTR expressing regions are located close to the ventricular or subarachnoid CSF (see 3.4.3 and Veening et al., 2010), and most of the pioneering research about the central effects of OT used i.c.v injections. The CSF levels of OT are not always correlated with the plasma levels (Amico et al., 1990), indicating a differential modulation (Veening et al., 2010). We already saw that OT can be somato-dendritically released without being neurosecreted and vice-versa. Furthermore, OT half-life in CSF is about half an hour compared to the few minutes in plasma (see 2.1.1.1). Some OT cells in the rodent brain seem to direct their dendrites towards the third ventricle (see FIGURE 23 on previous page), which might be a mechanism of release and sensing of OT in the CSF, in synergy with its diffusion from and towards the nearby extracellular matrix (Landgraf and Neumann, 2004). Interestingly, release of OT from



the dendrites into the third ventricle is an evolutionary old property of magnocellular neurons, which is less observed while moving along the phylogenic tree (see FIGURE 24). Since plasmatic OT cannot reach the CSF, some researchers argue that the high concentration observed in certain conditions in the CSF likely comes from a massive release of OT from OT dendrites, and not from its release and diffusion from OT axonal terminals. Veening and colleagues report that such concentrations could easily activate peri-ventricular brain areas through diffusion from the CSF (Veening et al., 2010).

Finally, Leng and Ludwig's main argument favoring a role in diffusion and transport through CSF of OT is based on the fact that parvocellular OT neurons are projecting to a restricted amount of brain areas, underpinning only some of the behavioral effects of OT, compared to the number of regions expressing the OTR and the various behaviors it modifies (Ludwig and Leng, 2006). However the "OT/TR mismatch argument" was recently undermined by the mapping of new OT forebrain projections arising from magnocellular OT collaterals (Knobloch et al., 2012; Ross et al., 2009). A wise explanation could be that both mechanisms are important and each have a role, a model defended by Landgraf and Neumann (Landgraf and Neumann, 2004). The diffusive process of OT could act on a slow time-scale, on a wide spatial range over entire brain areas, whereas the axonal release of OT would allow a focal, fast and highly constrained regulation at the cellular or microcircuit level (Stoop, 2012).

Actually, one observation could set a middle ground for those opposite modes of action. The evoked axonal effect of OT is not as fast as a synaptic release should be. When stimulating OT in *ex vivo* brain slices, responses in measured post-synaptic neurons are not within the milliseconds range, as one would expect for a (poly)synaptic effect, but in the seconds to even dozens of seconds range (personal observations and Knobloch and Grinevich, 2014). One explanation could be that OT is released locally from axons, but then acts through local diffusion over dozens of micrometers. Such delays were also observed in a study measuring cellular responses of cells located 100 micrometers away from magnocellular nuclei to an AVP evoked somato-dendritic release (Son et al., 2013). Furthermore, OT LDCVs are often not located in active zones of synapses in the SON or nucleus accumbens but are present all along the axons (Knobloch and Grinevich, 2014; Ross et al., 2009), indicating that OT axons might release OT through an en passant mechanism. This idea of a release of peptides containing LDCVs from virtually all part of the magnocellular neurons has already been proposed (Morris and Pow, 1991). OT cells might release OT from all part of the axons, and co-release glutamate and OT only in the synaptic terminals, as those contains small clear vesicles, are asymmetric in nature and do express VGlut2 (mentioned earlier) (Meeker et al., 1991; Navone and Di Gioia, 1988; Ross et al., 2009).

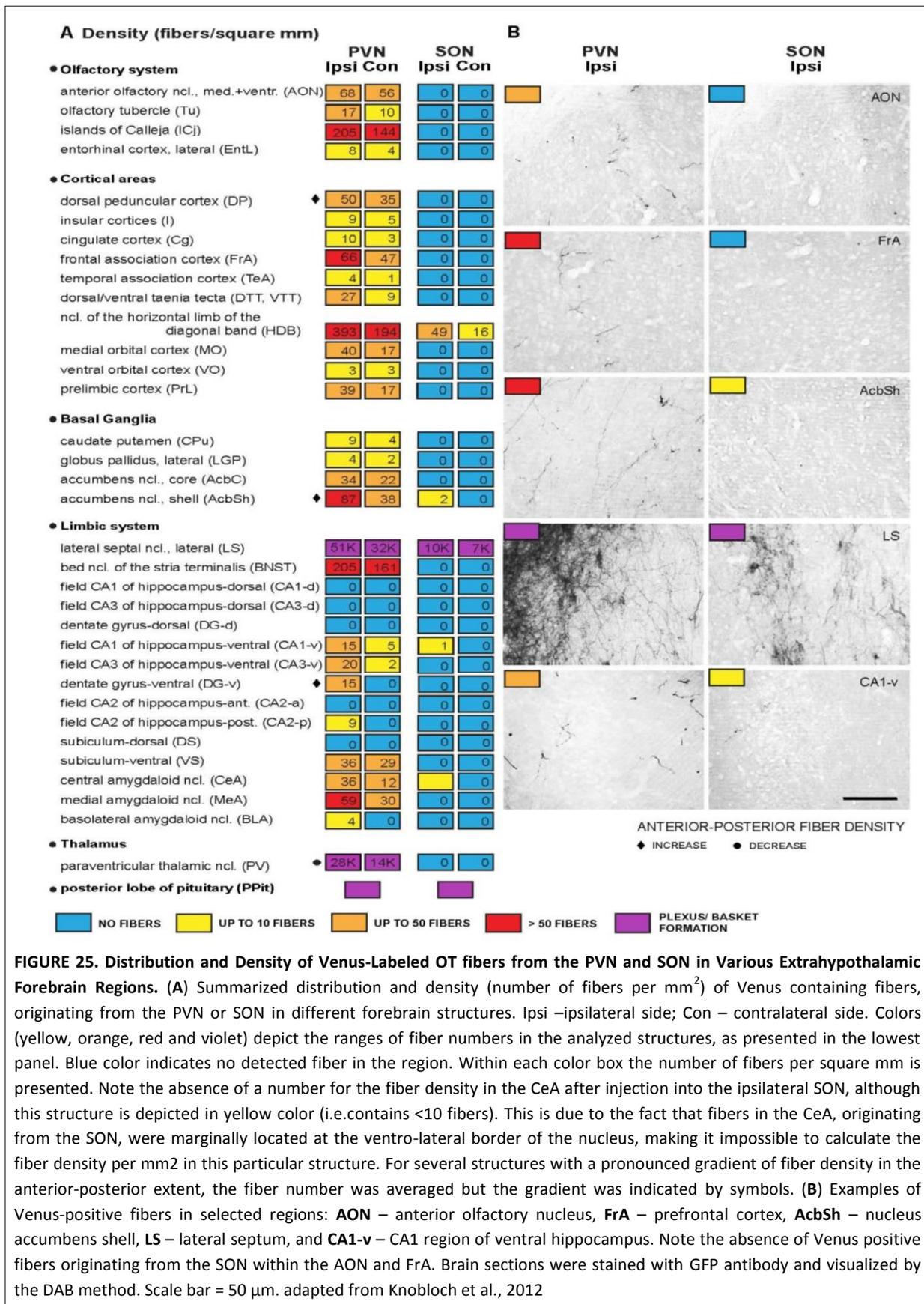
OT concentration measured in the CNS vary greatly from site to site (Russell et al., 1992). In order to fit with a volume transmission hypothesis, this could be explained by the regulation of ocytosinase levels, as mentioned earlier (see 2.1.1.1). But this would not explain the fact that OT concentration can, under certain conditions, reach values hundred times higher than basal ones, that in only one specific region of the brain (Beyer et al., 2010; Ebner et al., 2005). A simpler explanation of those observations is the focal and regulated axonal OT release in a given nucleus, supported by several results indicating a precise cellular and behavioral effect of OT axons stimulation, both *in vivo* and *in*

vitro (Knobloch et al., 2012; Mitre et al., 2016a; Oettl et al., 2016). Finally, depending on OT concentrations, differential G protein coupling could be favored: at high concentrations, $G_{i/o}$ coupling is also engaged by OT, when only G_q is at lower ones (Busnelli et al., 2012; Grinevich et al., 2016). This means that OT could have opposite or mixed effects on cellular networks in a given area after either a fast and focal axonal release of a high OT concentration and/or a slight and slow increase by diffusion from OT nuclei or spillover from OT synapses. Nevertheless, most data now point to an almost complete overlap between regions expressing OTR and their innervation by hypothalamic OT axons, with the exception of the olfactory bulb, which could be targeted by trans-ventricular pathways and regions very close to OT nuclei (ventral pallidum, medial preoptic area, and ventromedial hypothalamic nucleus), which could be efficiently modulated by simple diffusion from somato dendritically released OT (Grinevich et al., 2016)

3.4.2. OT Projections

Many anatomical studies in the 80s mapped the oxytocinergic projections using an immunohistochemical approach and described them in various brain areas (Buijs, 1983; Sofroniew, 1983) but could not always precisely determine the nuclei from which the fibers originated. A new method of mapping was employed by Knobloch and colleagues. They used viral transfection of OT cells to express the soluble fluorescent protein Venus under the OT promoter to specifically tag OT cells, and mapped them throughout the brain of the female rat. Their findings were in accordance with the data given by immunohistochemistry, but showed many more fine venus-expressing OT fibers than those previous studies. Through specific transfection of the fluorescent construct in only one of the nucleus, they could also discriminate the nucleus of origin for a given fiber, and doing so mapped the origins of all the projecting OT axons they found (see FIGURE 25 on next page). Interestingly, contrary to the common idea that only parvocellular OT neurons project centrally, they also found projections originating from the SON, composed only of magnocellular neurons. To further prove the magnocellular origin of those projections, they show that cells projecting to the central amygdala (CeA) also project to the pituitary. This finding is in accordance with the study of Ross and colleagues, who found that retrogradely labeled OT fibers innervating the nucleus accumbens originate from both the PVN and SON and simultaneously project to the pituitary in voles (Ross et al., 2009). Knobloch and colleagues study further demonstrated that stimulation of OT axons present in the CeA (which is not the most OT innervated region of CNS) was sufficient to elicit changes of the neuronal activity in slices containing the CeA, through actions on both OTRs and AMPARs. They further proved that the OT innervations of the CeA originate from all OT nuclei and that those axons possess varicosities only in the lateral part of the CeA (CeL). Using different genetic constructs, they show that those axons do en passant synaptic contacts on dendrites in the CeL

which express VGLUT2. Finally, the stimulation of OT axons in the CeA was sufficient to reduce the freezing behavior of rats in a contextual fear conditioning paradigm (Knobloch et al., 2012).



In light of those various findings, it is now clear that parvocellular neurons are not the only OT cells with central projections. The idea that magnocellular neurons project collaterals to both the pituitary and forebrain structures favor the hypothesis for a role for OT as a neuromodulator through focal and regulated axonal release in the CNS, as opposed to the hypothesized, yet still possible, action through volume transmission. A schematic map of OT Innervated brain areas is proposed in FIGURE 26.

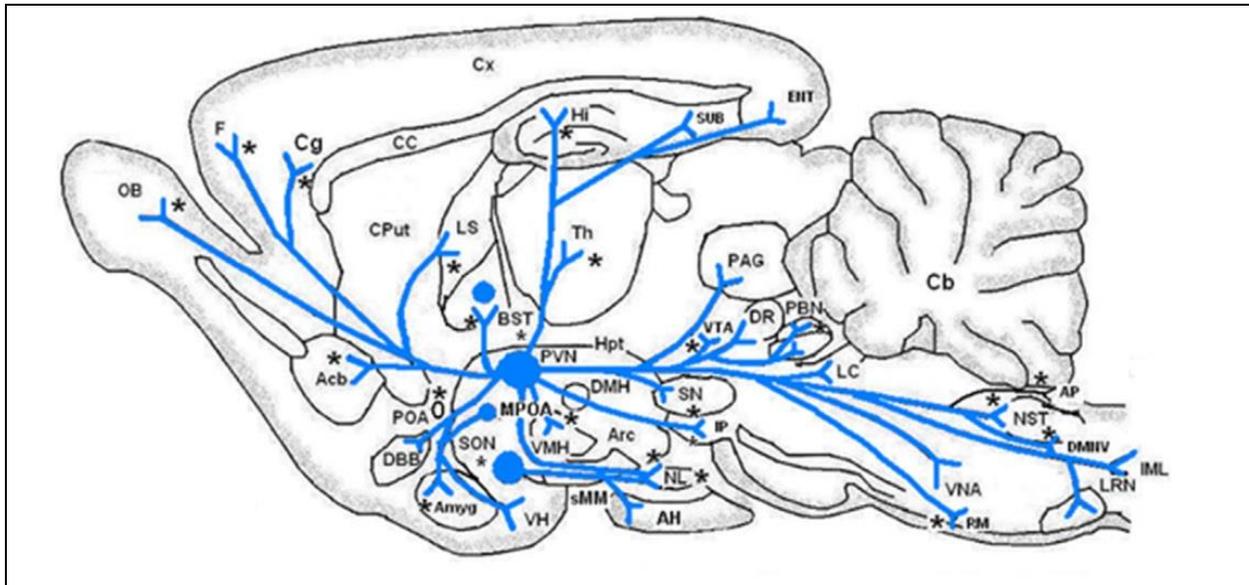


FIGURE 26. OT projections in the CNS. The blue circle represents the locations of OT cells somata, and the lines their projections. The asterisks represent sites where a concomitant presence of CCK endings and CCK-A type receptors was observed. Abbreviations: **Acb**, accumbens; **Amyg**, amygdala; **AP**, area postrema; **Arc**, arcuate nucleus; **BST**, bed nucleus stria terminalis; **Cb**, cerebellum; **Cg**, cingulate cortex; **CPut**, caudate and putamen; **Cx**, cortex; **DBB**, Broca's diagonal band; **DMH**, dorsomedial hypothalamic nucleus; **DMNV**, dorsomedial vagus nucleus; **DR**, dorsal raphe nucleus; **ENT**, entorhinal cortex; **F**, frontal cortex; **Hi**, hippocampus; **Hpt**, hypothalamus; **IML**, intermediolateral column autonomic neurons; **IP**, interpeduncular nucleus; **LC**, locus coeruleus; **LRN**, nucleus reticularis lateral; **LS**, lateral septum; **sMM**, supramammillary nucleus; **NL**, neural lobe; **NST**, nucleus solitary tract; **OB**, olfactory bulb; **OFc**, orbitofrontal cortex; **cPAG**, periaqueductal gray; **PBN**, parabrachial nucleus; **mPFC**, medial prefrontal cortex; **POA**, preoptic area; **PVN**, hypothalamic paraventricular nucleus; **RM**, raphe magnocellularis; **RN**, raphe nucleus; **SON**, supraoptic nucleus; **SN**, substantia nigra; **SU**, subiculum; **Th**, thalamus; **VMH**, ventromedial hypothalamic nucleus; **VMN**, ventromedial nucleus of the thalamus; **VP**, ventral pallidum; **VTA**, ventral tegmental area. Adapted from Vargas-Martínez et al., 2014.

3.4.3. OTR expression map in the CNS

The mapping of the pattern of expression of OTR in the CNS of rodents and other clades of mammals started in the 1980s. The tool of choice was (and can still be) the use of radiolabeled OTR ligands, the first used being a tritiated version of OT (see FIGURE 27 and for other references ANNEXE 1). The use of OT as a ligand raises the issue of cross-reactivity with AVP receptors V_{1A} and tritiated compounds require a long time of exposure, which led to the development of new radiolabeled ligands. The Iodinated OTR radioligand, ^{125}I -ornithine vasotocin analogue (^{125}I -OVTA) has a highly selective profile

(Elands et al., 1988b), that allowed for an efficient mapping of the OTR in a variety of rodent species. In line with autoradiography, still used today, several laboratories aim to develop fluorescently labeled, non-peptidic, BBB permeable OTR agonists, which will allow (cold) binding studies and with a high potential for *in vivo* binding assay (Karpenko et al., 2015). Nevertheless, the pioneering autoradiographic data allowed the deciphering of the role of OTR activation in targeted CNS areas, as opposed to studies which used nonspecific i.c.v injections. This led to the dissection of the

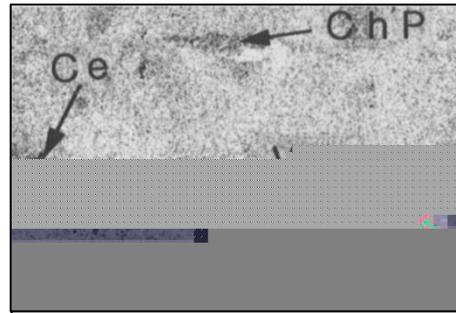


FIGURE 27 **Original pictures of an autoradiography** using [3H]-OT showing binding in CeA (CE), ventro-medial hypothalamus and choroid plexus. Adapted from Freund-Mercier et al., 1987.

neural substrates involved in the various effects of OT (and AVP) in the CNS, as will be discussed in part 3.5. One should note that mapping of the OTR in the primate brain using the same iodinated OTR ligand was first unsuccessful, due to the loss of specificity for the OTR. Using a new pharmacologically optimized method for competitive binding receptor autoradiography, this lack of specificity was overcome and used recently in at least three species of primates (Freeman and Young, 2016; Freeman et al., 2014a, 2014b). This (re-)mapping of the OTR in some non-human primates and its comparison to the mapping in rodents gave rise to interesting observations regarding the adaptation of the OT system for the processing of socially relevant information in each clade, which will be briefly discussed in 3.4.3.1. Gimpl and Fahrenholz have compiled the data from multiple binding assays in rat and human and *in situ* hybridization in rats in one table, reproduced as TABLE 2 (Gimpl and Fahrenholz, 2001). Of interest one should note that the OTR and AVP receptors binding patterns almost never overlap (Gimpl et al., 2001), and when both receptors are present in one area, they are spatially segregated, as exemplified in the CeA where OTR and AVP receptors are segregated in the lateral vs medial part of the CeA (Huber et al., 2005; Stoop, 2012 see also 3.6.1). Recently, Mitre and colleagues designed a new OTR antibody, proposed to be more specific to OTR, and used it to map the OTR expression in the mouse (Mitre et al., 2016b), as shown in FIGURE 28. Another group used a knock-in of the soluble fluorescent protein Venus to generate an OTR-expression map in mice, which corroborates the results from Mitre and colleagues (Yoshida et al., 2009). OTR localizations underlie OT putative functions in brain activity modulation and consequent behavioral modifications. Those will be discussed in part 3.5, but as a transition, we will discuss the interesting comparison of the patterns of OTR expression with an evolutionary perspective, which highlights its role in socially relevant information processing.

Brain Regions	Rat			Human
	mRNA	OT binding (infant)*	OT binding (adult)*	OT binding
Olfactory system				
Olfactory bulb	+	?	?	ND
Anterior olfactory nucleus	+++	++	++	?
Olfactory tubercle	+++	?	++	?
Islands of Calleja	ND	ND	+++	+
Piriform cortex	++	?	?	?
Entorhinal/perirhinal area	+	+	+	ND
Cortical areas				
Peduncular cortex	?	++	+++	(+)
Insular cortex	?	+	+	?
Cingulate cortex	+	+++	ND	?
Retrosplenial cortex	?	+++	ND	?
Frontal cortex	++	?	(+)	ND
Temporal cortex	(+)	?	+	ND
Taenia tecta	+++	?	(+)	?
Diagonal band of Broca	+	?	?	+
Basal nucleus of Meynert	ND	ND	ND	+++
Basal ganglia				
Caudoputamen	+++	+++	++	ND
Ventral pallidum cell groups	++	ND	+++	++
Globus pallidus	ND	+++	ND	++
Nucleus accumbens	+	?	+	ND
Limbic system				
Lateral septal nucleus	+	+	+	+++
Bed nucleus of stria terminalis (BNST)	+++	++	+++	ND
Amygdaloid-hippocampal area	+++	+	+	ND
Central amygdaloid nucleus	+++	++	+++	ND
Medial amygdaloid nucleus	++	+	+	ND
Basolateral amygdaloid nucleus	+++	+	+	ND
Parasubiculum and presubiculum	ND	++	++	ND
Dorsal subiculum	+++	+++	(+)	ND
Ventral subiculum	+++	+	+++	ND
Thalamus and hypothalamus				
Anteroventral thalamic nucleus	ND	+	ND	ND
Paraventricular thalamic nucleus	++	++	+	+
Ventromedial hypothalamic nucleus ^b	+++	ND	++	ND
Anterior medial preoptic area	+++	ND	ND	++
Supraoptic nucleus (SON)	+++	ND	(+)	ND
Paraventricular nucleus (PVN)	++	ND	(+)	ND
Medial tuberal nucleus	ND	++	++	+
Posterior hypothalamic area	+	ND	ND	++
Supramammillary nucleus	++	+	+	ND
Lateral mammillary nucleus	ND	+++	+	++
Medial mammillary nucleus	ND	+++	ND	+
Brain stem				
Substantia nigra pars compacta	++	ND	ND	+++
Ventral and dorsal tegmental area	++	ND	ND	ND
Central gray	+	ND	ND	+
Dorsal raphe nucleus	+	ND	ND	+
Reticular nuclei	+	ND	ND	ND
Medial vestibular nucleus	+	ND	ND	ND
Hypoglossus nucleus	++	ND	ND	++
Nucleus of the solitary tract	ND	ND	(+)	+++
Dorsal motor nucleus of the vagus nerve	+++	+	+	+
Inferior olive nucleus	ND	+	+	(+)
Substantia gelatinosa of trigeminal nucleus	+	+++	+	+++
Pituitary gland	ND	+	+	ND

TABLE 2. OTR mRNA expression and ligand binding levels in supra-spinal structures. Symbols corresponds to a semi-quantification of the levels of expression: +, low; ++, moderate; +++, high; ND, not detectable; (+), at the detection limit and/or not observed by all investigators; ?, not recorded. Adapted from Gimpl et al., 2001 and references therein.

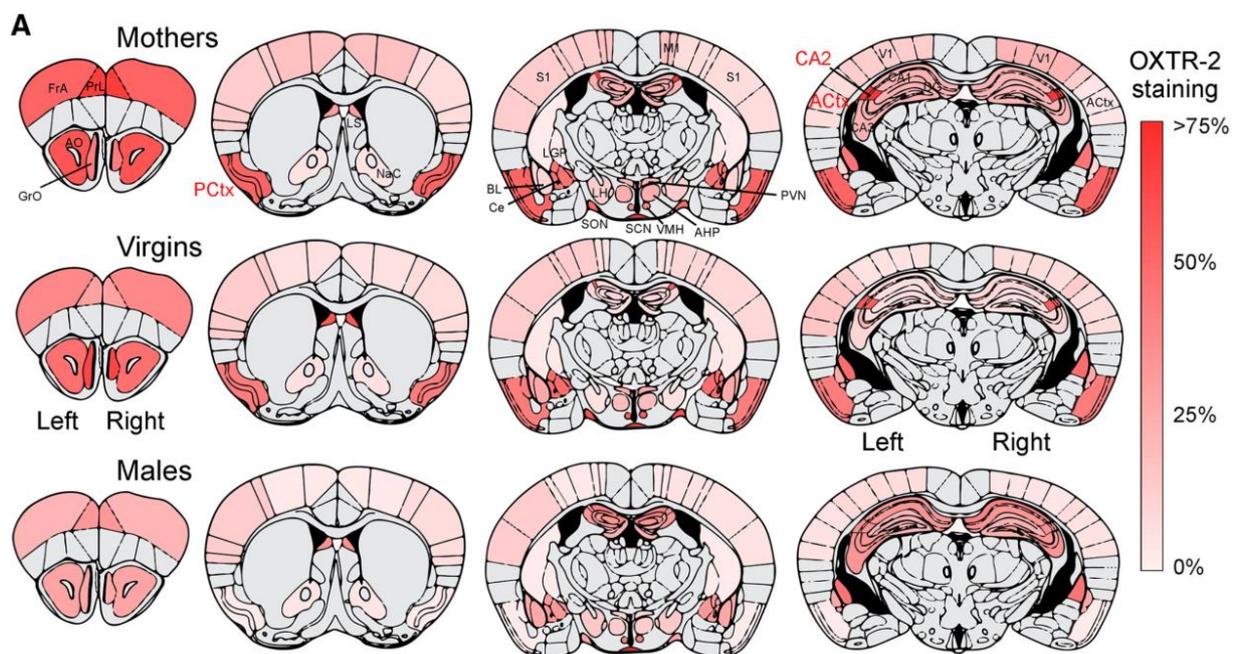


FIGURE 28. OTR expression profile in mouse for females (nultipare or not) and males. Schemes shown here represent four anterior–posterior coronal sections (from left: bregma 2.7 mm, interaural 6.5 mm; bregma 0.7 mm, interaural 4.5 mm; bregma 1.1 mm, interaural 2.7 mm; and bregma 2.3 mm, interaural 1.5 mm). Color indicates percentage of DAPI-positive cells that were OXTR positive per region. Abbreviations: auditory cortex (**ACTx**), anterior hypothalamus (**AHP**), basolateral amygdaloid nucleus (**BL**), central amygdaloid nucleus (**Ce**), anterior olfactory nucleus (**AO**), bed nucleus of stria terminalis (**BST**), hippocampal areas **CA1-CA3**, dentate gyrus (**DG**), frontal association cortex (**FrA**), globus pallidus (**LGP**), granular cell layer of the olfactory bulb (**GrO**), lateral hypothalamic area (**LH**), right lateral septum (**LS**), motor cortex (**M1**), nucleus accumbens core (**NaC**), piriform cortex (**PCTx**), prelimbic cortex (**PrL**), paraventricular nucleus of hypothalamus (**PVN**), median raphe (**RN**), somatosensory cortex (**S1**), supraoptic nucleus (**SON**), supraoptic nucleus of hypothalamus (**SON**), visual cortex (**V1**), and ventromedial hypothalamic nucleus (**VMH**). Gray areas may have expressed oxytocin receptors but were not quantified here. Adapted from Mitre et al., 2016b

3.4.3.1. *OTR expression pattern underpins its functions in various species*

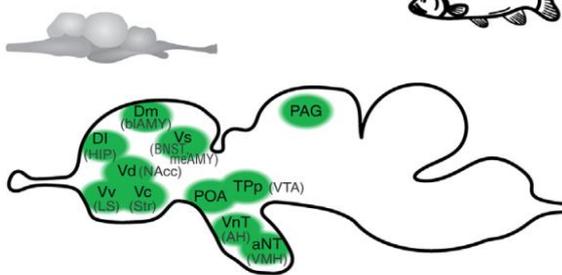
The conservation of the functions of OT and AVP analogs has been discussed above (see 1.2.2.2), with the notion that OT conserves its functions in being the “great facilitator of life” (Lee et al., 2009a). It facilitates life in the sense that on top of its function in the reproductive physiology, it promotes behaviors leading to a successful reproduction and *in fine* the perpetuation of the specie. From studies in various vertebrates, it seems that OTR is expressed by two networks of CNS regions termed together as being the mesolimbic social decision-making network. Species specific variation in OTR expression and OT effects is just a “variation on a theme of conserved neural and gene expression networks” (Cited from: O’Connell and Hofmann, 2012).

The first network regulates functions such as parental care, sexual and aggressive behavior, while the second network processes the salience of incoming stimuli (for the last see Shamay-Tsoory and Abu-Akel, 2016). Behaviors linked to reproduction and social interactions are often gender specific, therefore it is not surprising to find significant variations in the pattern of OTR depending on the gender. A comparison of the various areas expressing OTR and linked to one or the other network is presented in FIGURE 29 (Grinevich et al., 2016). A pattern that emerges while comparing OTR expression pattern across species is that OTR relocates in area processing the sensory stimuli that are the most important in regulating social approach, memory and resulting behaviors in a given specie, as exemplified by the strong expression of OTR in the olfactory bulbs in rodents and in the visual system in primates.

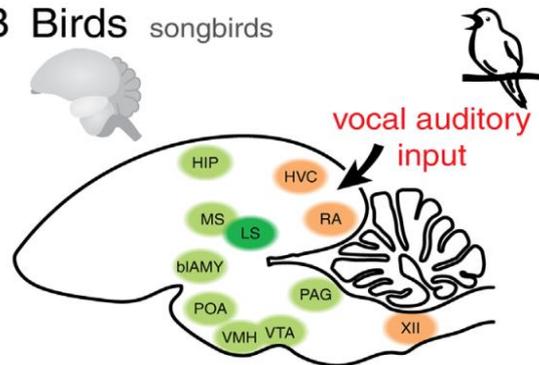
An often quoted example of the dramatic role of the subtle changes of OTR expression on reproductive / social behavior is the influence of a variation in OTR expression in the nucleus accumbens of voles (Insel and Shapiro, 1992). Briefly, they compared the pattern of OTR expression in both prairie and montane voles’ brains, which are either monogamous or polygamous, respectively. The two patterns of OTR superimpose poorly, but did superimpose with other vole species of corresponding pair bonding preferences. Furthermore, when comparing other systems linked to social behavior (benzodiazepines and μ -opioid systems), no differences were observed. This study gave birth to a big line of research exploring the role of OT and AVP in pair bonding behaviors and more generally mammalian sociality (Anacker and Beery, 2013; Gobrogge and Wang, 2015).

OTR expression in mesolimbic social and reward area

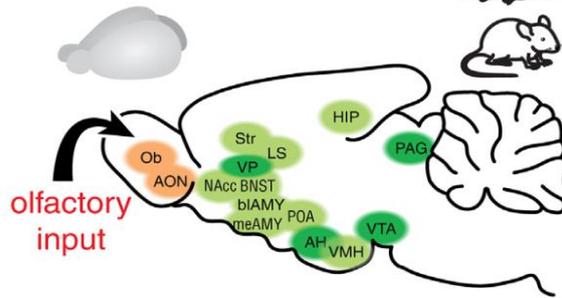
A Teleosts



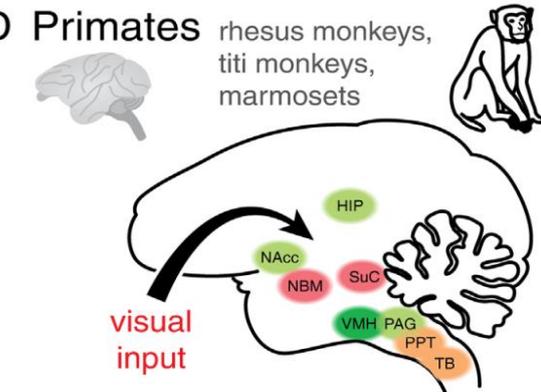
B Birds songbirds



C Rodents voles, rats, mice



D Primates rhesus monkeys, titi monkeys, marmosets



Areas of the mesolimbic social and reward networks

Other areas possibly involved in social behavior

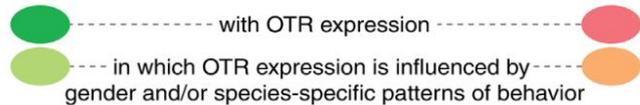


FIGURE 29. expression of OTRs in the mesolimbic social decision-making network compared between four classes of vertebrates. Grinevich and colleagues combines data from autoradiography, *in situ* hybridization and immunohistochemistry studies, referenced in their publication from which this figure is adapted. Abbreviations: mesolimbic social decision making network includes: nucleus accumbens (**NAcc**); ventral pallidum (**VP**); lateral septum (**LS**); preoptic area (**POA**); ventromedial nucleus of hypothalamus (**VMH**); basolateral amygdala (**biAMY**); medial amygdala, (**meAMY**); bed nucleus of the stria terminalis (**BNST**); anterior hypothalamus (**AH**); hippocampus (**HIP**); ventral tegmental area (**VTA**); periaqueductal gray (**PAG**); and striatum (**Str**). Teleost areas of the network (**A**) include: anterior nucleus tuberal (**aNT**), lateral pallial (dorsal) part of the telencephalon (**DI**); medial pallial (dorsal) part of the telencephalon (**Dm**); periventricular nucleus of the posterior tuberculum (**TPp**); central part of subpallial (ventral) telencephalon (**Vc**); dorsal nucleus of subpallial (ventral) telencephalon (**Vd**); ventral tuberal nucleus (**VnT**); supracommissural nucleus of subpallial (ventral) telencephalon (**Vs**); ventral part of subpallial (ventral) telencephalon (**Vv**). Other areas involved in social behavior depicted here include: medial septum (**MS**); high vocal center (**HVC**); robust nucleus of the arcopallium (**RA**); and motor nucleus of the XII cranial nerve (**XII**) in panel (**B**); olfactory bulb (**Ob**) and anterior olfactory nucleus (**AON**) in panel (**C**); nucleus basalis of Meynert (**NBM**); superior colliculus (**SuC**); pedunclopontine tegmental nucleus (**PPT**); and trapezoid body (**TB**) in panel (**D**). Adapted from Ginevich et al., 2016.

3.5. Central OT and its functions

In this part, some of the principal functions of OT in the CNS will be briefly described, with special emphasis on pain modulation, this function being at the center of the two projects developed during my PhD.

3.5.1. Cardiovascular function

OT containing axons, of parvocellular origin, have reciprocal connections with several brainstem and spinal cord (SC) nuclei involved in autonomous functions, notably cardio-respiratory control centers (Palkovits, 1999). PVN inputs from the medulla are activated during venous filling, which increases the blood volume, activating volume / baro receptors in venous-atrial junctions of the heart. Indeed, upon such stimuli PVN neurons show early genes expression. Then, OT parvocellular neurons projecting to the hindbrain and to the upper thoracic SC (to preganglionic sympathetic neurons) can increase the heart rate (Coote, 2005). Furthermore, OT terminals in the solitary vagal complex (NTS/DMV) modulate reflex control of the heart, through facilitation of vagal outflow and slowdown of the heart (Higa et al., 2002). Cardiovascular functions are also under the control of neuroendocrine actions of OT, which promotes release of ANP in the heart (see part 2.2.1.6 and Gutkowska et al., 1997). In general, OT can modulate autonomic activity, and doing so modulates many functions such as respiratory control, parasympathetic outflow to uterus, sexual activity, micturition etc. (Yang et al., 2013)

3.5.2. Motor activity

peripheral administration of OT increases general motor activity, in interaction with steroid hormone (Pettersson et al., 1998), as does central OT (Bodnar et al., 1984). OT has also been linked in regulation of cardiovascular responses elicited by contraction of skeletal muscles (Gimpl et al., 2001).

3.5.3. Osmosensation and osmoregulation

In rats, upon osmotic stress, OT (and AVP) magnocellular neurons show an increase firing rates leading to the release of endocrine OT (and AVP) to promote antidiuresis and natriuresis in the kidney. Their osmosensitivity is due to several factors, among which synaptic inputs from osmosensitive cell populations, for instance from the organum vasculosum laminae terminalis in the CNS and peripheral osmosensitive neurons via vagal and spinal pathways. Magnocellular neurons are also intrinsically osmosensitive. This involves the non-selective cation channels TRPV1. TRPV1 channels open through mechanical stimulation induced by cell shrinking, caused by the high osmolality of the extracellular medium. This leads to cations entry, depolarization and increase firing of OT (AVP) magnocellular neurons. Astrocytes also play a prominent role in this phenomenon. Upon

increase in osmolality, they release less taurine, a potent inhibitor acting on glycine inhibitory receptors, leading to a disinhibition of magnocellular neurons (Prager-Khoutorsky and Bourque, 2015).

3.5.4. Gastric motility , acid secretion control and energy balance

OT inputs to the brainstem dorsal vagal complex (NTS/DMV) also modulate feeding and gastric functions. In the DMV, OT increases gastric acid secretion (Rogers and Hermann, 1985). Generally, OT inputs in the dorsal vagal complex decrease the gastric tone and motility, and does so either by inhibiting cholinergic neurons of the DMV that project to the gastric tract, or by activating the NANC (non-adrenergic non-cholinergic) inhibitory neurons also projecting to the gastric tract (Holmes et al., 2013).

Central OT is also linked to regulation of food intake and generally the control of the energy balance. This fact is clear when looking at the phenotypes of OTR or OT-KO mice. Those mice suffer from obesity and a defective body temperature control when exposed to cold, resulting from both peripheral (adipose tissue) and central deficits (Nishimori et al., 2008). The control of feeding behavior is at least regulated by projections of OT neurons to the hindbrain, since lesions of PVN-hindbrain pathways lead to an increase in food intake and body weight in rats (Shor-Posner et al., 1985; Sims and Lorden, 1986). Release of OT and increased OT neurons activity is correlated to satiation linked termination of feeding, and central administration of OT has a powerful anorexigenic effect. These effects are mediated both by an endocrine effect (magnocellular neurons) and central projections of parvocellular OT neurons to the caudal brainstem, which modulates autonomic functions such as the gastrointestinal vago-vagal reflex (see above) (Sabatier et al., 2013). Parvocellular OT neurons receive projections from leptin, ghrelin, orexigenic, agouti-related peptide and pro-opiomelanocortin neurons, among others. All of those populations are linked to food-intake and energy balance homeostasis functions. Parvocellular projections to the anterior pituitary are also important in controlling release of several classes of hormones that regulate energy balance (*e.g.* thyrotropin releasing hormone). It is possible that magnocellular OT neurons also mediates central effects of OT on energy balance, by somato-dendritic release of OT in the VMH (Noble et al., 2014). Magnocellular neuron endocrine release of OT might be controlled by CCK release from the duodenum in response to food intake, through noradrenergic projections from the A2 cell groups (Onaka et al., 2012). An interesting point of view is given by Klockars and colleagues. They propose that OT anorexigenic effects are not directly linked to the maintenance of a proper energy balance, but more to calorie-intake independent parameters associated with consumption: excessive stomach distension and elevated plasma osmolality, and avoidance of toxic food (Klockars et al., 2015).

Furthermore, they propose that those anorexigenic actions might be of importance to favor sexual or maternal/paternal behavior over foraging ones, the former being maybe the primordial function of OT.

3.5.5. Reproductive function

OT is clearly involved both in the central and peripheral control of reproductive functions, from the sexual drive to the uterine contractions. First, it should be mentioned that OT is involved in the central control of the menstrual cycle through interaction with gonadotropin-releasing-hormone: neurosecreted OT modulates the estrous cycle length through influence on follicle luteinization in the ovary, and OT also influences ovarian steroidogenesis (McCracken et al., 1996), which then act on OT neurons through a feedback loop.

3.5.5.1. Somatic genital reflexes

OT is crucial in the somatic genital reflexes during mating. It involves the contractions of the genital striatal muscles, which are governed by a number of lumbosacral spinal and supraspinal systems. For instance, i.c.v OT administration reduces the ejaculation latency and the post-ejaculatory interval in male rats, and conversely an i.c.v OT antagonist injection blocks ejaculation. PVN and SON OT cells present sexually induced early gene expression. Furthermore in male rats, lesion of the parvocellular neurons of the PVN reduces OT contents in the spinal cord, which decreases the amount of semen emitted, but does not affect the ejaculation itself. The injection of OT antagonist in the level L6 of the spinal cord (and not T13) reduces the contractions of the bulbo-spongiosus muscle and consequent ejaculation, whereas OT injection in the lumbosacral and not thoracolumbar level of SC increases erection. The PVN receives genito-sensory inputs and projects to the nucleus paragigantocellularis, an important brainstem center for the control of genital reflexes that in both male and female (Normandin and Murphy, 2011; Yang et al., 2013). Some authors argue that upon sexual cues or behaviors, magnocellular OT neurons release OT both centrally in OT nuclei and peripherally in the bloodstream. The central OT affects parvocellular OT neurons, which in turn facilitates genital reflexes via the sacral parasympathetic nucleus in the SC. The systemic release of OT is also involved in the facilitation of ejaculation, whereas the central OT may be more involved in favoring the erection (Kita et al., 2006; Veening et al., 2015).

In females, i.c.v injections of OT stimulate the lordosis posture, but this effect is dependent on the ventricle in which OT is injected, and seems to rely on activity in the VMH and medial preoptic area, importantly, this effect seems in close interaction with estradiol and progesterone. This might explain the link between the estrous cycle in rats and the lordosis behavior. Furthermore, the VMH receives inputs from various regions that can convey sexually relevant cues. As in male, OT fibers descending

on the lumbosacral parts of SC are involved in the control of the pelvic organs for copulatory activities. Other brain regions are involved in the control of lordosis in female, but won't be discussed here (Lee et al., 2009a; Veening et al., 2015).

One should note that a high level of central OT might have opposite effects, and has been proposed to contribute to feelings of sexual satiety in male. Finally, OT does not convey those effects alone. For instance, testosterone is needed for OT effects on erection, as well as the NO synthesis in the PVN. Finally, there is a close interaction of the OT system with dopaminergic and serotonergic systems for the control of somatic genital reflexes (Lee et al., 2009a).

3.5.6. Social behavior

3.5.6.1. *OT and pair bonding*

We saw the importance of OT in the central control of genital organs for mating, but OT also has a clear role in the pair bonding formation. In monogamous pairs of humans or rodents, OT has well-documented affiliative effects (see Veening et al., 2015 and references therein). As mentioned above (see 3.4.3.1), there is a difference in OTR distribution between monogamous and polygamous voles. Such differences in the OT system have also been linked to partner fidelity in other species, including primates. Those pair bonds, either toward an offspring or a sexual partner can be very strong and if broken, have detrimental effects for the individual. The best term to describe this is a feeling of "love" which even if it sounds not so scientific, still perfectly defines it. Love is an evolutionary important feeling, with deep neurobiological roots (Carter and Porges, 2012). Generally, social attachment has strong similarities with addiction, and one can argue that the latter is a deleterious form of the former (the so-called "love for the product") (Zellner et al., 2011). In monogamous female voles, OT facilitates pair bonding, likely due to its release during mating (Lee et al., 2009a). Indeed, OT antagonist prevents partner preference in those female prairie voles (Cho et al., 1999). The ability of OT to favor pair bonding in females is dose-dependent and may require interaction with estradiol (Bales et al., 2007a; Engell et al., 2006). A similar role of OT in male is less clear, studies show either a facilitation or no effect on partner preference, and probably involves more the AVP system or an interaction between both (Bales et al., 2007b; Lee et al., 2009a).

3.5.6.2. *OT and maternal care*

We saw before that OT release in the periphery and inside OT nuclei is essential for lactation, as exemplified by the absence of milk letdown in mice deficient for OT (Nishimori et al., 1996). PVN and SON OT neurons also send collaterals in the nucleus accumbens, a key nucleus of the reward system in the CNS (Ross et al., 2009). The maternal behavior is complex and does not comprise only

nurturing of the offspring. It implies change in the olfactory system (recognition of the offspring), auditory system (hearing of the pups) food intake (for milk production), sexual behavior (rejection of interested males), aggression (to protect the offspring), cognitive and memory functions. Those changes clearly involve OT, in combination with other factors. Recently, a study dissected the role of OT in the modulation of the auditory cortex and showed how a lateralized OT system in the left auditory cortex enables pups retrieval by enhancing the salience of their calls (Marlin et al., 2015). Mitre and colleagues, using a newly developed antibody for the mouse OTR, depicted a distributed network of female mouse brain regions for maternal behaviors that are especially enriched in OTRs (Mitre et al., 2016b). The interested reader will find complete references regarding the change in the OT system during maternal care (and paternal care in some species) in the following reviews: Lee et al., 2009; Veening et al., 2015.

3.5.7. Social recognition and memory

In males, both AVP and OT play a role in social memory (the memory of conspecifics). One likely role of OT in social memory in male mice lies in the ability to recognize odors. Indeed, OT modulates cortical control of early olfactory processing which facilitates social recognition (Oettl et al., 2016). OT also induces noradrenaline release in the olfactory bulb, since OT infused in the olfactory bulb increases noradrenaline release and since the blocking of α -adrenoreceptor in the same nuclei prevents social recognition even in presence of OT (Dluzen et al., 1998, 2000). OT also plays a role in social recognition in other areas, such as the medial preoptic area. OT-KO mice fail to develop social memory, and comparison with WT mice showed that activation in the medial amygdala is necessary for this memory. But one should note that the deficits are specific to social memory and not sociability in general, as measured by the social approach task. The same deficit was observed for OTR-KO mice (Lee et al., 2009a).

In females, similar findings about the role of the OTR in medial amygdala in the modulation of social recognition were obtained (Choleris et al., 2007). Again, the role of OT is linked to the one of steroids, for instance, ER α and β -KO mice have similar deficit that OTR-KO ones (Choleris et al., 2003, 2006). Furthermore, the release of OT in the olfactory bulb after vagino-cervical stimulation is favored by high levels of estradiol during the proestrus and estrus phase, and increases social recognition (Larrazolo-López et al., 2008). An interesting review concerning the modulation of neural circuits for social behavior by OT is proposed in the following publication (Marlin and Froemke, 2017).

3.5.8. Stress and anxiety

A global observation about OT and stress responses is that it dampens them. First, OT acts on the hypothalamic-pituitary-adrenal axis. In general, higher plasma concentrations of OT are found following psychological and physiological stresses. Centrally, injection of OT antagonist in the PVN increases the basal or stress induced levels of adreno-corticotropin hormone (ACTH) (Lee et al., 2009a; Neumann et al., 2001). Secondly, OT is a potent anxiolytic. For instance, release of OT during mating reduce anxiety-like behavior in male mice, an effect reversed by OTR antagonist (Waldherr and Neumann, 2007). Bilateral OT infusions in the PVN have anxiolytic effects measured in both the elevated plus maze (EPM) and the light-dark box test (Blume et al., 2008). OT anxiolytic effects are also present in females. Indeed, following i.c.v injection of OT, a dose dependent decrease in plasma concentration of corticosterone was observed upon exposure to an auditory stressor. The same OT-induced anxiolysis was present upon exposure to a novel environment (Windle et al., 1997). OT was also anxiolytic in the EPM test in ovariectomized mice but only when combined with estradiol treatment, which highlight a dependency to sexual hormones (McCarthy et al., 1996). Furthermore, OT seems to reduce post-partum anxiety, since injection of an OTR antagonist in the ventro-caudal periaqueductal gray (PAG) increases the anxiety measured in the EPM in lactating dams, but not in virgin ones (Figueira et al., 2008).

Of interest for the work that will be presented here, the anxiolytic actions of OT are linked to the nonapeptide action in the amygdala. When OT is injected in the CeA of ovariectomized females, anxiety is reduced in the open field, and effect not present when OT is injected in the VMH (Bale et al., 2001). This experiment is crucial in that it demonstrates a key role for OT in the regulation of negative affects by its action in the amygdala, particularly in the CeA, which will be developed further in a few pages (see part 3.6). Furthermore, OT-KO mice display an anxious phenotype in the EPM, which is rescued by i.c.v OT infusion which acts in an OTR specific manner (Mantella et al., 2003). Furthermore, OT-KO mice have increased corticosterone levels only upon acute or chronic psychogenic stress and not in basal condition. This phenotype is not present with a physical stressor like insulin induced hypoglycemia, indicating a regulation of specific forms of stress by OT (Amico et al., 2008, 2004; Mantella et al., 2003).

To summarize the different behavioral effects discussed so far, Lee and colleagues propose the following table (TABLE 3). Note that this table also summarizes effects not discussed here: OT effects on learning and memory, aggression, and the known effects of OT on humans.

One crucial role of OT that was not discussed here is the functions of central OT in the regulation of nociception and pain, central to the work presented here, will be discussed in the next part.

Behavioral classes	Behaviors	Effects of Oxt in rodents	Effects of Oxt in humans
<i>Social behaviors</i>			
Social memory	Social recognition	-↑ odor processing in olfactory bulb -↑ social memory -↓ social recognition in Oxt KO mice - abnormal Bruce effect in female Oxt KO mice	-↓ amygdalar activation to social stimuli -↑ memory for faces
Affiliation	Sexual behavior	-↑ erections (with T) and ejaculation frequency in males -↑ receptivity (with E) in females	-↑ arousal in men and women -↑ uterine contractions at parturition
	Paternal behavior	-↓ parental behavior with concomitant Avp/Oxt antagonism -↓ adult paternal behavior with Oxt antagonist on PND1	no known effect
	Maternal behavior	- ↑ Oxt throughout the brain with onset of maternal behavior - necessary for lactation - induces full repertoire of maternal behaviors (in presence of E) - ↓ pup retrieval and pup survival in Oxt KO	no known effect
Aggression	Female aggression	-↑ Oxt levels in CeA correlated with aggression	no known effect
	Male aggression	- may have organizational effect during prenatal period	-↑ plasma Oxt levels in males with conduct disorder
<i>Non-social behaviors</i>			
Learning and memory	Non-spatial memory	-↓ memory in passive avoidance tasks	-↓ episodic memory in men and women -↓ verbal recall of certain categories of words
	Spatial memory	-↑ memory when injected into hippocampus - ↓ memory when injected into NBM	no known effect
Anxiety and depression	Anxiety	-↓ anxiety following Oxt administration -↑ anxiety in some Oxt KO mice; sexually dimorphic	-↓ amygdalar response to threatening stimuli -↓ anxiety to social stressors
	Depression	-↑ active/coping behaviors with i.p. Oxt administration	- ↓ plasma Oxt associated with major depression

TABLE 3. Behavioral effects of OT and OTR. This table reviews the principal effect of OT on some behaviors in rodents and humans. Adapted from Lee et al., 2009

3.5.9. Nociception and pain modulation: OT, the peptide that relieve

Pain is an ubiquitous experience in animals, described as a normal sensation triggered in the nervous system as an alert to the possibility of an injury and a need for rest and recuperation (Goodin et al., 2015). The international association for the study of pain uses the following definition: pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. The pain pathways consists of an ascending pathway, that convey the nociceptive signals from the periphery to the CNS, and a descending pathway, which projects onto different elements of the pathways to either increase or decrease the pain signals (see the scheme of general mechanisms of pain pathways in FIGURE 30 on next page).

OT is recognized as an important mediator of endogenous analgesia (Tracy et al., 2015). It acts on every level of nociception and pain processing, from the peripheral nervous system (see 2.2.2), to the spinal and finally the supraspinal levels. Next, we will examine the effects of OT on nociception from the spinal to the supraspinal level.

3.5.9.1. Anti-nociceptive effect of OT at the spinal level

PVN parvocellular OT neurons send axons to the laminae I and II of the dorsal horn, and around the central canal (Jójiárt et al., 2009; Puder and Papka, 2001; Swanson and McKellar, 1979). Those OT axons establish synaptic contacts with lamina I and II neurons of the dorsal horn and in the central gray (lamina X) (Moreno-López et al., 2013; Rousselot et al., 1990).

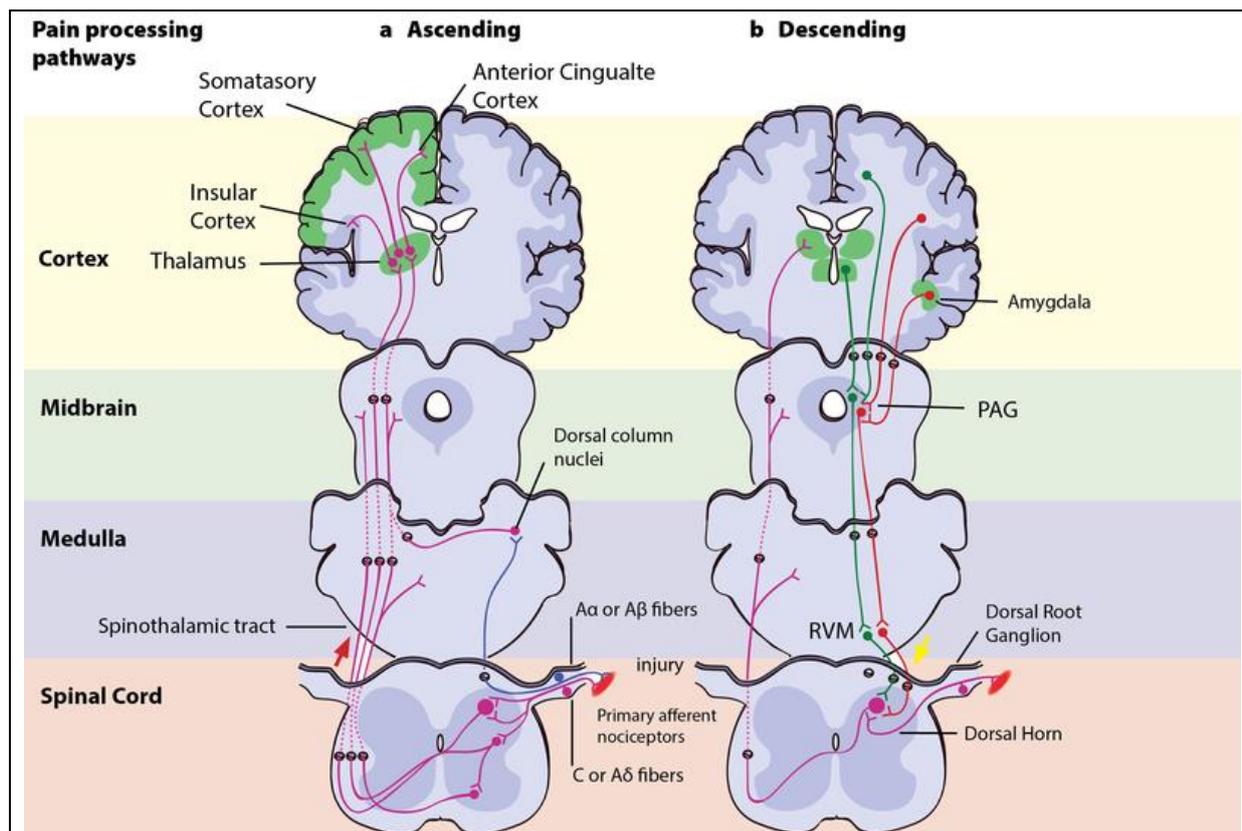


FIGURE 30 Pain processing pathways. **Left** - Ascending pain pathways: An injury is signaled simultaneously via fast-conducting A α or A β -fibres and slow-conducting C-pain or A δ -fibres. The fast A-fibres signal pressure, stretching and other tissue movements to the somatosensory cortex via the dorsal column nuclei. The C- and A δ -fibres sends pain information from nociceptors in the tissue or skin, and transmits these signals to second order neurons in the dorsal horn of the spinal cord. The second order neurons then cross over to the opposite side, where they form the ascending spinothalamic tract. This tract projects signals to nuclei in the medulla and midbrain on the way up to the thalamus (T). The thalamus relays the information to the somatosensory and insular cortex, as well as cortical regions mediating different aspects of the pain experience such as affective responses in the cingulate cortex. **Right** - Descending pain modulation pathways: Information from the environment and certain motivational states can activate this top-down pathway. Several areas in the limbic forebrain including the anterior cingulate and insular cortex, nuclei in the amygdala and the hypothalamus (H), project to the midbrain periaqueductal grey (PAG), which then modulates ascending pain transmission from the afferent pain system indirectly through the rostral ventromedial medulla (RVM) in the brainstem. This modulating system produces analgesia by the release of endogenous opioids (or other neuroactive substances like OT), and uses ON- and OFF-cells to exert either inhibitory (green) or facilitatory (red) control of nociceptive signals at the spinal dorsal horn. Adapted from nobaproject.com.

OTR is densely expressed in the superficial laminae of the dorsal horn, in good match with the presence of OT fibers (Reiter et al., 1994). Those anatomical observations are corroborated by experimental data. In a model of pain hypersensitivity induced by a spinal nerve ligation, dams that delivered had higher threshold for pain if they were lactating, in correlation with higher CSF levels of OT. Following separation of the pups, the hypersensitivity for pain increased again, an effect that could be reversed by intrathecal administration of OT. The other way around, an OTR antagonist abolished the increase in pain threshold of lactating dams. This indicates that there is a tonic release of OT in the SC, at least after delivery and during lactation, which can alleviate pain hypersensitivity (Gutierrez et al., 2013). *In vivo* unit recordings at different levels of the SC in rats showed that OT application could elicit either activation or inhibition of the recorded neurons. OT also reduced the

increased excitability evoked by glutamate application or nociceptive somatic stimulation. The differential activation or inhibition of the recorded cells suggest the role of inhibitory interneurons acting on second order neurons (Condés-Lara et al., 2003). Later, Breton and colleagues demonstrated through patch-clamp of lamina II neurons that OT actually activates a sub-population of glutamatergic interneurons, which allows the recruitment of GABAergic interneurons in the same lamina. This increased inhibition potentially inhibits incoming pain signal coming through A δ and C afferent (Breton et al., 2008). They also further showed that in the same lamina, OTR activation reduces the firing of neurons exhibiting a depolarization induced bursting firing pattern, and have no effect on the single spike firing pattern cells. They further proved that those OTR mediated change in firing pattern are mediated by a reduced amplitude and I_A inactivation of voltage-gated potassium currents (Breton et al., 2009). Of importance, contrary to the peripheral effect of OT on nociception, it seems that the AVP system is not involved in those effects of OT at the spinal level (Rojas-Piloni et al., 2010). Those effects of exogenous OT can also be observed through the stimulation of the descending OT projections from PVN. Indeed, while recording the nociceptive A δ and C evoked discharges, Condés-Lara and colleagues could reduce the duration of such discharges by electrical stimulations of the PVN. The effect of PVN stimulation is replicated by exogenous application of OT on the spinal site of recording, and both effects were reversed by application of an OTR antagonist. They further proved that, in a *in vivo* model of hyperalgesia and allodynia, intrathecal OT have clear anti-nociceptive properties (Condés-Lara et al., 2006), and that electrical stimulation of the PVN had a similar effect, both being dependent on OTR activation (Miranda-Cardenas et al., 2006). The same laboratory recorded PVN cells responses upon noxious mechanical and thermal stimuli, and found that a subpopulation of cells was responding to such stimuli and projected to the SC (Condés-Lara et al., 2009a). Furthermore, only stimulation in the anterior parts of the PVN can evoke an increase in spinal tissue OT contents, whereas posterior stimulations only increase OT in CSF (Martínez-Lorenzana et al., 2008). This could be explained by the sole stimulation of magnocellular neurons in posterior parts of the PVN, which do not project to the SC. Furthermore, stimulations of the PVN also evoke responses in the centrally projecting post-synaptic dorsal column neurons (Rojas-Piloni et al., 2008). *In vivo* studies by DeLaTorre and colleagues further showed that exogenous OT or endogenous OT released through PVN stimulation could reduce or prevent the LTP in spinal wide dynamic range neurons (WDR). Those neurons receive nociceptive and non-nociceptive sensory information and further project supra-spinally, and long term potentiation (LTP) in those cells can facilitate nociceptive evoked responses (DeLaTorre et al., 2009). In a rat model of inflammatory pain, PVN cells are activated as measured by an early genes expression and high levels endogenous levels of OT in SC. Furthermore, spinal OT exerts a tonic analgesia, as proven by the increase in pain thresholds after OTR antagonist injection. Interestingly, the long-lasting analgesia ensured by OT relies on

neurosteroidogenesis, which leads to increase GABA_A mediated inhibition in lamina II neurons (Juif et al., 2013). Those results indicate that long-lasting anti-nociceptive effects of OT might protect against central sensitization. Indeed, this model explains the transition from acute to chronic pain by potentiation (LTP) of nociceptive neurons after repeated noxious stimuli (or other mechanisms), which strengthen further the nociceptive signals. In humans, it was reported that in patients suffering from low back pain, intrathecal OT injections had a dose dependent analgesic effect (Yang, 1994). Of interest, spinal OT anti-nociceptive effects on somatic pain (used in previously cited studies) are also present for visceral pain, as the following study using bladder distention noxious stimulus highlighted (Engle et al., 2013).

As we already mentioned, a GABAergic interneurons population does respond indirectly or directly to OT application in SC (Breton et al., 2008) or PVN stimulation (Rojas-Piloni et al., 2007) to inhibit the activity of nociceptive centrally projecting cells. These results were further confirmed, but a participation of μ -opioid receptor was also involved. Indeed their blockade did partially block the inhibitory effects of OT application or PVN stimulation (Condés-Lara et al., 2009b; Miranda-Cardenas et al., 2006). Similarly, another study showed the participation of mu and kappa opioid receptors in the anti-nociceptive effect of OT in an inflammatory pain model (Yu et al., 2003). In an *ex vivo* preparation, mu-opioid agonist (as did OT and corticotrophin releasing factor) could decrease the spinal nociceptive inputs (Wilson et al., 2009). Furthermore, an *in vitro* study showed that evoked OT release from synaptosomes derived from spinal cord was decreased by dynorphin, suggesting that spinal OT release could be under inhibitory influence of κ -opioid receptor (Daddona and Haldar, 1994). Endogenous opioid systems constitute another descending pathway controlling nociception so it is reasonable to think that both pathways might actually be interacting. Rash and colleagues reviewed literature about the effect of OT on nociceptive processing and report several studies which demonstrated that antagonists of mu or kappa opioid receptors could partially block the anti-nociceptive effect of OT, but mostly through actions on supraspinal areas (Rash et al., 2014).

3.5.9.2. *Antinociceptive properties of OT in supraspinal structure*

Pain stimulation increases OT concentration in many supra-spinal areas such as: hypothalamic ventromedial nucleus, thalamic reticular nucleus, locus coeruleus, raphe magnus nucleus, caudate nucleus etc, that without elevating the plasma concentration of the nonapeptide. I.c.v OT can elevate pain threshold, whereas an anti-oxytocin serum reduces it (Yang et al., 2007a). In stressful situation like the force swim test, OT is released both centrally and peripherally from the PVN (Juif and Poisbeau, 2013; Juif et al., 2016; Robinson et al., 2002). Injections of OT antiserum, and not AVP one, inhibit the increase in pain threshold evoked by the chemical stimulation of the SON, an indication

that SON OT neurons are also involved in pain modulation (Yang et al., 2011a). In another study, an injury through incision of the skin decreased the OT content of the PVN indicating the release of OT, and i.c.v injection of OT elevated the mechanical threshold for pain in a dose-dependent manner while no effect of intrathecal OT injection was observed (Zhang et al., 2015). This result indicates independent mechanisms for spinal versus supraspinal nociception by OT. Anti-nociception by OT could also be observed while OT was injected in the cerebral cistern, PAG, nucleus raphe magnus, lateral ventricle and nucleus accumbens, but with the involvement of mu or kappa opioid receptors (Xin et al., 2017) notably in the PAG (Ge et al., 2002). The PAG and the rostral ventromedial medulla are highly connected, and are a key centers of descending pain pathways (Heinricher and Ingram, 2008). Many OT fibers and OT receptors can be found in the PAG (Figueira et al., 2008). Painful stimulations are able to elevate the OT content in the PAG and furthermore, intra-PAG injection of OT can increase the pain threshold and stimulate the release of endogenous opioid, whereas infusion of OT antagonist decreases the pain threshold and naloxone (an opioid receptors antagonist) also reduced the antinociceptive effect of OT (Yang et al., 2011b, 2011c). It should be added here that AVP could potentially have a nociceptive function by regulating the endogenous opioid system in the PAG (Yang et al., 2007b). Of importance for latter discussion, one should note that the PAG is massively interconnected with the CeA, which underpin the role of the CeA in pain modulation (Rizvi et al., 1991). The serotonergic system might also be involved in OT pain modulation, indeed the Raphe nuclei shows an increase in *c-fos* after PVN stimulation known to induce anti-nociception in SC (Condés-Lara et al., 2015). Serotonin given intrathecally can mimic the effect of OT and concomitantly given with the nonapeptide, potentiated OT anti-nociceptive effects. Furthermore, an antagonist of serotonergic receptors partially blocked the anti-nociceptive effect of PVN stimulation in the SC (Godínez-Chaparro et al., 2016). In an inflammatory pain model, the anti-hyperalgesic effect of i.c.v OT could be dampened by co-injection of an antagonist of endocannabinoid receptors CB1 or an opioid receptor antagonist (Russo et al., 2012). Those results highlight the implication of several neuromodulatory systems that cooperate with OT to produce analgesia. The anti-nociceptive effect of OT on the CeA, the latter being a key node in both the ascending and descending pathways of pain, has received only little attention. One study by Han and colleague shows that OT injected in the CeA has a dose-dependent and OTR specific effect on thermal and mechanical pain threshold (Han and Yu, 2009). The role of the CeA in the regulation of pain and the effect of OT on the activity of this nucleus will be discussed in part 3.6.

Another effect of OT on pain and analgesia might be more indirect. It could be that OT also has analgesic effects by improving mood. We saw that OT can interact with the serotonergic system, and in mice serotonergic neurons express the OTR and OT injection in the median raphe nucleus

reduces anxiety (Yoshida et al., 2009). OT is generally a potent downregulator of stress and anxiety levels (see 3.5.8 and Neumann and Landgraf, 2012). In humans, plasmatic OT levels are negatively correlated with depression and anxiety scores (Anderberg and Uvnäs-Moberg, 2000; Scantamburlo et al., 2007) and an intranasal OT administration study found decreased “fear-related” activation of the amygdala after OT application (Kirsch et al., 2005). Another placebo-controlled, double blind study in men showed that intranasal-OT resulted in greater calmness, less anxiety and a trend toward lower cortisol during the trier social stress test (Heinrichs et al., 2003). One study in human correlated low CSF levels of OT with high intent in suicidal subjects, indicating an implication of central levels of OT in mood disorders (Jokinen et al., 2012). Finally, we saw that in human, low plasma OT is generally associated with increased pain (see 2.2.2), but as already discussed, plasma OT concentration may or may not represent its central one (Kagerbauer et al., 2013; Rash et al., 2014; Wotjak et al., 1998) and humans studies considering plasma OT level as a reflect of the central one should be taken cautiously. Finally, chronic pain is linked with the development of anxio-depressive symptoms (Asmundson and Katz, 2009), which highlight the link between mood and pain, and knowing the various effect of central and peripheral OT on both, some crucial therapies might emerge through a better comprehension of the OT system in the CNS.

3.6. The Amygdala

The amygdala is an almond-shape set of neurons located deep in the brain's medial temporal lobe. It is part of the limbic system, and is a key component in the circuitry that assigns emotional significance and produces appropriate behavioral responses to salient external stimuli (Sah et al., 2003). On the next page you will find two figures extracted from the latter cited review, which depict the anatomy of the rat amygdala (FIGURE 31). We will next focus on the key role of the amygdala in pain and its modulation by OT.

3.6.1. Amygdala: A pain center

Pain is a multidimensional experience, which does not only comprise a nociceptive stimulus and nocifensive behavior, but also includes emotional-affective and cognitive components. We mentioned before that chronic pain leads to anxiety and depression, but the opposite is true: patients suffering from anxiety and depression are more prone to chronic pain (Neugebauer et al., 2009; Nicholson and Verma, 2004).

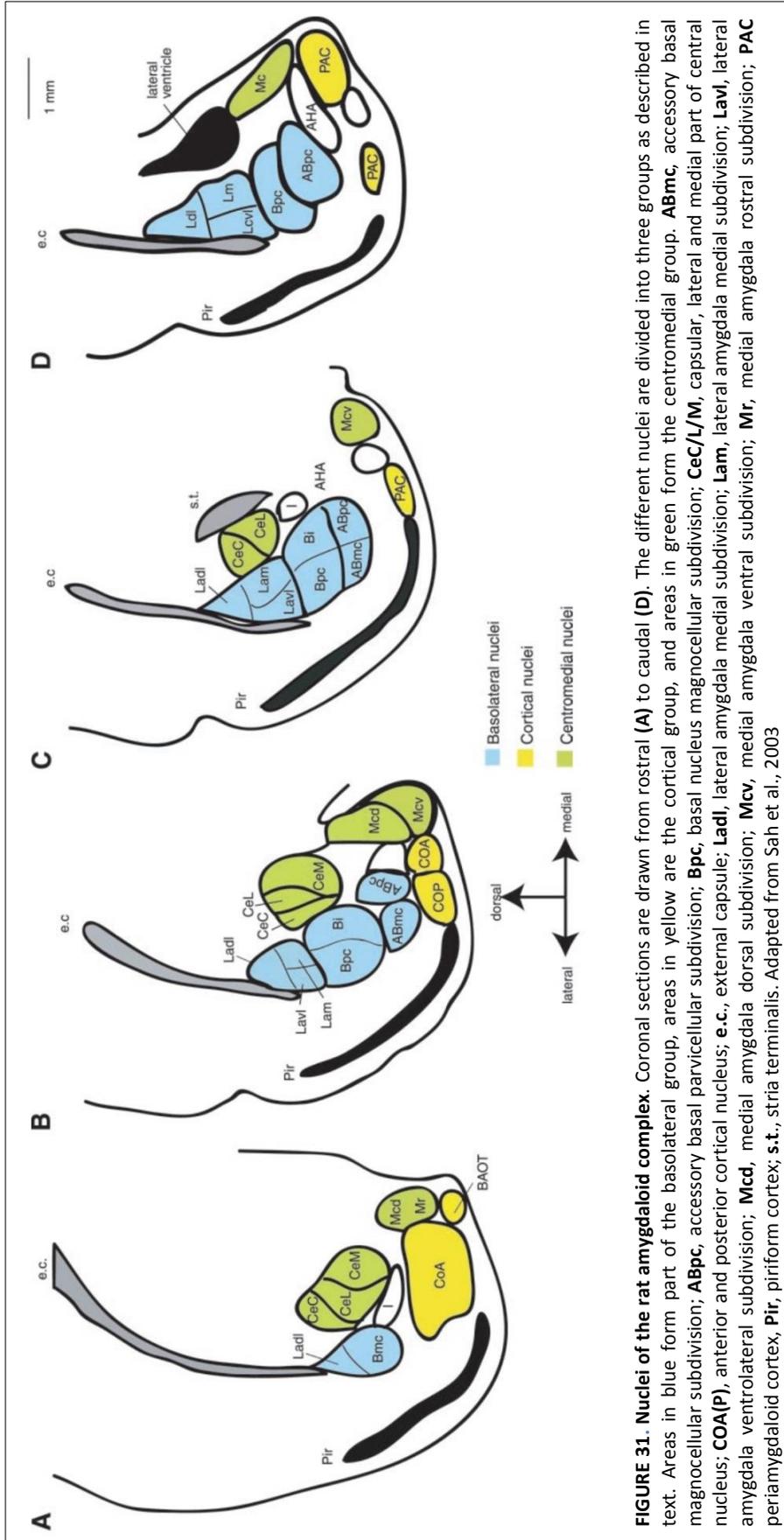


FIGURE 31. Nuclei of the rat amygdaloid complex. Coronal sections are drawn from rostral (A) to caudal (D). The different nuclei are divided into three groups as described in text. Areas in blue form part of the basolateral group, areas in yellow are the cortical group, and areas in green form the centromedial group. **ABmc**, accessory basal magnocellular subdivision; **ABpc**, accessory basal parvocellular subdivision; **Bpc**, basal nucleus magnocellular subdivision; **CeC/L/M**, capsular, lateral and medial part of central nucleus; **COA(P)**, anterior and posterior cortical nucleus; **e.c.**, external capsule; **Ladi**, lateral amygdala medial subdivision; **Lam**, lateral amygdala medial subdivision; **Lavl**, lateral amygdala ventrolateral subdivision; **Mcd**, medial amygdala dorsal subdivision; **Mcv**, medial amygdala ventral subdivision; **Mr**, medial amygdala rostral subdivision; **PAC** periamygdaloid cortex, **Pir**, piriform cortex; **s.t.**, stria terminalis. Adapted from Sah et al., 2003

The amygdala is part of a network of brain structures that process pain-related information, revealed notably by neuroimaging studies. This network is often referred as the pain matrix (Tracey and Mantyh, 2007) (FIGURE 32).

3.6.1.1. *The affective pain network*

The amygdala is part of the so-called secondary affect network of pain, in the sense it gives its affective dimension to nociception: the feelings of unpleasantness of pain. At the level of the neural correlates, there are two main pathways of input to the secondary affect network: i) spinal pathways projecting directly into limbic structures and medial thalamic nuclei pathways, which both provide direct sensory input to the secondary affect network, and ii) an indirect pathway through spinal pathways projecting into the somato-sensory thalamic and cortical areas, which then project through cortico-limbic pathways. The indirect pathways integrate contextual information and memories to provide a cognitive mediation of the pain affect (Price, 2000). This added affective component of pain contributes to the emotional and cognitive consequences of pain, and the other way around pain can also be modulated by previous emotional and cognitive states (Rhudy et al., 2008; Seminowicz and Davis, 2007; Seminowicz et al., 2004).

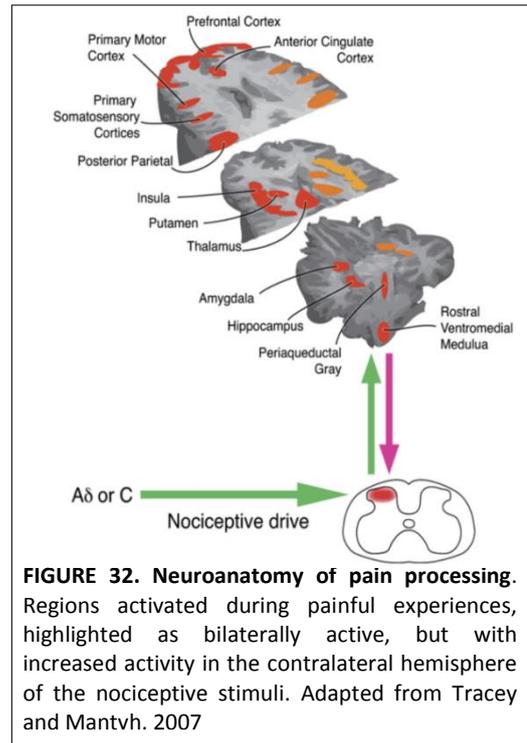


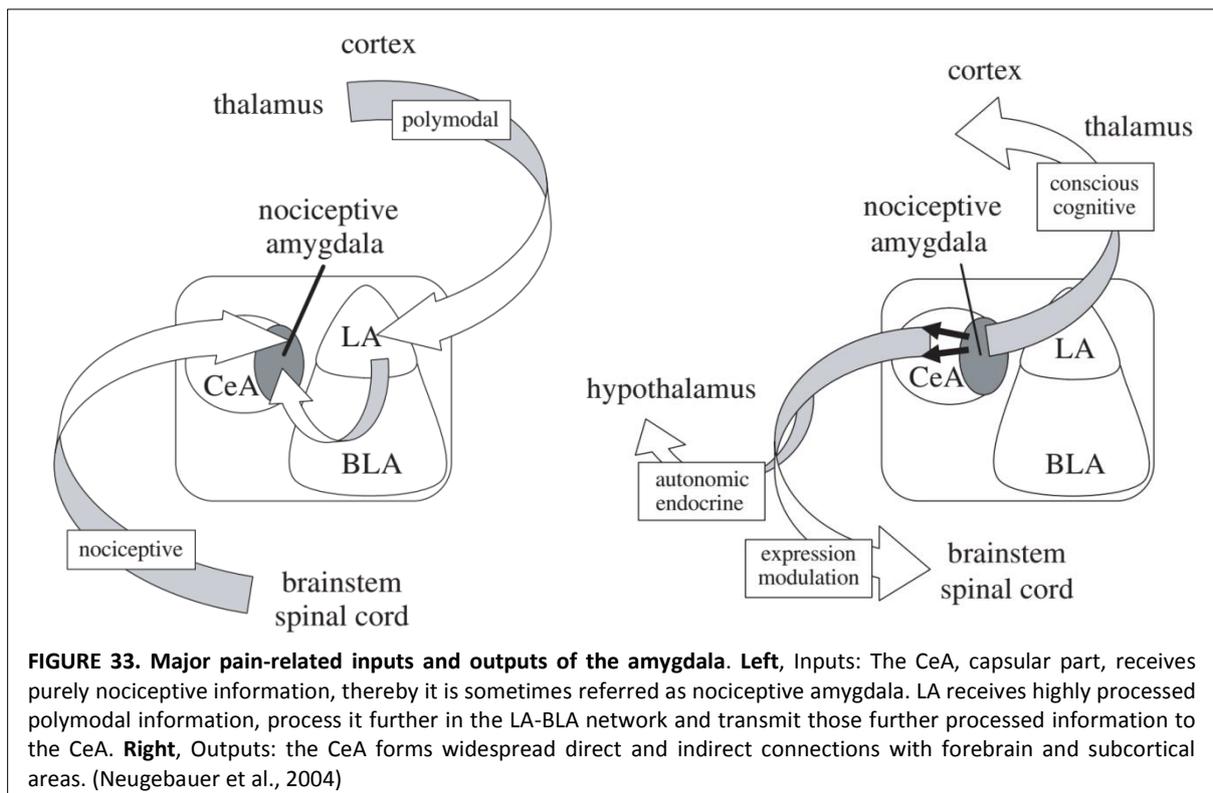
FIGURE 32. Neuroanatomy of pain processing. Regions activated during painful experiences, highlighted as bilaterally active, but with increased activity in the contralateral hemisphere of the nociceptive stimuli. Adapted from Tracey and Mantyh, 2007

3.6.1.2. *Anatomy of the painful amygdala*

The amygdala has long been known for its roles in emotions and affective disorders (Phelps and LeDoux, 2005) and in the emotional and affective dimension of pain (Carrasquillo and Gereau, 2007; Neugebauer, 2007). At the level of the amygdala subnuclei, the basolateral (BLA), lateral (LA) and central (CeA) amygdala nuclei are of particular importance in the processing of pain-related information. Purely nociceptive sensory information reaches the latero-capsular division of the central amygdala (referred as CeC or CeLC depending on authors), arriving directly from the spinal cord and the brainstem through the parabrachial area. Polymodal sensory information, including the nociceptive one, reaches the LA, through posterior areas of the thalamus and insular and association cortices. Therefore, the LA is believed to be the initial site of sensory convergence and integration in the amygdala. The LA-BLA network attaches emotional significance to sensory information, and transmit highly processed information to the CeA. The CeA is classically considered as the output

nuclei of the amygdala, projecting to descending pain centers in the hypothalamus (including PVN) and in the brain stem (including PAG, RVM, raphe magnus...) (Price, 2003). The following schemes (FIGURE 33) from Neugebauer and colleagues provides a summary of inputs and outputs of the “painful” amygdala (Neugebauer et al., 2004).

Note that the BLA is also interconnected with cortical areas, notably the prefrontal cortex, which is of critical importance regarding for the influence of pain (and generally affective states) on the decision making process based on risk anticipation, punishment avoidance etc. (see Ji et al., 2010; Pais-Vieira et al., 2009).



3.6.1.3. Pain induced plasticity In the amygdala

The central sensitization mechanisms of chronic pain include an enhanced neurotransmission in the amygdala, at both the BLA-CeL and parabrachial-CeL synapses, as shown in several model of chronic pain (Han and Neugebauer, 2004; Ikeda et al., 2007; Neugebauer et al., 2003) which are corroborated by the observed increase in amygdala activation in humans (Simons et al., 2014), for instance in patients suffering from osteoarthritis (Kulkarni et al., 2007). Decreasing amygdala activity through lesions or pharmacological interventions inhibits pain-related behaviors in different models (e.g. Fu et al., 2008; Palazzo et al., 2011) and activating the amygdala, even in normal conditions without lesions of some sort, can exacerbate or generate pain responses (Kolber et al., 2010; Li et al.,

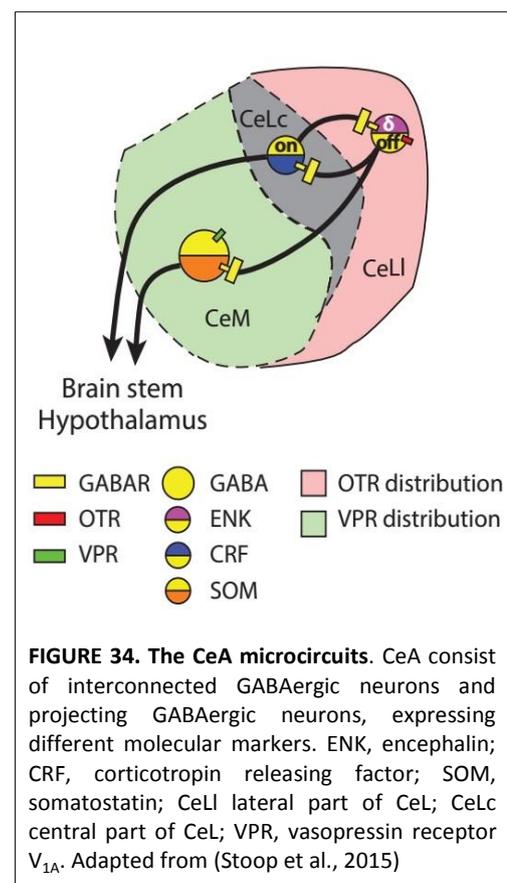
2011). In a way, one could consider the amygdala as a relay station for switching the pain ON and OFF as Rouwette and colleagues state in their review (Rouwette et al., 2012).

3.6.2. The CeA is not just an output nucleus

Nevertheless, the CeA do not act just as a switch for pain, it still processes the information it receives in internal microcircuits. When focusing solely on pain processing, the CeA is modulated by numerous neuronal and molecular actors, as the table provided in the review of Veinante and colleagues demonstrates (see next page, TABLE 4). CeA microcircuits are composed almost exclusively of inhibitory GABAergic (inter-)neurons (Cassell et al., 1999; Sun and Cassell, 1993) (FIGURE 34), which are mutually connected (Janak and Tye, 2015; Veinante et al., 2013) and respond each to various and different stimuli (Ciocchi et al., 2010). These microcircuits allow for a further integration and processing of signals, and project to and regulate the activity of various neural circuits and their functions (Keifer et al., 2015). One important pattern that emerges is that CeL neurons are highly interconnected and most project to the CeM where they regulate the firing of the output neurons of the CeM. But CeL cells also project outside of the CeA, and some CeM neurons project back to the CeL (not shown in figure), forming a complex circuits of mutual inhibitions (Badrinarayan et al., 2012; Pitkänen et al., 1997)

3.6.3. The CeA and OT

Among the sub populations of GABAergic neurons of the CeA, a clear distinction can be made while looking at the expression of OTR and V_{1A} . Huber and colleagues were the first to make the distinction between purely OTR and V_{1A} positive areas of the CeA, that previous autoradiography studies did not discriminate. They further demonstrated that OTR expressing CeL neurons respond to TGOT and doing so, inhibit the V_{1A} expressing neuron through GABA release. The reciprocal was not true, as AVP could excite the CeM neuron without affecting the CeL one, and those effects were inhibited by the appropriate antagonists. Finally, they demonstrated that activation of the GABAergic OTR expressing neuron of the CeL could efficiently reduce CeM neurons firing induced by stimulation of its afferent in BLA (Huber et al., 2005). The



Pain type ^a	Pain related outcome ^b		Reference
	Nociceptive behavior	Affective/emotional	
<i>1. CeA lesion</i>			
Naïve	- Reduced morphine-induced, stress-induced and conditioned hypoalgesia		[55,56,69]
Formalin	- Reduced morphine-induced and conditioned hypoalgesia	- Decreased pain-induced CPA	[70-73]
Acetic acid		- Decreased pain-induced CPA	[72]
<i>2. Injection of muscimol</i>			
Neuropathy	- Reduced mechanical hyperalgesia	- Decreased escape/avoidance	[74]
<i>3. Injection of NMDA antagonist</i>			
Neuropathy		- Decreased pain-induced CPA	[75]
<i>4. Injection of group I mGluRs ligands</i>			
Naïve	- Agonist induced visceral and mechanical hypersensitivity - Antagonist reduced visceral sensitivity		[76,77]
Formalin	- Antagonist reduced mechanical hypersensitivity		[77]
Arthritis	- Antagonist reduced mechanical hypersensitivity	- Antagonist decreased vocalizations	[78]
Neuropathy		- Agonist increased, and antagonist decreased, pain-induced CPA	[75]
<i>5. Injection of group III mGluRs agonists</i>			
Naïve	- Decreased mechanical sensitivity (mGluR7)	- Decreased vocalizations and anxiety	[79]
Arthritis	- Increased mechanical sensitivity (mGluR8)	- Increased vocalizations and anxiety	[79]
<i>6. Injection of cholinergic agonists</i>			
Naïve	- Decreased thermal sensitivity, reduced jaw opening reflex	- Decreased vocalizations	[63,65,66]
<i>7. Injection of noradrenergic α_2 ligands</i>			
Naïve	- Agonist induced mechanical and thermal hypoalgesia - Antagonist reduced stress-induced thermal hypoalgesia		[64,80]
Acetic acid		- Agonist decreased pain-induced CPA	[81]
<i>8. Injection of noradrenergic β antagonists</i>			
Acetic acid		- Decreased pain-induced CPA	[81]
<i>9. Injection of CGRP receptor ligands</i>			
Naïve	- CGRP decreased mechanical and thermal reflexes		[59]
Naïve	- CGRP increased mechanical reflexes	- CGRP increased vocalizations	[82]
Arthritis	- CGRP1 antagonist inhibited the enhanced reflex to mechanical stimulus	- CGRP1 antagonist decreased vocalizations	[83]
<i>10. Injection of CRF receptor ligands</i>			
Naïve	- CRF decreased mechanical and thermal sensitivity		[58]
Naïve	- CRF increases mechanical sensitivity	- CRF increased vocalizations	[84]
Arthritis	- CRF1 antagonist reduced mechanical hypersensitivity	- CRF1 antagonist decreased vocalizations and anxiety	[85,86]
<i>11. Injection of oxytocin, vasopressin, neurotensin, galanin</i>			
Naïve	- Decreased mechanical and/or thermal sensitivity		[57,60-62]
<i>12. Injection of opioid receptors ligands</i>			
Naïve	- Morphine and β -endorphin induced mechanical and thermal hypoalgesia	- Morphine decreased vocalizations	[47,66]
<i>13. Corticosterone implants</i>			
Naïve	- Sensitized visceromotor reflexes to colorectal and urinary bladder distension and to somatic mechanical sensitivity	- Increased anxiety	[87-90]

TABLE 4. Various effects of CeA manipulation on pain-related outcomes in different pain models. The multiple effects of various neuroactive substances injected in the CeA on the pain symptoms exemplify the numerous levels of regulations possible in the CeA. For references, see the review of Veinante et al., 2013.

inhibition of AVP responsive cells by OT responsive ones in the CeA might be an example of a general mechanism of neuropeptides action on behavior (Stoop, 2014; Viviani and Stoop, 2008). Further studies by Viviani and colleagues revealed that two populations of CeM neurons, one inhibited through action of OT in the CeL and one unaffected, were projecting to the PAG and to the dorsal vagal complex (DVG), respectively. Since the DVG is important for cardiovascular regulation and the PAG modulates the freezing behavior, the authors measured both outcomes through heart rate variability measurements and freezing behavior assessment in a contextual fear conditioning paradigm. They showed that TGOT could inhibit the freezing behavior, and that muscimol, a GABA agonist, had the same effect. But TGOT did inhibit freezing without modifying the heart rate variability, whereas muscimol changed it (Viviani et al., 2011). This study showed that OT responsive CeL interneurons can selectively gate different responses, to fear in this case, by acting on a subpopulation of projection neurons of the CeM. The physiological relevance of such regulation of CeA by OT was further tested. Using optogenetics to allow specific activation of the axons of OT neurons, Knobloch and colleagues showed that activation of OT axons in the CeL elicited the same responses in CeA GABAergic neurons as did exogenous applications of TGOT, but revealed a further glutamatergic component in those light-evoked responses. In a contextual fear conditioning paradigm, they could reduce freezing by optogenetic activation of OT axons in the CeL, demonstrating that the sparse OT fibers innervating the CeL are sufficient to elicit a drastic change in behavior in rats (Knobloch et al., 2012). Of interest, the onset and offset of behavioral changes after light stimulation of OT release were highly variable and sometimes delayed by several seconds after the beginning of the stimulation.

The study of the cellular mechanisms of OT effects in the CeA and how they affect pain and anxiety will be the focused of the second part of my work (ARTICLE 2). Please note that I also participated as a second author in a study about the importance of the local excitatory/inhibitory balance in the BLA in setting the mechanical nociceptive threshold. The corresponding published article is available in ANNEXE 3.

3.6.4. Amygdala and astrocytes

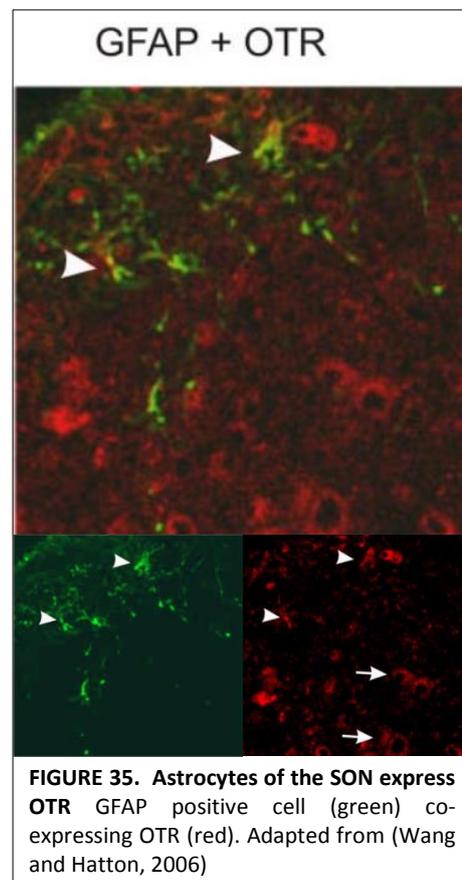
Not much is known about astrocytes in the amygdala and even less about CeA astrocytes. Sparse studies report that:

- Astrocytes are activated and proliferate in the amygdala after various seizures (Johnson et al., 2010).
- Impaired glutamate uptake and metabolism in the astrocytes of the amygdala could be involved in depression (Lee et al., 2007).

- Prenatal stress reduce the number of glial cells in the BLA and especially CeA at P25 (Kraszpulski et al., 2006).
- Astrocytic glycine may be released by astrocytes of the LA in response to afferent activity and participate in LTP, while astroglial cells tonically release D-Serine in basal, low afferent activity conditions (Li et al., 2013).
- Gliotransmitter release through astrocytic connexin 43 hemi-channels is necessary for memory consolidation in the BLA. (Stehberg et al., 2012)
- Olfactory bulbectomized rats show activated astrocytes in the amygdala (expressing more GFAP, sub-nuclei unknown) (Burke et al., 2013)
- A spared nerve injury of the sciatic nerve (neuropathy model) induced no change in astrocytes and microglial markers In the dorsal horn of the SC, but an increase in neurotransmitters transporter in all supraspinal regions measured (including amygdala), and astrocyte activation (increased GFAP) only in amygdala (Marcello and Cavaliere, 2012)

3.7. Which cells express the OTR?

The obvious response to this question is the neurons, as OT modifies their activity in numerous regions which results in a plethora of behaviors. Nevertheless, several studies demonstrate that they are also expressed at least by astrocytes. While working on the effect of OT on the production of prostaglandin in SON OT and AVP neurons, Wang and Hatton looked at various protein expression through immunohistochemistry, and found that GFAP positive cells of the SON express the OTR (FIGURE 35) (Wang and Hatton, 2006). Di Scala-Guenot and colleagues showed that hypothalamic cultured astrocytes express the OTR and they characterized OT binding to it, which is different than the one of cultured neurons and requires Mg^{2+} . They further showed that activation of cultured astrocytes OTR induces increase in $[Ca^{2+}]_i$, triggers oscillations of $[Ca^{2+}]_i$ in a few cells, which rely fully on intracellular IP_3 sensitive calcium stores. The astrocytic OTR desensitized through a PKC dependent phosphorylation and was recycled. Finally, they showed that co-culture with neurons without physical contacts between cells increased OTR expression in astrocytes, through actions of TGF β . But when neurons could contact astrocytes, it induced a decrease in both OTR binding and mRNA



contents (Mittaud et al., 2002; Di Scala-Guenot and Strosser, 1992, 1995a, 1995a, 1995b; Di Scala-Guenot et al., 1994). Furthermore, In cultured hypothalamic astrocytes, responses to OT seem linked to metabotropic glutamate receptors activation (Kuo et al., 2009). Using a novel antibody, Mitre and colleagues did an extensive mapping of OTR expression in the mice brain, and found somatic OTR expression on ~15% of glial cells of the auditory cortex (Mitre et al., 2016a). Finally, using a knock-in mouse in which the *OTR* gene was replaced by the Venus fluorescent protein, OTR expression was found to co-localize with GLAST positive cells in the SFO, OVLT and molecular layer of the cerebellar cortex (Yoshida et al., 2009).

4. PhD objectives

My PhD has been divided into two main research lines, with one common topic:

What underpins the mechanisms of action of OT in the CNS at the cellular and cell circuits' levels?

To tackle those questions a collaboration between the laboratory of Prof. Valery Grinevich from the DKFZ in Heidelberg (Germany) and my host laboratory in Strasbourg was established and is still tightly maintained. The interest of this association is multiple; the two research teams have a keen interest in the OT system in the CNS, and have a proven expertise in the field. At the technical skills level, our labs are complementary, Prof. Grinevich team being skilled in state of the art anatomical tracing and transgene delivery techniques, while our lab is more focused on functional and integrated neurosciences. This collaboration was continued all along my PhD, and helped to respond to an array of scientific questions.

4.1. OT neurons connectivity between hypothalamic nuclei: the role of a subpopulation of OT neurons.

We saw that OT is a potent endogenous analgesic and anti-nociceptive agent, which acts at several levels, from the periphery to the supraspinal circuits. It is still unknown how OT neurons coordinate themselves to mediate those functions. Are all OT neurons involved or are there specialized population and circuits for a given function? Or to formulate it differently, are there functional subpopulations and circuits among the OT cells of the CNS? Another unanswered question, secondary to the first one, is to know if there are precise anatomical and functional links between the two main types of OT cells, the parvocellular and magnocellular neurons. The literature describes them as two separated entities, morphologically but also on the functional aspects. Is it true, or some form of communication does exist between them?

The exploration of the role of a newly found parvocellular to magnocellular OT cells circuit has been the subject of a part of my PhD. I helped to tackle the functional role of this circuit in the modulation of inflammatory pain, using various electrophysiological and optogenetic tools, both *in vivo* and *ex vivo*. This work is presented in ARTICLE 1.

4.2. A role for astrocytes in OT modulation of CeA circuits ?

Astrocytes are active players of the information processing in the CNS, as we discussed in the introduction. In particular, they tightly regulate the magnocellular OT neurons, and express the OTR in some regions. OT is a potent modulator of CeA microcircuits, with drastic behavioral effects. But several observations point towards a peculiar neuromodulation of CeA circuits by OT. When applying exogenous TGOT or stimulating endogenous OT release, we observed very long and varying delays of action, both *in vitro* and *in vivo* (unpublished observations, except for the onset of behavioral effect in fig.5, C2, in Knobloch et al., 2012). Those variable and long onset times are peculiar, if one expects a direct action on the neurons. Several technical and physiological arguments could explain those delays, for instance in the case of evoked axonal release of OT, we might be in presence of a short-distance volume transmission through release of OT from the axons in the extracellular milieu, which would reach the neuronal targets through passive diffusion. But what if the OT message in the CeA was relayed through, and/or echoed and amplified by astrocytes? Indeed, if a temporal order of magnitude for neuronal synaptic transmission is the millisecond, astrocyte calcium excitability is a phenomenon in the second range, which fits with our observations of the onset of OT effects. This exciting hypothesis was proposed to me by Dr. Charlet as part of my PhD work. I used both established and new technical tools to tackle that hypothesis, both *in vivo* and *ex vivo*. The results of this work are presented in ARTICLE 2.

I. ARTICLE 1

1. Overview

INTRODUCTION

The parvocellular OT neurons have received few attention compared to their magnocellular neighbors. Not much is known about their precise role in various phenomena, and most of the studies so far based themselves on the observed projections in brainstem and SC to infer their functions. This lack of studies comes mostly from the absence of a specific molecular marker or identity gene that would allow a specific manipulation of parvocellular OT neurons. For instance, their putative intra- or inter-OT nuclei connectivity, notably with magnocellular OT neurons, remains unknown so far. One of the attributed functions of parvocellular OT neurons is an anti-nociceptive role through axonal projections in the spinal cord. But OT also modulates nociception in the periphery, or the release of OT in the bloodstream is undertaken by the sole magnocellular OT neurons. Hence, we characterized the connections between parvocellular and magnocellular OT neurons in the hypothalamus of the rat, and tested their respective role in nociception.

RESULTS

Using virally induced expression of fluorescent protein in the PVN neurons, we found that PVN cells contact both the contra- and ipsi-lateral SON through a dense plexus of axons. Those contacts in SON are found on OT expressing dendrites and cell body and are synaptic, probably of OT/glutamatergic and excitatory nature. We demonstrated that optogenetically driven excitation of OT axons of PVN origins in the SON could induce firing in SON units, putatively OT neurons, and that this excitation implicated the OT and AMPA receptors. We also showed that such stimulations could increase the release of OT in the blood stream, an indication that OT axons of the PVN can drive the neuroendocrine activity of SON magnocellular neurons. Using the retrogradely transported Cre expressing CAV2 (Canine adenovirus type 2) injected in the SON to induce the expression a Cre-dependent fluorescent protein only in the SON-projecting OT cells of the PVN, we anatomically identified the SON projecting PVN neurons as a small population of 30 neurons, morphologically corresponding to parvocellular neurons. Those cells did not uptake systemically injected fluorogold beads, which is one more indication that they are not pituitary-projecting neurons (i.e. magnocellular). Further electrophysiological recordings of those cells and of magnocellular ones nearby were in agreement with the anatomical conclusions.

Next, we assessed the role of this sub-population on nociception. Anatomical data showed that this sub-population of PVN cells also project in the SC, and produce synaptic contacts mostly on neurons

of deep layers, which co-express OTR and NK1 (substance P) receptors. Using *in vivo* unit recordings in SC deep layers, we identified wide dynamic range neurons (WDR) responding to nociceptive paw stimulations. The C-fiber-evoked increase in firing of those cells was further amplified by a NK1R agonist, and inhibited upon optogenetic stimulation of OT fibers of the SC. Through patch-clamp recordings in SC slices, we demonstrated that putative WDR of deep layers showed decrease excitability upon application of OTR agonist or biased agonist TGOT and atosiban, respectively. The injection of the CAV2 retrograde virus this time in the SC deep layers confirmed that the SC and SON projecting OT neurons of the PVN are the same cells, sending axon collaterals to both structures. Using the same recordings of WDR neurons in the SC, we showed that those parvocellular bi-projecting OT neurons produced a fast inhibition of C-fiber discharges when their SC projecting axon collaterals were stimulated, and produced a slower but longer inhibition, mediated by peripheral OTR, when the light stimulation occurred on their SON projecting axon collaterals.

Finally, we used the same viral strategy to express either channel-rhodopsin 2 or inhibitory Designer Receptor Exclusively Activated by Designer Drugs (DREADD) in this sub-population of bi-projecting parvocellular neurons. Using an inflammatory pain model in those animals, we demonstrated that optogenetic activation of this subpopulation had an anti-nociceptive effect, in an OTR specific manner, while clozapine-N-oxide (the DREADD agonist) decreased the pain threshold, which also indicates that this system of 30 parvocellular neurons was endogenously activated in those rats.

Therefore, we identified a small population of OT parvocellular neurons that coordinates efficiently with other OT neurons to control nociception through both central and peripheral mechanisms.

AUTHOR CONTRIBUTION

This study was obviously a group effort, for the sake of clarity I detail below my personal contribution to the following experiments (and consequent analysis):

- *In vivo* electrophysiological recordings, targeted drug infusions and optical stimulation of supraspinal structures.
- *Ex vivo* SC slices patch-clamp recordings.
- Stereotaxic surgeries for optical fiber implantation used in behavioral experiments.

A New Population of Parvocellular Oxytocin Neurons Controlling Magnocellular Neuron Activity and Inflammatory Pain Processing

Highlights

- Thirty parvocellular oxytocin neurons (ParvOT) alleviate acute pain
- ParvOT project to WDR sensory neurons in spinal cord (SC)
- ParvOT activate OT release from magnocellular OT neurons (magnOT)
- Dual pain suppression by peripheral magnOT and central SC OT

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In Brief

Eliava, Melchior, Knobloch-Bollmann, Wahis et al. demonstrate that thirty specialized oxytocin neurons in the rat hypothalamus coordinate activity of oxytocin neurons and deep dorsal horn spinal processing, as revealed by the repression of nociceptive messages and the promotion of analgesia.



A New Population of Parvocellular Oxytocin Neurons Controlling Magnocellular Neuron Activity and Inflammatory Pain Processing

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SUMMARY

Oxytocin (OT) is a neuropeptide elaborated by the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei. Magnocellular OT neurons of these nuclei innervate numerous forebrain regions and release OT into the blood from the posterior pituitary. The PVN also harbors parvocellular OT cells that project to the brainstem and spinal cord, but their function has not been directly assessed. Here, we identified a subset of approximately 30 parvocellular OT neurons, with collateral projections onto magnocellular OT neurons and neurons of deep layers of the spinal cord. Evoked OT release from these OT neurons suppresses nociception and promotes analgesia in an animal model of inflammatory pain. Our findings identify a new population of OT neurons that modulates nociception in a two tier process: (1) directly by release of OT from axons onto sensory spinal cord neurons and inhibiting their activity and (2) indirectly by stimulating OT release from SON neurons into the periphery.

INTRODUCTION

Oxytocin (OT), a neuropeptide that plays an important role in sociability, is produced in the brain exclusively in the hypothalamic paraventricular (PVN), supraoptic (SON), and intermediate accessory nuclei (Swanson and Sawchenko, 1983). OT neurons can be classified in magnocellular OT (magnOT) and parvocellular OT (parvOT) neurons, which are distinct in size and shape, subnuclear location, the amount of OT production, and involvement in distinct circuitries and functions (Armstrong et al., 1980; Swanson and Kuypers, 1980; Sofroniew, 1983; Swanson and Sawchenko, 1983).

According to a long-held dogma, magnOT neurons provide systemic OT supply by release into the blood via the posterior pituitary (Scharrer, 1928; Scharrer and Scharrer, 1940, Bargmann and Scharrer, 1951). Simultaneously, magnOT neurons innervate the forebrain, including the nucleus accumbens (Ross et al., 2009; Knobloch et al., 2012; Dölen et al., 2013) and the central nucleus of the amygdala (Knobloch et al., 2012). The forebrain fibers, as exemplarily studied in the central amygdala, allow for focal release and discrete, modulatory action of OT (Knobloch et al., 2012). These characteristics might account for the distinct impact of OT on numerous types of brain-region specific behaviors (Lee et al., 2010).

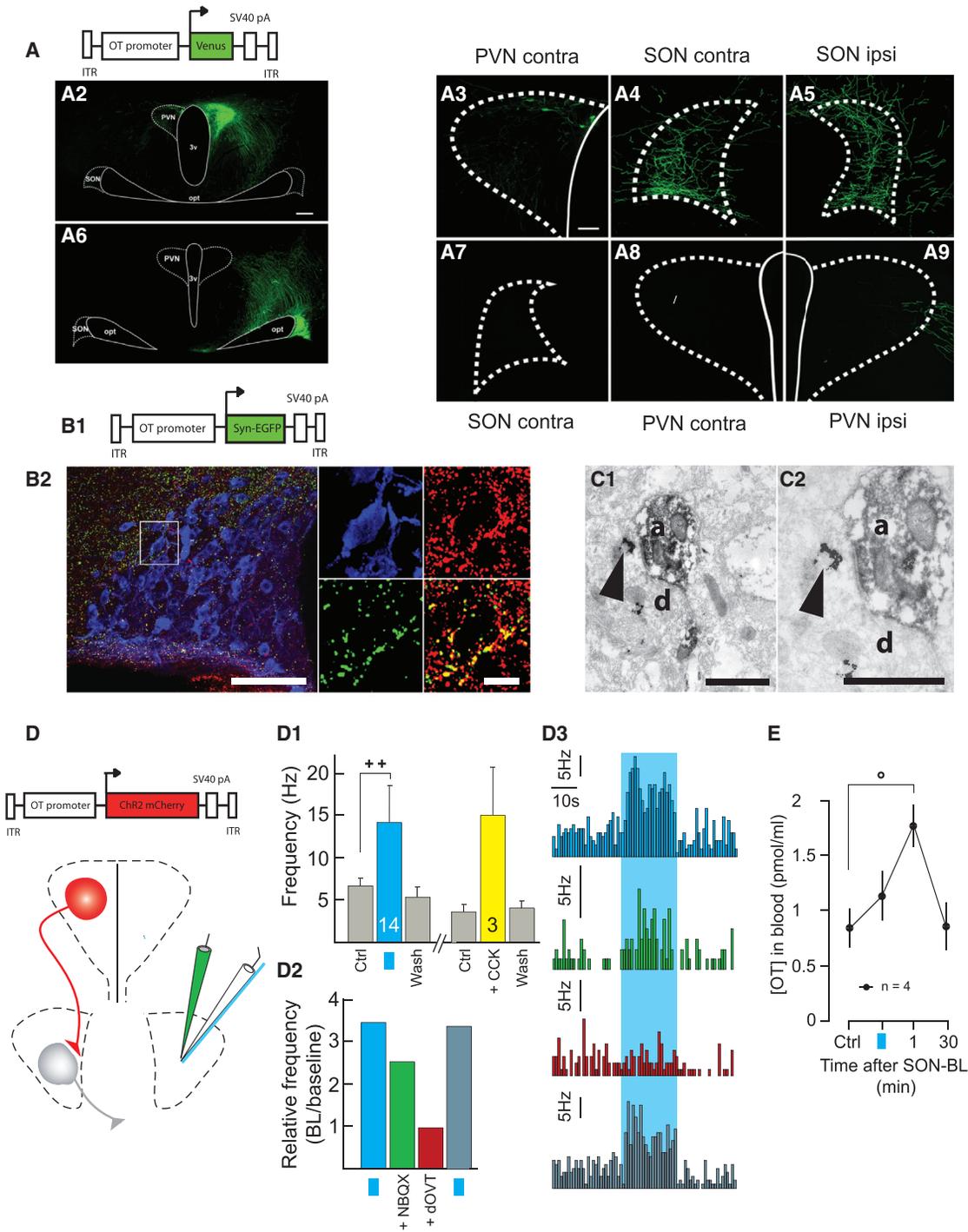


Figure 1. Anatomical and Functional Connectivity between OT Neurons of the PVN and SON

(A) OT projections from PVN to SON.

(A1) Scheme of the viral vector used to infect PVN neurons.

(A2–A9) PVN OT neurons infected with cell-type specific viral vector project Venus-positive axons to contralateral PVN (A3) and to contra- and ipsilateral SON (A4 and A5). OT neurons of the SON (A6) do not project Venus axons to contralateral SON (A7) or PVN (A8) and only marginally enter the external border of ipsilateral PVN (A9). The scale bars represent 200 μ m (left) and 50 μ m (right).

(B) OT axon terminals contain vGluT2.

(B1) Scheme of viral vector.

(B2) GFP-positive terminals in the area of the SON (left). In the magnified inset (right), the OT neuron (blue) is surrounded by GFP terminals, which also contain vGluT2 (red). Both of the immunosignals overlap (yellow) in virtually all of the terminals. The scale bars represent 100 μ m (left) and 25 μ m (right).

(legend continued on next page)

In contrast to magnOT neurons, parvOT neurons project to distinct brainstem nuclei and different regions of the spinal cord (SC) (Swanson and Sawchenko, 1983; Sawchenko and Swanson, 1982). Based on the location of parvOT axons and the effects of externally applied OT, it has been proposed that OT from parvOT axonal terminals contributes to modulation of cardiovascular functions, breathing, feeding behavior, and nociception (Mack et al., 2002; Petersson, 2002; Condés-Lara et al., 2003; Atasoy et al., 2012). However, no selective and specific genetic access to parvOT neurons has been available and, hence, there was no evidence for the capacity of parvOT axons to release endogenous OT and to selectively modulate the above-mentioned functions. Moreover, it has remained unknown how parvOT neurons are incorporated into the entire OT system and functionally interact with magnOT neurons.

Based on recent reports that OT-modulated nociception and pain response comprise a peripheral (Juif and Poisbeau, 2013) and a central component (Juif et al., 2013; González-Hernández et al., 2014), it is tempting to propose that these components are dependent on different OT cell types. The central component results from parvOT innervation of SC targets (Swanson and McKellar, 1979), whereas peripherally acting OT, in contrast, is provided to the blood stream by magnOT neurons and presumably targets C-type fibers in the dorsal root ganglion (DRG; Juif and Poisbeau, 2013). We therefore hypothesized that the complementary, analgesic OT action—at central and peripheral levels—depends on the communication between magnOT and parvOT neurons residing in spatially segregated OT nuclei. Our present results reveal that the modulation of pain signals by OT is triggered by only a handful of parvOT neurons that innervate simultaneously “sensory wide dynamic range” (WDR) neurons in the deep laminae of the SC, expressing neurokinin-1 (NK1R) and OT receptors (OTR), and SON neurons that secrete OT in the periphery. We show that these separate innervations underlie a two-tier modulation of pain by OT reaching the SC through fast, direct neuronal projections and a slower, indirect peripheral pathway.

RESULTS

Intrahypothalamic Axonal Trees of the OT System

To examine the intrahypothalamic OT system, we used recombinant adeno-associated virus (rAAV), allowing cell-type specific fluorescent labeling of OT neurons with 98%–100% cell-type specificity, as reported in Knobloch et al. (2012). To compare the OT system with the vasopressin (VP) system, we used AAV

carrying different fluorescent markers driven by an evolutionarily conserved VP promoter (for specificity, see Table S1; Figure S1A).

After injection of rAAV expressing Venus, under the control of an OT promoter (Figure 1A), we observed that OT neurons of the PVN give rise to fibers connecting to the ipsi- and even contralateral SON and form a pronounced plexus (Figures 1A4 and 1A5). Interconnections within the intrahypothalamic VP system, in contrast, were absent (Figure S1C). The OT plexus might stem from PVN OT neurons projecting above the third ventricle to the contralateral PVN (Figure 1A3). OT connectivity from the PVN to SON was present in females and males (Figure S2A). The connection between the OT nuclei was one-way: the SON-arising OT fibers reached only marginally the ipsi- (Figure 1A9) and never the contralateral SON (Figure 1A7) or PVN (Figure 1A8).

The OT PVN-SON connection was reconstructed using light sheet microscopy. As presented in Figure S2B, descending fibers from the PVN mainly project rostro-ventrally, turn horizontally at the level of the SON, and enter the SON from the rostral position, to run caudally along the whole extent of the nucleus.

PVN OT Neurons Innervate the SON and Control MagnOT Neuron Activity to Induce OT Release into Blood Circulation

At the light microscopic level, Venus-labeled OT axons that arose from the PVN formed tight appositions to dendrites and somata of magnOT SON neurons resembling synaptic contacts. To assess if synapses were present, we injected the PVN with rAAV that expresses the synaptic marker synaptophysin fused to the green fluorescent marker EGFP in PVN OT neurons (Figure 1B1). GFP-positive puncta were found in the SON. The vast majority of terminals with GFP signal overlapped with VGluT2 signal (red, Figure 1B2). GFP/VGluT2 terminals engulfed OT cell bodies and dendrites (blue, Figure 1B2). We found that EGFP signals overlapped with VGluT2 in 92.6% ± 8.3% of all terminals (Figure 1B2). These light microscopic observations suggested the presence of synaptic contacts, which we further confirmed at the electron microscopic level: EGFP-positive OT axons from the PVN (EGFP: greyish filling) formed asymmetric (presumably glutamatergic) synapses on OT dendrites of the SON (OT: dark aggregate in pre- and postsynaptic elements; Figures 1C1 and 1C2).

Based on the anatomical evidence for OT connections between PVN and SON neurons, we aimed for a functional characterization of these connections. We expressed the blue-light

(C–C2) Electron microscopy OT axon terminals (Venus visualized as diaminobenzidine [DAB] endproduct, OT, as a silver-gold-intensified DAB) form asymmetric synapses on OT-ir dendrite within the SON. The OT-immunoreactivity (clusters of silver particles, arrows) are shown in the presynaptic axon (a) terminal and postsynaptic dendrite (d) at lower (C1) and higher magnifications (C2). The scale bar represents 0.5 μm.

(D) Scheme of the viral vector and setup of in vivo electrophysiological recordings (white pipette) in SON, together with SON-BL stimulation (blue fiber) and drug infusion (green pipette).

(D1–D3) Functional connection between PVN and SON OT neurons. (D1) Average spike frequencies of SON OT neurons before (Ctrl), after either SON-BL (n = 14, blue bar) or systemic injection of CCK (n = 3, yellow bar), and after washout effect (Wash). (D2) Relative frequency increase induced by SON-BL in control condition (blue bar), after infusion of NBQX (1 μM, 0.5 μl; green bar), after additional infusion of dOVT (1 μM, 0.5 μl; red bar), and after 30 min washout of the drugs (dark blue bar). (D3) Histograms of the frequency rates recorded under conditions described in (D2).

(E) Effect of unilateral SON-BL effect on OT blood concentration at the end of SON-BL, 1 min and 30 min after (n = 4). All results are expressed as average ± SEM. The statistical significances: ++ p < 0.01 and Wilcoxon's test. (* p < 0.05, Friedman's test followed by Dunn post hoc test) The blue squares represent 20 s BL stimulation at 30 Hz with 10 ms pulses of BL stimulation.

(BL)-sensitive ChR2 protein (Nagel et al., 2003) fused to mCherry in PVN OT neurons (for construct validation, see Knobloch et al., 2012). In vivo extracellular recordings in anaesthetized animals revealed the expression of functional ChR2 in the PVN, as evident from BL-induced (PVN-BL, 20 s at 30 Hz with 10 ms pulses), reversible, and reproducible increases of spike frequencies in these PVN neurons (on average the frequency increased from 4.1 ± 0.7 to 7.8 ± 0.7 Hz; data not shown).

We then further tested in vivo whether exposure to BL of PVN-OT axons in the SON (SON-BL, scheme in Figure 1D) could also activate, ipsilaterally, SON neurons. SON-BL exposure evoked a reversible increase in spike frequencies of SON neurons, from 6.7 ± 1.5 to 14.1 ± 2.7 Hz, confirming that BL stimulation of parvOT PVN axon terminals could excite SON neurons (Figure 1D1). To verify that OT was the main transmitter involved, we recorded the response of a single neuron to the SON-BL in the absence of any drug, or after sequential infusion of AMPA and OTR antagonists (respectively, NBQX and dOVT) into the SON, and after their washout (Figures 1D2 and 1D3). Interestingly, while NBQX decreased the baseline frequency of SON neurons, SON-BL paired to NBQX application still efficiently increased the relative frequency of discharge of the recorded neuron. Subsequent dOVT infusion totally blocked the SON-BL response, with full recovery 30 min after washout (Figures 1D2 and 1D3). These results are in accordance with our previous observations in the central amygdala (Knobloch et al., 2012).

We aimed at providing functional evidence for the OT nature of the SON neurons that were contacted by the PVN. To this purpose, we first of all injected into the blood circulation cholecystokinin (CCK) a hormone inducing the activation of OT neurons (Verbalis et al., 1986). CCK induced a prominent increase in spike frequencies of SON-BL responding neurons from 3.6 ± 0.8 to 15.0 ± 5.9 Hz (Figure 1D1), establishing an indirect argument of the OT identity of the in vivo recorded SON neuron. Second, as magnOT neurons are known to release OT in the blood, we performed a time-dependent measurement of OT concentrations in plasma by mass-spectrometry after SON-BL. This revealed a significant increase of OT plasma concentrations at 60 s after SON-BL (from 0.84 ± 0.17 to 1.76 ± 0.22 pmol/ml; Figure 1E). Taken together, these findings provide evidence for an OT identity of the SON neurons that are activated by axonal terminals originating from OT neurons in the PVN.

OT Neurons Projecting to SON MagnOT Neurons Are ParvOT Neurons Displaying Distinct Anatomical and Electrophysiological Characteristics

To identify PVN neurons projecting to the SON, we injected into the SON retrogradely transported and monosynaptically transmitted canine adenovirus 2 (CAV2). After counting of sections containing the entire PVN, we identified in total a very small population of GFP/OT-positive neurons residing bilaterally (Table S2; 31.5 ± 8.5 neurons). CAV spread occurs within 200 μ m of the injection site (Schwarz et al., 2015), making unlikely the diffusion of the virus from the SON to PVN (the distance between these two nuclei is about 1.5 mm; see Paxinos and Watson, 1998).

To characterize the magno- versus parvocellular nature of back-labeled PVN cells, we combined CAV2 with systemic

administration of Fluorogold (Figure S3A). Fluorogold, when injected intraperitoneal (i.p.), is taken up by neurons projecting beyond the blood brain barrier, for example, by magnOT neurons, thus allowing to distinguish them from the parvOT (Fluorogold-negative) neurons (Luther et al., 2002; Table S2). Notably, all magnOT neurons of the SON were Fluorogold-positive (data not shown). After neuron counting in sections containing the entire PVN, we established that the vast majority of the 31.5 ± 8.5 GFP/OT-positive neurons (90%) did not contain Fluorogold (Table S2). In addition to the detection of back-labeled GFP-positive neurons in the PVN, we observed GFP neurons in other structures typically known to innervate the SON, further confirming the specificity of our retrograde labeling (Miselis, 1981; Cunningham and Sawchenko, 1988; Figure S3B).

To characterize the parvOT neurons projecting to the SON, we next injected into the SON CAV2 expressing Cre recombinase and into the PVN rAAV carrying a double-floxed inverted open reading frame (ORF) (DIO) of GFP under the control of the OT promoter (Figure 2A1). By this combination, we limited GFP expression exclusively to SON-projecting parvOT neurons. In line with previous results, this revealed a unique position of back-labeled GFP neurons in the dorso-caudal PVN (Figures 2A2, 2A3, S2B, and S2C). Individual GFP neurons have bipolar spindle-like morphology (Figure 2A4) distinct from neighboring magnOT neurons (Figure 2A5). The number of back-labeled PVN GFP (exclusively OT) neurons was comparable (33.4 ± 9.1), with the estimation of non-selectively labeled PVN neurons identified by costaining with OT antibodies.

OT neurons similar in morphology and location were obtained in our initial study (data not shown) with the application of latex retrobeads (Katz and Iarovici, 1990) in the SON, which, however, labeled only few cells in the PVN (and other structures innervating the SON; Figure S3), precluding quantitative analysis.

We determined the electrophysiological characteristics of fluorescently labeled neurons in the PVN to assess their parvocellular nature. We conducted whole-cell patch clamp recordings in slices (Figure 2B) in current clamp applying a protocol of depolarizing current injections (Figure 2B1). This was aimed to determine the presence of a transient outward rectification, which is typically found in magnOT, but not in parvOT neurons (Luther et al., 2002). We recorded in a total of seven animals 11 fluorescent putative parvOT, and found that none, as expected, exhibited a hyperpolarizing notch. Conversely, all of the 13 non-fluorescent neurons from the same region (putative magnOT) showed the typical transient outward rectifying current, as known as (aka) "notch". Quantification of these differences was made by analyzing the time to spike (spike delay) and rise slope, which was the slope measured between beginning of the depolarization and the peak time of the first action potential. Both of these parameters showed highly significant differences between the two groups of neurons (Table S4; Figure S4). Differences in the spike frequency also showed a tendency, though with less significance than previously reported (Luther et al., 2000). The electrophysiological responses were in agreement with the morphology of the cells. Neurons classified electrophysiologically as parvOT had a small soma and a more elongated shape, while the ones classified as magnOT had a big soma and were more rounded (Figure 2B2).

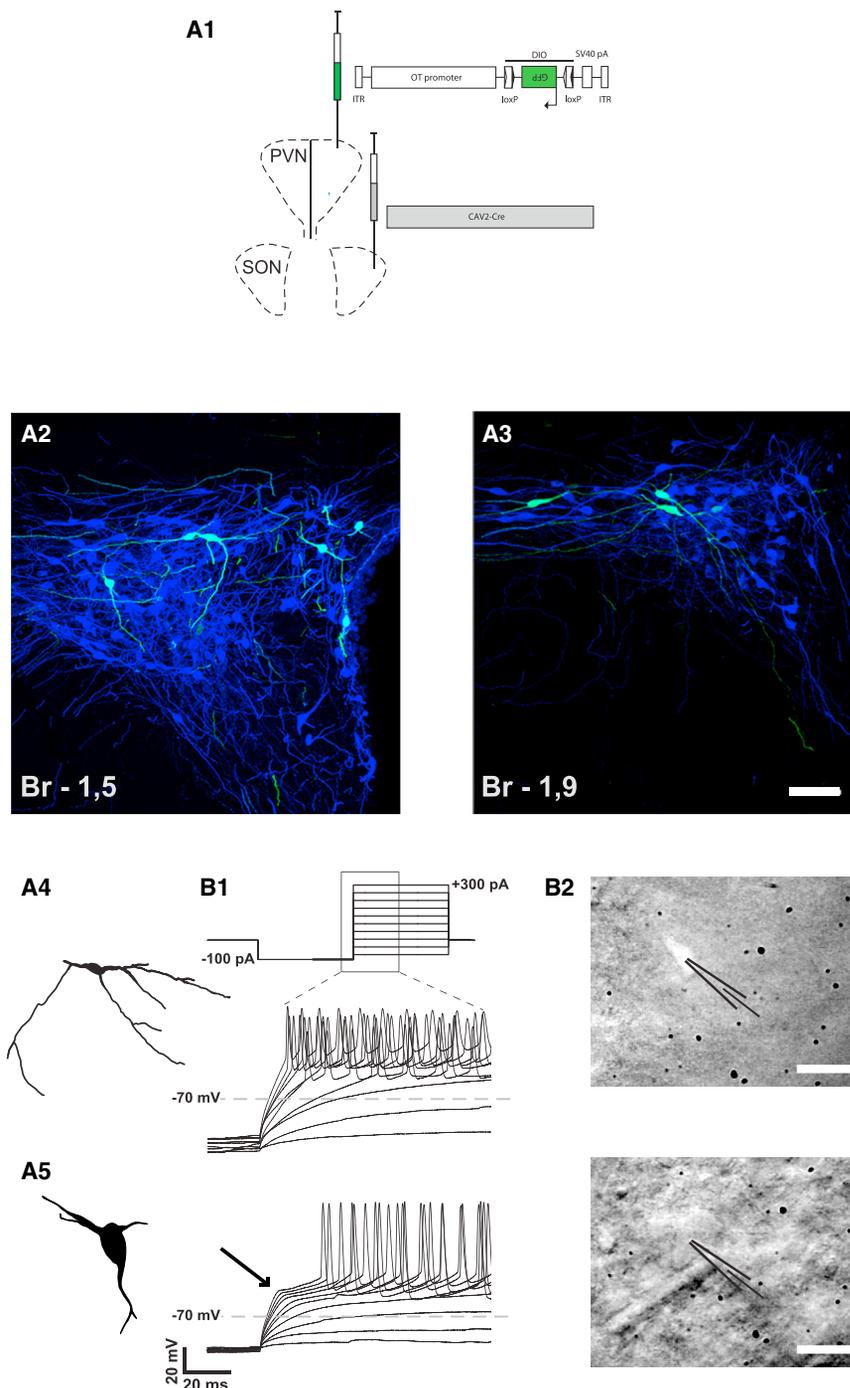


Figure 2. Anatomical and Electrophysiological Characteristics of PVN OT Neurons Projecting to the SON

(A) Identification of a subset of OT neurons projecting from PVN to SON.

(A1) Scheme showing the injection of viruses in the SON and PVN.

(A2 and A3) Defined subset of back-labeled OT neurons (green) in dorso-caudal PVN displays consistent morphology: small oval somas (12 to 20 μm in diameter) with predominantly longer horizontal axes. The scale bar represents 50 μm in (A2) and 50 μm in (A3).

(A4 and A5) The morphology of these cells is clearly distinct from the typical magnocellular neurons with large cell bodies and less branching processes (A5).

(B) Functional differentiation of this subset of PVN OT neurons.

(B1) Current steps protocol starting from a hyperpolarizing current chosen to reach -100 mV (here 100 pA) followed by progressively more depolarizing current injections (upper trace). The representative changes in membrane potential for the parvOT and magnOT PVN neurons during the part of the current steps as indicated by the zoomed area are shown (lower traces). The ParvOT neurons (middle trace) do not display the transient outward rectification specific for the magnOT neurons (lower trace, arrow).

(B2) Photographs of a GFP-fluorescent parvOT neuron (upper) in the PVN (labeled by injection of CAV2-Cre into the SON and OT-DIO-GFP AAV in the PVN) and in the same area a typical magnOT neuron (lower) as indicated by the patch pipettes. The scale bars represent 20 μm .

ParvOT Neurons Innervating MagnOT Neurons Also Project Specifically to NK1R/OTR Positive WDR Neurons in the Deep Layers of the SC

The above established exclusive labeling of parvOT neurons and all their processes using a combination of CAV2-Cre with OT cell typed-specific Cre-dependent rAAV (Figures 3A1–3A3) allowed us to follow projections of this OT cell population up to the distal (L5) segments of the SC. After labeling presumably all PVN OT neurons, axons can be visualized in both superficial and deep

of the antibody was confirmed in transfected HEK cells (Figure S5B1) and brainstem sections of OTR knockout mice (Figure S5B2), in agreement with a previous study using these antibodies in mouse cortex (Marlin et al., 2015). To show the colocalization of NK1R and OTR in the same cells, we performed fluorescent in situ hybridization and found the presence of respective mRNAs in the same neurons of deep layers of the SC (Figures 3B1–3B4). As a next step, we wanted to demonstrate that NK1R-positive neurons of deep SC laminae could

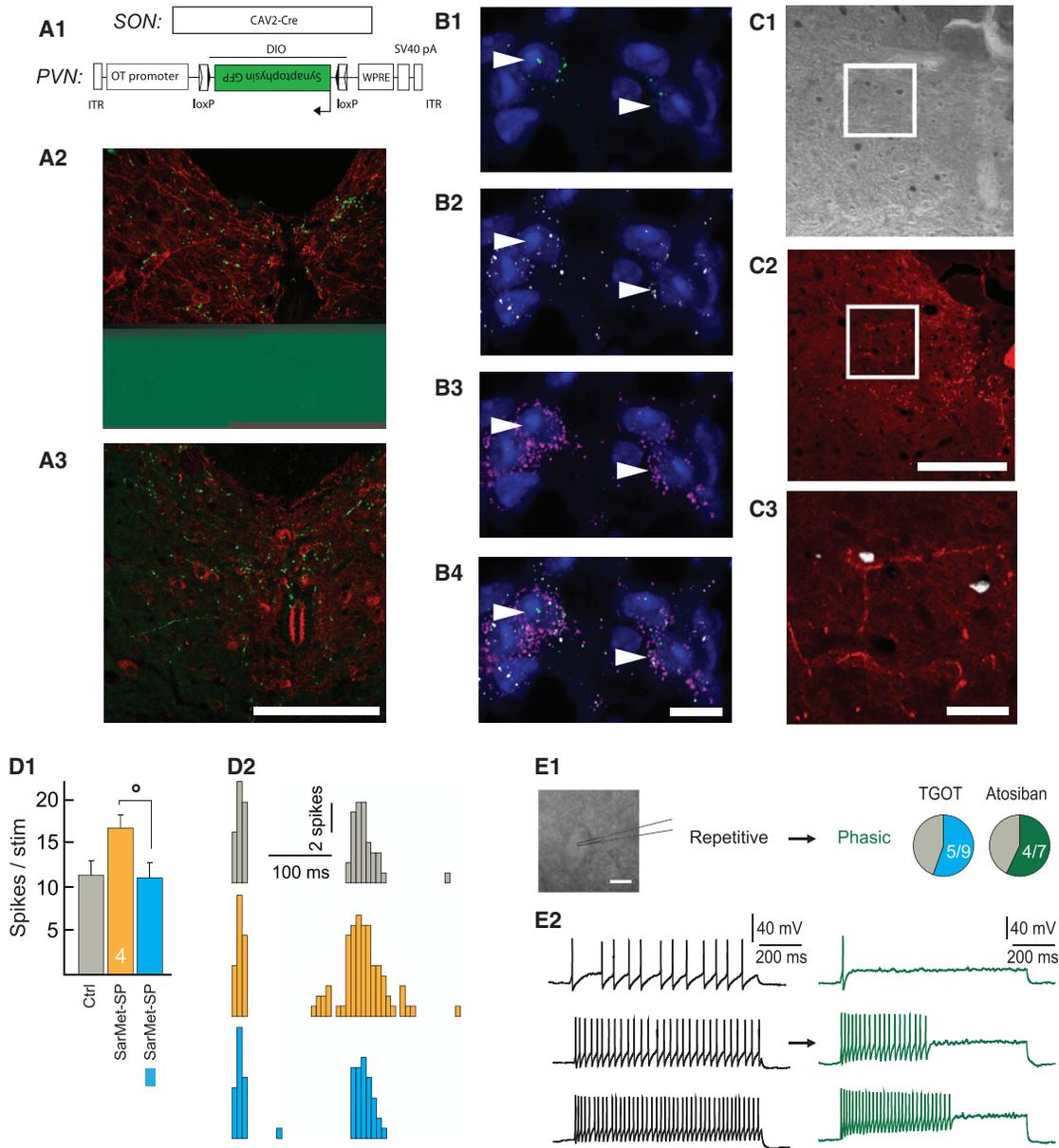


Figure 3. ParvOT Neurons Project to SC and Innervate NK1R/OTR WDR Neurons in Deep Laminae

(A) ParvOT projections to the SC.

(A1) Scheme of the viruses injected into the SON and PVN.

(A2) Detection of synaptophysin-GFP containing terminals (green) in close proximity to NK1R-positive neurons (red) in SC deep laminae.

(A3) Synaptophysin-GFP terminals locate close to OTR-positive neurons of deep laminae. The scale bars represents 500 μ m in (A2) and 500 μ m in (A3).

(B–B4) Colocalization of NK1R and OTR mRNAs in the same neurons of SC deep laminae. Immunofluorescent in situ hybridization revealed the presence of OTR mRNA (green dots; B1 and B4) and NK1R mRNA (white dots; B2 and B4) in the same neurons, which were visualized by detection of vGlut1/2/3 mRNAs in their somas (pink/violet dots; B3 and B4). The nuclei of cells were stained by DAPI. The arrow heads point NK1R/OTR double positive neurons. The scale bars represent 10 μ m.

(C–C3) NK1R-positive SC neurons start to express c-Fos after intraplantar injection of capsaicin in the hindpaw. The c-Fos signal (DAB) was detected in deep laminae of SC (C1), where the NK1R (red) were located (C2). The digital overlay of the two signals demonstrates localization of c-Fos in the NK1R-positive neuron (C3). The scale bars represent 500 μ m in (C1) and (C2) and 50 μ m in (C3).

(D) WDR C-fiber evoked spikes in response to a series of isolated hindpaw stimulations in control condition (Ctrl), during application of the specific agonist of NK1R SarMet-SP (orange), and during SarMet-SP paired with BL (blue).

(D1) Average of C-fiber evoked spikes (n = 5).

(D2) Representative traces.

(E) Discharge profile of putative WDR recorded in current clamp applying a protocol of depolarizing current injections before (black) and after bath application of 1 μ M TGOT (blue, n = 9) or 1 μ M Atosiban (green, n = 7).

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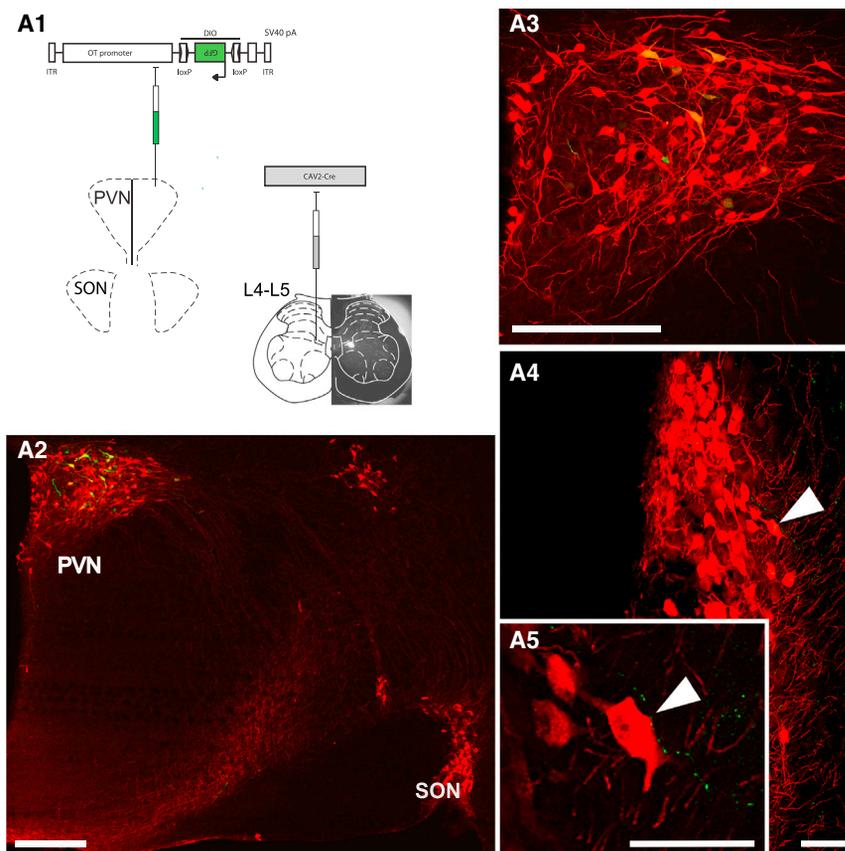


Figure 4. ParvOT-MagnOT-SC Anatomical Unit

(A) Scheme of viruses injected into the SC and PVN. The actual SC injection site (fluorescent latex bead accumulation) is shown as an insert underlying SC drawing.

(A2–A5) PVN parvocellular cells back-labeled from SC (green). The GFP-positive cell bodies were found in the caudal portion of the PVN and always colocalized OT (red) (A3, magnification from A2). Fibers, projecting from back-labeled PVN OT neurons to SON (arrow in A4, more caudal to A2) GFP-expressing varicose axons in close proximity to cell bodies and dendrites of SON magnocellular neurons (high magnification in A5) are shown. The scale bars represent 500 μm in (A2) and (A3) and 75 μm in (A4) and (A5).

Putative WDR neurons, identified by their large cell body and repetitive firing related to stimulation intensities (Figure 3E; Ritz and Greenspan, 1985), were located around the central canal (cc) and in deep layers V–VI. As expected, [Thr4,Gly7]-OT (TGOT) was able to change the firing properties of these neurons, from repetitive to phasic in 5/9 of recorded cells (Figure 3E), similar to what was described in superficial layers (Breton et al., 2009). Importantly, the application of a specific biased agonist

be activated by sensory/pain stimulation. Bilateral injection of capsaicin in the hindpaws indeed induced c-Fos expression in large NK1R neurons (Figures 3C1–3C3). Furthermore, back-labeled parvOT neurons were also activated by capsaicin (data not shown).

To show that NK1R WDR neurons are functionally modulated by both NK1R specific agonist (SarMet-SP) and parvOT-deriving OT, we measured in vivo the WDR C-fiber evoked spikes in response to a series of isolated hindpaw stimulations. We found that the C-fiber evoked spikes were increased in the presence of SarMet-SP, as expected (Budai and Larson, 1996). Interestingly, BL-activation of Chr2 expressing parvOT fibers in the SC (SC-BL; schemes in Figures 5A and 5B) upon SarMet-SP significantly reduced the number of C-fiber evoked spikes from 16.8 ± 1.1 to 11.2 ± 1.5 (Figures 3D1 and 3D2). These findings show that release of OT from parvOT axons can effectively inhibit the activity of WDR neurons potentiated by NK1R activation.

Then, we analyzed the inhibitory effect of OT on WDR neuron firing properties. To do so, we performed in vitro whole-cell patch clamp recordings in current clamp applying a protocol of depolarizing current injections (Figure 3E; Breton et al., 2009).

for OTR linked to G_i subunit, Atosiban (Busnelli et al., 2012), induced the exact same effects in 4/7 recorded cells (Figure 3E). This experiment demonstrates for the first time on living tissue that OTR can functionally bind a G_i protein, thus elucidating the inhibitory mechanism of OT on the firing properties of WDR neurons.

Finally, to demonstrate that the population of identified parvOT neurons is a single anatomical unit and that the same cells project collaterals to both the SON and SC, we injected a CAV2-Cre virus in deep laminae of L5 and Cre-responder AAV expressing GFP under the OT promoter in the PVN (Figure 4A1). We detected back-labeled cell bodies of GFP/OT neurons in the PVN and their axonal projections in close proximity to somas and dendrites of magnocellular neurons of the SON (Figures 4A2–4A5).

ParvOT Neurons Projecting to Magnocellular SON Neurons and NK1R/OTR Positive WDR Neurons in Deep Layers of the SC Control the Central Nociceptive Processing

To test whether the specific population of PVN-OT neurons projecting to both the SON and SC indeed acts on nociceptive input, we recorded SC neuronal responses in vivo during electrical stimulation of their hindpaw receptive field. The coding

(E1) Proportion of putative WDR neurons discharge pattern changed from repetitive to phasic after TGOT or Atosiban bath application.

(E2) Example response of putative WDR neuron to 20 pA (top), 40 pA (middle), and 60 pA (bottom) current injection before (black) and after (green) Atosiban bath application. The scale bar represents in (E1) 30 μm . All results are expressed as average \pm SEM. The statistical significance: $^{\circ} p < 0.05$, Friedman's test followed by Dunn post hoc test.

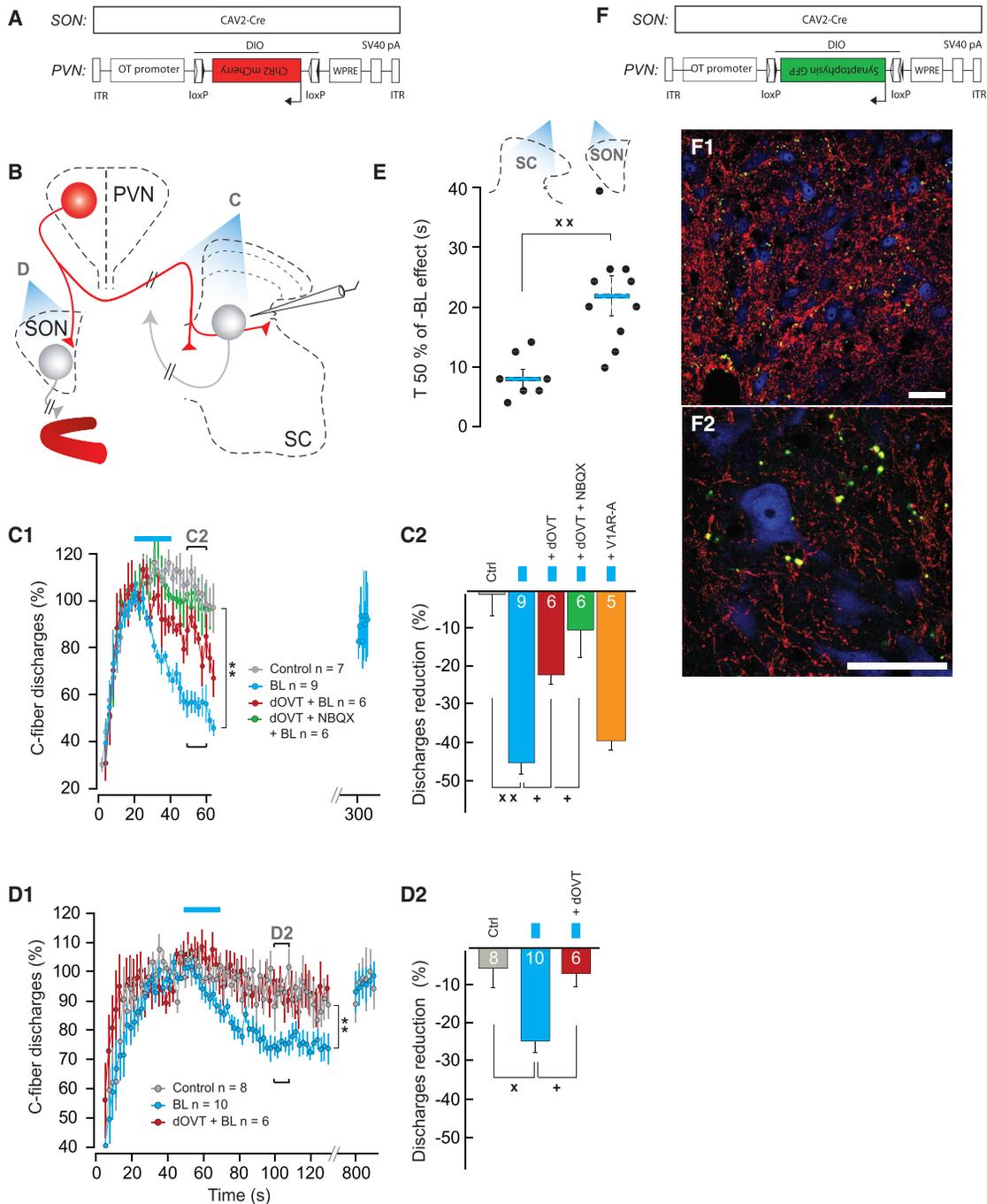


Figure 5. Stimulation of ParvOT PVN Axons in SON and SC Modulates Responses of WDR Neurons

(A) Viruses injected into the SON and PVN.

(B) Scheme of the experimental procedures.

(C) Effect of SC-BL on WDR-C discharges.

(C1) Time course of WDR-C in control condition (n = 7), when shining SC-BL alone (n = 9), after local dOVT application (n = 6), or local dOVT + NBQX application (n = 6).

(C2) Average discharge reduction of WDR-C on Ctrl (n = 7), when shining SC-BL alone (n = 9), after local dOVT application (n = 6), local dOVT + NBQX application (n = 6), or local V1AR-A application (n = 5). The statistical significance of drug modulation of the SON-BL effect was assessed by comparing the effect of SON-BL on the same neuron before and after drug injection.

(D) Effect of SON-BL on WDR-discharges.

(D1) Time course of WDR-C in control condition (n = 8), measured 30 s after shining SON-BL (as indicated in C1) alone (n = 10), or after systemic dOVT systemic injection (n = 6).

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properties and short-term potentiation (wind-up; WU) following repetitive receptive field stimulation were calculated from the response of WDR neurons in deep laminae. Recordings include the deep laminae, which integrate convergent peripheral sensory information from fast-conducting (A-type) and slow-conducting (C-type) primary afferent fibers (Figures 5 and S6).

We first tested the inhibitory action of OT released from parvOT-hypothalamo-spinal terminals by shining BL directly onto the dorsal surface of the SC (SC-BL). In this set of experiments, we used the same combination of viruses (CAV2-Cre and rAAV carrying OT promoter-DIO-ChR2-mCherry) to elicit OT release from parvocellular PVN fibers. SC-BL efficiently reduced the WDR discharges from C- ($-44.6\% \pm 3.7\%$; Figures 5C1 and 5C2) and A δ - ($-36.3\% \pm 4.5\%$), but not from A β - fibers ($-0.3\% \pm 2.8\%$; Figure S6G2). The half-efficacy of SC-BL inhibition was 8.3 ± 1.3 s (Figure 5E). The WU returned to control values ~ 300 s after SC-BL (Figure 5C1). SC-BL had no effect on superficial layer neuron activity in the same recording condition as for WDR neurons (Figure S6F). The OTR antagonist dOVT, directly applied to the surface of the SC, significantly, but not entirely, reduced A δ - and C-fiber mediated discharges (Figures 5C1 and 5C2). In contrast, the VP receptor type 1A antagonist applied on SC failed to change the SC-BL inhibition of WU intensity (Figure 5C2), whereas it could efficiently block the effect of exogenously applied AVP (data not shown). Since VGlut2 was detected in synaptophysin-GFP-containing (Figures 5F1 and 5F2; overlap of GFP and vGluT2 signals was found in $89\% \pm 7.4\%$ GFP terminals) axonal terminals of parvOT neurons near cell bodies of WDR-like neurons (Figure 5F2), we assessed the effect of NBQX in vivo. Coapplication of both dOVT and NBQX entirely blocked the SC-BL effects (Figures 5C1 and 5C2). Thus, stimulation of parvOT axons in SC deep layers leads to a fast, short-lasting decrease in nociceptive processing which is mediated by central OTR, and to a lesser extent by ionotropic Glut receptors.

We then assessed the efficiency of OT release from parvOT neurons onto magnOT SON neurons in modulating nociception (Figures 5B and S6A). Eliciting OT release from parvOT fibers in SON by BL (SON-BL) significantly reduced the WDR discharges evoked by slow-conducting C-type fibers ($-24.9\% \pm 3.1\%$; Figures 5D1 and 5D2) and fast-conducting fibers A δ - ($-30.0\% \pm 6.8\%$), but not by non-nociceptive, fast-conducting A β - fibers ($-6.4\% \pm 3.5\%$; Figure S6G1). The half-efficacy of SON-BL induced inhibition of WU was 22.2 ± 3 s (T 50%; Figure 5E), a value which was significantly higher than the SON-BL effect (Figure 5E). The WU intensity returned to control values only 800 s after SON-BL (Figure 5D1). Moreover, to further confirm that the reduction in

WU intensity was related to the elevated level of blood OT (see Figure 1E), we injected the OTR antagonist dOVT intravenously before applying SON-BL. As expected, this abolished the SON-BL inhibition of WDR discharges that were evoked by both A δ - and C-fibers (Figures 5D1, 5D2, and S6G1). Thus, the central release of OT from parvOT axons targeting magnOT SON neurons leads to a systemic release of OT, which reduces nociceptive processing by WDR neurons. This effect was slow to appear and long-lasting.

In summary, the subpopulation of PVN OT parvOT neurons projecting both to magnOT SON neurons and to NK1R/OTR WDR neurons from deep layers of SC exerts an inhibition of spinal nociceptive processing by fast action on SC neurons and a relatively slower effect on peripheral targets by stimulation of SON neurons and subsequent induction of OT release into blood.

Activation of ParvOT Neurons Results in Analgesia

In the last part of our work, we analyzed the functional importance of these parvOT neurons in the processing of inflammatory compared to nerve injury-induced neuropathic pain. To this purpose, we measured both the effects of stimulation or inhibition of parvOT neurons on the symptoms of either a peripheral painful inflammatory sensitization triggered by a single unilateral intraplantar injection of complete Freund adjuvant (CFA) or a nerve injury-induced neuropathy induced by the cuffing of the sciatic nerve (Cuff; Pitcher et al., 1999; Figure S7C1). To this purpose, we used rats that expressed either ChR2 or hM4Di (Zhu and Roth, 2014) restricted to parvOT PVN neurons synapsing on magnOT SON neurons (Figure 6A). The efficiency of ChR2-mediated activation and hM4Di-mediated inhibition of OT neurons was assessed respectively by targeting unilaterally the PVN by BL or by i.p. administration of CNO and was confirmed both in vitro (Figures S7A1 and S7A2) and in vivo (Figures S7B1–S7B4).

PVN-BL stimulation significantly, but not entirely, alleviated the CFA-mediated hyperalgesia by raising the threshold of response to both the mechanical (from 56.7 ± 7.6 g to 116.6 ± 16.4 g) and thermal hot stimulation (from 2.8 ± 0.2 to 4.8 ± 0.7 s; Figures 6C1 and 6C2). In contrast, PVN-BL failed to mitigate the mechanical hyperalgesia measured in condition of the Cuff peripheral neuropathy (Figures S7B2 and S7B3). Furthermore, return of the pain symptoms occurred after PVN-BL was fully blocked by i.p. injection of the blood brain barrier (BBB)-permeable OTR antagonist L-368,899 (Figures 6C1 and 6C2).

Conversely, CNO-induced inhibition of parvOT neurons significantly increased the CFA-mediated hyperalgesia by lowering

(D2) Average discharge reduction of WDR-C on Ctrl (n = 8), when shining SON-BL alone (n = 10), or after systemic dOVT injection (n = 6). The statistical significance of dOVT modulation of the SON-BL effect was assessed by comparing the effect of SON-BL on the same neuron before and after dOVT injection (n = 6).

(E) Comparison between individual (black dots) and average T 50% (blue bar) effect of SON-BL (n = 10) and SC-BL (n = 7) on recorded WDR.

(F) Viruses injected into the SON and PVN.

(F1 and F2) Axonal terminals containing synaptophysin-GFP fusion protein in proximity to SC L5 neurons.

(F1) Overview of fiber distribution within SC: VGlut2 (red), synaptophysin-GFP (green), and NeuN (blue).

(F2) A zoom-in shows the green signal (green) largely overlaps with the VGlut2 signal (red) in terminals surrounding cell bodies. The scale bars represent 50 μ m in (F1) and (F2). All results are expressed as average \pm SEM. The statistical significance: *p < 0.05, Friedman with Dunn post hoc test; + p < 0.05, ++ p < 0.01, and Wilcoxon's test; xx p < 0.01 and Kruskal and Wallis test; and ** p < 0.01 BL versus Control, two-way ANOVA.

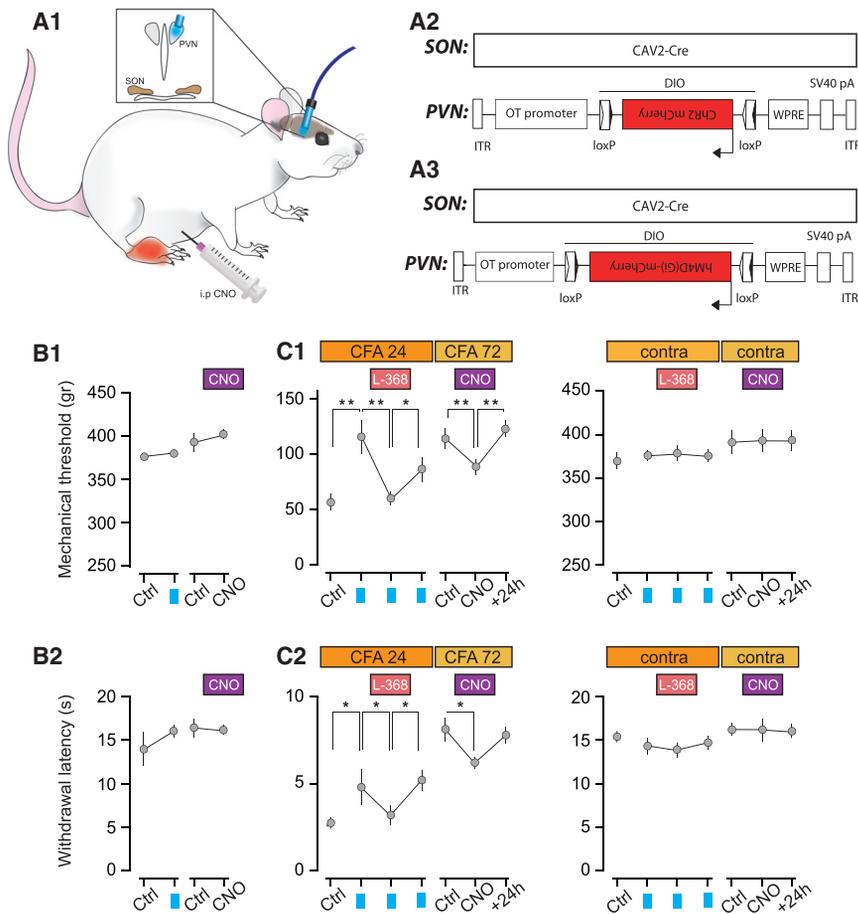


Figure 6. Activation/Inhibition of ParvOT PVN Neurons Modulates Mechanical Threshold and Thermal Hot Latency in Animals Subjected to Complete Adjuvant Injection

(A–A3) Scheme of the experimental procedure. The CAV2-Cre was injected in the SON and Cre-responding virus driving either (A2) ChR2 or (A3) hM4Di to achieve the expression of respective proteins in OT neurons of the PVN.

(B–B2) Mechanical thresholds and (B2) thermal hot latencies of naive animals before and after PVN-BL (ChR2, $n = 6$ and CNO, $n = 10$).

(C–C2) Mechanical thresholds and (C2) thermal hot latencies of the CFA-injected hindpaw (left graphs) and the contralateral hindpaw (right graphs). The effect of PVN-BL was assessed before, right after i.p. injection of OTR antagonist L-368,899 (1 mg/kg), and after its washout ($n = 6$). The effect of CNO (3 mg/kg) was measured 1 hr after i.p. injection and its 24 hr washout ($n = 10$). All results are expressed as average \pm SEM. The statistical significance: * $p < 0.05$, ** $p < 0.01$, and one-way ANOVA followed by Tukey's multiple comparison post hoc test.

the threshold of response to both the mechanical (from 115 ± 12.1 g to 88 ± 9.8 g) and thermal hot stimulation (from 8.1 ± 0.9 s to 6.3 ± 0.3 s; Figures 6C1 and 6C2). CNO had no effect in rats with the Cuff (Figures S7C2 and S7C3). These results from gain- and loss-of-function approaches highlight the role of parvOT control of peripheral painful sensitization, supported by our in vivo electrophysiological data.

In the course of our study, we observed that both PVN-BL and CNO failed to modify mechanical and thermal hot sensitivity in the absence of any peripheral sensitization, for example, in the contralateral paw or in naive animals (Figures 6B1, 6B2, 6C1, 6C2, S7C2, and S7C3).

Taken together, these findings provide evidence that 30 parvOT neurons are able to strongly promote analgesia in a pathological condition of inflammatory, but not nerve injury-induced neuropathic pain, presumably by both central (SC-mediated) and peripheral (SON-mediated) mechanisms.

DISCUSSION

Here, we identified, by a combination of latest state of the art viral-vector based (Grinevich et al., 2016a), anatomical, optogenetic, electrophysiological, and behavioral approaches, a small ($n \sim 30$) subpopulation of parvOT neurons in the PVN, which projects to magnOT neurons in the SON and to NK1R or

release from parvOT in the deep layers of the SC and from SON magnOT in the blood. Release in the SC is directly triggered from parvOT-spinal projections and follows a fast mode of action; release in the blood is indirectly triggered from SON magnOT neurons that are activated by parvOT projections and follows a slower time course. The functional role of this subpopulation of parvOT neurons was further confirmed in two rat models of peripheral painful sensitization, indicating that activation of parvOT neurons can decrease mechanical and thermal sensitivities in inflammatory, but not nerve injury-induced neuropathic pain.

Synaptic Crosstalk between OT Neurons

The question of how OT neurons in different nuclei within the hypothalamus interact with each other is a recurrent theme in past literature, but has not been elucidated experimentally. Belin and colleagues recorded pairs of OT neurons from SON and PVN and proposed an internuclear connection serving as a basis for synchronous firing during lactation (Belin et al., 1984, Belin and Moos, 1986). The hypothesis of an OT-mediated communication was stated already in the early 80's (Silverman et al., 1981), following observations that application of OT (or dOVT) into the third ventricle or in the SON synchronized (respectively, desynchronized) activity of OT neurons in PVN and SON (Freund-Mercier and Richard, 1984; Lambert et al., 1993). Furthermore,

the presence of synapses containing OT-immunoreactivity was demonstrated in the SON (Theodosios, 1985). Although we did not examine internuclear connectivity that underlies synchronized burst firing, our anatomical and functional data demonstrate that PVN-SON interconnectivity plays an important role in inhibiting spinal nociceptive processing and alleviation of inflammatory pain.

In an early study, lesion of the SON did not cause any loss of magnOT neurons in the PVN (Olivecrona, 1957), providing a first indication that parvOT PVN neurons might be at the basis of internuclear connection to the SON. However, as of today, the parvOT neurons in the PVN have remained much less studied than the magnOT neurons, mostly because of technical difficulties, specifically in labeling and modulating the activity of parvOT neurons. To our knowledge, the possibility to study a direct parvOT innervation of the SON by retrograde tracing techniques has seldom been discussed (e.g., Lambert et al., 1993) and any potentially involved parvocellular neurons have never been identified.

At the SON level, Bruni and Perumal (1984) have described an extensive network of small-diameter, beaded, unmyelinated fibers with no particular organizational pattern and of unknown origin that establishes functional axo-somatic and axo-dendritic contacts with magnOT neurons. At 30 years later, we reveal here a monosynaptic connection between parvOT PVN and magnOT SON neurons as respective pre- and postsynaptic components. The detection of a postsynaptic SON component was further confirmed by their stimulation through application of CCK (Renaud et al., 1987) and an increase in peripheral OT levels.

In contrast to the OT system, direct connectivity between VP-ergic neurons in rats has not been convincingly demonstrated and, accordingly, we were unable to find VP/Venus positive fibers descending the PVN in the SON and vice versa.

ParvOT Neurons Modulate NK1R Positive WDR Neurons

In addition to the control of magnOT activity, this newly described subpopulation of parvOT neurons densely projects exclusively to the deep layers (V, VI, and X) of the SC. Axonal terminals from parvOT were found in close appositions with NK1R positive WDR neurons, some of which are likely OTR-positives. However, we are not excluding projections of these parvOT neurons to non-WDR deep neurons. Nevertheless, their functional and selective inhibition of C- and A δ - mediated discharges in WDR suggest that nociceptive C-fiber project to deep layers, accordingly with models of dorsal horn circuits that include projections to the lamina V (Cervero and Connell, 1984; Ribeiro-da-Silva and De Koninck, 2008). Functionally, this fits with our results suggesting that OT modulates the excitability of WDR shown as an inhibition of discharges mediated by fibers containing substance P.

ParvOT Neurons Coordinate Neuroendocrine and Hardwired Inhibitory Pain Control

In accordance with our anatomical data, WDR action potential discharges in response to noxious peripheral stimulation are reduced by optogenetic manipulation of the subpopulation of OT neurons in the PVN and its subsequent stimulation at the level

of the SON. This reduction was selective to sensory information transmitted by A δ - and C-fibers, which are, in their majority, nociceptive-specific.

Regarding peripherally mediated OT effects, it has recently been shown that OTR could be expressed by non-peptidergic C-type sensory neurons in DRG (Moreno-López et al., 2013) and the in vitro application of OT suppresses their activity (Gong et al., 2015). Furthermore, intravenous administration of a selective OTR agonist induces an inhibition of discharges mediated by nociceptive-specific primary afferents (Juif et al., 2013). Our present work provides an additional support for this idea by selectively activating a circuit leading to release of OT to the blood (Figure 7). The effect was fully peripheral, since inhibition of nociceptive messages was completely abolished by the addition of the OTR selective antagonist dOVT in the blood flow.

Identification of a subpopulation of parvOT neurons projecting collaterals to both the SON and deep layers of the SC gave rise to the idea that these neurons may exert both a peripheral and central control by OT which we found to take place with a dual time course. This was confirmed by optogenetically stimulating parvOT PVN axons located either in the SON or in the SC. This stimulation led to a reduction of WDR discharges in response to a peripheral noxious stimulation, which was selective for A δ - and C-type nociceptive fibers. The effects in deep layers of the SC seemed to be mediated by the OTR, as we did not find any effects of VP V1a receptor similar to what has been reported (Qiu et al., 2014). As OT terminals on WDR-like neurons contained VGluT2, we assessed glutamate (Glu) and OT contribution to the SC-BL effect on WU intensity. This revealed that both OT and Glu participated to the inhibition of WU. These results are in accordance with our in vitro patch-clamp experiment and can be interpreted by a network effect as OT axons are likely to form en passant synapses (Knobloch et al., 2012), allowing local (micro)volumetric transmission from release sites (Knobloch and Grinevich, 2014; Grinevich et al., 2016b). The combination of two processes can then explain the observed effects: (1) OT acts on OTR in WDR neurons to inhibit them via G $_i$ intracellular pathway (Figure 3E) and (2) coreleased Glu either activates local GABA-interneurons in layers V–VI and around the cc (Schneider and Lopez, 2002; Deuchars et al., 2005), which, in turn, inhibits WDR neurons, or binds a mGluR leading to the direct inhibition of WDR neurons by a G $_{i/o}$ pathway (Gerber et al., 2000; Niswender and Conn, 2010).

Surprisingly, evoked spinal OT release by this subpopulation of parvOT did not modify nociceptive processing by neurons in superficial layers. This suggested that the OT inhibition of WDR firing was not induced by OTR activation in superficial layers, but only in deep dorsal horn layers. This was in agreement with our anatomical data describing the vast majority of parvOT neurons projecting to the deep layers. We failed to reveal any functional contribution of this subpopulation of parvOT projecting to SON and SC in a nerve-induced neuropathic pain, which may be modulated by OT projections to superficial layers of the dorsal horn. In contrast, they exerted a tonic inhibitory control on WU and pain symptoms in the peripheral inflammation. We speculate that the inflammatory component in pain state

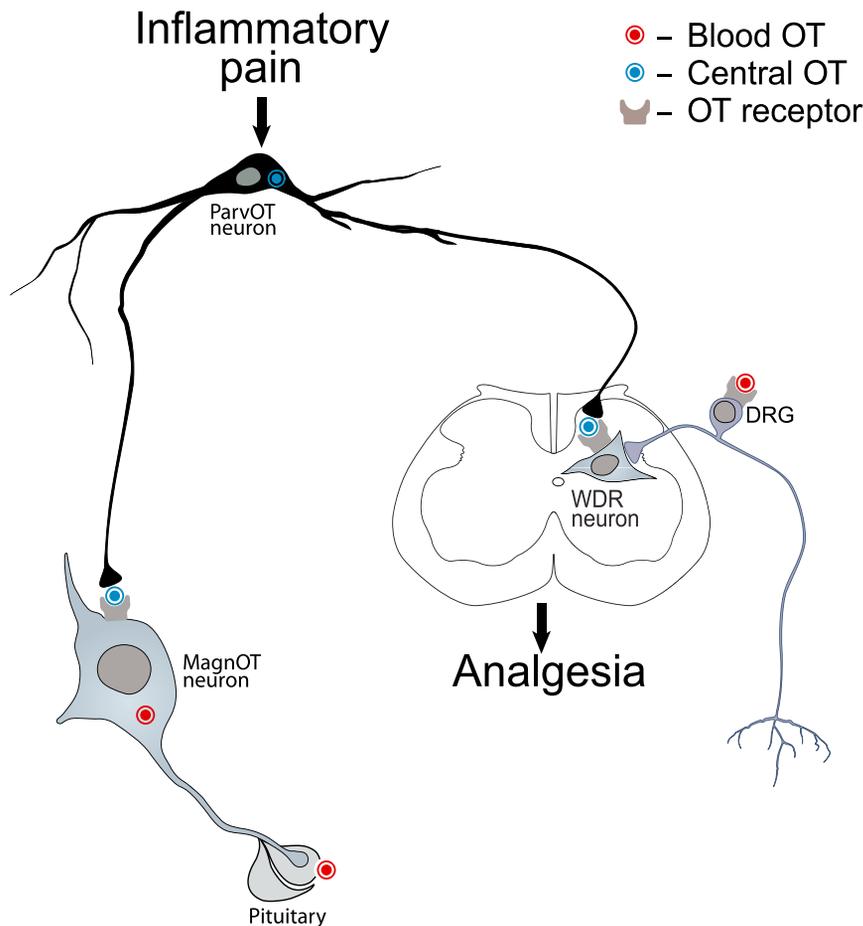


Figure 7. The Role of the Novel Type of ParvOT Neurons in Coordinating Central and Peripheral OT Release to Promote Analgesia

We hypothesize that pain stimulates the identified subset of parvOT PVN neurons, which simultaneously release OT in the SON and SC, exerting respectively delayed and longer lasting and immediate and shorter lasting analgesia. The peripheral analgesic effect of OT is likely mediated by its action on BBB-free sensory neurons of the DRG.

of OT neurons, which coordinate central and peripheral inhibition of nociception and pain perception, and hence, play a role in promoting analgesia (Figure 7).

EXPERIMENTAL PROCEDURES

Animals

Anatomical, electrophysiological, optogenetic, and behavioral studies were performed with Wistar rats (for details of the experiment see the respective figure legend). If not mentioned, rats were housed under standard conditions with food and water available ad libitum. All experiments were conducted under licenses and in accordance with EU regulations.

Viruses

rAAVs (serotype 1/2) carrying conserved regions of OT and VP promoters and genes of interest in direct or “floxed” orientations were cloned and produced as reported previously (Knobloch et al.,

2012). CAV2 equipped with GFP or Cre recombinase was purchased from the Institute of Molecular Genetics in Montpellier CNRS, France (Bru et al., 2010)

Neuroanatomy

To trace internuclear connections, rAAVs expressing Venus were injected into the PVN or SON to follow their axonal projections within the hypothalamus. Alternatively, CAV2-Cre was injected into the SON, while Cre-dependent floxed rAAV was injected into the PVN to identify OT PVN neurons synapsing onto SON neurons.

To trace hypothalamus-SC connections, a CAV2-Cre virus was injected into the SON and floxed rAAV, into the PVN or CAV2-Cre virus was injected in the SC, while Cre-dependent rAAV, into the PVN. This allowed us to visualize OT axon pattern in the SC and to identify projecting PVN OT neurons, respectively. After transcardial perfusion with 4% paraformaldehyde (PFA), brains and/or SC were sectioned and stained with antibodies against OT, VP, vGluT2, GFP, NeuN, NK1R, and OTR. Images for qualitative and quantitative analyses were taken on confocal microscopes Leica SP2 and SP5.

Electrophysiology Experiments

For in vitro patch-clamp recordings, 4 to 8 weeks after injection of virus in adult rats, brains were removed, the hypothalamus or lumbar SC was isolated, cut into 400 μm coronal slices, and kept in artificial cerebrospinal fluid (ACSF: 118 mM NaCl, 25 mM NaHCO₃, 10 mM glucose, 2 mM KCl, 2 mM MgCl₂·6H₂O, 2 mM CaCl₂·2H₂O, and 1.2 mM NaH₂PO₄) saturated 95% dioxygen (O₂), 5% carbon dioxide (CO₂). Visualized neurons were patched with borosilicate glass pipette (4–9 M Ω) filled with 140 mM KMeSO₄, 10 mM HEPES, 2 mM MgCl₂, 0.1 mM CaCl₂, 0.1 mM (1,2-bis(o-aminophenoxy)

regulates the excitability of this subset of parvOT neurons, as observed by c-Fos induction in parvOT neurons (data not shown). Our results indicate that the described subpopulation of parvOT neurons specifically targets NK1R and OTR positive neurons located in the deep layers of the SC to exert antinociceptive action on WDR to promote analgesia.

Described ParvOT Neurons as a New OT-Ergic Cell Type

Following pioneering works of Swanson and Kuypers (1980), Sawchenko and Swanson (1982), and Swanson and Sawchenko (1983), parvOT neurons have been considered as heterogeneous cell populations with descending projections to brainstem and/or SC regions. However, in the present study, we found a small group of parvOT neurons that forms a pathway distinct from the classical hypothalamo-neurohypophyseal axonal tract through the hypothalamus. Although we did not analyze in detail axonal collaterals of these parvOT cells in other forebrain regions, their existence in the hypothalamus adds a new feature to parvOT cells. In addition, these neurons simultaneously project collaterals to deep layers of the SC, representing a unique group of specifically located cells, which is likely distinct from the OT-immunoreactive neurons described by Jójárt et al. (2009) that massively project axons to the superficial layers of the SC. Based on the unique connectivity of the identified parvOT neurons, we speculate that they represent a new type

ethane-N,N,N',N'-tetraacetic acid) (BAPTA), 2 mM ATP Na salt, and 0.3 mM guanosine triphosphate (GTP) Na salt (pH 7.3), adjusted to 300 mOsm, and voltage-clamped at -60 mV.

For in vivo extracellular recordings, 4 to 8 weeks after injection of virus, adult animals were anaesthetized with 4% isoflurane and placed in stereotaxic frame. Extracellular neuronal activity was recorded using a stainless electrode with 10 M Ω impedance (FHC; UE(FK1)).

Behavioral Experiments

Mechanical allodynia was measured using a calibrated forceps (Bioseb). Thermal allodynia/hyperalgesia was measured using the Plantar test using Hargreaves method (Ugo Basile). Peripheral painful inflammatory sensitization was obtained by a single unilateral intraplantar injection of CFA (Sigma-Aldrich, 100 μ l in the right paw). Nerve injury-induced neuropathy was induced using the cuffing method.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2016.01.041>.

AUTHOR CONTRIBUTIONS

Conceptualization, A.C. and V.G.; Methodology, P.P., P.H.S., R.Stoop, A.C., and V.G.; Formal Analysis, M.E., M.Melchior, H.S.K.-B., J.W., and A.C.; Investigation—Neuroanatomy, M.E., H.S.K.-B., M.d.S.G., L.C.R., F.A., T.G., M.B., M.Mitre, and G.G.; Investigation—In Vivo Electrophysiology, M.Melchior, J.W., Y.T., and N.P.-D.; Investigation—In Vitro Electrophysiology J.W., A.C.C., and R.T.d.R.; Investigation—Behavior, M.Melchior, J.W., M.d.S.G., N.P.-D., and L.L.T.; Investigation—Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), V.C. and Y.G.; Resources, B.C., R.C.F., M.V.C., R.Sprengel, and R.K.; Writing—Original Draft, M.Melchior, H.S.K.-B., J.W., P.P., P.H.S., R.Stoop, A.C., and V.G.; Writing—Review & Editing, M.E., M.Melchior, H.S.K.-B., J.W., P.P., P.H.S., R.Stoop, A.C., and V.G.; Visualization, M.E., M.Melchior, H.S.K.-B., J.W., A.C., and V.G.; Supervision, A.C. and V.G.; Project Administration, R.Stoop, A.C., and V.G.; and Funding Acquisition, A.C. and V.G.

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Supplemental Information

A New Population of Parvocellular Oxytocin Neurons Controlling Magnocellular Neuron Activity and Inflammatory Pain Processing

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SUPPLEMENTAL MATERIALS

Marina Eliava, Meggane Melchior, H. Sophie Knobloch-Bollmann,

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**A new population of parvocellular oxytocin neurons controlling
magnocellular neuron activity and inflammatory pain processing**

SUPPLEMENTAL TABLES

Table S1 (corresponds to Figure 1 and S1):

**Specific Expression of VP Promoter, Virally Introduced to
Hypothalamic Nuclei of Naïve and Water Deprived Rats**

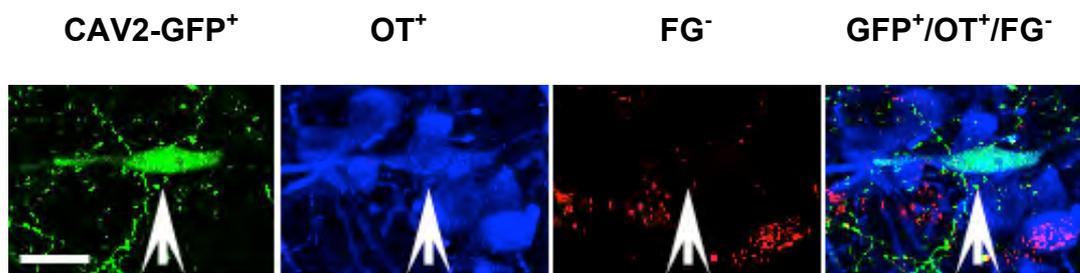
Animals	Comparison	SON	PVN
Naïve Rats	VP vs Venus	95.46% ± 5.53%	98.9% ± 1.74%
		n=552	n=858
	Venus vs VP	94.44% ± 3.29%	94.98% ± 3.26%
		n=552	n=858
Water deprived Rats	VP vs Venus	100% ± 0%	100% ± 0%
		n=1115	n=1169
	Venus vs VP	99.81% ± 0.56%	99.59% ± 0.52%
		n=1115	n=1169

Quantification of VP-immunopositive neurons infected with rAAV carrying VP promoter and Venus. n, absolute number of identified neurons per structure and condition. Results are presented as percentage ± SEM.

Table S2 (correspond to Figure 1 and S1):

Quantification of Back-labeled PVN ParvOT Neurons Projecting to the SON

Cell phenotype	GFP ⁺	GFP ⁺ /OT ⁺	GFP ⁺ /OT ⁺ /FG ⁺	GFP ⁺ /OT ⁺ /FG ⁻
n	52.2 ± 18.09	31.5 ± 8.5	3 ± 1	28.5 ± 6.5



The table shows the number of fluorogold (FG)-containing back-labeled PVN parvOT neurons projecting to the SON. n, absolute number of identified neurons per structure (6 sections per PVN of each rat, 3 rats).

Results are presented as Mean ± SEM.

Panel provides an example of a GFP positive (GFP⁺) back-labeled PVN cell, which contains OT immune-signal (OT⁺), but not Fluorogold (FG⁻), indicating that this is a parvOT neuron. Scale bar represents 10 μm.

Table S3 (corresponds to Figures 1-6):

**Experimental Procedures, Number of Animals, Target Structures,
Viral vectors, Expression time, and Corresponding Figure.**

Field	Name of experiment	Number of rats used	Viral vectors	Target structures	Expression time	Fig.
Anatomy	Intra-hypothalamic fiber trees of OT Neurons	10 5/nucleus	rAAV_Otpr_Venus	PVN or SON unilateral	4 weeks	1
Anatomy	Intra-hypothalamic fiber trees of AVP neurons	10 5/nucleus	rAAV_Vppr_Venus	PVN or SON unilateral	4 weeks	S1
Anatomy	Cell type specific expression of AVP promoter rAAV	5	rAAV_Vppr_tdTomato and rAAV_Otpr_Venus	PVN and SON bilateral	2 weeks	S1
Anatomy	Physiological responsiveness of AVP promoter rAAV	6 3 naïve, 3 water deprived	rAAV_Vppr_Venus	PVN and SON bilateral	2 weeks	S1
Anatomy	Light microscopic characterization of PVN OT contacts in the SON	10 5/viral approach	rAAV_Otpr_Venus or rAAV_Otpr_Syn-EGFP	PVN bilateral	4 weeks	1
Anatomy	Electron microscopic characterization of PVN OT contacts in the SON	3	rAAV_Otpr_Venus	PVN bilateral	4 weeks	2
Anatomy	Retrograde tracing with retrobead injection into SON and systemic fluorogold application	6	Rhodamin retrobeads and Fluorogold	SON (angled) unilateral and i.p.	10 days	3
Anatomy	Retrograde CAV-GFP virus application for visualization of PVN cells projecting to the SON	3	CAV2-GFP	SON unilateral	4-5 weeks	Table S2
Anatomy	Retrograde CAV-Cre virus application for Cre-dependent marker expression in PVN-SON connecting OT neurons to trace fibers into the SC	4	CAV2-Cre and Otpr_DIO_Venus	SON unilateral and PVN bilateral	4-5 weeks	S4
Anatomy	Translucent brain with unilaterally labeled PVN OT neurons and their fiber path	2	rAAV_OTpr_Venus	PVN unilateral	4 weeks	S2
Anatomy	Injection site size verification and virus spread evaluation in SON	10	CAV2mCherry and LV Ef1A	SON bilateral	2 weeks	3
Anatomy	Immunohistochemical identification of SC cells, found in close proximity to spinally projecting OT fibers	9	CAV-Cre and OTpr_DIO_GFP	SON bilateral and PVN bilateral	4 weeks	4
Anatomy	Identification, quantification and anatomical description of spinally projecting OT PVN cells, back-labelled after Cav-Cre virus delivery into lumbar segment of spinal cord	5	CAV-Cre and OTpr_DIO_GFP	L4-L5 unilateral and PVN bilateral	4 weeks	1 & 3
Anatomy	Immunohistochemical confirmation of glutamatergic nature of PVN-SON, and PVN-SC mutual synapses	3	OTp_iCreW and EF1a p_DIO_Synaptophysin-GFP	PVN bilateral	2 weeks	3

Anatomy	Detection of c-Fos in NK1R and OTR neurons in parvOT neurons after capsaicin application	9	CAV-Cre and OTpr_DIO_GFP	SON bilateral and PVN bilateral	4 weeks	1
Anatomy	Detection of NK1R and OTR mRNA by in situ hybridization	4	-	-	3 weeks	3
Electrophy. (in vivo)	SON-BL and SC/SON recording	9	Otp _r _Chr2-mCherry	PVN bilateral	4 weeks	1
Electrophy. (in vivo)	PVN stimulation and recording	8	Otp _r _Chr2-mCherry	PVN bilateral	> 8 weeks	-
Electrophy. (in vivo)	SC-BL with dOVT treatment	11	CAV2-Cre and rAAV- CAG-FLEX-ChR2-mCherry	SON bilateral and PVN bilateral	8-10 weeks	5
Electrophy. (in vivo)	SC-BL with V1aR antagonist treatment	2	CAV2-Cre and rAAV- CAG-FLEX-ChR2-mCherry	SON bilateral and PVN bilateral	> 8 weeks	S5
Electrophy (in vivo)	SC-BL with NBQX + dOVT and NBQX treatments	10	Cav-Cre and CAG-DIO-ChR2-mCherry	SON bilateral and PVN bilateral	> 8 weeks	5
Electrophy. (in vivo)	SON-BL and SON recording with NBQX and dOVT infusion in SON	5	Cav-Cre and CAG-DIO-ChR2-mCherry	SON bilateral and PVN bilateral	4 weeks	S5
Electrophy (in vivo)	SC-BL and SP treatments	5	Cav-Cre and CAG-DIO-ChR2-mCherry	SON bilateral and PVN bilateral	> 8 weeks	3
Electrophy (in vitro)	Validation of ChR2 approach and electrical differentiation of parvOT vs magnOT	8	Otp _p - hM4D(Gi)-mCherry	PVN bilateral	> 8 weeks	S6
Electrophy (in vitro)	Validation of DREADD approach	8	CAV2-Cre and Otp _p -DIO-hM4D(Gi)-mCherry	SON bilateral and PVN bilateral	> 8 weeks	S6
Electrophy (in vitro)	Direct action of OT on WDR neurons	14	-	-	> 8 weeks	3
Behavior	BL in CFA model	8	CAV2-Cre and rAAV- CAG-FLEX-ChR2-mCherry	SON bilateral and PVN bilateral	4 weeks	6
Behavior	DREADD in CFA model	10	CAV2-Cre and Otp _p -DIO-hM4D(Gi)-mCherry	SON bilateral and PVN bilateral	4 weeks	6
Behavior	DREADD in cuff model	12	CAV2-Cre and Otp _p -DIO-hM4D(Gi)-mCherry	SON bilateral and PVN bilateral	4 weeks	6
Behavior	BL in cuff model	10	CAV2-Cre and Otp _p -DIO-CHR2-mCherry	SON bilateral and PVN bilateral	4 weeks	6
Neuroendocrinology	OT content in plasma	5	CAV2-Cre and Otp _p -DIO-CHR2-mCherry	SON bilateral and PVN bilateral	4 weeks	1
TOTAL number of rats injected with viruses				224		

Table S4 (corresponds to Figure 2 and S2):

Passive and Active Properties of Parvocellular and Magnocellular Neurons, as Distinguished by the Presence or Absence of Fluorescent Labeling

Parameter analyzed	Parvocellular (n=11)	Magnocellular (n=13)	P value (unpaired)
Membrane potential (mV)	-56 ± 3	-59 ± 4	0.27
Input resistance (MOhm)	420 ± 65	390 ± 40	0.74
AP half width (ms)	0.49 ± 0.02	0.55 ± 0.06	0.40
Spike frequency (Hz)	20.4 ± 4.0	11 + 4	0.12
Spike delay (ms)	33 ± 4 ms	85 ± 19	0.04*
Rise slope (mV/ms)	1.2 ± 0.2	0.5 ± 0.2	0.02*

Quantified electrophysiological characteristics of parvocellular and magnocellular OT neurons in the PVN.

SUPPLEMENTAL FIGURES

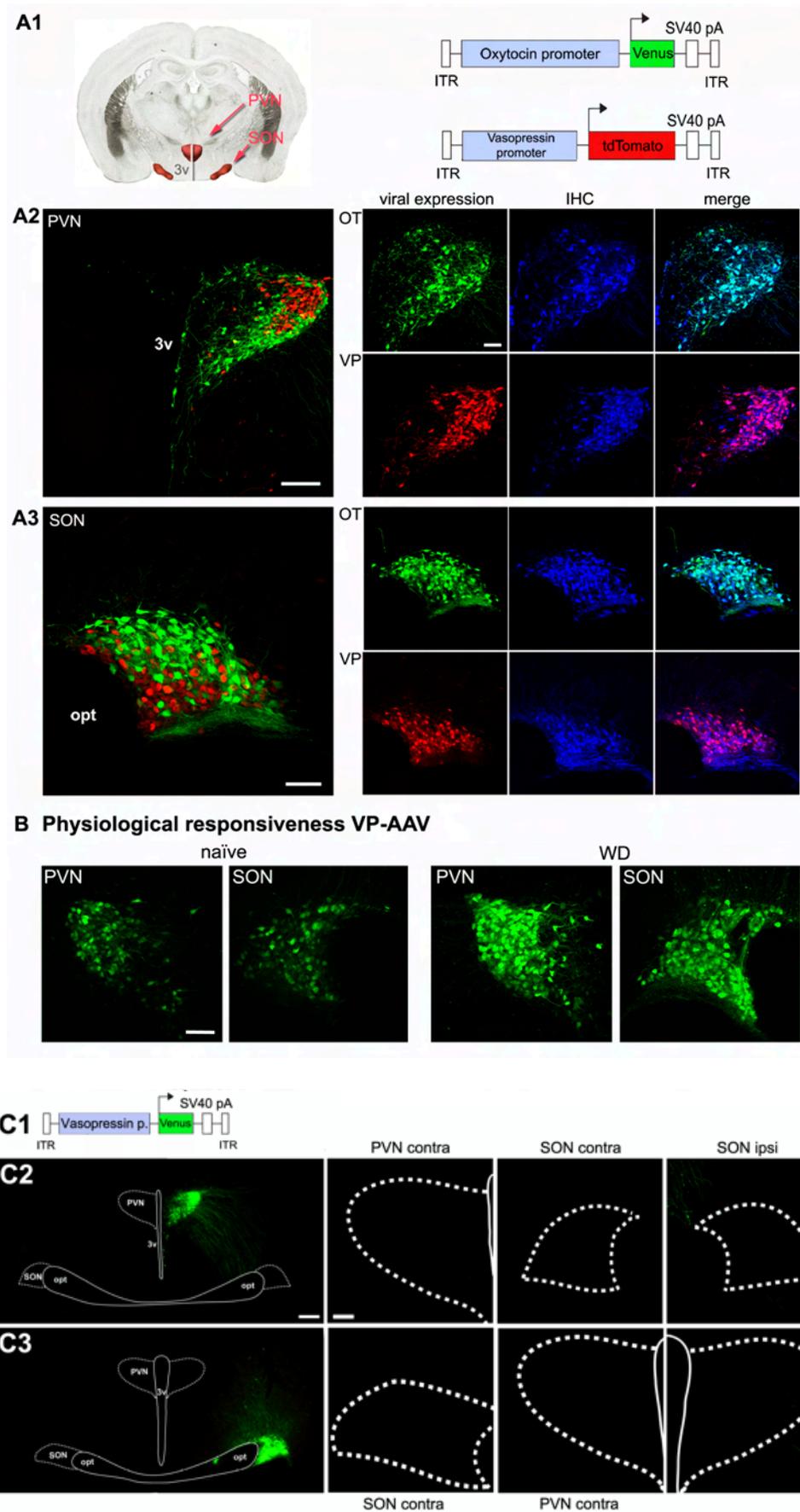


Figure S1 (corresponds to Figure 1). Cell Type Specific Expression and Physiological Responsiveness of VP Promoter rAAV, Projections of VP neurons.

(A) rAAV expressing Venus under the control of a 2.6 kb mouse OT promoter and tdTomato under the control of a 1.9 kb mouse VP promoter were injected concomitantly into PVN and SON (**A1**). Counterstaining for either OT or VP revealed a striking co-localization of the Venus (green) and OT signal (blue; **A2** and **A3**, upper rows) as well as tdTomato (red) and VP signal (blue; **A2** and **A3**, lower rows), indicating cell specific expression from both rAAVs in PVN and SON (further characterization of OT promoter rAAV in Knobloch et al., 2012). Scale bars represent 200 μm in A2 left panel, 100 μm in A3 left panel and 75 μm in right panels.

(B) After two days of water deprivation, the intensity of intrinsic Venus fluorescence increased compared to naïve conditions and indicates a preserved physiological responsiveness of the short virally delivered VP promoter (pictures taken with equal confocal settings). Scale bar represent 75 μm .

(C). Injection of AAV, expressing Venus under the control of VP promoter (**C1**) unilaterally in the PVN (**C2**) or SON (**C3**). No GFP-containing fibers were detected within the SON after PVN injection and vice versa as well as fibers were absent in contralateral hemisphere. Scale bars represent 200 μm in overview images (left panels), 50 μm in enlarged images of individual nuclei. 3v – third ventricle; opt – optic tract, SON - supraoptic nucleus.

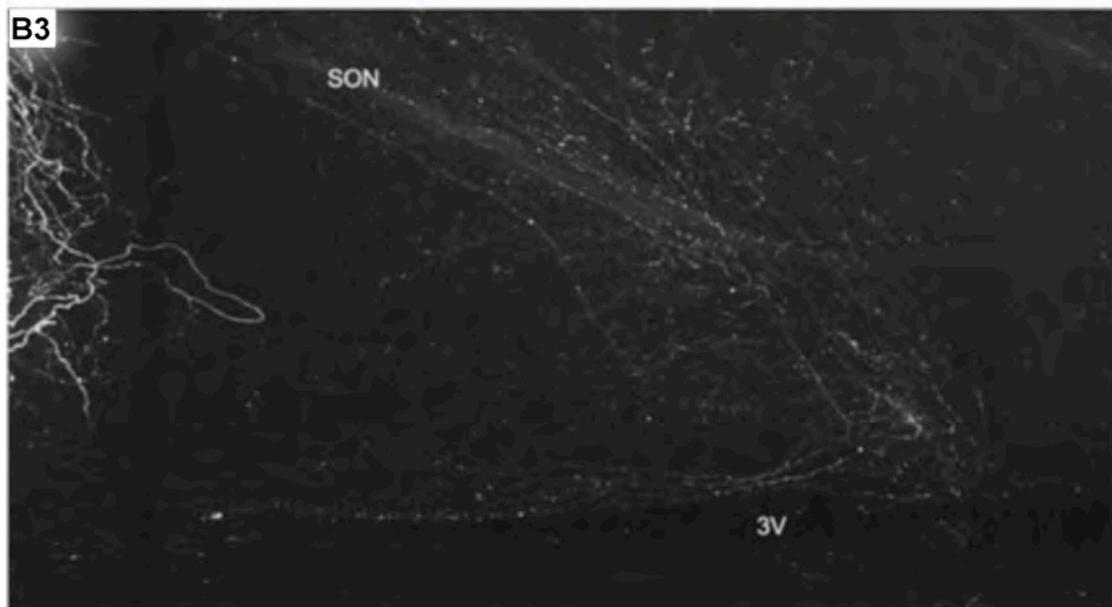
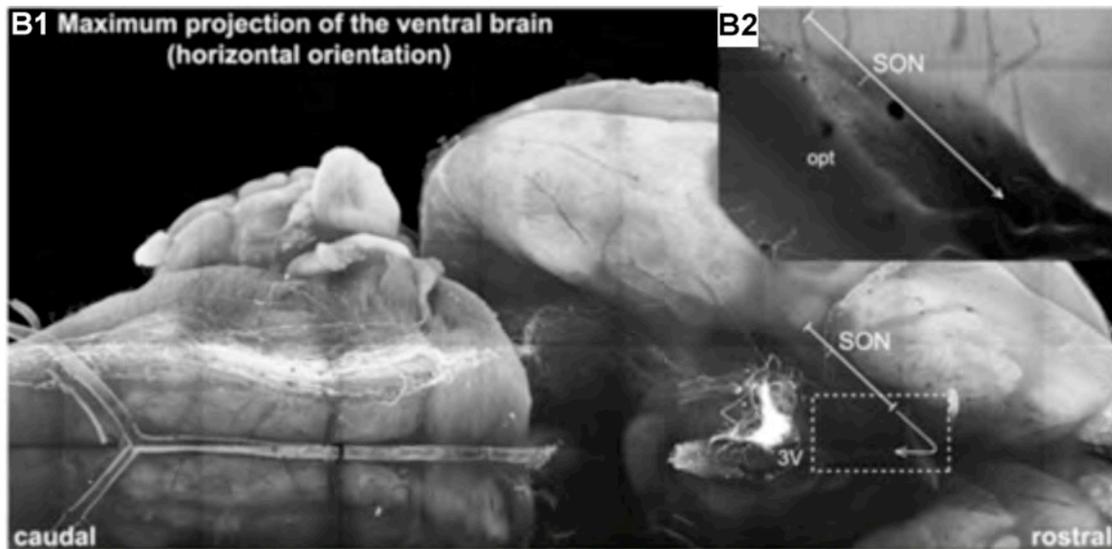
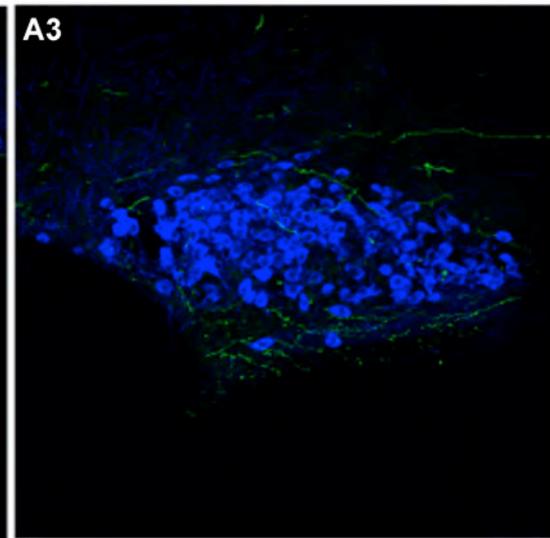
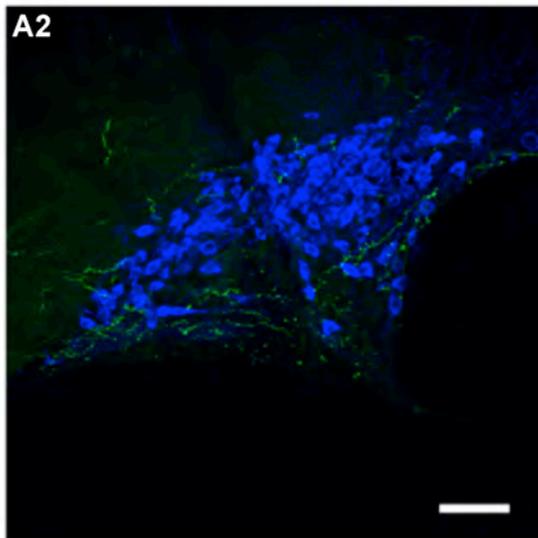


Figure S2 (corresponds to Figure 1). Reconstruction of OT Connections Between the PVN and SON.

(A) Injection of OT promoter-Venus AAV (A1) into the PVN of female (A2) and male (A3) rats resulted in the appearance of Venus-positive axonal terminals within the SON of rats of both genders. Scale bar: 50 μ m.

(B) The cleared rat brain (female) contains virally-mediated Venus labeling unilaterally in OT neurons of the PVN. Depicted are maximum projections of horizontal light sheet recordings of the ventral brain (B1: comprising the hypothalamus, B2: comprising the SON, B3: comprising the hypothalamus without the PVN). Fiber streaming towards the brainstem and SC are prominently labeled. Fine OT fibers, descending from the PVN, are recognizable in the SON, a 'banana-shaped' area bordering the optic tract (indicated in B1 and B2). (B2) Magnification of the dashed region of B1, revealing OT fibers stream longitudinal within the SON. (B3) Further magnification reveals that OT fibers stream beyond the rostral end of the SON to the brain midline (not crossing). There they kink and run ascending and caudally along the third ventricle to the PVN (compare to bent arrow in B1). The dashed square in B1 indicates the location of magnification B2.

3v – third ventricle; opt – optic tract, SON - supraoptic nucleus.

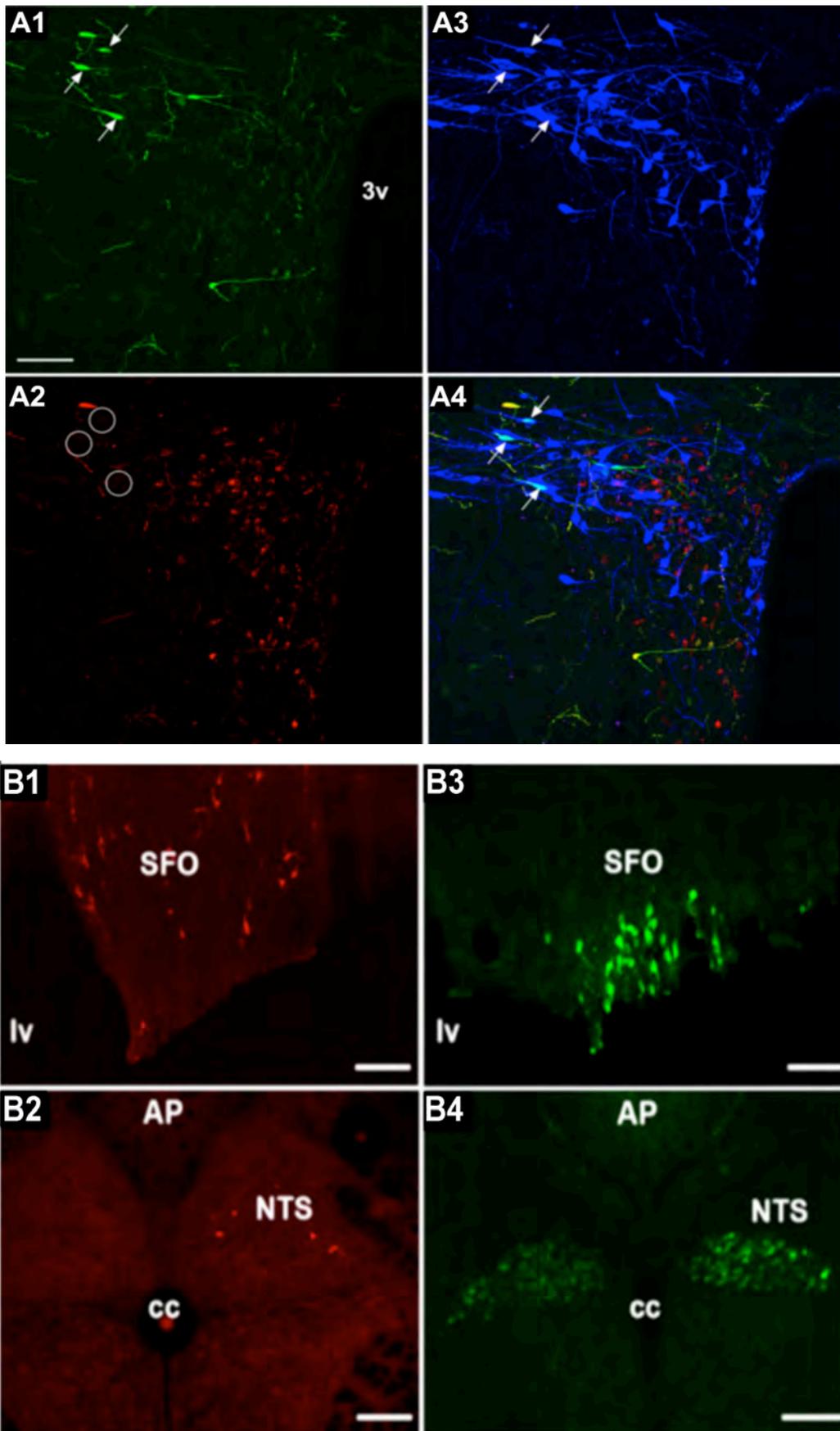


Figure S3 (corresponds to Figure 2). CAV2-based Labeling of PVN Neurons and Efficiency of CAV2 vs. Retrobeads.

(A) Overview of the PVN with GFP-positive back-labeled neurons after injection of CAV2-GFP into the SON. The cells positive for GFP (green, arrows, **A1**) and oxytocin (blue, arrows, **A3**) are negative for fluorogold (red, **A2**; respective areas are encircled). Overlay of the three channels is in **A4**.

3v – third ventricle, scale bar = 100 μ m.

(B) As proof of the method for uptake and traveling of Rhodamine-labeled Retrobeads (red), back-labeled neurons in the subfornical organ (**B1**) and nucleus of the solitary tract (**B2**), known to project to the SON, were detected in rats injected into the SON. (**B3, B4**) GFP-expressing neurons in the same brain regions after the injection of CAV2-GFP virus into the SON.

Scale bars represent 100 μ m.

AP – *area postrema*, cc – central canal, lv – lateral ventricle, NTS – nucleus of the solitary tract, SFO – subfornical organ.

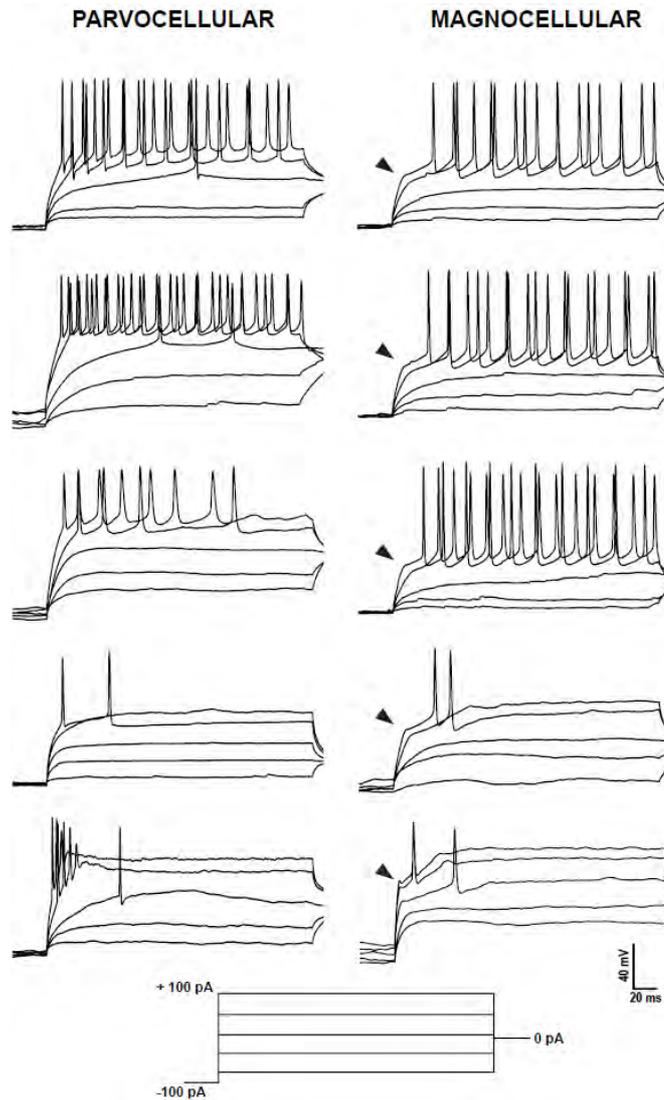


Figure S4 (corresponds to Figure 2). Electrophysiological Properties of ParvOT vs. MagnOT Neurons.

Example traces of spiking patterns of five putative parvo, (left column)- and magnocellular (right column) neurons following an initial hyperpolarizing step followed by depolarizing steps caused by current injections of different magnitudes (as shown below the traces). All putative magnocellular neurons (characterized by the absence of fluorescence) exhibited the typical "hyperpolarizing notch" as indicated by the arrows.

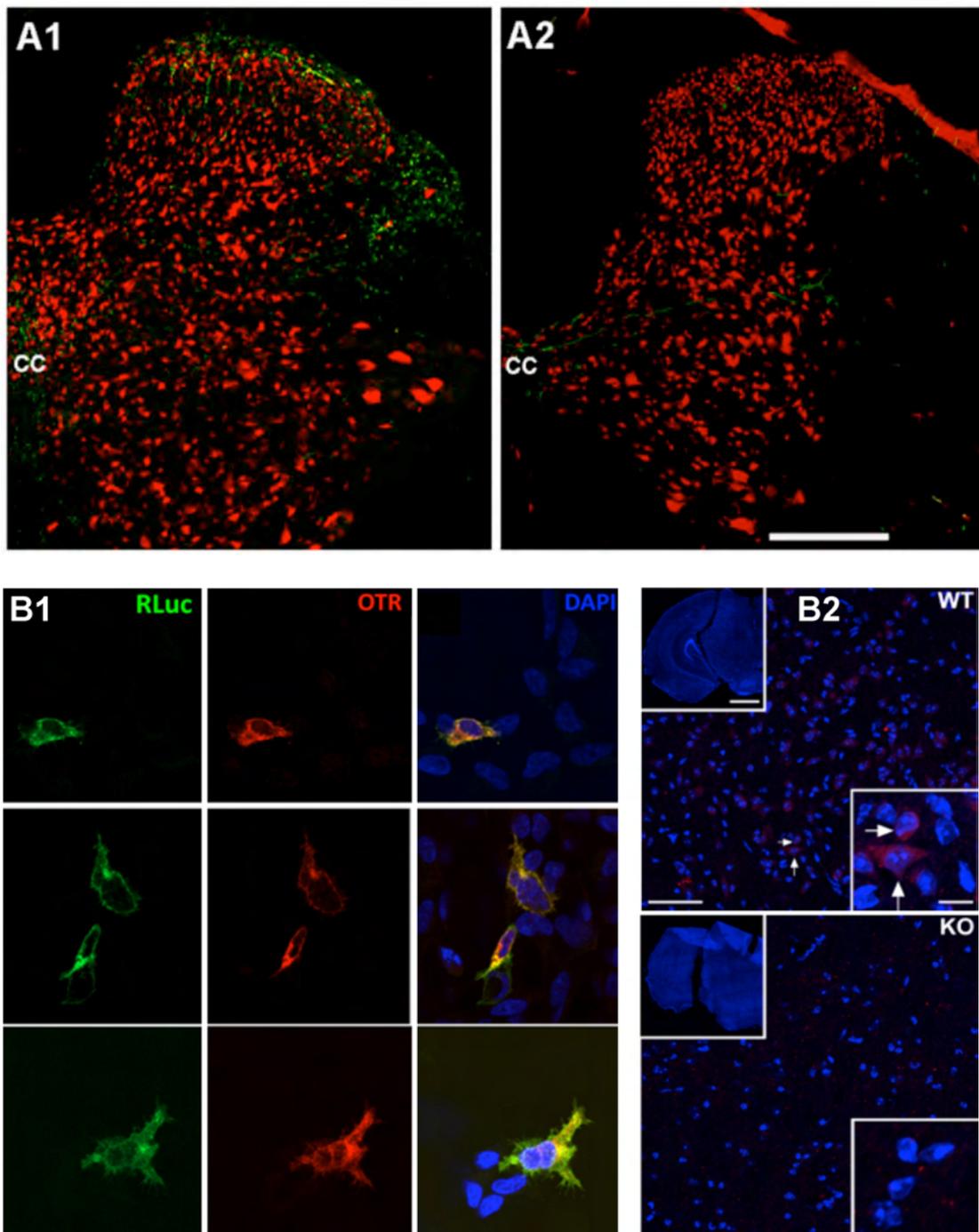


Figure S5 (corresponds to Figure 3). Distribution of GFP fibers on Coronal Spinal Cord Sections and Verification of Specificity of OTR antibodies.

(A) GFP fibers. (A1) Labeling all OT neurons (including all parvocellular neurons) resulted in appearance of OT/GFP axons in both superficial and deep layers. However, labeling only a fraction of parvOT neurons (also

projecting to the SON; **A2**) resulted in the appearance of GFP fibers only in deep layers. Cell bodies were visualized by NeuN immunostaining (red). Scale bar: 250 μ m. cc: central canal.

(B) Specificity of OTR antibodies. **(B1)** Transfected HEK cells: Mouse OTR fused to RLuc was stained with anti-RLuc antibody (Millipore mAb 4400) (green; RLuc) and the oxtr-2 antibody (red; OTR); nuclei were stained with DAPI (blue). Double staining was observed only in OTR-RLuc expressing cells in which plasma membrane and intracellular compartments were labeled. **(B2)** Brain sections of OTR KO mice: While in pons of WT mice (right panel) a population of neurons contains OTR immunosignal (red), in KO mice OTR immunoreactivity has not been detected. Cell nuclei were stained by DAPI (blue). Scale bars for left up inserts (overview of section) is 1000 μ m, for right low – 10 μ m, and for the central panel – 50 μ m.

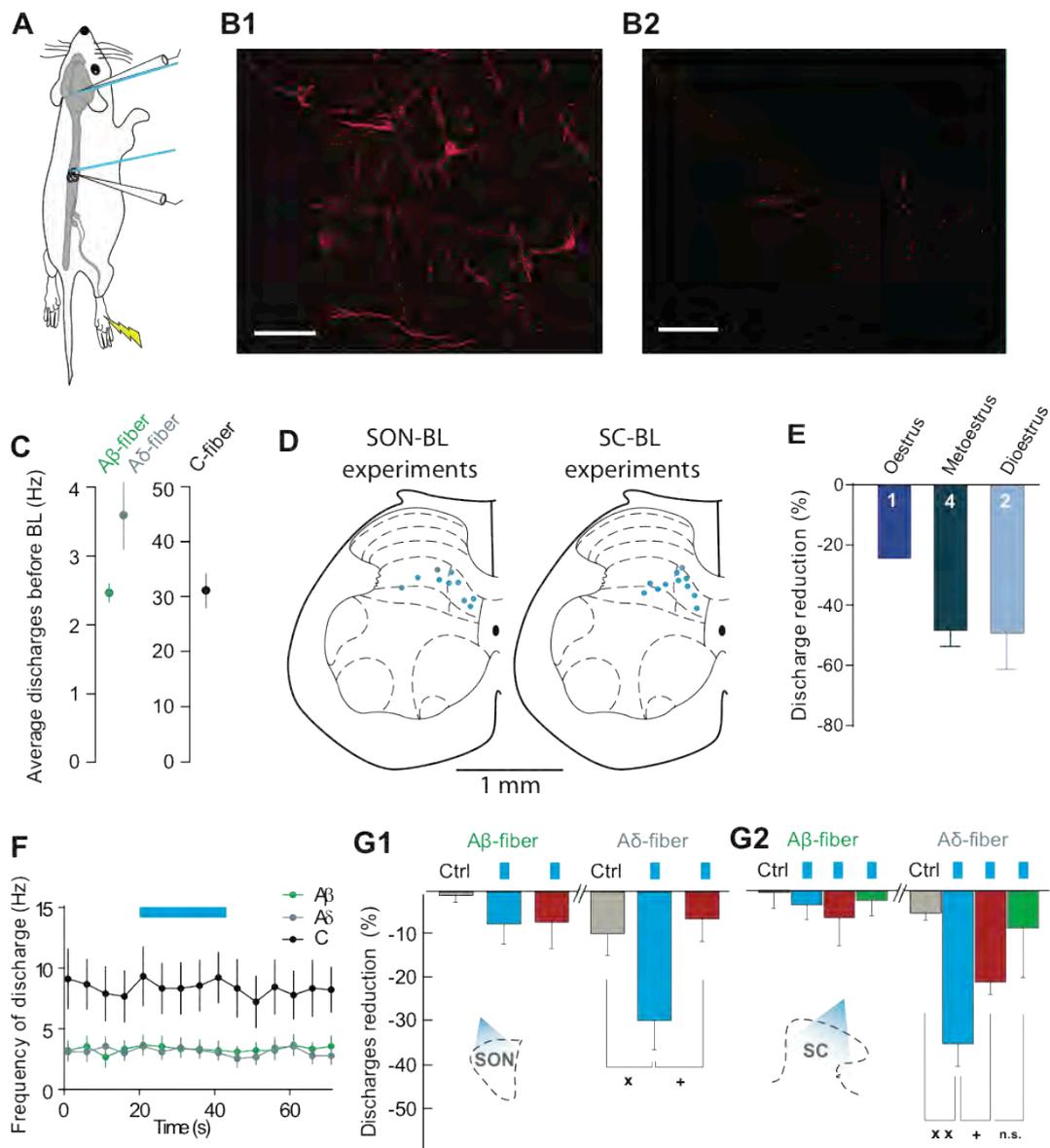


Figure S6 (corresponds to Figure 5). Responses of SC Neurons are Independent of Estrous Cycle and VP Receptors.

(A) Scheme of the experimental setup.

(B1) ChR2-mCherry expressing axons in the SON. **(B2)** Presence of the ChR2 expressing axons in the deep layers of lumbar segments of the spinal cord. Scale bars in **B1** and **B2** is 100 μ m.

(C) Basal frequencies of A β - (WDR-A β), A δ - (WDR-A δ) and C- (WDR-C) fibers per paw stimulation (1/s) upon stabilized wind-up (n = 31).

(D) Localization of the WDR neurons recorded for SON-BL and SC-BL experiments.

(E) Comparison of the SC-BL effect on WDR-C discharges regarding the estrous cycle (oestrus n = 1, metoestrus n = 4, dioestrus n = 2).

(F) Effect of SC-BL on superficial layer neurons (n=9).

(G1) Effect of SON-BL in WDR-Ab and WDR-Ad in non responding WDR neuron (gray, n=8), responding WDR neuron (blue, n=10) and in responding WDR neuron in presence of dOVT (red; n=6). (G2) Effect of SC-BL in WDR-Ab and WDR-Ad in non responding WDR neuron (gray, n=7), responding WDR neuron (blue, n=9) and in responding WDR neuron in presence of dOVT (red; n=6) or dOVT + NBQX (green; n=6).

BL – blue light, SON – supraoptic nucleus, SC – spinal cord, WDR – wide dynamic range.

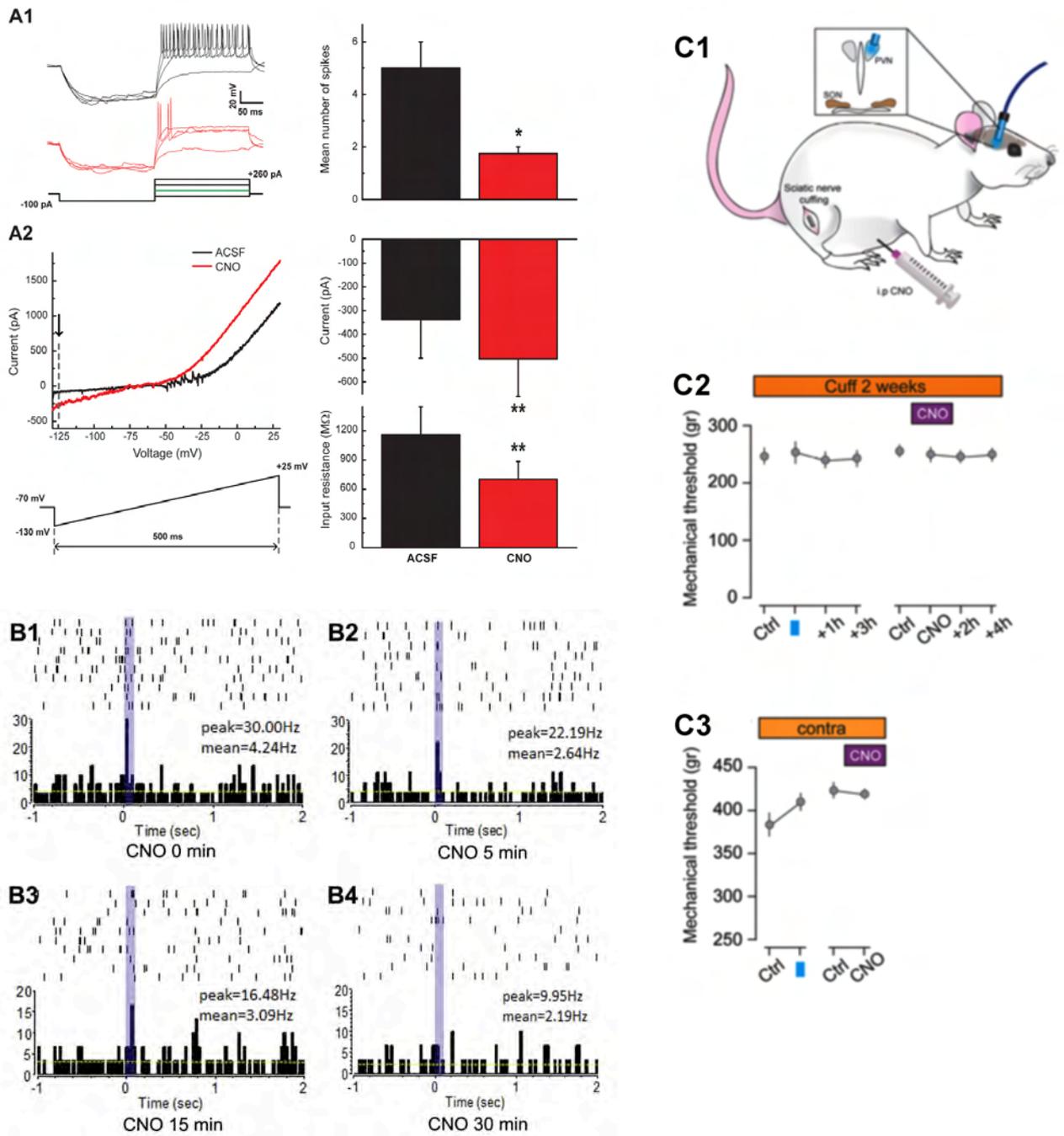


Figure S7 (corresponds to Figure 6). CNO Inhibits OT Neuron Activity *in Vitro* and *In Vivo* and Manipulation with ParvOT Neurons After Sciatic Nerve Cuffing.

(A1) Left panel: examples of voltage responses induced by current injections in the current clamp configuration, before (black) and during (red) CNO (5 min at 20 μ M). The protocol used is shown below the traces. Right

panel: CNO reduced the mean frequency of spikes induced by current injections (+40 pA), green line in protocol on the left, n=8. **(A2)** Left panel: example of current-voltage curve obtained from a 500 ms voltage ramp (shown below) from -130 to +25 mV, before (black) and during CNO (5 min at 20 μ M, red). Right panel: CNO increased the inward current (upper graph) measured at -125 mV (dashed line) during the voltage ramps and decreased the input resistance (lower graph) calculated from responses to hyperpolarizing voltage steps; n = 16, * p <0.05, ** p < 0.01, Student's paired t-test. Data represented as mean \pm SEM.

(B1) BL-induced OT neuron firing increases from basal rate of 1-3 Hz to 20 Hz *in vivo*. CNO (i.p. 3 mg/kg) gradually decreased basal and BL-evoked activity of the same OT neuron at 5 **(B2)**, 10 **(B3)** and 30 **(B4)** min after the injection.

(C1) Scheme of the experimental procedure for behavioral assessment of parvOT role in neuropathic pain model (cuff). **(C2)** Mechanical thresholds of the cuffed hindpaw two weeks after surgery. Effect of PVN-BL was measured 5 min, 1h and 3h after PVN-BL (n = 10; left panel). Effect of CNO (3 i.p. mg/kg) was measured 45 min, 2h and 4h after i.p. injection and its 24 h washout (n = 12; right panel). **(C3)** Mechanical thresholds of the contralateral hindpaw two weeks after surgery. Blue squares represent 20 s at 30 Hz with 10 ms pulses of BL stimulation.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals

Anatomical, electrophysiological, optogenetic and behavioral studies were performed with adult female Wistar rats except for the adult male Wistar rats in Figure S1 (for details of the experiment see the respective figure legend). Rats were housed under standard conditions with food and water available *ad libitum* (except for the water deprivation paradigm in Figure 1B; S1B and S1C) and maintained on a 12-hour light/dark cycle in accordance with EU rules. In total, we used 224 rats as described in Table S3.

Cloning of rAAV Vectors

The generation of rAAVs with specific expression in OT-cells is described in our previous work (Knobloch et al., 2012). Briefly, the conserved promoter region of 2.6 kb was chosen using the software BLAT from UCSC (<http://genome.ucsc.edu/cgi-bin/hgBlat>), was amplified from BAC clone RP24-388N9 (RPCI-24 Mouse, BACPAC Resources, CHORI, California, USA) and was subcloned into an rAAV2 backbone carrying an Ampicillin-resistance. Genes of interest introduced to the plasmid in floxed and unfloxed version are Venus, Channelrhodopsin2-mCherry, Synaptophysin-EGFP, and hM4Di. The rAAV vector that carries a conserved VP promoter sequence was designed in a similar way. It comprises a 1.9 kb sequence stretch (revealed by BLAT) that allows for cell-specific expression in hypothalamic VP neurons. As a gene of interest, Venus or tdTomato were introduced to the plasmid.

Production of Viruses

Production of chimeric virions (recombinant Adeno-associated virus 1/2; rAAV 1/2) was described in Knobloch et al., 2012. Briefly, human embryonic kidney cells 293 (AAV293; Agilent #240073) were calcium phosphate-transfected with the recombinant AAV2 plasmid and a 3-helper system (During et al., 2003). rAAV genomic titers were determined with QuickTiter AAV Quantitation Kit (Cell Biolabs, Inc., San Diego, California, USA) and are $\sim 10^{13}$ genomic copies per ml for all rAAV vectors used in this study. Canine Adeno Virus (CAV) serotype 2 (Bru et al., 2010; titer 2.5×10^{12} particles/ml) carrying the gene for Cre-recombinase (CAV2-Cre) was purchased from Institut de Génétique Moléculaire de Montpellier CNRS UMR 5535.

Stereotaxic Injections

Intrahypothalamic stereotaxic injections were performed as described (Grinevich et al., 2015) and coordinates were chosen in accordance to the rat brain atlas (Paxinos and Watson, 1998) for PVN (X axis: +/-0.4 mm; Y axis: -1.8 mm; Z axis: -8.0 mm) and SON (X: +/- 1.6 mm; Y: -1.4 mm; Z: -9.0 mm). Conducting the 15°-angled beads injections into the SON under rotation of the stereotax arm changed the coordinates accordingly (right hemisphere: X -4.44 mm; Y -1.3 mm; Z -8.48 mm). The injected volume for all viruses (rAAV and CAV2) was 300 nl (undiluted). Rhodamine conjugated Retrobeads (Lumaflyor Inc., Durham, NC, USA; Katz et al., 1984) were diluted 1:1 with 1x PBS and injected in a 75 nl volume.

Injection into the spinal cord. Animals have been injected unilaterally by Cav2-Cre virus into SC lumbar segment (L4 or L5 spines). The same animal received injection of Cre-dependent EGFP expressing virus was bilaterally to the rat PVN. The tip of injection pipette for SC injection was lowered at the stereotaxic coordinates used in our vivo recording experiments. To localize the Cav2-Cre injection site in SC we co-injected virus with a low volume of latex retrobeads, which formed a dense fluorescent beads in the injection site.

Fluorescence Immunohistochemistry

After transcardial perfusion with 1x PBS and 4% PFA, rat brains and SC were vibratome sectioned (50 μm) and stained with combinations of various primary antibodies against GFP (detects Venus; Abcam; ab 13970 chicken; 1:10.000), OT- and VP-Neurophysin (1:300; mouse; provided by Harold Gainer; Ben-Barak et al., 1985), NeuN (Chemicon; MAB377 mouse; 1:1000), VGlut2 (Synaptic Systems; 135403 rabbit. 1:5000), dsRed (detects tdTomato; Clontech; 632496 rabbit; 1:1000); Fluorogold (Millipore; AB153 rabbit; 1:3000), Cre (1:1000; rabbit; provided by Prof. Günther Schütz, DKFZ), and c-Fos (Santa-Cruz, 1:1000; rabbit). To label spinal cord neurons for OT or NK1 receptors rabbit polyclonal antibodies against oxytocin receptor (OTR, 1:1000; from Dr. Robert C. Froemke lab) and NK1R (ThermoFisher, Temecula, 1: 5000) were used.

The signals were visualized with the following secondary antibodies, FITC-conjugated, CY3-conjugated or CY5-conjugated antibodies (Jackson Immuno-

Research Laboratories, Inc.; 1:500) or Alexa 350 and 594 (Invitrogen; 1:500 or 1:200, respectively). All images were acquired on a Zeiss Axio Imager M1 light or a Leica TCS SP5 confocal laser scanning microscope. Digitized images were analyzed using Fiji (NIMH, Bethesda, MD, USA) and Adobe Photoshop CS5 (Adobe, Mountain View, CA).

Degree of VGluT2 and EGFP-colocalization in the varicosities of spinally projecting PVN axons was assessed by counting EGFP-immunoreactive (ir) varicosities within the area of 400 square μm ca. 500 μm lateral to the central canal of SC (3 rats, 3 planes for each, 680 ± 72 puncta per animal). Vast majority of EGFP-ir punctae ($89 \pm 7,4$ %) were positive for VGluT2.

Specificity of immunostaining with antibodies against OXTR

Cell cultures, cDNA constructs, transfections and immunofluorescent staining

HEK293T cells were maintained in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum, 200 U/ml penicillin, 200 mg/ml streptomycin, 2 mM L-glutamine (all purchased from Sigma). Cells were transiently transfected with cDNAs encoding for the mouse WT-OTR tagged at its N-terminus with RLuc (cloned into the pRLuc Perkin Elmer vector) and with the human WT-OTR tagged at its C-terminus with EGFP (cloned into the pEGFP Clontech vector). Cells were seeded in 6-well plates on glass coverslips (6×10^5 cells/cover slip), allowed to attach for 24 hours and then transfected with Turbofect (ThermoScientific). 48 hours after transfection cells were fixed for 20 minutes at room temperature

with 4% (w/v) paraformaldehyde and immediately processed for immunofluorescence.

For immunofluorescence, transfected cells were incubated with the 29300 antibody (Oxtr2; Froemle lab) diluted 1:100 in GDB buffer (20 mM sodium phosphate buffer, pH 7.4, 450 mM NaCl, 2% (w/v) gelatine, 10% (v/v) Triton X-100) + 0.2% (w/v) BSA, for 2.30 h at room temperature. The samples were then incubated for 1 h with secondary antibodies in GDB buffer (Alexa 488 goat anti-mouse and Alexa 555 goat anti rabbit from Molecular Probes). Glass cover slips were mounted on glass slides with Mowiol and analyzed under a confocal microscope LSM 510 Meta (Zeiss).

Staining with OTR in the brainstem of WT and OTR KO mice

Anesthetized animal with 1.5:0.5 mixture of ketamine-xylazine, were perfused with heparin-PBS until blood is flushed and perfuse for 10 min at room temperature (about 45 ml per mouse) with 4% paraformaldehyde (PFA) – PBS. After extractions of brains, they were postfixed for 2 hours-overnight at 4°C in 4% PFA and dehydrated in 30% sucrose for 18 – 24 hours at 4°C. Cryosections (thickness is 16 µm) were collected on SuperFrost Plus glass slides and stored at -80°C until immunostaining sprocedure. Briefly, sections were incubated PBS containing 0.2% v/v Triton X-100 and 5% v/v normal donkey serum and incubated with oxtr2 antibodies (dilution is 1:200) in 1% BSA on PBS for 24-48 h at 4C. Then sections were washed with PBS and incubated 1-2 h at RT with secondary Alexa–conjugated antibodies (dilution is 1:500). Finally, after washing in

PBS sections were stained with DAPI/Hoechst solution and coverslipped with Fluoromount.

Alignment of antibodies sequences with sequences of mouse and rat OXTR

Due to the fact that antibodies were generated against mouse OTR and the subject of our study was a rat, we compared sequences of two antibodies, oxtr1 (an N-terminal portion of the OTR; used in cell cultures) and oxtr2 (part of the 3rd intracellular loop of the OTR; used for staining in brain sections of mice and rats) with the rat OTR. Generation and initial testing of the antibodies has been described in Marlin et al., 2015.

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                OXTR 1
OXYR_MOUSE MEGTPAANWSIELDLGSGVPPGAEGNLTAGPPRRNEALARVEVAVLCLILFLALSGNACV 60
OXYR_RAT   MEGTPAANWSVELDLGSGVPPGEEGNRTAGPPQRNEALARVEVAVLCLILFLALSGNACV 60
          *****:***** ** *****:*****

OXYR_MOUSE LLALRTTRHKHSRLFFFMKHLSDLVVAVFQVLPQLLWDITFRFYGPDLLCRLVKYLQV 120
OXYR_RAT   LLALRTTRHKHSRLFFFMKHLSDLVVAVFQVLPQLLWDITFRFYGPDLLCRLVKYLQV 120
          *****:*****

OXYR_MOUSE VGMFASTYLLLLMSLDRCLAICQPLRSLRRRTDRLAVLATWLGCLVASVPQVHIFSLREV 180
OXYR_RAT   VGMFASTYLLLLMSLDRCLAICQPLRSLRRRTDRLAVLGTWLGCLVASAPQVHIFSLREV 180
          *****:*****

OXYR_MOUSE ADGVFDCWAVFIQPWGPKAYVTWITLAVYIVPVIVLAACYGLISFKIWQNLRLKTAATAAAA 240
OXYR_RAT   ADGVFDCWAVFIQPWGPKAYVTWITLAVYIVPVIVLAACYGLISFKIWQNLRLKTAATAAAA 240
          *****:*****

                OXTR 2
OXYR_MOUSE AAEGSDAAGGAGRAALARVSSVKLISKAKIRTVKMTFIIVLAFIVCWTPFFVQMWVSWD 300
OXYR_RAT   AAEGNDAAGGAGRAALARVSSVKLISKAKIRTVKMTFIIVLAFIVCWTPFFVQMWVSWD 300
          ****_*****

OXYR_MOUSE VNAPKEASAFIIAMLLASLNSCCNPWIYMLFTGHLFHELQVQRFCCSARYLKGSRPGETS 360
OXYR_RAT   VNAPKEASAFIIAMLLASLNSCCNPWIYMLFTGHLFHELQVRFCCSARYLKGSRPGETS 360
          *****:*****

OXYR_MOUSE ISKKSNSSTFVLSRRSSSQRSCSQPSSA 388
OXYR_RAT   VSKKSNSSTFVLSRRSSSQRSCSQPSSA 388
          :*****

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The alignment of the OTR mouse and rat sequences and labelled in bold our target sequence for the antibodies against oxtr1 and oxtr 2 in the mouse sequence. Aminoacids with a * underneath are the same between the mouse and rat oxtr.

As can be seen, oxtr 1 has 22/25 aminoacids overlap (88%) between mouse and rat, and oxtr 2 has 27/28 overlap (96%). The proteins seem overall very similar with only 9 different aminoacids out of 388, and most of the changes conserve the main characteristic (polar/charged vs. hydrophobic).

***In situ* Hybridization**

In situ hybridization (ISH) was performed on 25- μ m cryostat-cut coronal sections prepared from fresh-frozen mouse lumbar spinal cord L4/5 (male C57BL/6J, 19-20 weeks). Spinal cords were flushed out with PBS using a 23G needle and a 30 ml syringe, immediately frozen in Tissue-Tek O.C.T. compound and stored at -80 degrees Celsius. ISH was performed according to the manufacturer's instructions (Advanced Cell Diagnostics) for Fresh Frozen RNAscope Multiplex Fluorescent Assay. Spinal cord sections were (treated with positive and negative controls) and then examined for coexpression of OTR and NK1R using ACD designed target probes for OTR, NK1R and vGluT1/2/3, as well as the nuclear stain DAPI. Z-stacks were collected across a depth of 2 μ m at 1 μ m steps with an upright laser scanning microscope (LSM-710, Carl Zeiss) using a 63x-objective and keeping acquisition parameters constant.

Characterization of Cell Type Specificity and Physiological Responsiveness of the Virally-Delivered VP Promoter

The specificity of marker expression in VP neurons infected by VP promoter rAAVs was analyzed for PVN and SON separately. Injection of

300 nl rAAV mix per hypothalamic nucleus (titer VP virus: 5.8×10^{13} genomic copies/ml) resulted after two week expression in a confined appearance of tdTomato signal (red) exclusively in Vasopressin neurons (elaborated by counterstaining for VP in blue; merged color turquoise; Figure S1A) and Venus signal (green) exclusively in OT neurons (blue; merged color magenta; Figure S1A). A subsequent quantification of the expression specificity (performed with the brighter marker Venus; viral titer 2.7×10^{13} genomic copies/ml) was performed in three naïve and three physiologically stimulated (two days water deprived) male rats (Table S1). Per rat and nucleus, Venus and VP positive neurons were counted (after immunohistochemical staining) on three sections of different rostro-caudal localization (Bregma levels PVN: -1.5, -1.8, -2.0 mm; SON: -1.1, -1.4, -1.7 mm). Results were calculated as percentage \pm SEM. Preserved physiological responsiveness was assessed after two days water deprivation at the end of the two-week expression period. Expression differences are visualized as intensity differences of the intrinsic fluorescence at equal Bregma levels taken as single layer confocal microscopy pictures with identical microscope settings (Figure S1B).

Translucent Brain Sample Preparation and Imaging

Rats were injected unilaterally into the PVN with rAAV OT promoter-Venus and perfused four weeks later with 1x PBS and 4% PFA, followed by 15 hours postfixation in 4% PFA/PBS. Dehydration and optical clearing was performed at 30°C with increasing concentrations of *tert*-butanol in water (30%, 50%, 70%, 80%, 96%, 100%,100%; v/v, two days each step)

followed by incubation in clearing solution (BABB: 1 vol. benzyl alcohol + 2 vol. benzyl benzoate; cf. (Dodt et al., 2007)), for one week, with the pH of dehydration and clearing solutions adjusted to 9.5 with triethylamine (Schwarz et al., 2015). Venus fluorescence (excitation: 514 nm; detection: 520-550 nm) was recorded from the uncut translucent brain on a custom-built light sheet microscope controlled by custom acquisition software based on Labview 8.6 (Schwarz et al., manuscript submitted) as xy mosaics of z stacks (xyz voxel size: 1.613 x 1.613 x 3.226 microns). Individual image planes were saved as 16bit TIFF files. Mosaic tiles were stitched with a plugin written for ImageJ/Fiji (Niedworok et al., 2012; www.fiji.sc). For analysis of PVN-SON connectivity, a gamma function (gamma=0.125) was applied to the resulting data stacks to enhance dim signals, followed by conversion to 8bit TIFF format. After another conversion to Knossos cube format, axons were traced with Knossos software (Helmstaedter et al., 2011).

Electron Microscopy

Rats were perfused transcardially with 4% PFA in phosphate buffer containing 0.05% glutaraldehyde at pH7. The 50 μ m coronal brain sections containing the SON were incubated with rabbit polyclonal anti-GFP antibodies (Molecular Probes; 1:5000). The GFP signals were visualized using the standard avidin-biotin complex (ABC) protocol and DAB chromogen. Sections were further intensified by silver-gold in accordance with Liposits and colleagues (1986).

Fluorogold

Rats received an i.p. injection of 4% Fluorogold in 0.9% NaCl (20 mg/kg body weight) to label all neuroendocrine cells protruding beyond the blood-brain-barrier (most importantly, magnOT neurons). Ten days later, rat brains were transcardially perfused, sectioned and stained as described above. The Fluorogold (own emission: 530-600 nm upon UV excitation) was visualized with Alexa 350 (Invitrogen; 1:500) to shift the detectable emission to ~440 nm (microscope requirement).

Counting of ParvOT Neurons Projecting to the SON

Three weeks after injection of CAV2-GFP virus into the SON, the total number of GFP-positive neurons within the PVN was counted in six sections (section thickness 50 μ m), containing the entire PVN in three rats. Another three rats received i.p. injection of Fluorogold (FG, see above) three weeks after injection of CAV2-GFP virus and were killed three weeks later. Three sections containing rostral, middle and caudal part of the PVN were stained for GFP, OT and FG and the total number of GFP⁺/OT⁻, GFP⁺/OT⁺, GFP⁺/OT⁺/FG⁻ and GFP⁺/OT⁺/FG⁺ was counted (see Table S2). In similar manner we counted the number of back labeled OT neurons, using CAV2-Cre virus injection in the SON and Cre-dependent rAAV, expressing GFP under the control of OT promoter in the PVN. Using combination of two viruses we achieve expression of GFP exclusively in OT PVN neurons.

Surgical Procedure for Catheter and Optic Fiber Implantations

Implantation of catheters in the jugular vein was performed under isoflurane anesthesia (2% in pure oxygen at a flow rate of 1 l/min). The jugular vein was exposed and a small incision was made in the vessel with iridectomy scissors. The catheter (PE50, Warner Instruments, Hamden, USA), filled with saline and soaked at the tip in heparin (Heparin-Natrium Braun, 10.000 IU/ml), was inserted into the vein and ligated to the vessel. The wounds were loosely closed with a suture (Silkam 4-0, Unodis, Hagenau, France).

For *in vivo* behavioral experiments, we used a blue laser (λ 473nm, output of 100 mW/mm², DreamLasers, Shanghai, China) coupled with optical fibers (BFL37-200-CUSTOM, EndA=FC/PC, and EndB=Ceramic Ferrule; ThorLabs, Newton, New Jersey; final light intensity ~ 10 mW/mm², 30 Hz, 10 ms pulses, 20 s duration), which were connected to a chronically implanted optic fiber to target the PVN (CFMC12L10, Thorlabs). Optic fibers were chronically implanted under isoflurane anesthesia (4% induction, 2% maintenance) at stereotaxic positions of -1.8 mm antero-posterior and 3 mm lateral from Bregma with a 21° angle to avoid previous injection scars and were stabilized with dental cement. This should lead to a specific stimulation of the PVN, as prevalent measurements with blue laser stimulations in rodent brain have shown that the blue light of the laser does not penetrate the tissue further than 500 μ m (Yizhar et al., 2011).

OT blood concentration

For measurement of OT concentrations, blood samples were obtained using a catheter in the femoral artery implanted under isoflurane anesthesia. The femoral artery was exposed, and a small incision was made with iridectomy scissors. The catheter (PE50, Warner Instruments, Hamden, USA) filled with saline and soaked at the tip with heparin was inserted into the artery and ligated to the vessel. A first sample of around 500 μ l of blood was made before the BL stimulation. Then BL was applied for 20 seconds. Three other blood samples were made: just after BL stimulation, 2 min and 30 min after BL stimulation. Blood samples were centrifugated at 4°C, 3000 rpm for 15 min, the plasma was collected and stored at -80°C.

Plasma preparation for LC-MS/MS analysis

5,76 pmole of D5-oxytocine internal standard was added to 50 μ l of lithium heparin plasma. Plasma was acidified up to 1 % H_3PO_4 (v/v) in a final volum of 400 μ l and was centrifuged (14,000 x g, 5min). The resulting supernatant was collected prior to solid phase extraction (SPE). SPE procedure was performed with a positive pressure manifold (Thermo Electron). OASIS HLB SPE-cartridges (1 cc, 30 mg, Waters, Guyancourt France) first activated with 1 ml of acetonitrile (ACN) and then washed with 1 ml of H_2O 99 % / H_3PO_4 1 % (v/v). The sample was loaded and the SPE-cartridge was washed with 1 ml of H_2O 99 % / H_3PO_4 1 %. After a 2 ml wash with H_2O /formic acid 0.1 % (v/v) and with 1 ml of ACN 5 % / H_2O 94.1 % / formic acid 0.1 % (v/v/v), elution was performed with 500 μ l of

acetonitrile 60% / H₂O 40% (v/v). Eluates were collected and dried under vacuum prior to mass-spectrometry (MS) analysis (see below).

LC-MS/MS instrumentation and analytical conditions - LC-analyses were used to determine the presence of oxytocin in the selected reaction monitoring mode (SRM). Analyses were performed on a Dionex Ultimate 3000 HPLC system (Thermo Scientific, San Jose, CA, USA) coupled with a triple quadrupole Endura (Thermo Scientific). The system was controlled by Xcalibur v. 2.0 software (Thermo Scientific).

Extracted plasma samples were solubilised in 100 µl of H₂O/formic acid 0.1 % (v/v) and 20 µl of the solution were loaded into an Accucore RP-MS column (ref 17626-102130; 100 x 2.1 mm 2.6 µm, Thermo Electron) heated at 35 °C. Oxytocin and D5-oxytocin elutions were performed by applying linear gradient of buffers A/B. Buffer A corresponded to H₂O 99.9 % / formic acid 0.1 % (v/v), whereas buffer B was ACN 99.9 % / formic acid 0.1 % (v/v). A linear gradient of 20 % - 95 % of solvent B at 400 µL / min over 2.5 min was applied followed by a washing step (0.5 min at 85 % of solvent B) and an equilibration step (1 min of 20 % of buffer B).

Qualitative analysis and quantification were performed in SRM using an Endura triple quadrupole mass spectrometer and deuterated internal standards. For ionization, 3500 V of liquid junction voltage and 292 °C capillary temperature were applied. The selectivity for both Q1 and Q3 was set to 0.7 Da (FWHM). The collision gas pressure of Q2 was set at 2 mTorr of argon. For oxytocin and D5-oxytocin, the selection of the monitored

transitions and the optimization of the collision energy were preliminary and manually determined. The transitions and the corresponding collision energies (CE) used for SRM were the following: m/z 504.3 \rightarrow m/z 86.3 (CE = 39 eV), m/z 504.3 \rightarrow m/z 285.2 (CE = 30 eV) for oxytocin with 2 charges, and m/z 506.7 \rightarrow m/z 86.4 (CE= 32 eV), m/z 506.7 \rightarrow m/z 290.3 (CE= 26 eV) for D5-oxytocin (with 2 charges). Identification of the compounds was based on precursor ion (with 2 charges), selective fragment ions and retention times obtained for oxytocin and D5-oxytocin internal standard. Quantification of oxytocin was done using the ratio of daughter ions response areas of the D5-oxytocin.

Determination of the Phase of Estrous Cycle

To test if the estrous cycle could modify the answer of WDR neurons to BL, we analyzed the estrous phase by using vaginal smear cytology (Marcondes et al., 2002). At the end of the *in vivo* recording experiment, a micropipette filled with 100 μ L of saline solution (NaCl 0.9%) was placed in the rat vagina. Cells were removed and dissociated by pipetting up and down for at least three times. A drop of the smear was placed on a glass slide and observed by a light microscope with a 40x or a 100x objective lens. Estrous phase was determined based on the proportion of leukocytes, nucleated epithelial cells and anucleate cornified cells.

Pharmacological inactivation of OT neurons: Verification of the DREADD technique

In vitro electrophysiological recording

To test effects of CNO *in vitro*, we used rats that had been infected with a combination of CAV2-Cre (SON) and Cre-dependent virus, expressing hM4Di under the control of OT promoter. 6-8 weeks after infection coronal slices were prepared from the paraventricular nucleus and fluorescent neurons (indicative of the expression of the virus) were selected for whole-cell patch-clamp recordings. After establishing the clamp, neurons were either recorded in current or voltage clamp mode.

In current clamp mode, cells were subjected to a hyperpolarizing current injection (bringing the membrane potential close to -100 mV), followed by progressively more depolarizing current injections (see Figure 2B1) which triggered changes in membrane potential characteristic of parvocellular neurons (as shown previously in Figure 2) and causing progressively more action potentials. Application of CNO led to a decrease in input resistance as a result of the opening of inward rectifying potassium channel leading to a decrease in number of action potentials (see example traces in left part figure S7A1, before CNO in black, during CNO in red). To quantify the effects of CNO, the number of action potentials were counted at one specific current injection (shown in green) and averaged over several neurons, plotted in the right part of figure S7.

In voltage clamp mode, cells were subjected to a 500 ms voltage ramps starting from -125 going up to +25 mV. The voltage ramp and an example of the resultant current are plotted in figure S7A2 (left part, resp lower and

upper panels). As can be observed, application of CNO caused an increase in the ramp evoked current (in red) both at negative and positive potential, indicating the opening of inward rectifying potassium channels. The currents and changes in membrane resistance averaged over several neurons are plotted in the right parts of figure S7A2 (resp. upper and lower panels).

Taken together, these experiments show the inhibitory effects of CNO application on the excitability of virus-infected neurons as a result of the activation of inward rectifying potassium channels.

In vivo optoelectrode recording

Animals were anesthetized in stereotaxic frame by 1.5% isofluran mixed with oxygen and NO₂. A mixture of AAVs, carrying either ChR2 or hM4Di driven by the OT promoter was unilaterally injected into the PVN. (Bregma -1.8 Lateral - 0.3 Deep 8.0). Six screws (two with ground wires) were drilled into skull around the injection site together with dental cement (Tetric Evoflow) were used to anchor the base for electrode implantation. After this procedure, electrodes with assembled optic fiber implanted 0.5 mm above the PVN. After 2 week recovery the tetrodes were gradually lowered into the PVN under laser stimulation (100 Hz, 5 ms, 10 sys, 40 mW) until an OT neuron was identified by PSTH. CNO (1mg/kg) was injected i.p. and recording of OT cells was continued during 30 min.

Eight custom made nickel tetrodes were arranged in order, surrounded by the optic fiber (Thor Lab, Φ 200 μ m) and assembled in the 32-channel microdrive. Tetrodes impedance was measured between 0.3m Ω -0.5m Ω before the surgery. Data acquire system was built on OpenEphys Acquisition board (v2.2), head stage connected with 32-channel preamplifier and Ultra thin SPI cable (Intan Tech) with sampling rate at 30k Hz, low-pass filter set to 600 Hz, high-pass filter 6000 Hz in OpenEphys GUI. Laser stimulation was triggered by waveform generator 33220A (Keysight Tech) and Laser generator BL473T5-200FC (Shanghai Laser & Optics Century Co., Ltd); wave length 473nm, output power 245mW, which together generate 450 nm blue light stimulation at programmed pattern. Additional I/O board allows synchronized digital signal of optogenetic stimulation in data acquire board. Data analysis: OpenEphys GUI 0.3.5 is used in online data analyse. Matlab, Simpleclust 0.5, and Neuroexplorer 3.2 are used in offline data sorting and analyses.

***In vitro* Electrophysiology**

Slice Preparation. Four-to-eight weeks after injection of virus in adult rats, brains were removed, the hypothalamus or lumbar spinal cord was isolated, cut into 400 μ m coronal slices, and kept in artificial cerebrospinal fluid (ACSF, consisting of 118 mM NaCl, 25 mM NaHCO₃, 10 mM glucose, 2 mM KCl, 2 mM MgCl₂*6H₂O, 2 mM CaCl₂*2H₂O, 1.2 mM NaH₂PO₄) saturated with oxycarbon gas (95%O₂, 5%CO₂). Fluorescent cells were identified by fluorescence microscopy in combination with oblique illuminated infrared video microscopy.

Electrophysiological Recordings. Whole-cell patch-clamp recordings were visually guided by infrared oblique light videomicroscopy (DM-LFS; Leica), using 4–9 M Ω borosilicate pipettes filled with 140 mM KMeSO₄, 10 mM HEPES, 2 mM MgCl₂, 0.1 mM CaCl₂, 0.1 mM BAPTA, 2 mM ATP Na salt, 0.3 mM GTP Na salt (pH 7.3), adjusted to 300 mOsm, and amplified with an Axopatch 200B (Axon Instruments). Data were lowpass filtered at 5 kHz with the amplifier and sampled at 10 kHz using pClamp 10 data-acquisition and analysis software (Axon Instruments). The liquid junction potential (12 mV) was corrected for according to Neher (1992).

Spinal cord recording and WDR depolarization protocol. Lamina V-VI and around the central canal putative WDR neurons were recorded based on the visualization of their large body size (~30 μ m) and further electrophysiologically identified through their increasing firing rate with the intensity of stimulation. In current-clamp mode, the membrane potential was adjusted at -60mV. Firing profiles were then characterized using current step injections (from -80 to 200pA, 10pA increments for 900ms, repeated every 20s). This characterization was performed 5-10min after the beginning of the recording in order to ensure stable recordings and optimal dialysis of the intracellular compartment. To investigate the possible role of washout on cell firing properties, some neurons were recorded for more than an hour and no significant changes in the firing profiles (spike frequency and spike amplitude), passive membrane properties and resting membrane potential were noted indicating that the intracellular dialysis with our experimental intracellular solution had no effect on the passive and active membrane properties of deep

laminae neurons (not shown). Neurons were kept for analysis only if they displayed stable resting membrane potentials more negative than -50mV and proper spike overshoots (>15mV).

Hypothalamus recording and electrophysiological identification of parvOT vs magnOT. In order to distinguish between the parvocellular and the magnocellular neurons from the PVN we used a current step protocol which has been previously shown to reliably allow identification of this type of cells. Initially described by Tasker and Dudek (Tasker and Dudek, 1991), this method has been used in several other studies in order to allow discrimination between parvocellular and the magnocellular neurons (Brisson et al., 2013; Chu et al., 2013; Luther and Tasker, 2000; Yuill et al., 2007). Passive and active electrophysiological parameters of neurons were analyzed using Clampfit 10.0 (Axon Instruments, USA). The "rise slope" was analyzed using the same fraction of the trace as for the spike delay, that is, from the start of the depolarizing step until the beginning of the first action potential.

***In vivo* Electrophysiology**

Single-unit Extracellular Recording of Dorsal Horn Spinal Neurons in vivo

Adult Wistar rats were anesthetized with 4% isoflurane in pure oxygen and a laminectomy was performed to expose the L4-L5 SC segments. During the procedure, the isoflurane level was reduced to 2%. The animal was then placed in a stereotaxic frame with the L4-L5 region being held by two clamps

placed on the apophysis of the rostral and caudal intact vertebrae. The dura mater was removed and the SC covered with a thin oil layer. To record wide-dynamic-range neurons (WDR), a stainless electrode (FHC, USA; UE(FK1)) was lowered into the medial part of the dorsal horn of the SC, at a depth of around 500 -1100 μm from the dorsal surface (see Figure S8 for localization of recorded WDR). We recorded WDR neurons of lamina V, receiving both non-noxious and noxious information from the ipsilateral hind paw. A few superficial layers (I-II) neurons were also recorded (Figure S8F). Voltage changes were amplified and filtered (low 300 Hz, High 3000 Hz) by a DAM-80 differential amplifier (WPI, Aston, UK). Recordings were analyzed using the spike 2 software, collecting data through a CED 1404 analog-to-digital interface (CED, Cambridge, UK). The stimulating thresholds to evoke action potential, resulting from the stimulation of the peripheral receptive fields on the rat hindpaw, were attributed to the recruitment of A- and C- type sensory fibers based on their latency from the stimulus artifact as follows: A β - 20 ms, A δ - 20-90 ms, C- 90-300 ms and post discharge 300-800 ms.

Single-unit Extracellular Recording of PVN Neurons in vivo

Adult Wistar rats were anaesthetized with 4% isoflurane and the animal was placed in a stereotaxic frame. During the procedure, the isoflurane level was reduced to 2%. Then a stainless steel electrode coupled with an optical fiber was inserted into the PVN in order to stimulate parvOT neurons expressing ChR2 and record their activity. Hardware and software were the same as previously mentioned. To confirm that the activation of SON neurons was OTR

related, we stereotaxically infused 0.5 μ l / 2 min of dOVT 1 μ M, or NBQX 1 μ M directly in the SON and repeated the recording session.

Wind-up Protocol

We measured the WDR action potential firing responses induced by a stimulation of the hindpaw inducing a short-term plasticity named Wind-up (1 ms pulse duration, frequency 1 Hz, intensity corresponding to 3 times the C-fiber threshold). Wind-up (WU) is a property of WDR neurons, which answer by an increased firing when they are stimulated repeatedly at specific intensities and frequencies (Mendell and Wall, 1965; Schouenborg, 1984). As WU is dependent on C-fiber activation, it can be used as a tool to assess nociceptive information in the SC and OT antinociceptive properties.

Nociceptive Behavioral Tests

Mechanical Allodynia

In all experiments, we used a calibrated forceps (Bioseb, Chaville, France) previously developed in our laboratory to test the animal mechanical sensitivity (Luis-Delgado et al. 2006). Briefly, the habituated rat was loosely restrained with a towel masking the eyes in order to limit stress by environmental stimulations. The tips of the forceps were placed at each side of the paw and a graduate force is applied. The pressure producing a withdrawal of the paw, or in some rare cases vocalization, corresponded to the nociceptive threshold value. This manipulation was performed three times for each hind paw and the values were averaged.

Thermal Allodynia/Hyperalgesia

To reveal the animals' heat sensitivity, we used the Plantar test using the Hargreaves method (Ugo Basile, Comerio, Italy). We compared the response of each hindpaw (Hargreaves et al. 1988) when testing healthy animals and animals having received unilateral intraplantar CFA injection (Sigma-Aldrich, 100 μ l in the right hindpaw). Exposed to a radiant heat, the latency time of paw withdrawal was measured three times per hind paw and the values were averaged.

Optic Stimulation of OT Neurons

For optic stimulation of OT release at fibers descending parvOT PVN cells, ChR2 was expressed from Cre-dependent rAAV (CAG-DIO-ChR2-mCherry; obtained from Deisseroth Lab). Cre was retrogradely transported to respective PVN neurons from the CAV2-Cre injected SON site. Optical stimulation was provided using a blue laser (λ 473 nm, output of 100 mW/mm², DreamLasers, Shanghai, China) coupled with optical fibers (BFL37-200-CUSTOM, EndA = FC/PC, and EndB = Flat cleave; ThorLabs, Newton, New Jersey) to activate ChR2-containing neurons at the level of SON, SC or the PVN as described below.

For the SON-BL experiment, the optical fiber was lowered in the SON together with a stainless steel electrode (FHC, USA; UE(FK1) to activate the axons of PVN-OT neurons projecting to the SON. We recorded WDR neurons in the SC during the following protocol: 50 s WU, 20 s SON-BL (10 ms pulses at 30 Hz, \sim 10 mW/mm²) plus WU, 50 s WU, for a total of 130s recording session.

Recovery of the control WU was tested every five min after the end of the first recording session. In order to confirm that the reduction in WU intensity was related to an OT release in the blood circulation and acting on peripheral OTR, we injected intravenously 100 μ l of 1 μ M dOVT (d(CH₂)⁵-Tyr(Me)-[Orn⁸]-vasotocine; Bachem, Weil am Rhein, Germany), and repeated the stimulation protocol after 15 min.

For the SC-BL experiment, the optical fiber was placed on the surface of the SC in order to activate the fibers of the parvOT PVN neurons that express ChR2 (hence projecting both to the SON and the SC), a few millimeters over the tungsten electrode. BL was applied in 10 ms pulses at 30 Hz. We recorded WDR neurons in the SC during the following protocol: 20 s WU, 20 s SC-BL (10 ms pulses at 30 Hz, \sim 10mW / mm²) plus WU, 20 s WU, for a total of 60 s recording session. Recovery of the control WU was tested every five min after the end of the first recording session. To confirm that the reduction in WU intensity was OTR related, we infused 500 μ l of dOVT 1 μ M, AV_{1A}R 1 μ M (Phenylacetyl¹,O-Me-d-Tyr²,Arg^{6,8},Lys⁹]-vasopressin amide; AV_{1A}R: Sigma Aldrich, Saint-Louis, France) or NBQX 1 μ M directly on the SC for 20 minutes and repeated the recording session. For the superficial neurons, which do not express WU, we used repeated hindpaw stimulation (1 ms pulse duration) at a frequency of 0.5 Hz and an intensity corresponding to three times the threshold for C-type sensory fiber activation. We used the following protocol: five hindpaw stimulations alone, five stimulations under BL, and five stimulations without BL.

Statistical Analysis

Friedman test with Dunn post-hoc test was used to compare the average level of blood OT in function of time after SON-BL. Two-way repeated ANOVA was used for the assessment of BL effects on WU efficacy. Wilcoxon's test was used to compare the average variation of spikes frequencies measured for the same neurons in control, BL, and antagonists + BL conditions. Kruskal and Wallis test was used to compare the average variation of spikes frequencies measured for different neurons in control and BL conditions. One-way ANOVA, followed by multiple comparison post-hoc Tukey test, was used to analyze behavioral data. Differences were considered significant for $p < 0.05$.

RRIDs

Compound	Compagny	Proper citation
GFP chicken	Abcam; ab 13970	(Abcam Cat# ab13970, RRID:AB_300798)
VP-Neurophysin mouse	Harold Gainer	
NeuN mouse	Chemicon; MAB377	(Millipore Cat# A60, RRID:AB_2314889)
VGluT2 rabbit	Synaptic Systems	(Synaptic Systems Cat# 135 103, RRID:AB_2315570)
dsRed rabbit	Clontech; 632496	(Clontech Laboratories, Inc. Cat# 632496, RRID:AB_10015246)
Fluorogold rabbit	Millipore AB153	(Millipore Cat# AB153, RRID:AB_90738)
Cre rabbit	Schutz	
c-Fos rabbit	Santa-Cruz AB_2106765	(Santa Cruz Biotechnology Cat# sc-7202, RRID:AB_2106765)
NK1R rabbit	Thermo Fisher AB_1958926	(Thermo Fisher Scientific Cat# PA1-32236, RRID:AB_1958926)
Wistar rats		RGD:7241799

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J. ARTICLE 2

1. Overview

Astrocytes are essential to the proper functioning of the CNS. On top of their long-known homeostatic roles, numerous studies now attribute a direct role of astroglia in the information processing by the CNS. Astrocytes can participate to synaptic transmission and shape it, through various actions. Especially, astrocytes are known regulator of magnocellular OT neurons of the hypothalamus. Through morphological and molecular adaptations, they can modify the excitability of magnocellular OT neurons and therefore modify their physiological functions. Few data have shown the expression of the OTR in astrocytes, both in cell culture and brain tissue. OT magnocellular neurons are not just neuroendocrine cells, they project in the forebrain and notably in the central amygdala, a key nucleus of emotional processing, notably of pain and anxiety. The release of OT in the CeA modulates its local neuronal circuitry and consequently the output neurons of the CeA, found in its medial subdivision (CeM). The role of astrocytes in the regulation of microcircuits of the CeA has never been assessed, left alone their role in the effect of OT in this area. Here we propose to test the implication of astrocytes in the effect of OT on CeA micro-circuitry, through *ex vivo* and *in vivo* experiments.

RESULTS

OTR binding is present in the lateral part of the CeA (CeL) therefore we sought for OTR expression in astrocytes of this area, using *in situ* hybridization in the CeA of mice. The OTR mRNA was found in cells co-expressing GFAP (putative astrocytes), and in cells without GFAP expression (putative neurons). Next, we tested the functionality of this glial OTR on living astrocytes in rat brain slices. Using calcium imaging coupled to a marker of living astrocytes in slices containing the CeL, we observed that optogenetically evoked activation of OT fibers of the CeL could provoke oscillating and long-lasting calcium transients in astrocytes of the same area. Pharmacological activation of OTR through exogenous application of TGOT could evoke virtually the same oscillating calcium transients, in an OTR specific manner. Those transients were independent of neuronal network activity, since the pre-incubation of tetrodotoxin (TTX) had no influence on the responses of astrocytes to OTR activation. TGOT-evoked calcium transients rely on both intra and extracellular sources of calcium since extracellular calcium was found to be mandatory and the pre-emptying of endoplasmic reticulum stores diminished both the proportion of responding astrocytes and the magnitude of those responses. The entry of calcium from the extracellular medium relied partially on voltage gated calcium channels of the Ca_v3 family, which are expressed in astrocytes. Partially, because the initial

response to TGOT was still present when Ca_v3 were blocked, but not the long-lasting oscillations of $[Ca^{2+}]_i$. Using biased agonist of OTR, we showed that both G_q and G_i proteins are functionally coupled to the astrocytic OTR. Furthermore, phospholipase C signaling is involved to some extent, but not protein kinase C. To assess the potential role of astrocytes in modulating the known effect of OT on CeA neuronal circuits, we used whole cell patch-clamp of the output neurons of the CeA, found in the CeM. The inactivation of astrocytes with fluorocitrate or another gliotoxin blocked the neuronal effects of TGOT without affecting basal synaptic transmission. The other way around, specific optogenetical activation of astrocytes could mimic TGOT effect on synaptic transmission. By conducting further pharmacological experiments on slices, we highlighted the involvement of neuronal NMDA receptors in the response to TGOT, and identified D-Serine as a potential gliotransmitter. Finally, we tested the importance of astrocytes in the analgesic and anxiolytic effects of OT in the CeA. In a model of chronic pain, the pharmacological inactivation of astrocytes through intra-CeL injections of fluorocitrate blocked the analgesic effect of TGOT, and dampened its anxiolytic effect.

From those results, it seems clear that astrocytes are crucially involved in the effect of OT on the activity of the CeA and the behaviors it modulates.

AUTHOR CONTRIBUTION

As for the first study, I want to detail my personal contribution. I conducted all the experiments and analysis in this study, except for:

- The *in situ* hybridization.
- The production and stereotaxic injections of the opsins expressing rAAVs virus.
- I took only a minor part in the behavioral experiments conducted by Dr. Alexandre Charlet.
- I produced the astrocytes patch-clamp data with the help of Benjamin Bellanger, as those experiments were part of his master internship.

Title: A key role for amygdala astrocytes in regulation of negative affective processing by oxytocin.

Short Title: Oxytocin-sensitive astrocytes in affect regulation.

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Abstract:

Complex integrations by astrocyte-neuron networks in both the temporal and spatial domains are crucially involved in brain circuits and functions. Oxytocin orchestrates social and emotional behaviors through modulation of brain circuits such as the central amygdala (CeA). We hypothesize that oxytocin effects in the CeA arise from modulation of such astro-neuronal networks. We found that a subpopulation of CeA astrocytes expresses the oxytocin receptor and responds to its activation by long-lasting increase in calcium transients. Those responses are necessary for oxytocin effects on neuronal activity in the CeA and astrocytic light-evoked calcium transients are sufficient to drive the neuronal network activity. Astrocyte-neuronal communication occurs through release of D-Serine and subsequent activation of NMDA receptors. Accordingly, impairing astrocytes in the CeA abolishes effects of oxytocin on both pain and anxiety. Thus, astrocytes play a critical role in the oxytocinergic modulation of CeA neuronal network and its beneficial modulation of emotional experiences.

One Sentence Summary:

Astrocytes of the CeA have complex responses to oxytocin receptor activation which modulate local neuronal network, leading to decreased pain and anxiety.

Main Text:

The neuropeptide oxytocin is involved in the regulation of many neurophysiological functions, among which anxiety and pain modulation, notably through its action on CeA microcircuits (1–4). A possible role of astrocytes in conveying neuromodulatory effects of oxytocin in the central

nervous system has rarely been explored (5, 6), despite numerous findings of their active involvement in the regulation of neural circuits (7–10) with yet many controversies about the mechanisms involved (11, 12). Moreover, astrocytes seem to be key players in the etiology of chronic pain pathologies (13) as does the CeA (14). Here we tackle the role of astrocytes in the pain and anxiety relieving effects of oxytocin in the CeA of rats, through patch-clamp of output neurons of the CeA, found in its medial part (CeM), and calcium imaging or *in vivo* manipulation of astrocytes from the lateral and capsular part of the CeA (CeL), where oxytocin receptor (OT-R) are found (1).

As the cell types bearing the OT-R in CeL remain unknown, we performed *in situ* hybridization (ISH) to detect OT-R and glial fibrillary acidic protein (GFAP) mRNAs, and found cell transcribing both or only one (Fig. 1A). This indicates that OT-R is expressed in at least astrocytes of the CeL.

To test whether OT-R found in astrocytes could elicit astrocytic responses, we expressed CIV1(t/t) under the oxytocin promoter through rAAVs injected in the paraventricular and supraoptic nuclei, enabling activation of oxytocin neurons and their distant axons present in CeL (3, 4) (Fig. 1B, C). Spinning disk calcium imaging of the calcium indicator Oregon Green® 488 BAPTA-1 loaded in astrocytes, identified by sulforhodamine 101 labelling (Fig. 1D, S1) showed that light-driven activation of CIV1 expressing axons evoked long-lasting calcium transients in astrocytes (Fig 1E). Presence of TTX excludes that responses of astrocytes are secondary to neuronal network activity.

Specificity of OT-R activation as the sole mechanism inducing increase in calcium transients in astrocytes was confirmed by the use of the exogenous agonist [Thr4Gly7]OT (TGOT) alone. TGOT responses were prevented by incubation of OT-R antagonist [d(CH2)5,Tyr(Me)2,Orn8]-

vasotocin (dOVT) but remained unchanged in the presence of TTX (Fig 2.A), which was used in all following calcium imaging experiments with OT-R agonists. This indicates a direct, OT-R specific, modulation of astrocytic calcium transients by oxytocin.

Hence we sought to elucidate the intracellular mechanisms underlying astrocytes responses upon OT-R activation. Calcium imaging experiments were conducted using calcium-free ACSF, which almost prevents any responses, in a partially reversible manner. Internal stores of calcium from the endoplasmic reticulum were also involved since thapsigargin, a SERCA pump blocker, diminished both the proportion of responding astrocytes and the magnitude of those responses (Fig. S2A). TGOT responses of astrocytes being dependent on extracellular calcium, we looked for functional calcium channels in CeL astrocytes. In a subpopulation of astrocytes, specific blockade of voltage gated calcium channels of the Ca_v3 family with TTA-P2 prevented the occurrence of calcium oscillations in response to TGOT, as illustrated by a decrease of area under the curve (AUC), but not of maximal amplitude (Fig. 2B). Immunohistochemistry confirmed the presence of $Ca_v3.3$ on CeL astrocytes (Fig. 2C), and further experiments using blockers of the Ca_v1 family excluded implication of those calcium channels in TGOT induced calcium transients increase (Fig. S2B). OT-R activation in astrocytes elicited complex intracellular signaling, raising the question of the identity of one of the primary effectors of G protein coupled receptors activation, the G_α subunit. We used recently described biased agonists for OT-R: atosiban, biased towards $G_{i/o}$ linked OT-R, and carbetocin, biased towards G_q ones (15, 16). Both elicited calcium transients with similar value to TGOT. Furthermore, the use of N-ethylmaleimide (NEM) (17) proved that astrocytes responding to TGOT require a functioning set of $G_{i/o}$ (Fig 2D). Conversely, interfering with G_q linked pathways using the broad spectrum protein kinases inhibitor staurosporine diminished the proportion and amplitude of TGOT responses in astrocytes

(Fig S2C). Further experiments showed that protein kinase C is not involved, but phospholipase C is, to some extent (Fig S2C). These pharmacological experiments highlight the complex intermingling of both G_i and G_q signaling pathways in OT-R induced calcium transients in astrocytes (Fig. 2E)

To address the role of astrocytes in amygdala microcircuits, we conducted *ex vivo* patch-clamp of CeM projection neurons, the functional output neurons of the CeA (1, 2). We first confirmed that repeated TGOT applications consistently induced an increase in inhibitory post synaptic currents (IPSCs) frequencies (Fig. S3A). We then asked whether astrocytes are necessary to support the oxytocinergic modulation of neuronal network. When astrocyte activity was abolished *ex vivo* using the specific gliotoxin fluorocitrate (FC), TGOT effects on CeM IPSCs frequencies were drastically reduced (Fig. 3A), a result confirmed with the use of another gliotoxin, L- α -amino adipate (Fig. S3B). The inhibitory action of FC on astrocytic TGOT induced calcium transients was confirmed through calcium imaging experiments (Fig. S3C). This indicates the necessary presence of functional astrocytes to mediate oxytocin modulatory effects. Finally, we elicited calcium transients specifically in CeL astrocytes using rAAVs mediated expression of C1V1(t/t)-mCherry driven by the GFAP promoter (Fig. 3B). Light-driven calcium transients in CeL astrocytes led to an increase in CeM neurons IPSCs frequencies (Fig. 3C), proving that astrocytes alone are sufficient to modulate CeA neuronal networks.

We next tackled the question of how astrocytes do transmit information to the neuronal network and identified the activation of neuronal *N*-methyl-D-aspartate receptor (NMDAR) as the key component of astrocyte to neuron communication. Indeed, the blockade of NMDAR or its co-agonist site, through (2R)-amino-5-phosphonopentanoate (AP5) or 5,7-dichlorokynurenic acid (DCKA), respectively, decreased TGOT evoked increase of IPSCs frequencies (Fig. 3D-E). On

contrary, the blockade of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) had no effect (Fig. 3F), neither did ifenprodil, an antagonist specific to NR2B containing NMDAR (Fig. S3D). Moreover, AP5 did not impair astrocytic responses to TGOT (Fig. S3E), confirming that the TGOT message is conveyed directly from astrocytes to neurons through neuronal NMDAR activation. To identify the molecular actor released from astrocytes onto neuronal NMDAR, we used D-amino-acid oxidase (DAAO) to selectively degrade the NMDAR co-agonist D-Serine found in astrocytes (18). TGOT had no effect after DAAO incubation, but subsequent incubation of the same cells in D-Serine containing ACSF rescued the TGOT mediated increase in IPSCs frequency (Fig. 3G).

To ask whether those *ex vivo* conclusions were also relevant *in vivo*, we tested the effect of TGOT on the increased pain sensitivity and anxiety levels in rat model of neuropathy using the spared nerve injury model. Cannulae were implanted to infuse drugs micro volumes into the CeL (Fig. 4A, B). Infusion of TGOT alone did reduce the mechanical hyperalgesia in neuropathic animals, an effect that lasted for up to 4 hours. If prior to TGOT, FC was injected in the CeL, the pain relieving effect of TGOT was abolished (Fig. 4C). Later, using a similar injection procedure when anxiety levels were assessed, we found that TGOT did relieve anxiety in neuropathic animals, an effect diminished by prior injection of FC (Fig. 4D).

Oxytocinergic regulation of neural circuits and their physiological functions is under intense scrutiny (19–21). Here we show a new mechanism of oxytocin action, which induces complex modifications of astrocytic calcium transients, leading to changes of CeA neural network outputs and their behavioral correlates. The different characteristics of astro-neuronal networks dynamics provide new means for regulation of brain functions (22–24), on a different spatio-temporal scale than neuro-neuronal networks, with a complexity yet to unravel (25). Intracellular pathways

leading to the increase in somatic calcium concentrations in astrocytes after OT-R activation are complex and involve more than one classical pathway. This might explain some reports that sole activation or inactivation of G_q signaling in astrocytes was insufficient to evoke any effect on synaptic activity (26, 27). In a larger perspective, with growing evidence showing that oxytocin dampens down inflammatory processes and modulates innate host defense mechanisms (28), our data revealing an astrocytic regulation of oxytocin-induced relief in anxiety and pain potentially offer a novel insight in the neurobiological mechanism underpinning the placebo response (29). Finally, given that oxytocin modulates emotions and behaviors through a variety of peripheral and central nervous systems actions (4, 19, 30), our study highlights a pivotal role of astrocytes in oxytocinergic neuromodulation and opens a general question about the role of astro-neuronal networks in the modulation of emotional experiences by neuropeptidergic systems.

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Conceptualization, AC; Methodology, AC, BBo, CLM, ID, JYC, PP, RS, VG; Analysis, AC, BBe, BBJ, CML, HSKB, JW; *In situ* hybridization, HSKB; Immunohistochemistry, JW, MdSG, ME; *Ex vivo* patch-clamp electrophysiology, AC, BBe, JW, MA; *Ex vivo* calcium imaging, CLM, JW; Behavior, AC, BBJ; viruses validation, MdSG, ME, VG; Spared nerve injuries, MP; Writing, AC, BBo, JYC, JW, PP, RS, VG; Supervision, AC; Project administration, AC; Funding acquisition, AC.

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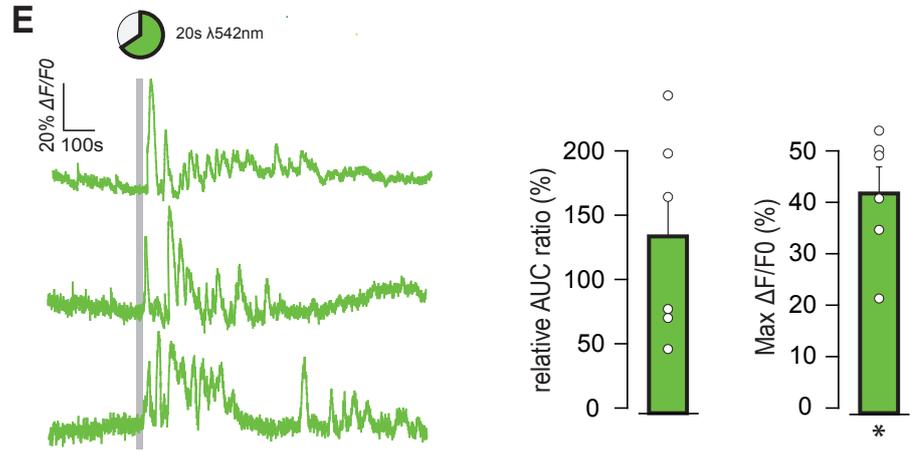
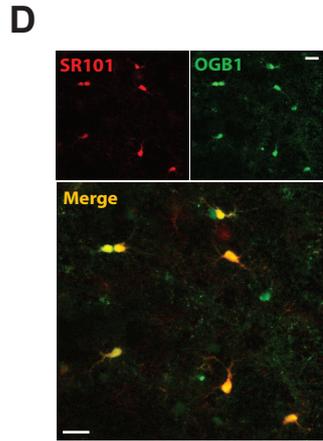
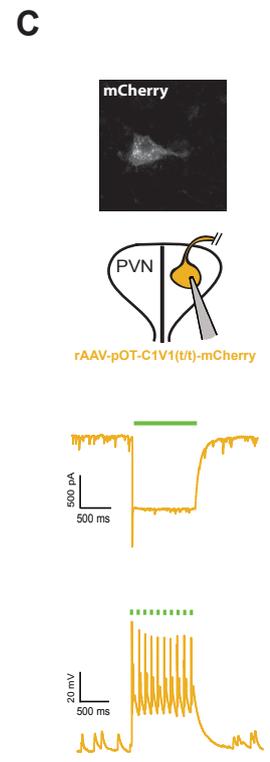
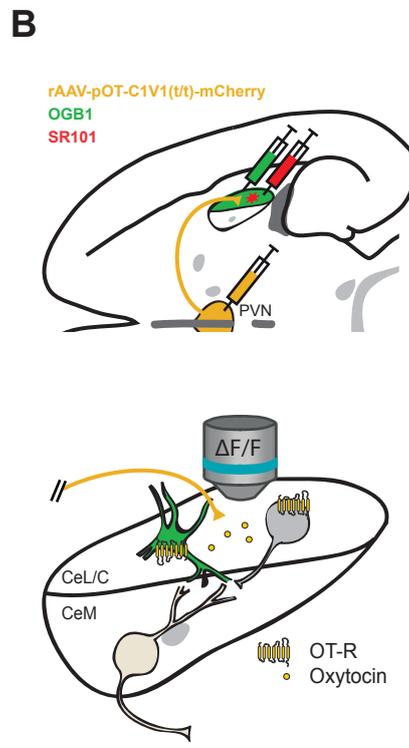
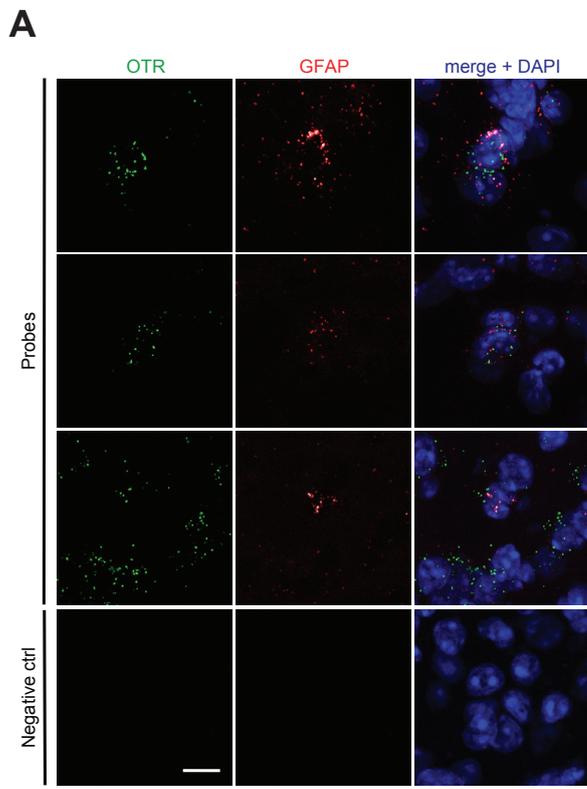


Fig. 1. CeL astrocytes express OT-R mRNA and respond to light-driven activation of oxytocinergic axons of the CeL. (A) RNAscope in situ hybridization showing GFAP (red) and OTR (green) expressing cells in CeA. Merged images include DAPI stain (blue). (Bottom) Negative control probe targeting the bacterial gene DapB. Scale bar 10 μ m. (B) Experimental scheme showing PVN nucleus, in which rAAVs were injected to induce expression of C1V1(t/t)-mCherry in oxytocinergic neurons, some of which project to CeA. On CeA slices, co-incubation of SR101 and OGB1 allows calcium imaging of astrocytes. (C) (Top) C1V1-mCherry expressing oxytocin neurons of the PVN in which (bottom) λ 542nm light exposure induced depolarizing currents, enabling precise spiking control. (D) Typical confocal image of CeL astrocytes co-labeled with SR101 and OGB1. Scale bar 20 μ m. (E) Effect of activation of oxytocinergic axons in the CeL through a 20s λ 542nm light pulse on calcium transients of CeL astrocytes. (Left) Pie charts of the proportion of responsive astrocytes and typical $\Delta F/F$ traces; (right) ratios of $\Delta F/F$ AUCs before and after light-driven activation of CeL oxytocinergic axons and maximal peak values (n slices (n_s)=6, n astrocytes (n_a)=37). Data are expressed as means across slices plus SEM. White circles indicate averages across responding astrocytes per slices. Values were statistically compared to TGOT+TTX ones found in figure 2. * P <0.05, independent samples Student's t-test.

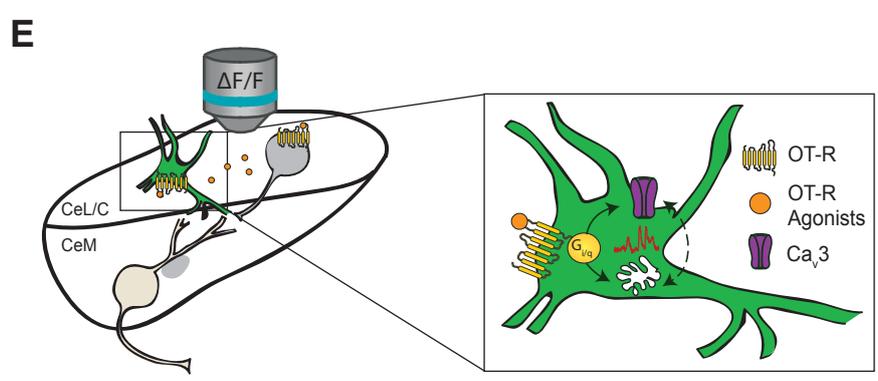
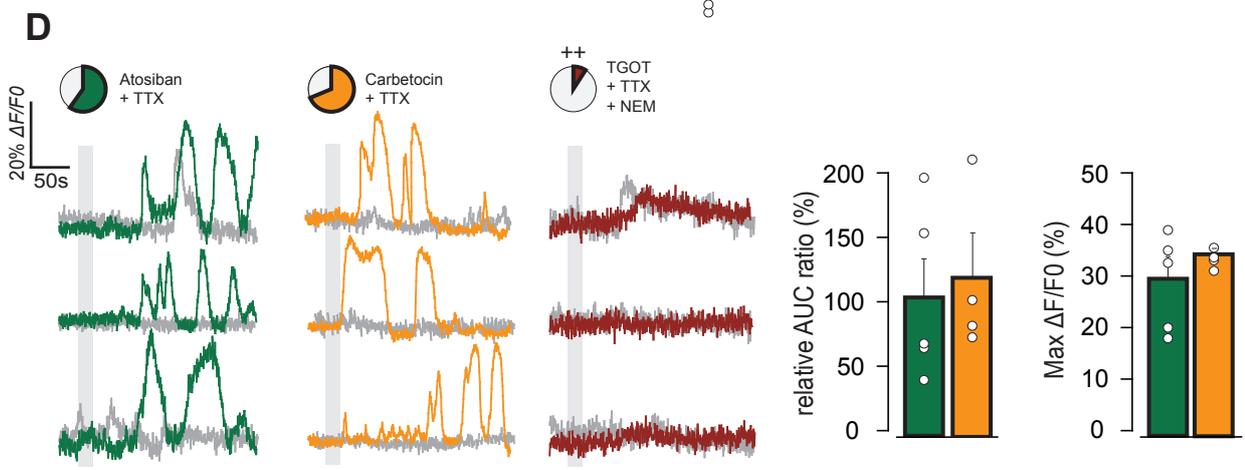
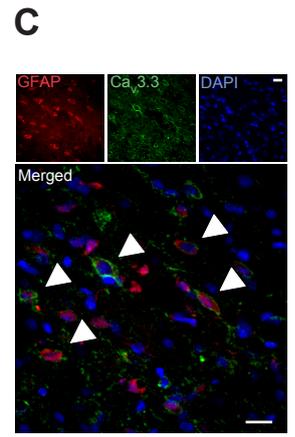
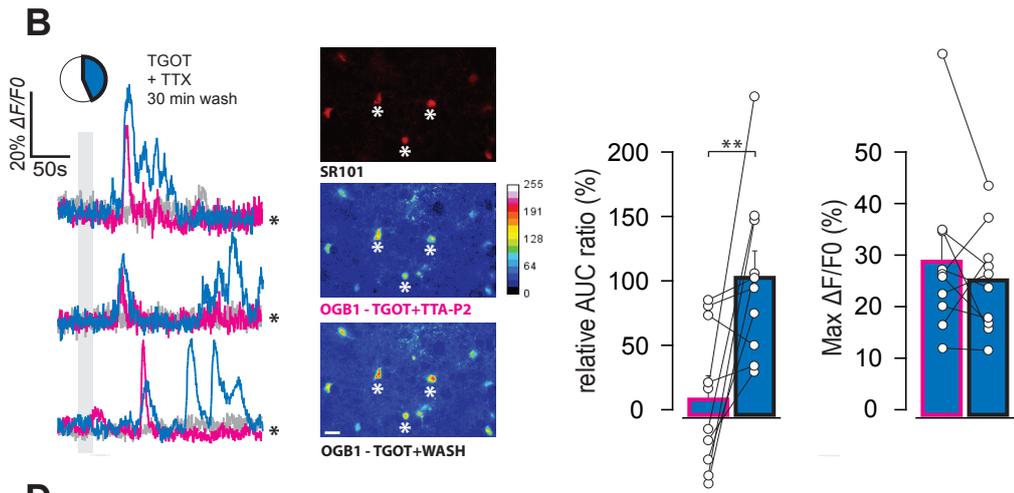
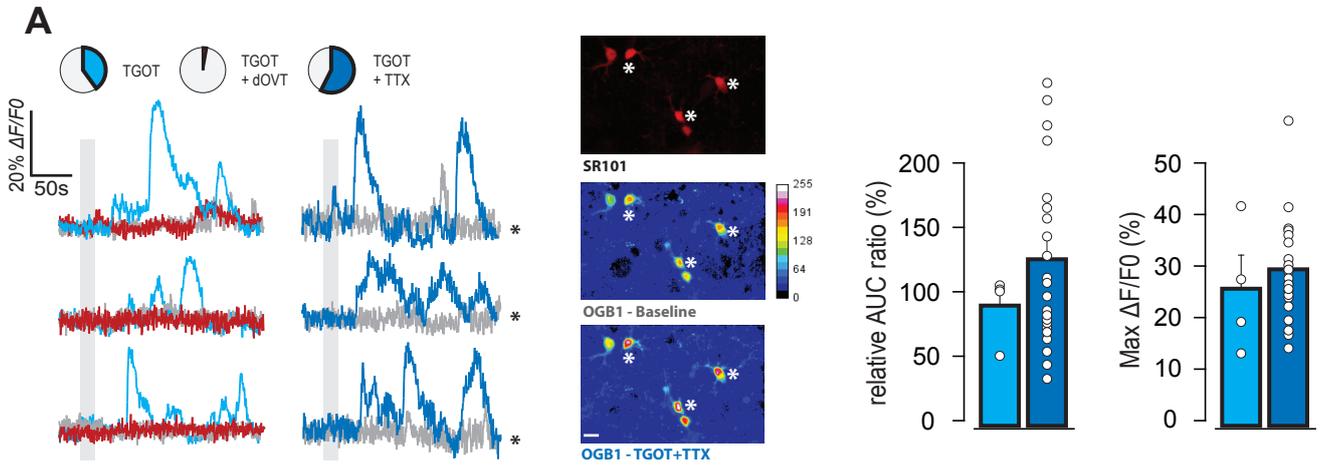


Fig. 2. OT-R activation evokes astrocytic calcium transients, independent of neuronal network activity and partly dependent on CaV₃ channels. (A to B) (Left) Pie charts of the proportion of responsive astrocytes and typical $\Delta F/F$ traces; (middle) Astrocytes identified through SR101 (red) and corresponding pseudo-color images of OGB1 fluorescence during baseline and after drug application (stacks of 50 images/25s of recording). Asterisks indicate astrocytes from which the example $\Delta F/F$ traces are shown; (Right) ratios of $\Delta F/F$ AUCs before and after drug application and maximal peak values upon exposition to: (A) TGOT alone (400nM, $n_s=4$, $n_a=39$), with dOVT (1 μ M, $n_s=3$, $n_a=34$) or TTX (1 μ M, $n_s=22$, $n_a=182$); (B) TGOT+TTA-P2, followed by a 30 min wash (1 μ M, $n_s=3$, $n_a=23$). Only astrocytes that responded after wash are represented here. White circles here represent individual astrocytes values (C) Immunohistochemistry showed Ca_v3.3 expression in GFAP positive cells of the CeL (white arrows). (D) (left) Pie charts of the proportion of responsive astrocytes and typical $\Delta F/F$ traces; (right) ratio of $\Delta F/F$ AUCs before and after drug application and maximal peak value upon exposition to: atosiban (2 μ M, $n_s=5$, $n_a=47$), carbetocin (10 μ M, $n_s=4$, $n_a=50$) or NEM+TGOT (100 μ M, $n_s=7$, $n_a=52$). (E) Scheme summarizing conclusions of previous calcium imaging experiments. Data are expressed as means across slices plus SEM, White circles indicate averages across responding astrocytes per slices (except TTA-P2, see B). All values were statistically compared to those of TGOT+TTX, $^{++}P<0.01$, independent samples Mann-Whitney U test except for TTA-P2 experiments: $^{**}P<0.01$, paired samples Student's t-test. Scale bars 20 μ m

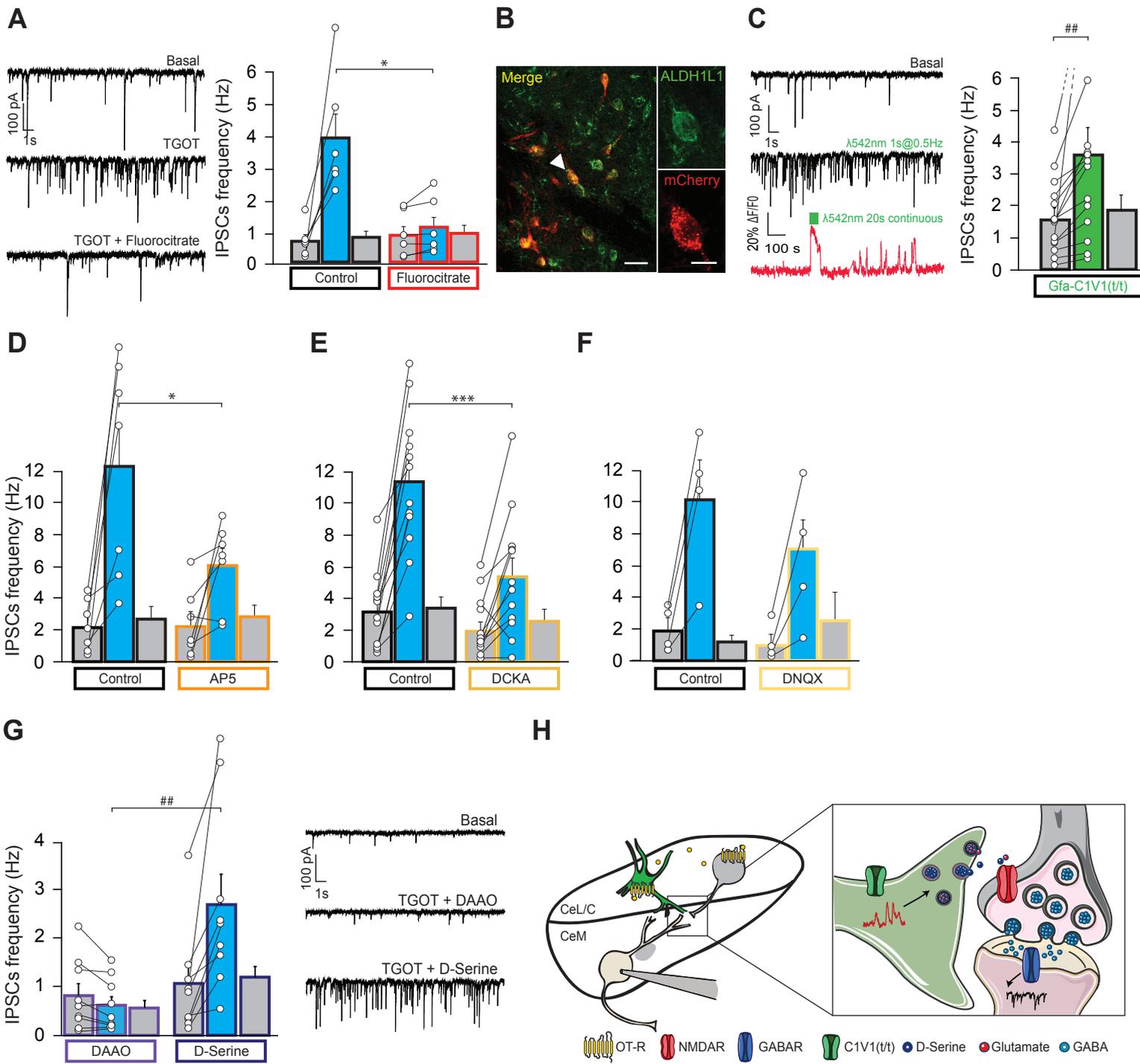


Fig. 3. Astrocytes are sufficient and necessary to the OT-R mediated modulation of CeA neuronal network activity. (A) Effect of fluorocitrate on TGOT induced increase of IPSCs frequencies (TGOT 400nM, fluorocitrate 100 μ M, 1h, n=6). (B) Immunohistochemistry for CeL cells infected with AAV-Gfa-C1V1TsmCherry with co-labeling for ALDH1L1. White arrow shows one cell expanded in insets. Scale bars 25 and 10 μ m. (C) (Top) Effect of light induced activation of C1V1 expressing astrocytes on IPSCs frequencies (n=19). (Bottom) Effect of a 20s, continuous, λ 542nm light exposure on the $\Delta F/F$ of a C1V1 expressing astrocyte. (D-E) Effect of AP5 (50 μ M, n=7) or DCKA (10 μ M, n=12) on TGOT induced increase in IPSCs frequencies. (F) DNQX (25 μ M, n=4) effect on TGOT induced increase in IPSCs frequencies. (G) Effect of DAAO (0.15U/ml, incubation time >1h30), followed by 20min incubation in D-Serine (100 μ M) on TGOT induced increase in IPSCs frequencies (n=9). (H) Scheme summarizing conclusions of previous patch-clamp and calcium imaging experiments. Data are expressed as averaged frequencies plus SEM before, during and after TGOT effect. Linked white circles represent individual cell values before and during TGOT effect. * P <0.05, *** P <0.001, paired samples Student's t-test. ## P <0.01 Wilcoxon signed rank test.

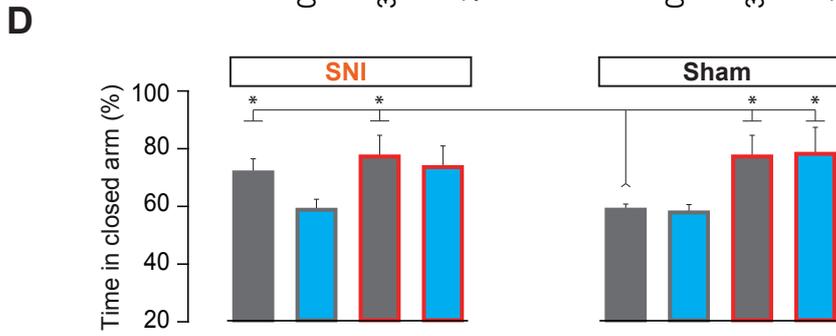
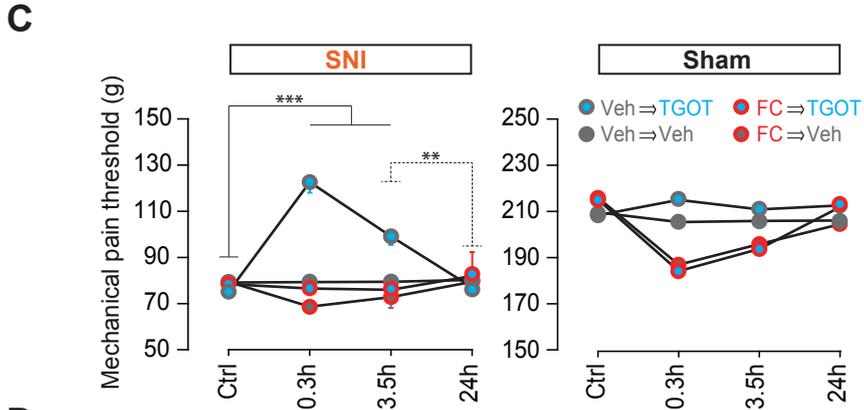
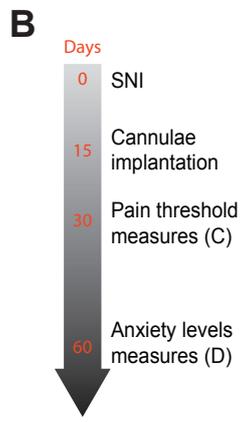
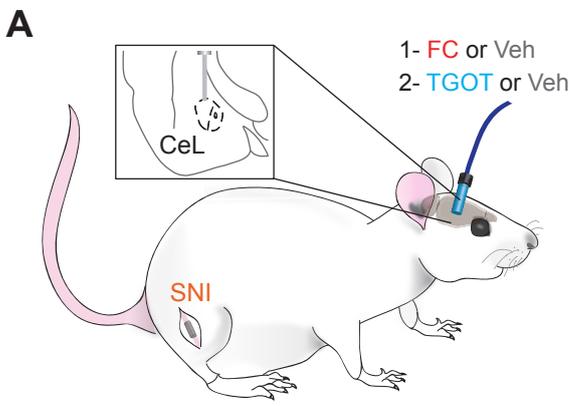


Fig. 4. CeL astrocytes are necessary for beneficial effect on pain and anxiety of OT-R activation. (A) Scheme of surgical procedures and drug injections in CeL. FC or vehicle were administered 1h before TGOT or vehicle, which were injected 20min before behavioral tests. (B) Timeline of the different procedures and behavioral tests. (C) On day 30 post SNI surgery, mechanical pain threshold was assessed on the neuropathic paw before (ctrl) and after drugs injections for sham and SNI animals. (D) On day 60 post-surgery, anxiety levels were assessed through measurements of the time spent in closed arms of the elevated plus maze after drugs injections for sham and SNI animals. n=4-12 per group. Data are expressed as averages across rats plus SEM. Pain threshold measures: ** $P < 0.01$, *** $P < 0.001$, mixed-design ANOVA followed by posthoc Bonferroni tests. Elevated plus maze: * $P < 0.05$, ANOVA on log transformed data followed by posthoc Dunnett t-tests using Sham-Vehicle-Vehicle group as control.



Supplementary Materials for

A key role for amygdala astrocytes in regulation of negative affective processing by oxytocin.

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This PDF file includes:

Materials and Methods
Figs. S1 to S4

Materials and Methods

Animals

Animals were housed under standard conditions with food and water available *ad libitum* and maintained on a 12-hour light/dark cycle and all experiments were conducted in accordance with EU rules and approbation from French Ministry of Research (01597.05). For *ex vivo* and *in vivo* experiments, male (if not stated otherwise) wistar rats were used. *Ex vivo* experiments used animals between 18 and 25 days old, except in experiments where rAAVs were injected, in which case animals were between 2 to 6 months old. *In vivo* experiments used animals of 2 months old at the moment of the first surgery.

Cloning and production of rAAV Vectors:

To construct the OTp-C1V1(t/t)-TS-mCherry AAV vector we used previously cloned OTp-DIO-GFP-WRE plasmid (4), equipped with characterized 2.6 kb OT promoter (3). In this plasmid the DIO-GFP sequence was replaced by C1V1 (t/t)-TS-mCherry from the pAAV CaMKIIa-C1V1(t/t)-TS-mCherry (Addgene, plasmid #35500). To generate GfaP-C1V1(t/t)-TS-mCherry AAV vector we replaced CamKIIa promoter from the pAAV CaMKIIa-C1V1(t/t)-TS-mCherry by the Gfa promoter from the pZac2.1 gfaABC1D-tdTomato (Addgene, plasmid: 44332). The cell type specificity of the rAAV carrying the Gfa promoter was recently confirmed (31).

Injections of rAAV viruses

Stereotaxic surgery was performed under deep ketamine-xylazine anesthesia, using KOPF (model 955) stereotaxic system. 3 female Wistar (180-200g, age 8 weeks) were injected with ca. 200 nl of rAAV serotype 1/2 (AAV-Gfa-C1V1TSmCherry, GFAP promoter containing plasmid obtained from Addgene) unilaterally at the coordinates corresponding to CeA: rostro-caudal: -2,7mm, medio-lateral: 4,2mm, dorso-ventral: 8mm (From Paxinos and Watson Atlas).

Horizontal and coronal slices preparation

In all cases, animals were anaesthetized using ketamine (Imalgene 90 mg/kg) xylazine (Rompun, 10 mg/kg) administered intra-peritoneally. Transcardial perfusion was then performed using one of the following artificial cerebro-spinal fluids (ACSFs) dissection solutions. For rats between 18 and 25 days old, an ice-cold sucrose based dissection ACSF was used containing (in mM): Sucrose (170), KCl (2.5), NaH₂PO₄ (1.25), NaHCO₃ (15), MgSO₄ (10), CaCl₂ (0.5), HEPES (20), D-Glucose (20), L-ascorbic acid (5), Thiourea (2), Sodium pyruvate (3), N-acetyl-L-cysteine (5), Kynurenic acid (2). For rats between 2 to 6 months old, an ice-cold NMDG based ACSF was used containing (in mM): NMDG (93), KCl (2.5), NaH₂PO₄ (1.25), NaHCO₃ (30), MgSO₄ (10), CaCl₂ (0.5), HEPES (20), D-Glucose (25), L-ascorbic acid (5), Thiourea (2), Sodium pyruvate (3), N-acetyl-L-cysteine (10), Kynurenic acid (2). In both cases, pH were adjusted to 7.4 using either NaOH or HCl, this after bubbling in 95% O₂-5% CO₂ gas, bubbling which was

maintained throughout the duration of use of the various ACSFs. Those ACSFs formulae were based on the work of (32). Following decapitation, brain was swiftly removed in the same ice-cold dissections ACSFs as for transcardial perfusion, and 350 μm thick horizontal slices containing the CeA obtained using a Leica VT1000s vibratome. For experiments in Fig. 1C, coronal slices of the same thickness containing the PVN were used. Upon slicing, brain slices were hemisected and placed, for 1 hour minimum before any experiments were conducted, in a room tempered holding chamber, containing normal ACSFs. For slices of 2 to 6 month old rats, slices were first let for 10 minutes in 35°C NMDG ACSF before placing them in the holding chamber at room temperature. Normal ACSF, also used during experiments, is composed of (in mM): NaCl (124), KCl (2.5), NaH_2PO_4 (1.25), NaHCO_3 (26), MgSO_4 (2), CaCl_2 (2), D-Glucose (15), adjusted for pH values of 7.4 with HCL or NaOH and continuously bubbled in 95% O_2 -5% CO_2 gas. All ACSFs were checked for osmolarity and kept for values between 305-310 mOsm/L. In experiment using 0 Ca^{2+} ACSF, calcium was replaced by equimolar concentration of EGTA. In electrophysiology or calcium imaging experiments, slices were transferred from holding chamber to an immersion recording chamber and superfused at a rate 2 mL/min with normal ACSFs unless indicated otherwise.

Calcium imaging and astrocytes identification

To identify astrocytes, SR101 (1 μM) was added to ACSF in a culture well and slices incubated for 30 minutes. The specificity of SR101 to astrocytes of the CeL was verified through patch-clamp experiments, which results can be found in Fig. S1. After SR101 loading, the synthetic calcium indicator OGB1 was bulk loaded following an adapted version of the method described previously (33) reaching final concentrations of 0.0025% (~20 μM) for OGB1, 0.002% Cremophor EL, 0.01% Pluronic F- 127 and 0.5% DMSO in ACSF, for 50 to 60 minutes at 35°C. Upon incubation time, slices were washed in ACSF for at least an hour before any recordings were performed. Astrocytes recorded for this study were then those co-labeled for SR101 and OGB1, as shown in the picture in Fig. 1D. Spinning disk confocal microscope used to perform astrocyte calcium imaging was composed of a Zeiss Axio examiner microscope with a 40x water immersion objective (numerical aperture of 1.0), mounted with a X-Light Confocal unit – CRESTOPT spinning disk. Images were acquired at 2Hz with either a Rolera em-c² emCCD or an optiMOS sCMOS camera (Qimaging, BC, Canada). Cells within a confocal plane were illuminated for 100 to 150 ms for each wavelength (SR101: 575 nm, OGB1: 475 nm) using a Spectra 7 LUMENCOR. The different hardware elements were synchronized through the MetaFluor software which was also used for online and offline quantitative fluorescence analysis. Astrocytic calcium levels were measured in hand drawn ROIs comprising the cell body plus, when visible, proximal processes, without distinctions. $[\text{Ca}^{2+}]_i$ variation were estimated as changes in fluorescence signals over the baseline ($\Delta\text{F}/\text{F}$). Baseline was established for each ROI as the average fluorescence over all pictures. To take into account micro-movements of the specimen on long duration recordings, the $\Delta\text{F}/\text{F}$ values were also calculated for SR101 and subsequently subtracted to the ones of OGB1. Bleaching was corrected using a linear regression on the overall $\Delta\text{F}/\text{F}$ trace for each astrocyte, which values were then subtracted to the $\Delta\text{F}/\text{F}$. Upon extraction of data, calculations and corrections of $\Delta\text{F}/\text{F}$ for each astrocyte, the area under

the curve before and after drug application (or light pulse for optogenetics experiments) was calculated and proportionally corrected relative to the different recording durations. An astrocyte was considered as being responsive if the relative ratio of AUCs after drug/light application over baseline was 20% greater than in baseline conditions. The relative AUCs ratios values were used for quantitative analysis and called “relative AUC ratio”. Maximal peak reached after drug/light application was also measured in responsive cells and used in quantitative analysis. Data were averaged across astrocytes per slices, which were used as the statistical unit. Image J software was also used on SR101 / OGB1 pictures to produce illustrative pictures such as the one in fig. 2A-C. All calcium imaging experiments were conducted at room temperature.

Electrophysiology

Whole cell patch-clamp recordings of CeM neurons and CeL astrocytes were visually guided by infrared oblique light visualization of neurons, completed by SR101 fluorescence for astrocytes. Whole-cell voltage clamp recordings were obtained with an Axon MultiClamp 700B amplifier coupled to a Digidata 1440A Digitizer (Molecular Devices, CA, USA). Borosilicate glass electrodes ($R = 3.5 - 7 \text{ M}\Omega$) with inner filament (OD 1.5 mm, ID 0.86 mm; Sutter Instrument, CA USA) were pulled using a horizontal flaming/brown micropipette puller (P97; Sutter Instrument, CA, USA). Pipettes were filled with an intracellular solution containing (in mM): KCl (150), HEPES (10), MgCl_2 (4), CaCl_2 (0.1), BAPTA (0.1), ATP Na salt (2), GTP Na salt (0.3). For patch-clamp of astrocytes, the intracellular solution was composed of (in mM) KMeSO_4 (135), NaCl (5), MgCl_2 (2), HEPES (10), ATP Na salt (2), GTP Na salt (0.3). For both solutions, pH was adjusted to 7.3 with KOH and osmolarity checked to be between 290-295 mOsm/L, adjusted with sucrose if needed. All cells were held at a membrane potential of -70 mV, astrocytes -80 mV. Series capacitances and resistances were compensated electronically throughout the experiments using the main amplifier. Recordings were filtered at 2 kHz, digitized at 40 kHz and stored with the pClamp 10 software suite (Molecular Devices; CA, USA). Analysis of patch-clamp data were performed using Clampfit 10.6 (Molecular Devices; CA, USA) and Mini analysis 6 software (Synaptosoft, NJ, USA) in a semi-automated fashion (automatic detection of events with chosen parameters followed by visual inspection). Average events frequencies per cells were calculated on 20s windows, chosen for TGOT or light stimulation at maximal effect, as determined by the visually identified maximal slope of the cumulative plot of the number of events. Baseline and recovery frequencies were measured at the beginning and end of each recording. All patch-clamp experiments were conducted at room temperature.

Optogenetics

We used C1V1(t/t) , a ChR1/VChR1 chimera with the combined mutations E122T/E162T (for more details, see (4)). Optogenetic green light stimulation of C1V1(t/t) was performed using either the Spectra 7 LUMENCOR ($\lambda 542 \text{ nm}$) or light source X-Cite® 110LED from Excelitas Technologies through a Cy3 filter, controlled via MetaFluor or Clampex driven TTL pulse, respectively.

Spared nerve injury procedure

Animals were randomly separated in two groups to undergo SNI or sham procedure. Animals were anaesthetized using isoflurane 1.5–2.5%. Incision was made at mid-thigh level using the femur as a landmark and a section was made through the biceps femoris. The three peripheral branches (sural, common peroneal and tibial nerves) of the sciatic nerve were exposed. Both tibial and common peroneal nerves were ligated using a 5.0 silk suture and transected. The sural nerve was carefully preserved by avoiding any nerve stretch or nerve contact (34). For animals undergoing sham surgery, same procedure was performed but the nerves remained untouched. Animals were routinely observed daily for 7 days after surgery and daily tested by the experimenter (Fig. S4). Besides observing weight, social and individual behavior, the operated hindpaw was examined for signs of injury or autotomy. In case of autotomy or suffering of the animal, the animal was euthanized in respect of the ethical recommendations of the EU. No analgesia is provided after the surgery in order to avoid interference with chronic pain mechanisms and this is in accordance with our veterinary authorization. Suffering is minimized by careful handling and increased bedding.

Cannulae implantation

Animals were bilaterally implanted with guide cannulae for direct intra-central amygdala infusions. As guide cannulae we used C313G/Spc guide metallic cannulae cut 5.8 mm below the pedestal. For this purpose, animals were deeply anesthetized with 5% isoflurane in pure oxygen and their heads were fixed in a stereotaxic frame. The skull was exposed and two holes were drilled according to coordinates that were adapted from a rat brain atlas (2.3 mm rostral-caudal; 4 mm lateral; 7.5 mm dorso-ventral relative to bregma) by comparing the typical bregma-lambda distance (9 mm) with the one measured in the experimental animal. Two screws were fixed to the caudal part of the skull in order to have an anchor point for the dental cement. The acrylic dental cement was finally used to fix the cannulae and the skin was sutured. In case of long lasting experiment (neuropathy-induced anxiety) with a cannulae implantation at distance of the behavioral assay (> 4 weeks) the cannulae were sometime lost and animals excluded from testing.

Drugs infusions through cannulae

Bilateral injections of 0.5 μ l containing either vehicle (NaCl 0.9%), or oxytocin agonist TGOT (1 μ M) or fluorocitrate (0.1 μ M) dissolved in NaCl 0.9%. For this procedure two injectors (cut to fit 5.8 mm guide cannulae protruding 2 to 2.5 mm beyond the lower end of the cannulae in older animals and 1.8 mm in 3-4 week old rats) were bilaterally lowered into the guide cannulae, connected via polyten tubing to two Hamilton syringes that were placed in an infusion pump and 0.5 μ l of liquid was injected in each hemisphere over a 2 minute period of time. After the injection procedure, the injectors were kept in place for an additional minute in order to allow a complete diffusion of liquid throughout the tissue. Rats were subsequently left in the home cage for 15 minutes to recover from the stress of the injection for and then handled for mechanical threshold or elevated plus maze assessment.

Mechanical sensitivity assessment

In all experiments, we used a calibrated forceps (Bioseb, Chaville, France) previously developed in our laboratory to test the animal mechanical sensitivity (35). Briefly, the habituated rat was loosely restrained with a towel masking the eyes in order to limit stress by environmental stimulations. The tips of the forceps were placed at each side of the paw and a graduate force is applied. The pressure producing a withdrawal of the paw, or in some rare cases vocalization, corresponded to the nociceptive threshold value. This manipulation was performed three times for each hind paw and the values were averaged.

Elevated plus maze experiment

Following protocol from (36), the arena is composed of four arms, two open (without walls) and two closed (with walls, 30 cm high). The arms are 10 cm wide, 50 cm long and elevated 50 cm off the ground. Two intensity adjustable up to 50 watts lamps positioned on the top of the maze uniformly illuminate it. Animals were video tracked using a video-tracking system (Ethovision Pro 3.16 Noldus, Wageningen, Netherlands). To avoid interaction and noise with the animal, the operator and the computer were physically separated from the maze by a black curtain and the test session occurred in a separated room from the housing room. After each trial, the maze was cleaned with 70% ethanol and dry with towel paper. Twenty minutes after intracerebral injections, animal was let to the center of the plus maze facing the open arm opposite to where the experimenter is and was able to freely explore the entire apparatus for six minutes. The total time and the time spend in closed and open arms were recorded in seconds and the percentage of time spent in closed arms was calculated as a measure of anxiety. Rats falling from the apparatus during the test, or freezing more than 50% of the total time were removed from the analysis.

Immunohistochemistry

Ca_v3.3: Immunohistochemistry for Ca_v3.3 was performed on cryostat-cut 20 μm in width horizontal sections prepared from fresh-frozen 20 days old rat brains, previously transcardially perfused with 4% paraformaldehyde PBS, after anesthesia. Slices were washed in PBS (6 times 5 min) then blocked 1 hour at room temperature using PBS containing 1% bovine serum albumin and a 10% mixture of normal serum from the species secondary antibodies were raised in. Primary antibodies were then added in the same medium and incubated overnight at 4°C. The following antibodies were used: Chicken raised antibody for GFAP (1:500) (ref: ab4674 from abcam), Rabbit raised antibody for Ca_v3.3 (1:200) (ref: ACC-009, Alomone).

The day after, upon washes of primary antibodies solutions, secondary antibodies were added for 1 hour at room temperature, using Alexa 555 against chicken (1:1000) and Alexa 488 against rabbit (1:500) (purchased from abcam), then washed (6 times 5 minutes). DAPI (1:2000) was added on the 5 out of 6 washes. Slices were then mounted using aqua-polymount (Polysciences, Inc) and pictures taken using the confocal spinning disk microscope (cf. Calcium imaging part).

AAV-Gfa-CIVITSmCherry specificity: After 3 weeks of virus expression in the brain, the rats were transcardially perfused with 4% paraformaldehyde solution. Tissue blocks, containing CeA were dissected from the fixed brain and Vibratome-cut into 50 µm thick free-floating sections. After several rinse steps sampled sections were blocked with 5% NGS in PBS and incubated for 48 h at 4°C with polyclonal rabbit anti-ALDH1L1 antibody (1:500, Abcam) in 1% Triton-PBS buffer, containing 0,1 % NGS. Appropriate secondary antibody (AlexaFluor488 conjugated goat anti-rabbit (1:1000, LifeTechnologies) was used for further antigene detection. Intrinsic mCherry fluorescence of virus- expressing cells was strong enough to detect them in the tissue without any additional antibody enhancement. The immunolabeled sections were mounted onto Superfrost slides, cover-slipped with Moviol, analyzed and documented using LEICA SP5 confocal microscope.

In situ hybridization

In situ hybridization (ISH) in fig. 1A was performed on 25-µm cryostat-cut coronal sections prepared from fresh-frozen mouse brain (male C57BL/6J, P22). After extraction, brains were immediately frozen in Tissue-Tek O.C.T. compound and stored at -80 degrees Celsius. ISH was performed according to the manufacturer's instructions (Advanced Cell Diagnostics) for Fresh Frozen RNAscope Multiplex Fluorescent Assay. Treatment of amygdala containing sections were adjusted with the 3-plex negative control and then coexpression of OTR and GFAP examined using ACD designed target probes as well as the nuclear stain DAPI. Single plan images were collected with an upright laser scanning microscope (LSM-710, Carl Zeiss) using a 40x-objective with keeping acquisition parameters constant between control and probe treated sections.

Statistical analysis

All parametrical statistical tests presented in figures captions were performed following correct verification of the assumptions on the distribution of data, and if not either transformations of data or non-parametric tests were used. Tests were performed using either SPSS 23 (IBM) or statview 5 (SAS institute Inc.).

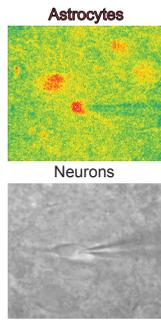
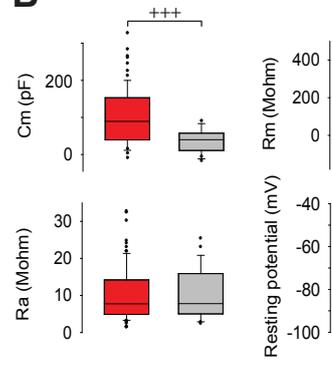
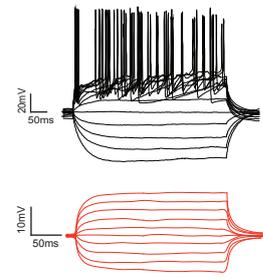
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Fig. S1. SR101 is specific to astrocytes (A) Pseudo-color pictures of an SR101 positive cell identified as an astrocyte compare to neurons identified under oblique infrared light. (B) Electrophysiological properties of patched SR101⁺ (red, n=84) and SR101⁻ cells (grey, n=21). (C) Responses to 20pA current steps of SR101⁺ (red) and SR101⁻ cells (black).

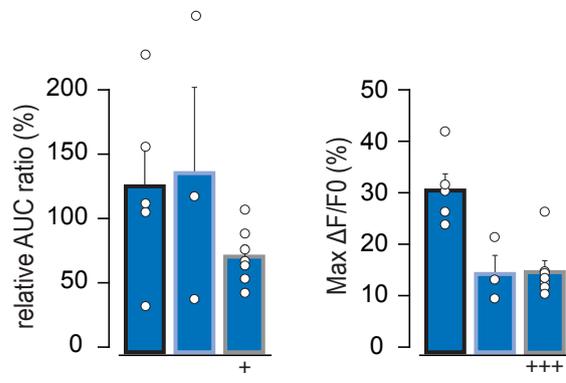
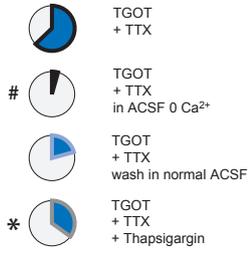
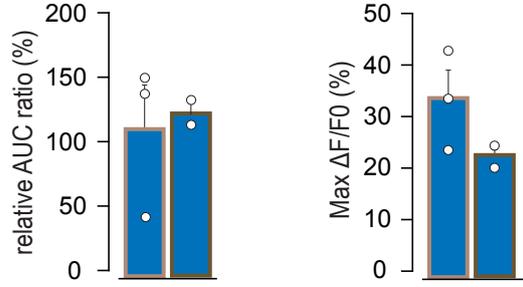
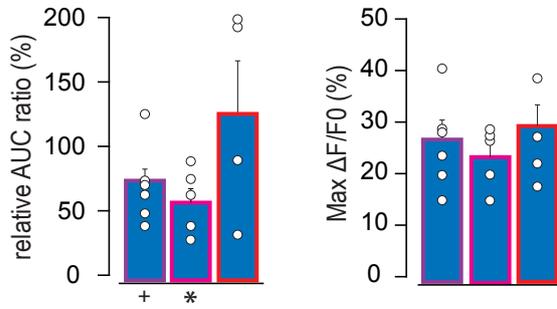
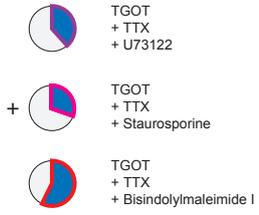
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Fig. S2. Intracellular signaling involved in astrocytes responses to OT-R activation. (A to B) (Left) Pie charts of the proportion of TGOT+TTX responsive astrocytes and their (right) ratios of $\Delta F/F$ AUCs before and after drug application and maximal peak values upon exposition to: (A) removal of extracellular calcium ($n_s=6$, $n_a=55$. AUCs and Max values not shown, only 2/55 astrocytes still classified as responsive) followed by normal ACSF ($n_s=3$, $n_a=29$); thapsigargin ($1\mu\text{M}$, $n_s=7$, $n_a=58$); (B) verapamine ($50\mu\text{M}$, $n_s=3$, $n_a=28$) or nimodipine ($10\mu\text{M}$, $n_s=2$, $n_a=20$). (C) U73122 ($10\mu\text{M}$, $n_s=6$, $n_a=45$), staurosporine ($1\mu\text{M}$, $n_s=5$, $n_a=43$) or bisindolylmaleimide-I ($1\mu\text{M}$, $n_s=4$ slices, $n_a=30$ astrocytes); Data are expressed as mean across slices plus SEM. White circles indicate averages across responding astrocytes per slices. * $P<0.05$, ** $P<0.01$ independent samples Students's t-test, + $P<0.05$, +++ $P<0.001$ independent samples Mann-Whitney U test, # $P<0.05$ Wilcoxon signed rank test.

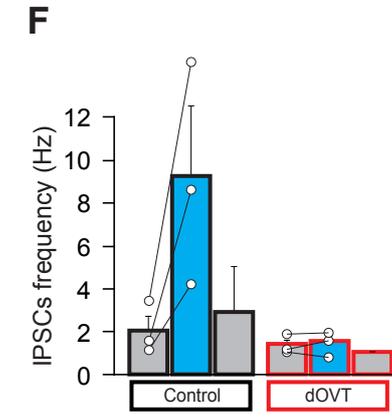
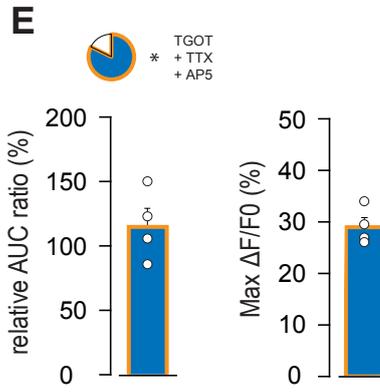
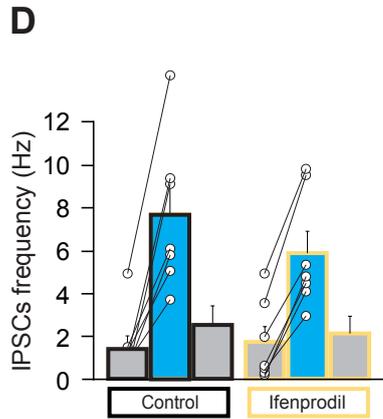
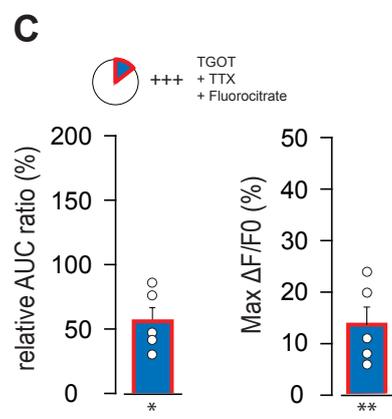
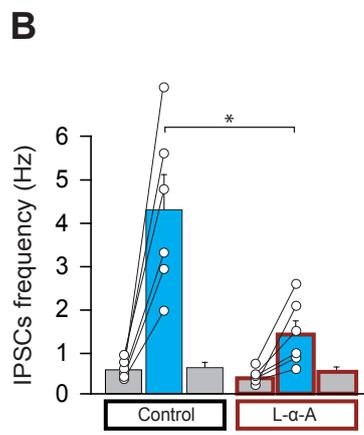
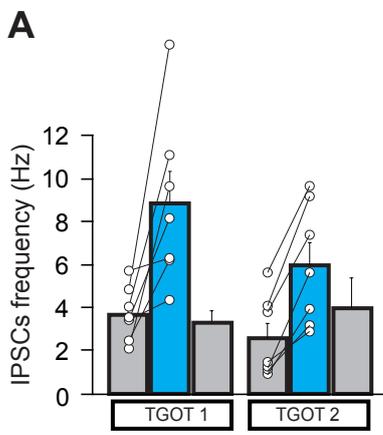


Fig. S3. TGOT effect on CeM neurons is repeatable, specific to OT-R and does need astrocytes as intermediary through non NR2B containing neuronal NMDA-R signaling. (A) Effect of double (20min apart) application of TGOT on IPSCs frequencies (n=7). (B) L- α -aminoadipate (100 μ M, 40min, n=6) effect on TGOT induced increase in IPSCs frequencies. (C) (Top) pie chart of the proportion of TGOT+TTX responsive astrocytes and (bottom) ratio of $\Delta F/F$ AUCs before and after drug application and maximal peak value upon exposition to fluorocitrate (200 μ M for 30min, n_s=8, n_a=81). (D) ifenprodil (3 μ M, n=7) effect on TGOT induced increase in IPSCs frequencies. (E) (Top) pie chart of the proportion of TGOT+TTX responsive astrocytes and (bottom) ratio of $\Delta F/F$ AUCs before and after drug application and maximal peak value upon exposition to AP5 (50 μ M, n_s=4, n_a=35). (F) dOVT (1 μ M, n=3) prevents the effect of TGOT on IPSCs frequencies. Patch-clamp experiments: data expressed as averaged frequencies plus SEM before, during and after TGOT effect. Linked white circles: individual cell values before and during TGOT effect. Calcium imaging experiments: data expressed as means across slices plus SEM. White circles: averages across responding astrocytes per slices. * P <0.05, ** P <0.01 independent samples (calcium-imaging) or paired (patch-clamp) Students's t-test, +++ P <0.001 independent samples Mann-Whitney U test.

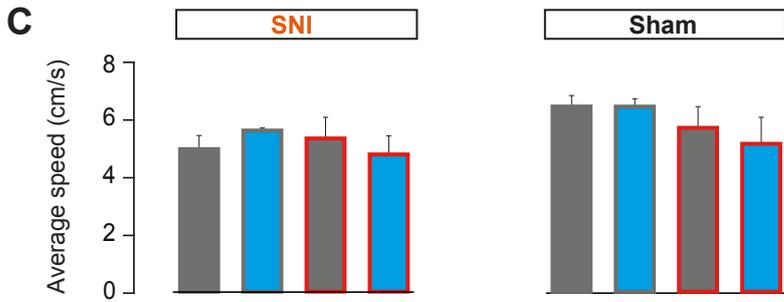
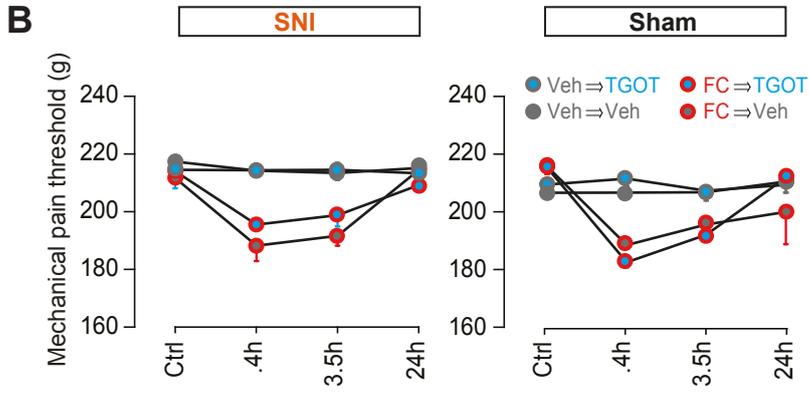
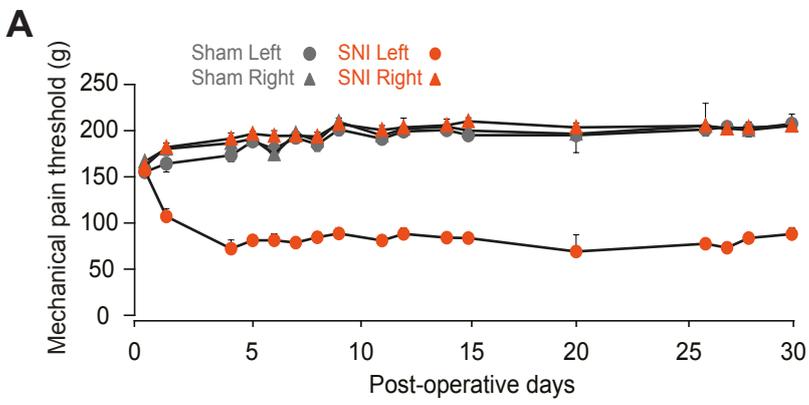


Fig. S4. SNI neuropathic pain model is specific to the injured paw and doesn't impair locomotion speed. (A) 30 days' time course of mechanical pain threshold evolution across the different groups (B) On day 30 post-surgery, mechanical pain threshold was also assessed on the non-injured paw. Different combination of FC or vehicle followed by TGOT or vehicle were administered in the CeL and mechanical pain threshold assessed again at different time points for sham and SNI animals. (C) On day 60 post-surgery, locomotion was assessed through measurements of the average speed during the time of the elevated plus maze experiment, after administration of different combination of FC or vehicle followed by TGOT or vehicle. n=4-12 per group. Data are expressed as averaged frequencies plus SEM. Pain threshold measures: Mixed-design ANOVA followed by posthoc Bonferroni tests. Elevated plus maze: Independent samples Kruskal-Wallis test.

K. DISCUSSION

During this thesis, I participated in studies unraveling new mechanisms of OT action in the CNS. As often, those findings open up to more questions and give several perspectives. I will divide this discussion following the two main studies I exposed in this manuscript.

1. On the new role of a sub-population of parvocellular OT neurons

In this study, we unveiled the role of a small population of ~30 parvocellular neurons in the modulation of pain. This population is able to produce a measurable analgesia in rats via a dual mechanism: i) a direct action on the pain pathways in the SC and ii) through stimulation of the endocrine release of OT by magnocellular OT neurons. This dual action is interesting in its temporality, in the sense that it provokes a fast anti-nociceptive action through axonal projection in SC, followed by a long-lasting anti-nociception, through a systemic action of OT. The direct action of parvocellular OT neurons on spinal pain processing has been suspected for long, yet it had not been proven *per se*, and had not been precisely dissected. For instance, it was surprising to find that those particular parvocellular neurons project mainly to deep layers of the spinal cords, whereas Jójárt (and others) described many OT fibers projecting to the superficial layers of the dorsal horn (Jójárt et al., 2009). It would then be interesting to use the same techniques to decipher the cell-body location(s) and the circuits regulating those superficial layers projecting OT neurons.

The second role of those few parvocellular OT neurons, and maybe the most important in term of physiological mechanism, is to coordinate the activity of neurosecretory magnocellular neurons of the SON. This is the first demonstration, to my knowledge, of a functionally proven synaptic communication between two OT expressing neurons of different nuclei, despite speculations about such connections coming from early anatomical and functional data (Lambert et al., 1993; Silverman et al., 1981). We demonstrated that parvocellular neurons of the OT nuclei, on top of their known function on autonomic responses notably, are regulators of magnocellular neurons activity. By making a synaptic link between the PVN and SON nuclei, those neurons may allow for a coordinated OTergic modulation of several forebrain areas (where magnocellular OT neurons project) with the spinal networks and the peripheral systems, nervous or not. In this hypothesis, parvocellular neurons would then act as true coordinators of the OT system and its “body wide” functions. Therefore future studies might unravel new specialized subgroups of parvocellular OT neurons, governing one or several of the many functions of OT cells. In such studies, it would be interesting to map the targets, but also the specific inputs of those sub-populations of neurons, among which the one we unraveled. Coming back on the subject of inter-nuclei OT neurons connectivity, it would be interesting to test

the plasticity of such PVN to SON connections during the drastic changes of OT nuclei activity observed in several physiological challenges. For instance in the introduction we evoked the synchronization of OT neurons bursts during lactation and its proposed mechanisms (see 3.2.2). What might happen to the parvocellular (sub)population(s) in this state? One could make an educated guess by speculating that they might drive the synchronization of firing between nuclei, in addition to the other mechanisms already identified. This idea might also be supported by the fact that we observed no inter-nuclei connections between AVP cells. Yet, there is no known burst synchronization between AVP cells, not at the same level than OT cells during lactation. Taken together, those two observations might imply that some parvocellular OT neurons are mediators of the synchronization between OT neurons of different OT nuclei.

We identified an average of only 30 parvocellular neurons supporting a measurable analgesia. One may criticize that such a small population supporting an important function is not reliable in terms of resilience of neural circuits. What would happen if those cells are impaired? One explanation might be that at any given time, an ensemble of only 30 neurons is implicated in the coordination of spinal and peripheral anti-nociception by OT, but if those cells are silenced or destroyed, another ensemble of cells could replace them, in a form of resilience of the function. One could test that hypothesis by using the same viral tools to specifically destroy or block synaptic activity of those cells via targeted expression of the tetanus toxin light chain (Martin et al., 2002) or suicide genes, or through inhibition of their electrical activity through DREADDs or other methods (White et al., 2001). In the same line of discussion about the role of a few numbers of neurons in the regulation of a given function, it would be of importance to know if this is a general mechanism in OT nuclei. The recruitment of virtually all OT cells during lactation might be the exception, and happens only because it requires the simultaneous release of massive amounts of OT in the bloodstream by magnocellular neurons. But OT also regulates very specific behaviors, such as pups retrieval by dams (Marlin et al., 2015). One could use the viral techniques developed in this study to identify the role of other subpopulations of OT neurons in the regulation of a given function, and manipulate them through expression of various proteins of interest. The high level of specialization of the population we identified here is highlighted by the fact that we could not see any effect of the stimulation of those 30 neurons on the pain thresholds of neuropathic animals using the cuff model (see Yalcin et al., 2014). This implies that those OT cells are able to discriminate between several forms of pain, and specifically address them. The observation of OT cells responding only to an inflammatory pain might be explained by the fact that OT has well characterized anti-inflammatory effects and more generally modulate immune responses (Li et al., 2017; Wang et al., 2015b). Therefore, its endocrine secretion upon injury would also be required to resolve the injury on top of dampening the evoked pain. In the second study

presented here, we highlight the role of OT in reducing the allodynia evoked by injury to the sciatic nerve, through local action in the CeA, an analgesic effect also present in non-neuropathic animals for mechanical and “acute heat” pain (Han and Yu, 2009). This highlights again the existence of specialized OT circuits for a given pain modality.

1.1. Perspectives

These results push the idea that OT neurons must be able to discriminate between different sources of nociceptive signals, and respond appropriately through specialized OT circuits targeting other specialized neural circuits and peripheral targets. It would be advantageous to isolate genetical/molecular markers of those specialized population, which would allow their specific targeting through genetic or molecular tools, enabling their easy manipulation. Doing so, and if such circuits are also present in the human hypothalamus, one might imagine new ways to attain targeted reduction of specific types of pain, with fewer side effects than current treatments. In conclusion, those findings adds another layer of complexity to the OT neural circuits, and opens up the way for further dissection of OT neural pathways.

2. On the role of astrocytes in OT modulation of CeA circuits

In this study, we propose that astrocytes are a necessary component of the modulatory effects of OT on the CeA microcircuits and their behavioral correlates. We demonstrated on more than 1200 astrocytes that around 50% of the astroglial cells of the CeL are equipped to respond actively and independently of neuronal network activity to OTR activation. The calcium responses of astrocytes could be blocked by gliotoxins infusion, and under such conditions, TGOT had no visible effect on the neuronal activity in CeM. The other way around, we showed that the selective activation of astrocytes could mimic the effect of TGOT on the synaptic activity in the CeA, therefore demonstrating that astrocytes are both necessary and sufficient to convey the neuromodulatory effects of OT, at least *ex vivo*. We confirmed the active implication of astrocytes to OT actions in CeA by behavioral experiments, in which the inactivation of astrocytes abolished the dampening effect of TGOT on pain and anxiety levels. In addition, we showed that TGOT evoked astrocytic calcium transients rely both on intra and extracellular calcium sources, and partially uncovered the intracellular mediators of OT effect in astrocytes. Furthermore, we found that the signaling pathways of OTR in those cells involve at least two different G_{α} protein coupling. Finally, we proposed that the OT message is conveyed from astrocytes to neurons through the release of D-serine, a known gliotransmitter.

The primordial role of astrocytes in the modulation of neurotransmission has been demonstrated in numerous studies over the past years, but it is to our knowledge the first time that they are involved in both the synaptic regulation in central amygdala and in the neuronal effect of OTR signaling. We propose that astrocytes are necessary messengers of the OT signal, acting both as sensors and amplifiers of the OT message in the CeA. Indeed the CeA is not the most densely OT-innervated area of the CNS (Knobloch et al., 2012) but still show a high quantity of binding site for OTR in rat (Freund-Mercier et al., 1987), data that were corroborated by a new OTR mapping through immunohistochemical detection and a transgenic model in mice (Mitre et al., 2016c; Yoshida et al., 2009). Synapses formed by OT axons are enriched in glutamate transporter, and clear vesicles can be found in OT synapses, whereas OT containing LDCVs are present all along OTergetic axons and can be released from virtually all part of OT cells (see 3.4.1 and Morris and Pow, 1991). Knowing that, a mechanism of OT neuromodulation in the CeA could be the following: upon OT neurons activity, OT could be released all along the axons at various sites in the CeL, while both OT and glutamate would be released only in synapses with GABAergic interneurons. Synaptic OT and glutamate release would directly and quickly activate these GABAergic interneurons of the CeL, while OT released from the axons en passant through the CeL would slowly diffuse. Through this diffusion, it could activate the high affinity OTRs on astrocytes which evokes long-lasting $[Ca^{2+}]_i$ oscillations therein. Astrocytes could then release gliotransmitters, at least D-Serine, in the proximity of synapses either on excitatory inputs of CeL interneurons or pre-synaptically on the GABAergic CeL-CeM synapses. This would consequently potentiate the activity of the GABAergic transmission in the CeA, thus inhibiting the output activity of the CeA. This speculative mechanism could produce an amplification of the OT effects on the CeA neuronal activity, both in space and time.

Nevertheless, many questions are left open in view of those results. First, one should measure the consequences of OT evoked calcium transients in astrocyte on the CeL GABAergic interneuron itself. Here we choose to measure only the synaptic activity of the post-synaptic output neurons of the CeM, and inferred a pre-synaptic action based on previous published findings. Hence it would be interesting to further confirm those mechanisms through experiments looking this time at the GABAergic interneuron of the CeL itself. Through whole cell patch-clamp recordings of those neurons and selective activation of astrocytes, ideally OTR expressing ones, one could characterize the mechanisms through which astrocytes increase the CeL neuron activity and its consequent GABAergic inputs into CeM neurons. Notably, one could look at the NMDAR currents like the slow inward currents, linked to astrocytic activation of neuronal extra-synaptic NMDAR (Pál, 2015). Yet, note that we excluded the involvement of the NR2B subunit containing NMDAR in our study, which is

thought to be expressed mostly in extra-synaptic NMDARs (Li et al., 2002), but not only, and other subunits exist in such NMDARs (Oliet and Papouin, 2014).

We identified D-Serine as a necessary element of the OT effect on CeA neurons, through its selective degradation and consecutive exogenous replenishment. Despite numerous proofs, the fact that astrocytes are the sole producer of the co-agonist D-Serine is still disputed by some researchers in the field (see Wolosker et al., 2016). It should then be confirmed that astrocytes synthesize D-Serine, by looking at D-Serine presence in those cells through immunohistochemistry or indirectly by detecting the presence of serine racemase, the D-Serine synthesizing enzyme. Another way to prove our astrocytic D-Serine hypothesis could be to compare the amount of D-Serine in CeA slices extracellular medium both before and after application of TGOT, through HPLC/mass spectrometry. Then repeat the experiment but this time with previous inactivation of astrocytes through gliotoxin incubation. If an increase of extracellular D-Serine happens in slice with viable astrocytes and not in slices with impaired astrocytic function, we would have a good indication that D-Serine is of astrocytic origin.

One other gliotransmitter that is often characterized is ATP (Butt, 2011), which acts on neuronal or astroglial purinergic receptors (P2). Using the wide range P2X receptors antagonist PPADS or the P2X₇ specific antagonist A438079, we recently observed the same effect than with NMDA antagonists: a significantly decreased effect of TGOT on synaptic transmission, and unaffected astrocytic responses to TGOT (data not shown). Nevertheless, since the degradation of D-Serine totally prevented TGOT effects, it seems to be solely implicated. One hypothesis could reconcile both findings: D-Serine might be released through astrocytic P2X₇, which are known to form pores in those cells (Pan et al., 2015; Volonté et al., 2012). There are several mechanisms of release of neuroactive substances from astrocytes: vesicular exocytosis, large vesicles, connexin 43 or pannexin hemi-channels etc. (Giaume et al., 2013; Kang et al., 2013; Montero and Orellana, 2015; Sahlender et al., 2014). It seems that the mechanisms of release of neuroactive substances by astrocytes are quite versatile and participate to what some call the gliocrine system (Verkhratsky et al., 2016). In our case, the precise mechanisms of release of D-Serine remain to be more conclusively determined.

Astrocytes are known to form gap-junction between each other, and doing so form a syncytium through which calcium transients can propagate. We have not tested the involvement of gap junctions in our study, but a preliminary result seems to

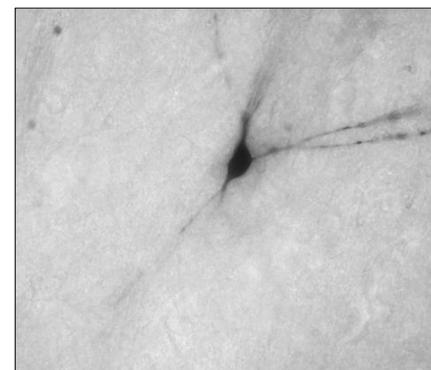


FIGURE 36. A biocytin infusion in a patched astrocyte did not diffuse in neighboring cells.

indicate that CeL astrocytes are not interconnected, since biocytin infusions in patched astrocytes did not diffuse to other cells (FIGURE 36). But this experiment lacks a sufficient number of repetitions before being conclusive. Another experiment could use gap junctions blockers to assess their implication in the propagation of astrocytic calcium transients in response to TGOT application (Orthmann-Murphy et al., 2008).

Interestingly, we identified the implication of both intra and extracellular calcium sources in the OTR mediated calcium transients of astrocytes. We characterized the need for voltage gated calcium channels (VGCCs) Ca_v3 for the maintenance of the calcium oscillations, at least in a sub-population of TGOT responding astrocytes. The existence of VGCCs on astrocytes is not news (Parpura et al., 2011), and CeL astrocytes can present small and punctual depolarization upon application of TGOT (Data not shown, B.Bellanger Master thesis) that could be sufficient to open such channels and provoke the long-lasting and oscillating calcium transients seen in CeL astrocytes. The implication of Ca_v3 channels in the generation of oscillatory increases in intracellular calcium concentration is well characterized in neurons, in which it can modulate phasic bursting behaviors (Cain and Snutch, 2013; Chevalier et al., 2006), but also in astrocytes (Parpura et al., 2011) and other cell types (e.g Brown et al., 2016). Therefore their implication in the generation of oscillatory calcium transients in astrocytes in response to OTR activation is not surprising. A mechanism very similar the one we propose was proved to underlie the release of glutamate and consequent neuronal NMDARs activation by astrocytes of the thalamus, but implicated L-type (Ca_v1) VGCCs (Parri et al., 2001), which implication was excluded here. One could also argue that OTR activation provokes the activation of store operated calcium entry, which has been proposed to be mediated by the transient receptor potential in astrocytes (Papanikolaou et al., 2017; Verkhratsky and Parpura, 2014). One could test such hypothesis in CeA astrocytes responses to TGOT using various pharmacological agents known to block such mechanisms.

One feature that we could not test due to technical limitations is the possibility of uncoupled calcium transients between astrocytic processes and cell bodies. Many studies now reveal that the important calcium transients also happen in the fine processes of astrocytes (Bazargani and Attwell, 2016; Shigetomi et al., 2016) and can be independent of both somatic increases of calcium (Otsu et al., 2014) and endoplasmic reticulum stores (Rungta et al., 2016). They involve extracellular calcium (Rungta et al., 2016) entry and mitochondrial stores of calcium (Agarwal et al., 2017; Reyes and Parpura, 2009). We could not resolve such fine processes, but it would be interesting to observe the effects of TGOT on the calcium dynamics in various morphological parts of the astrocytes of the CeL.

In our study, we also deciphered part of the intracellular signaling involved in the response to OTR activation in astrocytes. Using the biased agonist properties of both carbetocin and atosiban, we showed that both G_q and G_i subunits are functionally coupled to the OTR. This finding is peculiar, since the global cellular effects of the pathway linked to those G_α subunits are mostly opposed. Yet, G_i linked pathways have been shown to induce calcium transients in astrocytes (Mariotti et al., 2016; Ödemis et al., 2012) and the $G_{\beta\gamma}$ subunit of G_i coupled (mutated) OTR also leads to increase calcium in cultured cells (Hoare et al., 1999). We tested the involvement of $G_{\beta\gamma}$ in the effect of TGOT on astrocytes using the blocker gallein but it did not affect in any way the responses of astrocytes (data not shown). The use of pertussis toxin is difficult in living slices and even after several hours of incubation, the responses to TGOT were still present. Nevertheless, it might be that pertussis toxin incubation time was insufficient to totally block G_i linked signaling. One solution could be to stereotaxically inject it the day before *ex vivo* experiment. We choose to use the “not so specific” N-ethylmaleimide as a blocker of G_i (with short incubation time) and could block TGOT induced responses in astrocytes, yet with a questionable specificity. Regarding the G_q pathway, we isolated some of its downstream effectors as partial mediators of TGOT effect on astrocytic calcium transients, as described in the article. One untested component that could be involved in the responses of astrocytes to TGOT is the cAMP regulation, tightly linked to G_i signaling notably (in an inhibitory fashion). Then, it might be interesting to test the involvement of the cAMP pathways in astrocytic responses, since for instance it has been linked the mechanisms of gliotransmitter release in a few studies (Vardjan and Zorec, 2015). The co-existence of G_q and G_i coupling (and maybe G_s , which was not tested) to astrocytic OTR might involve complex intercommunication between classical signaling pathways, that might be interesting to explore further.

2.1. Perspectives

Our study deciphered a new mechanism of action of OT in the CNS, and while it still needs a good amount of research to be better characterized, it brings several perspectives. One may ask if this mechanism is a general feature of OT signaling in the CNS, or a CeA-only mechanism. Conceptually, it is interesting to think of astrocytes as mediators of OT effect in CNS. An astrocytes-mediated regulation of neural circuits by OT could fit well with the various proposed mode of action of OT, namely the volume transmission versus axonal release hypothesis. Astrocytic activity might be finely regulated by slow changes in the OT concentration through OT volume transmission, which would modulate their calcium excitability, resulting in fine tuning of the neurotransmission on a slow time scale. As proposed earlier, they would also help to modulate neuronal responses to synaptic OT/glutamate inputs. While having a direct, “classical” synaptic effect, OT axons could also release OT out of synapses in locally high concentration, thereby activating all surrounding OTR expressing

astrocytes, which would then be able to shape the potentiation of OT-sensitive neural circuits. Therefore it would be highly interesting if such a mechanism can be found elsewhere in the CNS.

Notably in the SC, astrocytes have been shown to be of primordial importance in the central sensitization mechanisms of chronic pain, notably through increased release of D-Serine inducing LTP in nociceptive pathways neurons, and are then proposed to be major actors in chronic pain etiology (Hansen and Malcangio, 2013; Ji et al., 2013; Lefèvre et al., 2015) especially in the SC (O'Callaghan and Miller, 2010). Astrocytes could even mediate the anti-nociceptive effect of OT in the spinal cord in the same way than in the CeA, for instance Jójárt and colleagues found astrocytes in close apposition to OT endings in the SC central canal (Jójárt et al., 2009). Furthermore, one should highlight that the remodeling and activation of astrocytes and other glial cells in the amygdala has been linked to the establishment of neuropathy in animals (Burke et al., 2013; Marcello et al., 2013; Panigada and Gosselin, 2011).

Therefore, astrocytes might be crucially involved in several areas of the pain matrix, and be key actors of the pathophysiology of pain disorders.

L. CONCLUSION

During this thesis, I could participate in the exploration of various aspects of OT action in several regions of the CNS. Those studies led to the discovery of novel and promising pathways of pain mitigation by the uterotonic nonapeptide discovered more than 110 years ago. As often, the road from fundamental research to the clinical use is a tortuous journey disseminated with pitfalls. Nevertheless, the design of new OTR ligands with coupling profile selectivity and improved BBB permeability is on the good path (Manning et al., 2012b). The OT system is a new target in many disorders, from cancer research (Imanieh et al., 2014) to psychiatric disorders (Shamay-Tsoory and Young, 2016; Zik and Roberts, 2015), notably autism spectrum disorders (Guastella and Hickie, 2016). Hence, oxytocin is a very important neuropeptide whose thorough study will help to have a better understanding of the nervous system and how it regulates its own circuits and peripheral targets.

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N.ANNEXES

1. ANNEXE 1: List of different OTR agonists and antagonists

AGONISTS

LIGAND	SPECIES	ACTION	AFFINITY	UNIT	REFERENCES
oxytocin	Hs	Full agonist	8.2 – 9.6	pK _i	3,22-24,42,48
vasopressin	Hs	Partial agonist	7.3 – 9.3	pK _i	3,15,21-23,29,42,48
desmopressin	Hs	Full agonist	6.7 – 7.6	pK _i	22,48
dAVP	Hs	Full agonist	8.3	pK _i	21
d[Leu4]LVP	Rn	Full agonist	7.2	pK _i	68
d[Leu4]AVP	Hs	Full agonist	6.7	pK _i	21
d[Leu4,Dap8]VP	Rn	Full agonist	6.9	pK _i	68
d[Cha4]LVP	Rn	Full agonist	6.2	pK _i	68
d[Cha4]AVP	Hs	Full agonist	7.1 – 7.7	pK _i	29,42
d[Cha4,Dab8]VP	Rn	Full agonist	6.4	pK _i	68
arginine vasotocin	Hs	Full agonist	9.4	pK _i	22-23
[Thr4,Gly7]OT	Hs	Full agonist	8.2 – 8.4	pK _i	23,32,48
[Phe3]OT	Hs	Full agonist	8.8	pK _i	22-23
[3H]OT (human, mouse, rat)	Hs	Full agonist	8.2 – 9.5	pK _d	22,39,48,52
[3H]AVP (human, mouse, rat)	Hs	Partial agonist	8.8 – 8.9	pK _d	15,22

Hs: Homo Sapiens, Rn: Rattus Norvegicus. Reference numbers correspondence can be found on <http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=369#references>

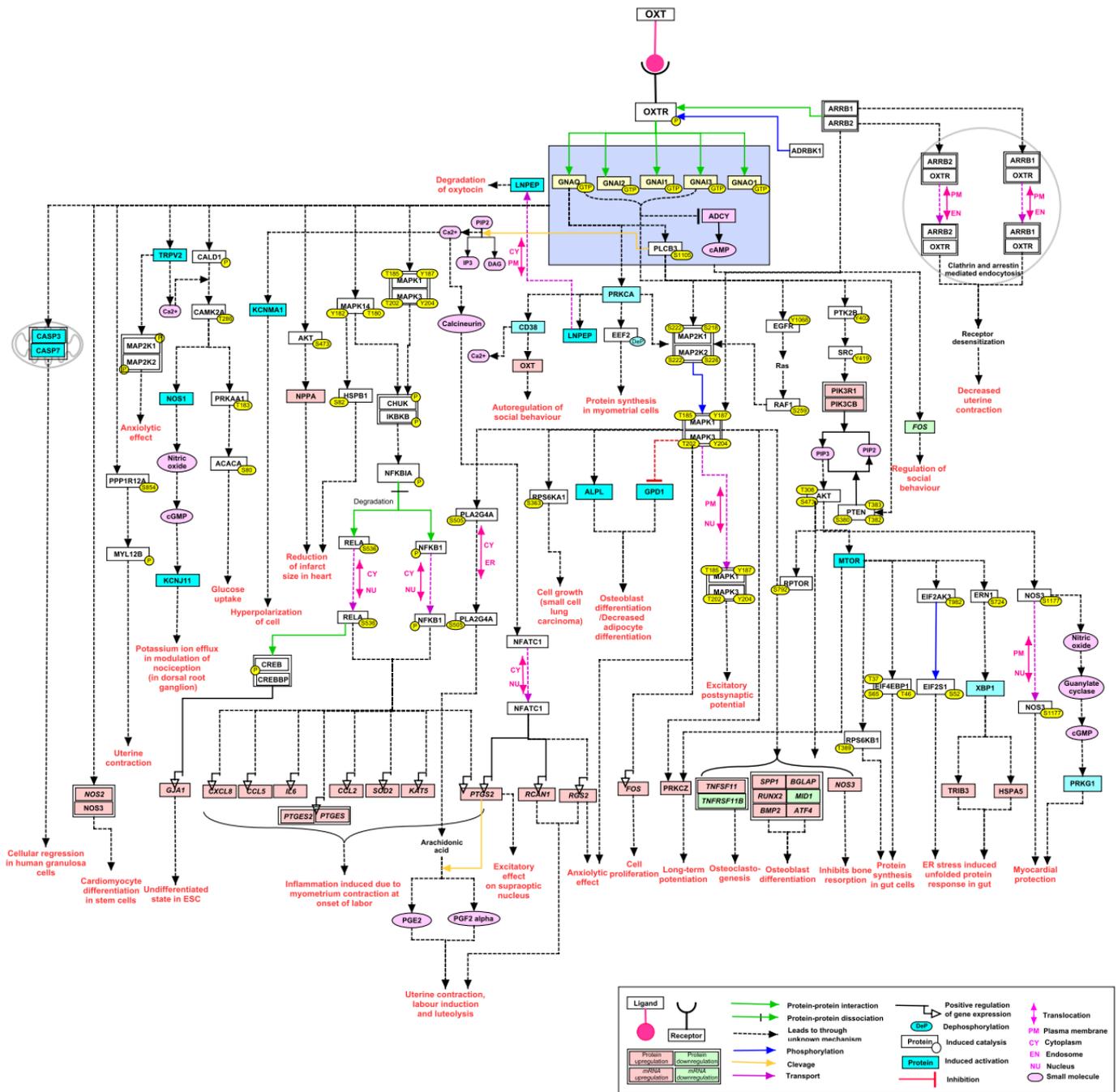
Adapted from Daniel Bichet, Michel Bouvier, Bice Chini, Gerald Gimpl, Gilles Guillon, Tadashi Kimura, Mark Knepper, Stephen Lolait, Maurice Manning, Bernard Mouillac, Claudine Serradeil-Le Gal, Melvyn Soloff, Joseph G. Verbalis, Mark Wheatley, Hans H. Zingg. ;

<http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=369>.

ANTAGONISTS

LIGAND	SPECIES	ACTION	AFFINITY	UNIT	REFERENCES
[35S]non-peptide OT antagonist	Hs	Antagonist	10.4	pK _d	55
[Mpa1,D-Tyr(Et)2,D-Tic7,Aib9]OT	Hs	Antagonist	7.1	pIC ₅₀	38
[Mpa1,D-Tyr(Et)2,D-Tic7,D-Tic9]OT	Hs	Antagonist	7.6	pIC ₅₀	38
[Mpa1,D-Tyr(Et)2,D-Tic7]OT	Hs	Antagonist	7.3	pIC ₅₀	38
atosiban	Hs	Antagonist	6.0 – 7.6	pK _i	3,24,48,59,76
compound 37 [PMID: 16250654]	Hs	Antagonist	8.9	pK _i	13
d(CH2)5[Tyr(Me)2,Thr4,Phe(3I,4N3)-NH29]OVT	Hs	Antagonist	9.0	pK _i	15
d(CH2)5[Tyr(Me)2,Thr4,Phe(3I25I,4N3)-NH29]OVT	Hs	Antagonist	9.6	pK _d	15
d(CH2)5[Tyr(Me)2,Thr4,Tyr(3I25I)-NH29]OVT	Hs	Antagonist	9.7 – 10.0	pK _d	15,22-23
d(CH2)5[Tyr(Me)2,Thr4,Tyr-NH29]OVT	Hs	Antagonist	9.2 – 10.0	pK _i	42,59
d(CH2)5[Tyr(Me)2]AVP	Hs	Antagonist	8.3	pK _i	48
L023103	Hs	Antagonist	9.2	pK _i	71
L023103	Rn	Antagonist	9.2	pK _i	71
L-365,209	Hs	Antagonist	8.5	pK _i	48
L-366,509	Hs	Antagonist	6.6	pK _i	48
L-366,682	Hs	Antagonist	8.4	pK _i	48
L-366,875	Hs	Antagonist	9.5	pK _i	48
L-366,948	Hs	Antagonist	8.6	pK _i	48
L-367,773	Hs	Antagonist	7.6	pK _i	48
L-367,938	Hs	Antagonist	7.9	pK _i	48
L-368,228	Hs	Antagonist	8.4	pK _i	48
L-368,899	Hs	Antagonist	8.1	pK _i	48
L-368,930	Hs	Antagonist	8.5	pK _i	48
L-369,020	Hs	Antagonist	8.5	pK _i	48
L-371,257	Hs	Antagonist	8.8	pK _i	42
L-372662	Hs	Antagonist	8.4	pK _i	11
LS-192629	Hs	Antagonist	7.6 – 7.8	pK _i	24
LS-192629	Rn	Antagonist	6.9	pK _i	24
nelivaptan	Hs	Antagonist	6.8 – 8.8	pK _i	42,77
OPC-21268	Hs	Antagonist	6.8	pK _i	48
relcovaptan	Hs	Antagonist	6.5 – 7.0	pK _i	3,42
retosiban	Hs	Antagonist	9.2	pK _i	60
Ro5028442	Hs	Antagonist	5.0	pK _i	73
SSR126768A	Hs	Antagonist	8.8 – 9.1	pK _i	76

2. ANNEXE 2: An overview of the OT-OTR signaling network



OT-OTR signaling network obtained through a systematic review of published signaling information. All references and particular information for each interaction is available freely on netpath.org, accession number NetPath_169. See URL below. Adapted from Chatterjee et al., 2016.

http://www.netpath.org/pathways?path_id=NetPath_169

3. ANNEXE 3: Publication non directly linked to the thesis

NEUROSYSTEMS

Favouring inhibitory synaptic drive mediated by GABA_A receptors in the basolateral nucleus of the amygdala efficiently reduces pain symptoms in neuropathic mice

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Abstract

Pain is an emotion and neuropathic pain symptoms are modulated by supraspinal structures such as the amygdala. The central nucleus of the amygdala is often called the 'nociceptive amygdala', but little is known about the role of the basolateral amygdala. Here, we monitored the mechanical nociceptive thresholds in a mouse model of neuropathic pain and infused modulators of the glutamate/GABAergic transmission in the basolateral nucleus of the amygdala (BLA) via chronically-implanted cannulas. We found that an *N*-methyl-D-aspartate-type glutamate receptor antagonist (MK-801) exerted a potent antiallodynic effect, whereas a transient allodynia was induced after perfusion of bicuculline, a GABA_A receptor antagonist. Potentiating GABA_A receptor function using diazepam or etifoxine (a non-benzodiazepine anxiolytic) fully but transiently alleviated mechanical allodynia. Interestingly, the antiallodynic effect of etifoxine disappeared in animals that were incapable of producing 3 α -steroids. Diazepam had a similar effect but of shorter duration. As indicated by patch-clamp recordings of BLA neurons, these effects were mediated by a potentiation of GABA_A receptor-mediated synaptic transmission. Together with a presynaptic elevation of miniature inhibitory postsynaptic current frequency, the duration and amplitude of GABA_A miniature inhibitory postsynaptic currents were also increased (postsynaptic effect). The analgesic contribution of endogenous neurosteroid seemed to be exclusively postsynaptic. This study highlights the importance of the BLA and the local inhibitory/excitatory neuronal network activity while setting the mechanical nociceptive threshold. Furthermore, it appears that promoting inhibition in this specific nucleus could fully alleviate pain symptoms. Therefore, the BLA could be a novel interesting target for the development of pharmacological or non-pharmacological therapies.

Introduction

The amygdaloid complex (or amygdala) is composed of a dozen subcortical nuclei within the temporal lobe. It can be subdivided anatomically and functionally into four distinct subregions: superficial, basolateral, central and medial (Veinante *et al.*, 2013). Functionally, it is an important structure of the limbic system providing an emotional value to sensory information and leading to adaptive behavioural responses. In line with our study, the amygdala plays a key role linking pain sensation and emotional responses as demonstrated by anatomical, electrophysiological, pharmacological and behavioural approaches (for review see Neugebauer *et al.*, 2009).

The central nucleus of the amygdala (CeA), often referred to as the 'nociceptive amygdala', serves as the output nucleus for major

amygdala functions and regulates behavioural responses through projections to hypothalamic nuclei and various brainstem areas (Veinante *et al.*, 2013). This nucleus receives nociceptive information from two major pathways. The first pathway is composed of the spino-parabrachio-amygdaloid projections (Gauriau & Bernard, 2002). It provides nociceptive information of different modalities originating from a large number of tissues (e.g. skin, viscera, joints and muscles) and is mostly associated with an increase in the activity of CeA neurons. The second pathway is involved in the processing of polymodal nociceptive information originating from the thalamus and cerebral cortex. This sensory information is initially processed by neuronal circuits in the basolateral nucleus of the amygdala (BLA) before reaching the CeA, directly or indirectly via a relay in the intercalated cell clusters. Transfer of this information is ensured by a subpopulation of glutamatergic BLA neurons establishing excitatory synapses with the target neurons. In comparison to the direct BLA–CeA projections, which generate excitation, synaptic

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connections to intercalated GABAergic neurons provide an inhibitory interface capable of generating feed-forward inhibition (Royer *et al.*, 1999). Interestingly, lesion of the BLA has been recently proposed to prevent the development of chronic pain states (Li *et al.*, 2013).

The contribution of the BLA to nociceptive processing cannot be restricted to an input provider to the CeA. Its role in the control of analgesic responses has been demonstrated after local infusion of a wide variety of pharmacological substances (Veinante *et al.*, 2013). Injection of morphine into the BLA suppresses formalin-induced conditioned place aversion, at least in part, through inhibition of glutamatergic transmission mediated by *N*-methyl-D-aspartate (NMDA) receptors (Deyama *et al.*, 2007). From this study, it is also clear that the place aversion induced by intraplantar formalin is associated with excessive glutamate levels in the BLA. Of particular interest for our present study, stress-induced analgesia has been significantly reduced after bilateral injection into the BLA of the GABA_A receptor agonist, muscimol, or of the positive allosteric modulator, diazepam (DZP) (Helmstetter, 1993; Rea *et al.*, 2011). These experiments and others dealing with the modulation of anxiety strongly indicated that GABAergic inhibitory transmission in the BLA plays a key role in the processing of sensory information including nociception.

In addition to the influence of GABAergic intercalated cell cluster neurons, the coding properties of glutamatergic BLA projection neurons can be placed under the control of local GABAergic interneurons. Although not fully characterized, they account for about 10–15% of the neuronal population and are tentatively classified based on expression of neurochemical markers such as parvalbumin, somatostatin, cholecystokinin, calbindin, calretinin and vasoactive intestinal peptide [see references in Ehrlich *et al.* (2009)]. The majority (50%) are parvalbumin-positive interneurons that establish synaptic contacts on the soma and proximal dendrites of projection neurons. The location of these inhibitory synapses is of course expected to play a critical role for the coding properties of principal BLA neurons (Veres *et al.*, 2014). This may easily explain the anxiolytic efficacy of benzodiazepine (Nagy *et al.*, 1979; Shibata *et al.*, 1982), which may preferentially target BLA sites (Niehoff & Kuhar, 1983; Thomas *et al.*, 1985; Hodges & Green, 1987). Based on these studies, anxiolytic effects have been attributed to the recruitment of $\alpha 2/3$ -containing GABA_A receptors expressed by BLA neurons. It is interesting to note here that intercalated cell cluster neurons express large amounts of extrasynaptic $\alpha 4/\delta$ -containing GABA_A receptors, known to be extremely sensitive to neurosteroids, which contribute greatly to a tonic inhibition of principal BLA neurons (Marowsky & Vogt, 2014).

To date there is still little data demonstrating the influence of inhibitory/excitatory BLA microcircuits on nociceptive processes and pain responses. We therefore used a mouse model of neuropathic pain and intra-BLA injections to manipulate the balance between excitatory and inhibitory controls. Focusing mostly on GABAergic inhibitory controls, we characterized the analgesic action of benzodiazepine and non-benzodiazepine allosteric modulators of GABA_A receptor function after *in vivo* intra-BLA injections. The associated changes in GABAergic synaptic transmission were also studied using patch-clamp recordings of pyramidal neurons of the BLA in brain slices.

Materials and methods

Animals and ethical issues

In the present study, behavioural testing was performed on 8–12-week-old C57Bl6J male mice (20–30 g; Janvier, Le Genest St.

Isle, France), housed under standard conditions (room temperature, 22 °C; 12/12-h light/dark cycle) with *ad libitum* access to food and water. All animals were habituated to the room and to the tests at least 1 week before starting the experiments. For electrophysiological recordings, postnatal day 21–28 C57Bl6J mice of both sexes were used and housed in the same conditions. All procedures were performed in accordance with the recommendations of the directive (2010/63/EU) of the European Parliament and of the Council (September 22, 2010). The project received authorization from the French Department of Agriculture (license number 67-116 to P.P.) and from the regional ethic committee (CREMEAS AL/12/15/03/07).

Surgical procedures

All surgeries were performed under aseptic conditions and with ketamine/xylazine anaesthesia (ketamine, 17 mg/mL, i.p.; xylazine, 2.5 mg/mL, i.p., 4 mL/kg; Centravet, Taden, France).

Cannula implantation and drug infusion

The animals were anaesthetized with intraperitoneal injection of a mixture containing ketamine (75 mg/kg) and medetomidine (1 mg/kg). At the time of surgery, the animals were implanted with two 6-mm 23-gauge stainless-steel guide cannulas (outer diameter, 0.64 mm; inner diameter, 0.32 mm; PlasticsOne, USA). All animals were bilaterally implanted with cannulas targeting the basolateral amygdala (BLA relative to bregma; AP, −1.8 mm; ML, ± 3.1 mm; DV, −4.2 mm) by stereotaxic instruments according to the mouse brain atlas of Paxinos & Watson (1998). After surgery, animals received Atipamezole (10 mg/kg, i.p.) and Ketofene (5 mg/kg, i.m.), after which they had 7 days recovery before starting the experiments. Prior to drug injection, animals were lightly anaesthetized with isoflurane (2%) and 30-G cannulas (outer diameter, 0.3 mm; inner diameter, 0.15 mm; length, 6 mm; PlasticsOne) were inserted. Drugs (0.5 μ L) were infused over a period of 5 min (0.1 μ L/min) with a syringe pump (CMA 400; CMA Microdialysis, Sweden). The injection needles were left in place for an additional 5 min to allow diffusion. Animals were tested at 15 min after infusion after visual confirmation of full recovery from anaesthesia. After finishing the test sessions, bilateral injections of a 1% Chicago Sky Blue (Sigma) solution were targeted to the BLA (0.5 μ L each side) as described in the Drug Application section below. After 30 min, the animals were anaesthetized with isoflurane (2.5%) and decapitated. Brains were removed, frozen in isopentane (Sigma) and stored at −80 °C. Sectioning (40 μ m) was performed with a cryostat (Microm HM560, Thermo Scientific, USA). The sites of injections were verified according to the atlas of Paxinos & Watson (1998). Animals with injection sites located outside the BLA regions were not used in the analysis.

Neuropathic pain model

To produce a controlled constriction of the sciatic nerve, we used the cuff model, which has been well characterized previously (Benbouzid *et al.*, 2008). The common branch of the right sciatic nerve was exposed and a 2-mm-long split section of polyethylene tubing (inner diameter, 0.38 mm; outer diameter, 1.09 mm; PE-20, Harvard Apparatus, Les Ulis, France) was placed around it (Cuff group). The shaved skin layer was sutured closed. Sham-operated mice underwent the same surgical procedure as described above but without implantation of the cuff (Sham group).

Measures of mechanical nociception and pain symptoms

The mechanical threshold was measured using von Frey (VF) filaments (Bioseb, Chaville, France) as previously described (Chaplan *et al.*, 1994). Mice were placed in clear Plexiglass boxes (7 × 9 × 7 cm) on an elevated mesh screen, and were allowed to habituate for 15 min before testing. Filaments were applied to the plantar surface of each hindpaw in a series of ascending forces. We approached the filament toward the plantar surface slowly until it slightly bent at contact. At that point the pressure was immediately removed. Each filament was tested five times per paw and the threshold was defined as three or more withdrawals observed among the five consecutive trials. The results were expressed in grams and both hindpaws (ipsilateral allodynic right paw and contralateral control left paw) were tested on cuff and sham animals.

Patch-clamp recordings

Slice preparation and solutions

Coronal slices containing the BLA were prepared from C57Bl6J mice (21–28 days old). Briefly, after ketamine anaesthesia (2.5 mg/100 g, *i.p.*, Centravet), animals were decapitated, and brains were removed quickly and immersed in 4 °C Mg-artificial cerebrospinal fluid containing (in mM): 124 NaCl, 10 MgSO₄, 2.7 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 2 CaCl₂, continuously bubbled with 95% O₂/5% CO₂ leading to pH 7.4. The brain was glued on the slicer platform, and coronal brain slices (325 µm thick) were prepared with a tissue slicer (1200vs, Leica, Germany). Slices were hemisected and incubated for 45 min at 34 °C in artificial cerebrospinal fluid containing (in mM): 124 NaCl, 2.7 KCl, 1.25 NaH₂PO₄, 1.3 MgSO₄, 2.0 CaCl₂, 26.0 NaHCO₃, bubbled with 95% O₂/5% CO₂. After another hour at room temperature, in artificial cerebrospinal fluid containing 0.5 µM tetrodotoxin (Abcam Biochemicals, UK) and 2 mM kynurenic acid (Sigma-Aldrich) to block sodium voltage-dependent ionic channels and ionotropic glutamatergic receptors, a single slice was transferred to the recording chamber.

Electrophysiological recordings and data acquisition

Whole-cell voltage-clamp recordings were obtained with an amplifier (MultiClamp 700B, Axon Instruments, Foster City, CA, USA). Borosilicate glass electrodes ($R = 3\text{--}8\text{ M}\Omega$) with an inner filament (outer diameter, 1.2 mm; inner diameter, 0.69 mm; Harvard Apparatus Ltd, UK) were pulled using a horizontal laser puller (P2000; Sutter Instruments, USA). Pipettes were filled with an intracellular solution containing (in mM): 125 KCl, 2 MgCl₂, 10 HEPES, 2 MgATP, 0.2 Na-GTP (pH adjusted to 7.3 with KOH, osmolarity 295 mOsm adjusted with sucrose). Due to the chloride reversal potential of 0 mV and holding potential of -60 mV, inward synaptic currents corresponded to GABA miniature inhibitory postsynaptic currents (IPSCs). Series capacitances and resistances were compensated electronically throughout the experiments using the main amplifier. Recordings were filtered at 2 kHz, digitized at 10 kHz and stored with PCLAMP 8.0 software (Molecular Devices, Sunnyvale, USA) before analysis. All recordings were from neurons identified visually as having a large, pyramidal-like soma corresponding to glutamatergic BLA output neurons. Our patch-clamp configuration for miniature IPSCs did not allow us to record action potential firing; therefore, these neurons could not be identified by their accommodating firing pattern (Sah *et al.*, 2003). In a subset of experiments, however, we found that large pyramidal-like cells

indeed displayed accommodated spiking when tetrodotoxin and kynurenic acid were absent from the extracellular medium (data not shown). In addition, biocytine was included in the pipette solution identifying that these pyramidal neurons had axons targeting the CeA (data not shown).

Data analysis

Synaptic currents were detected and analysed using the Strathclyde electrophysiology software packages WINEDR and WINWCP (courtesy of Dr J. Dempster, University of Strathclyde, Glasgow, UK). Detection of each single event was further confirmed by visual inspection. For each synaptic current, the peak amplitude was measured as well as the exponential decay time constant (τ) and the overall inhibitory charge (area under the curve). In some experiments KCl was replaced by K MeSO₄ in patch pipette solution and the excitatory : inhibitory (E : I) ratio was calculated. For each recorded cell, the excitatory charge per time unit measured at E_{Cl} was divided by the inhibitory charge per time unit measured at E_{cation} .

Drug application

We used bicuculline methiodide (10 µM; Sigma-Aldrich) or MK-801 (1 µM; Sigma-Aldrich) as antagonists of GABA_A receptor and NMDA glutamate receptor currents, respectively. They were prepared as 1000 times concentrated solutions in water. DZP (Roche, France) was dissolved in absolute ethanol at a final concentration of 10 µM (final ethanol concentration < 0.1%). Etifoxine (EFX) (2-ethylamino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride) (Stresam™, Biocodex, Gentilly, France) was prepared in dimethylsulphoxide (Sigma-Aldrich) and injected at a final concentration of 60 µM (final concentration of dimethylsulphoxide < 0.1%). Drugs were either infused (0.5 µL) in the basolateral nuclei *in vivo* or bath-applied at the same concentration on amygdala slices *in vitro*. Finasteride (FIN) [1,(5 α)-androst-4-ene-3-one-17 β -(N-tert-butyl-carboxamide)] (Steraloids, Newport, RI, USA) solubilized in ethanol and diluted in olive oil was used to inhibit 5 α -reductase activity. It was subcutaneously injected (100 µL, 25 mg/kg) three times every 2 days.

Statistics

Data are presented as mean \pm SEM. Statistical analyses were performed with Prism software (GRAPHPAD) using multifactor ANOVA or *t*-test. The Tukey *posthoc* test and Bonferroni comparisons were used when appropriate for multiple comparisons in the behavioural as well as the electrophysiological experiments. Differences were considered to be statistically significant when $P < 0.05$.

Results

The influence of inhibitory and excitatory controls in the BLA was characterized in a mouse model of mono-neuropathy resulting from a chronic constriction of the sciatic nerve. As shown in Fig. 1A, the surgery aimed at installing a polyethylene cuff around the main branch of the right sciatic nerve triggered a significant ipsilateral (right paw) reduction in the VF threshold as a measure of mechanical nociceptive thresholds (repeated-measures two-way ANOVA, time \times paw side, $F_{51,561} = 4.406$, $P < 0.0001$). Compared with the value before surgery (4.75 ± 0.55 g, $n = 12$), the threshold was strongly reduced to 0.62 ± 0.04 g ($n = 12$, Tukey, $P < 0.0001$) after 7 days and remained significantly lower for > 70 days (Fig. 1A). During this period, the VF values of the ipsilateral paw

were significantly different for every time-point from the respective mean values of the contralateral paw and of both paws of sham-operated mice. This result is fully in agreement with the presence of a mechanical allodynia resulting from the 'cuff' compression of the right sciatic nerve.

Excitation/inhibition balance control of nociception and pain in the basolateral nucleus of the amygdala in mice

Using mice preimplanted with BLA cannulas and bilateral injections, the effects of drugs modulating the balance between fast excitation and inhibition were analysed at 2 weeks after the cuff surgery. As shown above, VF thresholds measured at 2 weeks after surgery were significantly different (repeated-measures two-way ANOVA, time, $F_{5,90} = 21.9$, $P < 0.0001$; Bonferroni, $P < 0.0001$) between intact left and cuffed right paws. As early as 15 min after

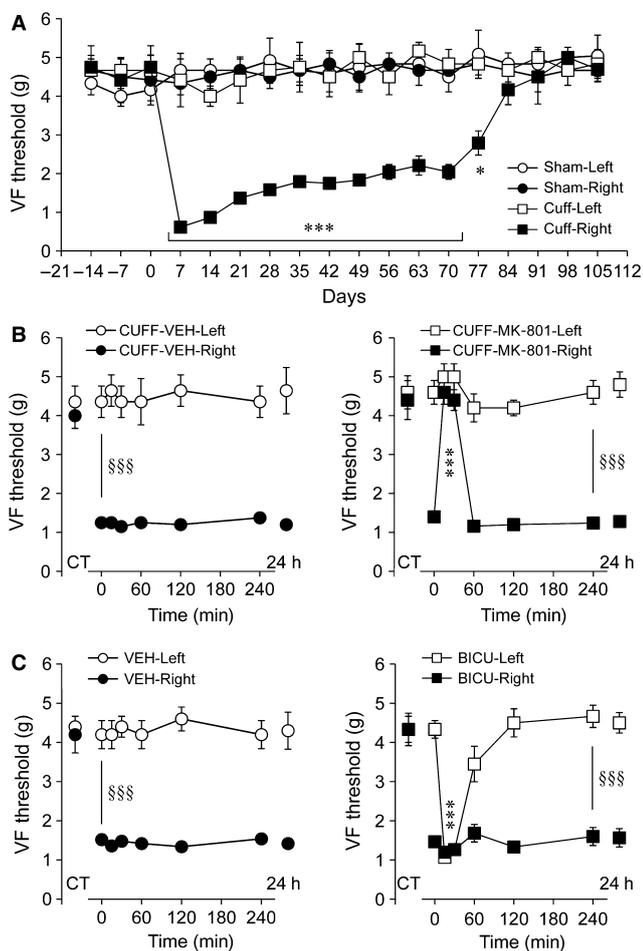


FIG. 1. Changes in mechanical nociceptive thresholds measured with VF filaments in mice that were sham-operated (Sham) (A) or with a chronic compression of the right sciatic nerve (Cuff) (A–C). Values are shown for left and right hindpaws. (A) Time-course of VF threshold before and after surgery (day 0) for both hindpaws in sham and cuffed mice. Note that mechanical allodynia developed rapidly for the right hindpaw of cuffed mice and persisted for about 3 months. (B and C) Changes in VF thresholds in neuropathic mice after a bilateral BLA injection at time 0 of the vehicle (VEH) solutions (left graphs): 1 μM MK-801 (B, right graph) or 10 μM bicuculline (BICU) (C, right graph). VF thresholds are also given in control (CT) (i.e. before cuff surgery). Tukey (*compared with paw value at time 0) and Bonferroni (§ for a time-point between paws) multiple comparisons are indicated: ***,§§§ $P < 0.001$, * $P < 0.05$.

BLA injection of the NMDA receptor antagonist MK-801 (1 μM , Fig. 1B), the VF threshold increased significantly from 1.36 ± 0.04 to 4.62 ± 0.31 g ($n = 10$; Tukey, $P < 0.001$) and also remained not significantly different from the intact contralateral paw after 30 min (Bonferroni, $P = 0.073$). This strong analgesic effect was transient and thresholds returned to basal (neuropathic) levels at 60 min after the BLA infusion (1.16 ± 0.07 g, $n = 10$; compared with before injection: Tukey, $P = 0.9$). We next used bicuculline to inhibit GABA_A receptor-mediated inhibition in the BLA (Fig. 1C). If bicuculline (10 μM) had no significant effect on the allodynic right paw (from 1.47 ± 0.12 to 1.20 ± 0.06 g after 15 min, $n = 12$; Bonferroni, $P = 0.99$), it induced a rapid decrease in the mean VF threshold of the left (intact) hindpaw (from 4.33 ± 0.22 to 1.07 ± 0.04 g after 15 min, $n = 12$; repeated-measures two-way ANOVA, time \times paw side, $F_{5,45} = 25.21$, $P < 0.001$; Tukey, $P < 0.0001$). These low VF thresholds were not different from the cuffed paw at 15 and 30 min after bicuculline injection (Bonferroni, $P = 0.95$). Note that the effects of MK-801 and bicuculline were short-lasting (< 1 h) but particularly efficient at producing analgesia or allodynia, respectively. This effect seemed specific as vehicle injections were ineffective at modulating VF thresholds in all cases ($n = 10$ for MK-801 and $n = 12$ for bicuculline).

Analgesia mediated by the potentiation of inhibition in basolateral nucleus of the amygdala microcircuits

As the function of BLA microcircuits also relies on the activity of GABAergic interneurons, we next evaluated the consequences of injecting GABA_A receptor-positive modulators on mechanical nociception and allodynia. We first used the classical benzodiazepine DZP (Fig. 2A, left graph). The injection of DZP at 1 μM (final volume 0.5 μL) was associated with a rapid and significant increase in the mean VF threshold for the left and right hindpaws (repeated-measures two-way ANOVA, time \times paw side, $F_{5,55} = 17.97$, $P < 0.0001$). This increase was modest but statistically significant in the case of the left hindpaw as the maximal antinociceptive effect was reached at 30 min after injection of DZP when the VF mean threshold reached 6.33 ± 0.22 g (compared with before injection: 4.67 ± 0.28 g, $n = 12$; Tukey, $P < 0.0001$). The DZP analgesic effect was more pronounced for the ipsilateral (allodynic) paw (from 1.33 ± 0.04 to 5.33 ± 0.38 g, 30 min after DZP, $n = 12$; Tukey, $P < 0.001$). VF threshold changes followed a similar time-course in both paws. The DZP-induced analgesic effect was still present at 1 h after the injection and thresholds were back to their respective basal values after 2 h. We also used the recently described non-benzodiazepine compound EFX (Fig. 2B, left graph), which potentiates GABA_A receptor function after binding to a site distinct from the benzodiazepines. Injected at a concentration of 60 μM , we found that it significantly increased the VF threshold of the allodynic paw (repeated-measures two-way ANOVA, time \times paw side, $F_{5,45} = 10.08$, $P < 0.0001$) but was without apparent effect on the left paw (from 4.80 ± 0.33 to 5.20 ± 0.33 g, 2 h after EFX, $n = 10$; Tukey, $P = 0.99$). The mean VF thresholds of the right hindpaw were increased from 1.28 ± 0.06 g to a maximal value of 4.40 ± 0.40 g ($n = 10$; Tukey, $P < 0.0001$) at 2 h after EFX bilateral injection. No statistical differences could be found between the two paws of the cuffed EFX-treated mice at 60 and 120 min. It should be noted here that the antiallodynic effect was much slower to establish, compared with DZP, but was also transient as thresholds were back to their original level at 4 h after the injection.

Apart from a direct allosteric modulation of GABA_A receptor function, DZP and EFX are also known to bind to mitochondrial

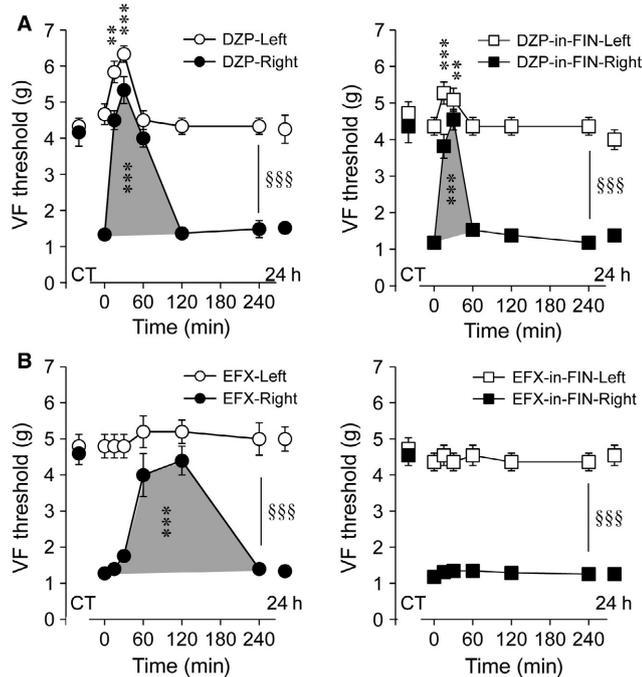


FIG. 2. Effects of BLA bilateral injections of DZP (1 μ M) (A) and EFX (60 μ M) (B) at time 0 on VF thresholds. The graphs on the right were obtained in FIN-treated animals. In all cases, VF thresholds are also given in control (CT) (i.e. before cuff surgery). Tukey (*compared with paw value at time 0) and Bonferroni (§ for a time-point between paws) multiple comparisons are indicated: ***,§§§ $P < 0.001$, ** $P < 0.01$.

translocator protein (TSPO). In several brain structures, this action has been shown to promote the intracellular synthesis of allopregnanolone-like compounds (neurosteroids), which are the most potent endogenous modulators of GABA_A receptor function (Poisbeau *et al.*, 2014). To verify this hypothesis, the synthesis of allopregnanolone-like compounds was inhibited by treating the mice with three subcutaneous injections of FIN every 2 days before BLA infusions (see Materials and methods). As illustrated in Fig. 2 (right graphs), DZP analgesia persisted in FIN-treated mice (repeated-measures two-way ANOVA, time \times paw side, $F_{5,50} = 18.51$, $P < 0.0001$), whereas EFX action was fully prevented (repeated-measures two-way ANOVA, $F_{5,50} = 0.20$, $P = 0.99$). As the DZP analgesic effect was shorter (< 1 h) in FIN-treated mice, this result suggests that part of the transient analgesic effect was also controlled by endogenous neurosteroid. In the case of EFX and at this concentration, the endogenous production of allopregnanolone-like compounds was necessary to observe the antiallodynic effect.

Modulation of inhibitory synaptic controls by etifoxine in basolateral nucleus of the amygdala neurons in mice

To go one step further, the effect of these modulators was more directly characterized by using patch recording of BLA pyramidal neurons and analysis of GABA_A receptor-mediated synaptic currents (GABA_A receptor IPSCs) (Fig. 3). As expected, DZP applied at 1 μ M on BLA neurons potentiated the apparent affinity of GABA_A receptors. This was clearly seen by a prolongation of the decaying time constant (from 16.84 ± 2.17 to 30.62 ± 3.82 ms, $n = 6$; Student's t -test, $t = 6.21$, $df = 5$, $P = 0.0016$). If no significant change could be seen for the mean amplitude (from 15.54 ± 3.27 to 22.39 ± 4.31 pA, $n = 6$; Student's t -test, $t = 1.77$, $df = 5$,

$P = 0.13$), a clear increase was observed for the frequency of occurrence (from 2.78 ± 0.32 to 5.21 ± 0.88 ms, $n = 6$; Student's t -test, $t = 3.65$, $df = 5$, $P = 0.015$). In the case of EFX, potentiation was associated with an increase in the mean frequency of occurrence of spontaneously-occurring GABA_A IPSCs and of their amplitude and duration (see Fig. 3 and Table 1). This corresponded to an overall increase in the inhibitory charge (e.g. increase of both amplitude and duration) carried by individual GABA_A IPSCs from 222.6 ± 20.6 to 334.9 ± 26.7 pA.ms ($n = 17$; one-way ANOVA, $F_{3,32} = 6.344$, Tukey, $P = 0.037$). When applied to BLA slices and within the time-course of a patch-clamp recording (about 1 h), we failed to see a proper washout of EFX. However, similar experiments performed on slices collected from FIN-treated mice revealed that the EFX increase of IPSC inhibitory charge was absent (Table 1). This was not the case for the transient increase in IPSC frequency, which persisted in the FIN condition.

To investigate, *in vitro*, the consequences of EFX on BLA network activity, we calculated the E : I ratio in control condition ($n = 13$) or in the presence of BIC ($n = 4$), EFX ($n = 6$) or MK-801 ($n = 6$) in 23 cells. In control conditions, excitation was more important than inhibition, and the mean E : I ratio was 2.62 (ranging from 0.99 to 6.71). Bicuculline fully suppressed inhibition. EFX, as well as MK-801, reversed the ratio, but in different ways (mean EFX E : I ratio, 0.26 ranging from 0.025 to 0.48; mean MK-801 E : I ratio, 0.31 ranging from 0.054 to 0.64). EFX increased the inhibitory charge by 66.51%, whereas the excitatory charge decreased (-605.72%) due to the large overall network inhibition. For its part, MK-801, as expected, decreased the excitatory charge (-89.71%) but also reduced the inhibitory charge (-15.93%) to lead to a net imbalance in favour of inhibition.

Discussion

The BLA is the initial site of sensory convergence and integration in the amygdala. Indeed, infusion in the BLA of mu opioid receptor agonist decreases thermal nociceptive sensitivity (Helmstetter *et al.*, 1995; McGaraughty & Heinricher, 2002). Moreover, the BLA controls stress-induced and fear-induced analgesia, which are both reduced by intra-BLA infusions of the GABA_A receptor agonist muscimol (Rea *et al.*, 2011) or by the allosteric modulator DZP

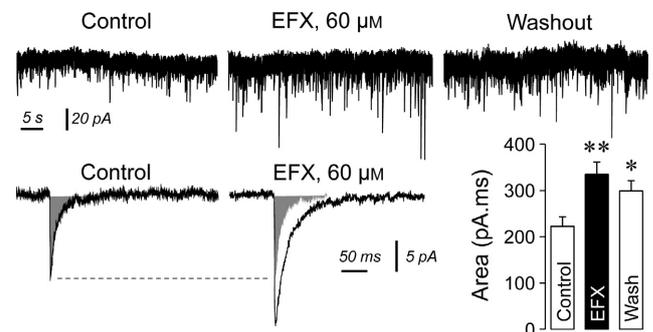


FIG. 3. Properties of GABA_A receptor-mediated IPSCs recorded in BLA neurons in amygdala slices from 21–28-day-old mice using the whole-cell patch-clamp technique. As illustrated with the raw traces, bath application of EFX increased the frequency of occurrence of IPSCs and their amplitudes (see also Table 1). Histogram gives the mean inhibitory charge carried by the GABA_A receptor IPSCs (area) in control, EFX and after washout of the drug. Note that, during the course of the recording (maximum recording time of 40 min), increase in inhibitory charge was hardly reversible after EFX bath application. Statistical significance with Tukey *posthoc* test: ** $P < 0.01$, * $P < 0.05$.

TABLE 1. Changes in miniature GABA_A-mediated IPSCs after application of EFX (60 μ M) in the absence (top three lines) and presence (bottom three lines) of the 5 α -reductase inhibitor finasteride (FIN, 10 μ M)

Conditions	Freq. (Hz)	Area (pA.ms)	Amp. (–pA)	Decay (ms)	N
Control	3.2 \pm 0.5	222.6 \pm 20.6	13.6 \pm 1.2	17.3 \pm 1.3	17
EFX (60 μ M)	5.4 \pm 0.8***	334.9 \pm 26.7**	18.3 \pm 1.3**	21.0 \pm 1.2*	17
Washout	3.9 \pm 0.5	299.0 \pm 22.6*	15.1 \pm 1.3	21.3 \pm 2.0*	13
Control in FIN	2.8 \pm 0.8	192.3 \pm 27.8	15.3 \pm 2.9	17.8 \pm 1.2	8
EFX (60 μ M) in FIN	5.6 \pm 1.0*	209.9 \pm 33.5	14.5 \pm 2.7	17.6 \pm 1.2	8
Washout in FIN	2.5 \pm 0.6	206.8 \pm 31.8	16.6 \pm 2.3	17.7 \pm 1.6	7

Mean frequency of occurrence (Freq.), inhibitory charge carried by IPSCs (Area), absolute current amplitude (Amp.) and half decay time are given for the IPSCs in the two different conditions. Data in bold were found to be statistically different at *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ with the Tukey *posthoc* test after one-way ANOVA. Statistical analysis was performed on BLA neurons submitted to at least 10 min of EFX washout (i.e. 13 out of 17 in control; 7 out of 8 in finasteride). In finasteride condition (FIN), note that only changes in frequency persisted during the application of EFX.

(Helmstetter, 1993). Based on this experimental evidence, it can be concluded that GABAergic inhibition in the BLA controls nociceptive responses as well as analgesia induced by fear or stress. Among many possibilities, BLA inhibition is likely to affect CeA neuronal activity, which has been shown to modulate pain behaviour through projections to descending pain control centres in the brainstem (Neugebauer *et al.*, 2009) and to control pain modulation systems (Helmstetter *et al.*, 1998). To fully understand the involvement of BLA inhibition in pain modulation, we also have to take into account the excitatory/inhibitory balance regulating BLA network excitability and therefore its impact on the activity of downstream structures. To date, no studies have shown any modulation of excitation in the BLA in pain conditions or in stress-induced or fear-induced analgesia. However, it has been shown that infusion of NMDA and non-NMDA receptor antagonists in the BLA reduced anxiety (for review, see Bergink *et al.*, 2004), demonstrating that modulation of excitation in the BLA can influence behaviour. Accordingly our results show that altering the BLA excitatory/inhibitory balance either alleviates neuropathic pain symptoms or induces pain. Indeed, a direct blockade of NMDA receptor suppressed mechanical allodynia, whereas a blockade of GABAergic inhibition produced allodynia in the healthy paw.

Based on these results and focusing on GABAergic transmission, we have explored the consequences of an increase of this neurotransmission on neuropathic-induced allodynia. Previously, it has been shown that BLA infusion of DZP attenuated stress-induced analgesia (Helmstetter, 1993) or produced antinociceptive effects (Jimenez-Velazquez *et al.*, 2010). Our results confirmed that DZP alleviates mechanical allodynia and produces analgesia in both neuropathic and healthy paws. In addition, we used another allosteric modulator of GABA_A receptor (i.e. EFX, a non-benzodiazepine anxiolytic), which has a different pharmacological profile and binding sites that are distinct from those of benzodiazepines (Hamon *et al.*, 2003). A single infusion of EFX produced a longer-lasting alleviation of neuropathic pain symptoms without effect on the healthy paw. As shown previously in a model of neuropathic pain, this effect is likely to be contributed by the action of EFX on neurosteroidogenesis through its action on the mitochondrial translocator protein TSPO (Poisbeau *et al.*, 2014). Indeed, blockade of a key enzyme in the production of neurosteroid by FIN fully suppresses the antiallodynic action of EFX. A similar mechanism has already been proposed by our group (Zell *et al.*, 2014, 2015) to explain analgesia produced by high plasma corticosterone levels through the modulation of spinal GABAergic inhibition. We may then conclude that EFX, by increasing mitochondrial cholesterol entry, increases the synthesis of neurosteroids that are, in turn, potent positive modu-

lators of GABAergic synaptic transmission (Poisbeau *et al.*, 1997) resulting in an increased inhibition within the BLA neuronal network.

To dissect the action of EFX further, we recorded GABAergic synaptic transmission on BLA output neurons and compared the effect of the well-characterized benzodiazepine DZP with that of EFX. DZP prolonged the decay time constant and increased the amplitude and frequency of IPSCs. The increase in IPSC duration and amplitude are mostly contributed by postsynaptic mechanisms (Vicini *et al.*, 1986), whereas the frequency increase generally results from a presynaptic mechanism as demonstrated for glycine receptors in rat spinal sacral dorsal commissural nucleus neurons (Jeong *et al.*, 2003). Presynaptic GABA_A receptors have previously been identified in the BLA where they produced excitation (Woodruff *et al.*, 2006). Indeed, depolarization of the presynaptic terminal is expected to increase synaptic release and neuronal excitability, especially if the neurotransmitter is excitatory (Pugh & Jahr, 2011; Dellal *et al.*, 2012). In comparison with DZP, the effects of EFX are likely to be similar, as seen by the increase in frequency, amplitude and duration of synaptic currents. Our results show that the latter two depend greatly on EFX-induced neurosteroidogenesis, whereas the presynaptic increase of synaptic current frequency is not affected by FIN. Accordingly, at the network level, the cellular effect of EFX on synaptic transmission impacts network excitability. Indeed, EFX increases network inhibition, which in turn drastically reduces network excitation leading to a change of network excitatory/inhibitory balance and a decrease in network excitability.

Altogether, this overall increase of GABAergic inhibition impacts CeA neuronal activity. However, the nature of the impact on CeA neuronal activity is difficult to predict. There are many pathways between the BLA and CeA, either direct from glutamatergic output BLA neurons or through a relay in the intercalated mass (Veinante *et al.*, 2013). In the former case, the working hypothesis will predict that a decrease in BLA excitatory drive will cause a smaller activation of the GABAergic CeA neuronal network. In the latter case, a decrease of the excitatory drive on inhibitory intercalated cells will reduce their feed-forward inhibition on CeA neurons. To add layers of complexity, it has also been shown that feed-forward inhibition is itself controlled by inhibition coming from neighbouring intercalated cells (Royer *et al.*, 1999, 2000) and that some specific intercalated cells may respond to noxious stimuli and project back on BLA interneurons (Bienvenu *et al.*, 2015). Therefore, a large amount of work remains to be done in order to fully dissect the BLA–CeA microcircuits. However, our *in vivo* experiments clearly demonstrate that stimulating endogenous GABAergic inhibition in the BLA with DZP or EFX alleviates allodynia in neuropathic mice. This analgesic

action is mediated by a direct and indirect (i.e. neurosteroid-based) potentiation of GABA_A receptor function at presynaptic and postsynaptic sites.

Conflict of interest

P.P. received financial support from Biocodex to establish its molecular mechanisms of action. The other authors declare no conflict of interest.

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Abbreviations

BLA, basolateral nucleus of the amygdala; CeA, central nucleus of the amygdala; DZP, diazepam; E : I, excitatory : inhibitory; EFX, etifoxine; FIN, finasteride; ISPC, inhibitory postsynaptic current; NMDA, *N*-methyl-D-aspartate; VF, von Frey.

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Etude des mécanismes d'action de l'ocytocine sur la modulation des circuits astro-neuronaux de régulation de la douleur.

Résumé

La douleur est un phénomène complexe régi par le système nerveux. De nombreuses molécules modulent la douleur par des mécanismes complexes et variés. L'une d'elle, l'ocytocine, est plus connue pour ses rôles dans la reproduction et les interactions sociales, mais est pourtant un puissant agent analgésique endogène. Au cours de cette thèse, j'ai cherché à comprendre comment l'ocytocine régulait la douleur dans deux différentes régions du cerveau, l'hypothalamus et l'amygdale. Ces travaux ont permis de mettre à jour un petit groupe de neurones ocytocinergiques de l'hypothalamus, qui contrôle la douleur par une action double, à la fois en inhibant la transmission du message douloureux dans la moelle épinière et en contrôlant en même temps l'activité d'autres neurones ocytocinergiques qui sécrètent alors de l'ocytocine dans la circulation sanguine. Dans l'amygdale, nous avons pu montrer que l'effet analgésique de l'ocytocine nécessitait le bon fonctionnement d'un type de cellule non neuronal, l'astrocyte, qui répond à la présence d'ocytocine et, ce faisant, permet l'activation de circuits de neurones contrôlant la douleur.

Mots-clefs : Douleur, ocytocine, astrocyte, hypothalamus, amygdale, moelle épinière, analgésie, électrophysiologie, imagerie calcique.

Summary

Pain is a complex phenomenon arising from the nervous system. Numerous molecules modulate pain through complex and various mechanisms. One of those, oxytocin, is more famous for its roles in reproduction and social interactions, but is also a potent endogenous analgesic. During this thesis, I tried to understand how oxytocin modulates pain in two brain regions, the hypothalamus and the amygdala. This work unveiled a small group of oxytocinergic neurons in the hypothalamus which control pain through a dual action, firstly by inhibiting the pain signals in the spinal cord and secondly by activating at the same time another population of oxytocinergic neurons, which then secrete oxytocin in the bloodstream. In the amygdala, we showed that the analgesic effect of oxytocin required the proper functioning of a non-neuronal cell type, the astrocyte, which responds to oxytocin and, doing so, allows the activation of neural circuits which modulate pain.

Key-words: Pain, oxytocin, astrocyte, hypothalamus, amygdala, spinal cord, analgesia, electrophysiology, calcium imaging.