Distribution of oxytocin and co-localization with arginine vasopressin in the brain of mice

Marcos Otero-García ²

Carmen Agustín-Pavón ¹

Enrique Lanuza ²

Fernando Martínez-García ^{1,*}

Phone: +34964 387457

Email: femartin@uji.es

¹ Lab. of Functional Neuroanatomy (NeuroFun), Unitat Predepartamental de Medicina, Facultat de Ciències de la Salut, Universitat Jaume I, Av. de Vicent Sos Baynat, s/n, 12071 Castelló de la Plana, Spain

² Departaments de Biologia Cel·lular i de Biologia Funcional, Facultat de Ciències Biològiques, Universitat de València, València, Spain

Abstract

Oxytocin (OT) and vasopressin (AVP) play a major role in social behaviours. Mice have become the species of choice for neurobiology of social behaviour due to identification of mouse pheromones and the advantage of genetically modified mice. However, neuroanatomical data on nonapeptidergic systems in mice are fragmentary, especially concerning the central distribution of OT. Therefore, we analyse the immunoreactivity for OT and its neurophysin in the brain of male and female mice (strain CD1). Further, we combine immunofluorescent detection of OT and AVP to locate cells co-expressing both peptides and their putative axonal processes. ~~The results indicate that OT is present in the neurosecretory paraventricular (Pa) and supraoptic nuclei (SON) hypothalamic cells~~ The results indicate that OT is present in cells of the neurosecretory paraventricular (Pa) and supraoptic hypothalamic nuclei (SON). From the anterior SON, OTergic cells extend into the medial

amygdala, where a sparse cell population occupies its ventral anterior and posterior divisions. Co-expression of OT and AVP in these nuclei is rare. Moreover, a remarkable OTergic cell group is found near the ventral bed nucleus of the stria terminalis (BST), distributed between the anterodorsal preoptic nucleus and the nucleus of anterior commissure (ADP/AC). This cell group, the rostral edge of the Pa and the periventricular hypothalamus display frequent OT + AVP double labelling, with a general dominance of OT over AVP immunoreactivity. Fibres with similar immunoreactivity profile innervate the accumbens shell and core, central amygdala and portions of the intervening BST. These data, together with data in the literature on rats, suggest that the projections of ADP/AC nonapeptidergic cells onto these brain centres could promote pup-motivated behaviours and inhibit pup avoidance during motherhood.

AQ1
AQ2
AQ3
AQ4
AQ5

Keywords

- Amygdala
- Bed nucleus of the stria terminalis
- Accumbens
- Maternal behaviour
- Sociosexual brain
- Neurosecretory cells

Abbreviations

10N	Dorsal motor nucleus of the vagus
12N	Hypoglossal nucleus
3V	Third ventricle
4n	Trochlear nerve or its root
4V	4th ventricle
A1	A1 no adrenaline no radrenaline cells
AC	Anterior commissural nucleus

AC	Anterior commissural nucleus
aca	Anterior commissure, anterior part
Acb	Accumbens nucleus
AcbC	Accumbens nucleus, core
AcbSh	Accumbens nucleus, shell
ACo	Anterior cortical amygdaloid nucleus
acp	Anterior commissure, posterior part
AD	Anterodorsal thalamic nucleus
ADP	Anterodorsal preoptic nucleus
AHA	Anterior hypothalamic area, anterior part
AHP	Anterior hypothalamic area, posterior part
AIP	Agranular insular cortex, posterior part
AM	Anteromedial thalamic nucleus
Amb	Ambiguus nucleus
AN	Accessory nuclei
AP	Area postrema
APir	Amygdalopiriform transition area
Aq	Aqueduct (Sylvius)
Arc	Arcuate hypothalamic nucleus
AStr	Amygdalostratial transition area
AV	Anteroventral thalamic nucleus
AVP	Arginine vasopressin
AVPe	Anteroventral periventricular nucleus
AVP-ir	Arginine vasopressin immunoreactive
AVV	Anteroventral thalamic nucleus, ventral part
BAC	Bed nucleus of the anterior commissure
BAOT	Bed nucleus of the accessory olfactory
Bar	Barrington's nucleus
BLA	Basolateral amygdaloid nucleus, anterior
BLV	Basolateral amygdaloid nucleus, ventral
BMA	Basomedial amygdaloid nucleus, anterior
BST	Bed nucleus of the stria terminalis
BSTA	Bed nucleus of the stria terminalis, anterior part

BSTIA	Bed nucleus of the stria terminalis, intraamygdaloid division
BSTLD	Bed nucleus of the stria terminalis, lateral division, dorsal part
BSTLP	Bed nucleus of the stria terminalis, lateral division, posterior part
BSTLV	Bed nucleus of the stria terminalis, lateral division, ventral part
BSTMA	Bed nucleus of the stria terminalis, medial division, anterior part
BSTMP	Bed nucleus of the stria terminalis, medial division, posterior part
BSTMPI	Bed nucleus of the stria terminalis, medial division, posterointermediate part
BSTMPL	Bed nucleus of the stria terminalis, medial division, posterolateral part
BSTMPM	Bed nucleus of the stria terminalis, medial division, posteromedial part
BSTMV	Bed nucleus of the stria terminalis, medial division, ventral part
BSTS	Bed nucleus of the stria terminalis, supracapsular part
cc	Corpus callosum
CC	Central canal
Ce	Central amygdaloid nucleus
CeC	Central amygdaloid nucleus, capsular part
CeL	Central amygdaloid nucleus, lateral division
CeM	Central amygdaloid nucleus, medial division
CeMAD	Central amygdaloid nucleus, medial division, anterodorsal part
CeMAV	Central amygdaloid nucleus, medial division, anteroventral part
CGPn	Central grey of the pons
Cl	Clastrum

CL	Centrolateral thalamic nucleus
CM	Central medial thalamic nucleus
cp	Cerebral peduncle, basal part
CPu	Caudate putamen (striatum)
cst	Commissural stria terminalis
Cu	Cuneate nucleus
CxA	Cortex–amygdala transition zone
D3V	Dorsal third ventricle
DEn	Dorsal endopiriform nucleus
DG	Dentate gyrus
DLPAG	Dorsolateral periaqueductal grey
DM	Dorsomedial hypothalamic nucleus
DMPAG	Dorsomedial periaqueductal grey
DMTg	Dorsomedial tegmental nucleus
DP	Dorsal peduncular cortex
DpMe	Deep mesencephalic nucleus
DRC	Dorsal raphe nucleus, caudal part
DRI	Dorsal raphe nucleus, interfascicular part
DTg	Dorsal tegmental nucleus
DTgP	Dorsal tegmental nucleus, pericentral part
DTM	Dorsal tuberomammillary nucleus
DTT	Dorsal tenia tecta
ECu	External cuneate nucleus
EW	Edinger–Westphal nucleus
f	Fornix
F	Nucleus of the fields of Forel
FG	Fluorogold
fi	Fimbria of the hippocampus
fmi	Forceps minor of the corpus callosum
fr	Fasciculus retroflexus
GrDG	Granular layer of the dentate gyrus
Gus	Gustatory thalamic nucleus
	Nucleus of the horizontal limb of the diagonal

HDB	band
I	Intercalated nuclei of the amygdala
IAD	Interanterodorsal thalamic nucleus
ic	Internal capsule
ICj	Islands of Calleja
ICjM	Islands of Calleja, major island
ICjvm	Islands of Calleja, ventromedial island
icp	Inferior cerebellar peduncle
IG	Indusium griseum
IL	Infralimbic cortex
IM	Intercalated amygdaloid nucleus, main part
In	Intercalated nucleus of the medulla
IO	Inferior olive
IOD	Inferior olive, dorsal nucleus
IPAC	Interstitial nucleus of the posterior limb of the anterior commissure
IPACL	Lateral interstitial nucleus of the posterior limb of the anterior commissure
IPACM	Medial interstitial nucleus of the posterior limb of the anterior commissure
IRt	Intermediate reticular nucleus
KF	Kölliker-Fuse nucleus
La	Lateral amygdaloid nucleus
LA	Lateroanterior hypothalamic nucleus
LaDL	Lateral amygdaloid nucleus, dorsolateral part
LaVL	Lateral amygdaloid nucleus, ventrolateral part
LaVM	Lateral amygdaloid nucleus, ventromedial part
LC	Locus coeruleus
LDTg	Laterodorsal tegmental nucleus
LDTgV	Laterodorsal tegmental nucleus, ventral part
LEnt	Lateral entorhinal cortex
LGP	Lateral globus pallidus
LH	Lateral hypothalamic area

LHb	Lateral habenular nucleus
lo	Lateral olfactory tract
LPBE	Lateral parabrachial nucleus, external part
LPBS	Lateral parabrachial nucleus, superior part
LPBV	Lateral parabrachial nucleus, ventral part
LPMR	Lateral posterior thalamic nucleus, mediodorsal part
LPO	Lateral preoptic area
LRt	Lateral reticular nucleus
LS	Lateral septum
LSD	Lateral septal nucleus, dorsal part
LSI	Lateral septal nucleus, intermediate part
LSV	Lateral septal nucleus, ventral part
LV	Lateral ventricle
maopt	Medial accessory optic tract
MCLH	Magnocellular nucleus of the lateral hypothalamus
MCPO	Magnocellular preoptic nucleus
MD	Mediodorsal thalamic nucleus
MdD	Medullary reticular nucleus, dorsal part
MdV	Medullary reticular nucleus, ventral part
Me	Medial amugdaleid amygdaloid nucleus
Me5	Mesencephalic trigeminal nucleus
MeA	Medial amygdaloid nucleus, anterior part
MeAD	Medial amygdaloid nucleus, anterior dorsal part
MeAV	Medial amygdaloid nucleus, anteroventral part
MePD	Medial amygdaloid nucleus, posterodorsal part
MePV	Medial amygdaloid nucleus, posteroventral part
mfb	Medial forebrain bundle
MGP	Medial globus pallidus (entopeduncular nucleus)
MHb	Medial habenular nucleus
ml	Medial lemniscus
mlf	Medial longitudinal fasciculus
MnPO	Median preoptic nucleus

MPA	Medial preoptic area
MPB	Medial parabrachial nucleus
MPO	Medial preoptic nucleus
MPOL	Medial preoptic nucleus, lateral part
MPOM	Medial preoptic nucleus, medial part
MS	Medial septal nucleus
mt	Mamilliothalamic Mammillothalamic tract
mtg	Mamillotegmental Mammillotegmental tract
MTu	Medial tuberal nucleus
MVe	Medial vestibular nucleus
mvStP	Medioventral striato-pallidum
NADPHd	Nicotinamide adenine dinucleotide phosphate diaphorase
Ns ns	Nigrostriatal bundle
O	Nucleus O
opt	Optic tract
OT	Oxytocin
<u>OT-ir</u> Here we should add the abbreviation OTR for "Oxytocin receptor"	Oxytocin-like immunoreactive (referring to immunostaining using antibodies raised against oxytocin or against its specific neurophysin) OTR Oxytocin receptor
PaAP	Paraventricular hypothalamic nucleus, anterior parvicellular
PAG	Periaqueductal grey
PaL	Paraventricular hypothalamic nucleus, lateral part
PaM	Paraventricular hypothalamic nucleus, medial part
PaPo	Paraventricular hypothalamic nucleus, posterior part
PaV	Paraventricular hypothalamic nucleus, ventral part
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PC	Paracentral thalamic nucleus

Pc	Posterior commissure
PCom	Nucleus of the posterior commissure
Pe	Periventricular hypothalamic nucleus
PeF	Perifornical nucleus
PF	Parafascicular thalamic nucleus
PH	Posterior hypothalamic area
Pir	Piriform cortex
PLCo	Posterolateral cortical amygdaloid nucleus
PMCo	Posteromedial cortical amygdaloid nucleus
PMD	Premammillary nucleus, dorsal part
PMn	Paramedian reticular nucleus
PMnR	Paramedian raphe nucleus
PMV	Premammillary nucleus, ventral part
PnC	Pontine reticular nucleus, caudal part
PO	Periolivary region
Pr5	Principal sensory trigeminal nucleus
PRh	Perirhinal cortex
PrL	Prelimbic cortex
PSTh	Parasubthalamic nucleus
PT	Paratenial thalamic nucleus
PV	Paraventricular thalamic nucleus
PVA	Paraventricular thalamic nucleus, anterior part
Py	Pyramidal cell layer of the hippocampus
Rad	Stratum radiatum of the hippocampus
Re	Reuniens thalamic nucleus
RI	Rostral interstitial nucleus of the medial longitudinal fasciculus
RLi	Rostral linear nucleus of the raphe
RMg	Raphe magnus nucleus
RPF	Retroparafascicular nucleus
RRF	Retrosubthalamic field
rs	Rubrospinal tract
Rt	Reticular thalamic nucleus

SCh	Suprachiasmatic nucleus
SCO	Subcommissural organ
scp	Superior cerebellar peduncle
SFi	Septofimbrial nucleus
SFO	Subfornical organ
Shi	Septohippocampal nucleus
SHy	Septohypothalamic nucleus
SI	Substantia innominata
SL	Semilunar nucleus
SLu	Stratum lucidum, hippocampus
SM	Nucleus of the stria medullaris
sm	Stria medullaris of the thalamus
SN	Substantia nigra
Sol	Nucleus of the solitary tract
sol	Solitary tract
SON	Supraoptic nucleus
SOR	Supraoptic nucleus, retrochiasmatic part
SP	Substance P
sp5	Spinal trigeminal tract
st	Stria terminalis
StA	Striatal part of the preoptic area
Su3	Supraoculomotor periaqueductal grey
Su5	Supratrigeminal nucleus
SubB	Subbrachial nucleus
SubC	Subcoeruleus nucleus
TBS	TRIS-buffered saline
TC	Tuber cinereum area
Te	Terete hypothalamic nucleus
Tu	Olfactory tubercle
Unc	Uncinate fasciculus
V1aR	Arginine vasopressin receptor type 1a
V1bR	Arginine vasopressin receptor type 1b
VA	Ventral anterior thalamic nucleus

VDB	Nucleus of the vertical limb of the diagonal band
VEn	Ventral endopiriform nucleus
VLPO	Ventrolateral preoptic nucleus
VM	Ventromedial thalamic nucleus
VMH	Ventromedial hypothalamic nucleus
VMHC	Ventromedial hypothalamic nucleus, central part
VMHDM	Ventromedial hypothalamic nucleus, dorsomedial part
VMHVL	Ventromedial hypothalamic nucleus, ventrolateral part
VMPO	Ventromedial preoptic nucleus
VOLT	Vascular organ of the lamina terminalis
VP	Ventral pallidum
vsc	Ventral spinocerebellar tract
VTa	Ventral tegmental area
VTg	Ventral tegmental nucleus
VTM	Ventral tuberomammillary nucleus
VTT	Ventral tenia tecta
Xi	Xiphoid thalamic nucleus
ZI	Zona incerta

Introduction

The nonapeptides oxytocin (OT) and vasopressin (AVP) are two well-characterized and closely related neurohypophyseal peptides. They evolved by duplication from an ancestor gene, only differ in two amino acids, and their functions are highly conserved among vertebrates (Caldwell and Young 3rd 2006; Choleris et al. 2013). Both nonapeptides are synthesized in the hypothalamus and released into the bloodstream through the posterior pituitary gland. As hormones, OT promotes uterine contraction during childbirth and milk ejection during lactation, whereas AVP increases blood pressure via antidiuretic and vasoconstrictor effects. In addition, they act centrally as neurotransmitters and neuromodulators (Stoop 2012). Although they cannot cross the blood–brain barrier (Neumann and Landgraf 2012), OT and AVP are known to be released

from the soma and dendrites of neuroendocrine cells (Sabatier et al. 2004; Ludwig and Leng 2006; Tobin et al. 2012) and also from axons using synaptic mechanisms. Studies over the last decades have provided solid evidence supporting a central role of OT and AVP in the control of different aspects of a wide range of social and non-social behaviours, including pair bonding, maternal attachment, olfaction and sensory processing, fear and anxiety, stress coping, homeostasis, and learning and memory in multiple vertebrate species for excellent reviews see (Donaldson and Young 2008; Carter et al. 2008; Meyer-Lindenberg et al. 2011; Neumann and Landgraf 2012; Stoop 2012). To understand the neurobiology of social behaviour and its concomitant physiological components, we are in need of a detailed description of the central distribution of both neuropeptides and their receptors.

Rodents have been extensively used for research on the neurobiology of social behaviour (Toth and Neumann 2013; Bosch 2011; Nephew and Bridges 2008; Takahashi and Miczek 2013). The large amount of neuroanatomical data available from rats confirm that the neural network that controls socio-sexual behaviour, the so-called socio-sexual brain (as defined by Newman 1999), shows a remarkable density of receptors for AVP and/or OT (Veinante and Freund-Mercier 1997) as well as important innervation by AVPergic fibres (DeVries et al. 1985). Although OT-cell bodies have been mapped (e.g. Hou-Yu et al. 1986), data on the distribution of OT fibres are scarce, even in the brain of rats.

Mice are becoming the species of choice for the research on social neurobiology. On the one hand, pheromones eliciting specific socio-sexual responses are being identified for the mouse (Chamero et al. 2007; Nodari et al. 2008; Roberts et al. 2010; Isogai et al. 2011) and, on the other, the use of genetically engineered mice are generating very useful data (Caldwell et al. 2008; Nishimori et al. 2008; Pobbe et al. 2012; Egashira et al. 2007; Bielsky et al. 2005; Dölen et al. 2013; Jin et al. 2007). However, neuroanatomical data are fragmentary and scarce in mice, as compared to rats. Concerning nonapeptides, several good descriptions of the distribution of AVP have recently been published (Rood and de Vries 2011a; Rood et al. 2013; Otero-Garcia et al. 2014). However, OT distribution was only described by Castel and Morris (1988) using immunohistochemistry for the accompanying neurophysin (type I). A reanalysis of this issue in the light of new anatomical and functional data is

urgently needed. Concerning the receptors for nonapeptides, Dubois-Dauphin et al. (1996) studied the binding sites for radiolabelled AVP agonists in mice, and Insel et al. (1993) and Hammock and Levitt (2013) have reported maps of the pattern of OT receptor binding in the brain of embryos, and postnatal and adult mice.

As discussed by Otero-Garcia et al. (2014), a comparison of the distribution of the OT/AVP receptors and nonapeptidergic innervation evidences a certain level of mismatch between the pre- and post-synaptic elements. Such mismatches can be attributed to receptor promiscuity, but also to extra-synaptic slow signalling through volume transmission. In fact, neuropeptides possess some peculiarities with respect to canonic neurotransmitters: they are not recycled at the synapse (Liu et al. 1994) and may diffuse through the extracellular fluid (Zoli and Agnati 1996). In addition, they can be released extra-synaptically by exocytosis and spread through the extracellular space, triggering long duration events on large groups of distant target neurons (Landgraf and Neumann 2004; Ludwig and Leng 2006; Trueta and De-Miguel 2012). Landgraf and Neumann (2004) proposed that the combination of wired and diffuse signalling confers the high regulatory capacity that neuropeptides need to mediate long-term modulation of complex neural systems.

In a recent review, Stoop (2012) demonstrated a close interplay between AVP and OT modulatory action in different functional systems of the brain, including relay stations of the olfactory systems, the key nuclei for social recognition and mating (medial amygdala), as well as in the central amygdala, where they may be involved in the decrease of anxiety associated with motherhood (Knobloch et al. 2012). In this scenario, identifying the site of release of both nonapeptides and their relative amounts is crucial to understand their functionality (Merighi et al. 1989). This is further complicated by a certain promiscuity of OT receptors, as they bind AVP and OT with similar affinity (see Manning et al. 2012), so that OTR can be considered as a receptor for both neuropeptides.

In this respect, it must be emphasized that although AVP and OT are usually considered to be expressed in different, segregated populations of neurons, there is evidence that the majority of magnocellular neurons co-express both peptide messenger RNAs in varying amounts (Glasgow et al. 1999; Xi et al. 1999). This co-expression is functionally relevant as

demonstrated by the fact that it is regulated during physiologically relevant conditions like water deprivation (Telleria-Diaz et al. 2001) or maternity (Mezey and Kiss 1991). However, [a description of the basal pattern of co-expression of both peptides in neurosecretory and centrally projecting cells and their processes is still lacking.](#)

Therefore, the aim of the present work is to provide a precise characterization of the distribution of OT-immunoreactive elements in the brain of mice and compare their distribution with that of AVP. Also, we analyse the possible co-localization of AVP and OT in the neurosecretory cell groups of the hypothalamus as well as in centrally projecting cells and their processes in different regions of the brain.

Materials and methods

Animals

For the present study, we used 16 adult mice (12 virgin females, 9–16 weeks of age; 4 males of the same age) of CD1 strain (Harlan, Barcelona, Spain; Janvier-Europe, Le Genest Saint Isle, France). The animals were treated according to the European Union Council Directive of June 3, 2010 (6106/1/10 REV1) and the procedures were approved by the Committee of Ethics on Animal Experimentation of the University of València.

Perfusion and sectioning

The animals were deeply anaesthetized using an overdose of pentobarbital (i.p. injection of 9 $\mu\text{L/g}$ of body weight) of the solution as reported by Shipley and Adamek (1984) and transcardially perfused with saline solution (5.5 ml) followed by 66 ml of 4 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at a rate of 5.5 ml/min. Brains were carefully removed from the skull, postfixed in the same fixative for 4 h and placed in 30 % sucrose (in 0.01 M phosphate-buffered saline, pH 7.6, PBS) until they sank. A longitudinal incision was performed in the right cerebral cortex to identify that side of the brain in each histological section. Then, brains were frozen and 40- μm -thick coronal sections were obtained with a freezing microtome (Microm HM-450, Walldorf, Germany). Free-floating sections were collected in four parallel series and frozen in phosphate-buffered 30 % sucrose (0.1 M pH 7.4) for their later processing.

ABC immunoperoxidase staining

For the description of the distribution of OT immunoreactivity, we obtained permanent immunoperoxidase preparations from ten virgin females and two virgin males using the indirect avidin–biotin complex procedure. Briefly, sections were incubated sequentially in: (1) 1 % hydrogen peroxide (H_2O_2) in 0.05 M TRIS-buffered saline, pH 7.6 (TBS), for 20 min at room at 25 °C for endogenous peroxidase inactivation; (2) diluted primary antibody (Table 1) in TBS, with 0.3 % Triton X-100, 1 % bovine serum albumin (BSA) and 4 % normal goat serum overnight at 4 °C; (3) diluted biotinylated secondary antibody (Table 1) in TBS for 90 min at room temperature (25 °C); and (4) avidin–biotin–peroxidase complex (ABC Elite kit; Vector Labs, PK-6200) in TBS for 90 min at room temperature. After each step, the sections were washed in TBS (4×15 min). After ABC incubation, the sections were rinsed in TBS (2×15 min) and TRIS buffer 0.05 M, pH 8 (TB, 2×10 min), prior to the histochemical detection of the resulting peroxidase activity. This was done by incubation in 0.003 % H_2O_2 and 0.025 % 3,3'-diaminobenzidine (Sigma) in TB for about 20 min. The sections were rinsed thoroughly in TB and mounted onto gelatinized slides, dehydrated in ethanol, cleared with xylene and coverslipped with Entellan.

Table 1

Antibodies and immunoprocures

Primary antibody	Dilution	Secondary antibody	Dilution	Tertiary
<i>Mouse anti-Oxytocin</i> , monoclonal IgG (Dr. Harold Gainer, NIH Cat#PS38) Antigen: Oxytocin-specific neurophysin Immunogen: Rat posterior pituitary extract, KLH coupled	1:600	Biotinylated goat anti-mouse IgG (Vector Labs, BA-9200)	1:200	ABC Elite kit (Vector Labs, PK-6200)
	1:200	Rhodamine Red X-conjugated goat anti-mouse IgG (Invitrogen R6393)	1:250	
<i>Rabbit anti-Vasopressin</i> IgG (Millipore Cat#AB1565) Antigen: Full-length vasopressin Immunogen: Synthetic	1:2500	Alexa Fluor 488-conjugated Goat anti-rabbit IgG (Jackson ImmunoResearch, 111-545-003)	1:300	

vasopressin conjugated to thyroglobulin				
<i>Rabbit anti-Oxytocin</i> IgG (Millipore Cat#AB911) Antigen: Full-length oxytocin Immunogen: Synthetic oxytocin conjugated to thyroglobulin	1:25,000	Biotinylated goat anti-rabbit IgG (Vector Labs, BA-1000)	1:200	ABC Elite kit (Vector Labs, PK-6200)
	1:8000	Alexa Fluor 488-conjugated Goat anti-rabbit IgG (Jackson ImmunoResearch, 111-545-003)	1:300	

One of the four parallel series of each specimen was processed for the immunodetection of OT. In 6 out of the 12 specimens, we used the polyclonal primary antibody raised against OT from Millipore (Cat#AB911; see Table 1); in the remaining six animals, we employed the monoclonal primary antibody raised against OT-specific neurophysin from H. Hainer (NIH Cat#PS38; see Table 1). As explained in “Results”, immunolabelling with both primary antibodies was indistinguishable. In two females and two males, a second of the four parallel series was also processed for OT immunodetection.

Immunofluorescence

For simultaneous immunolabelling of oxytocin and vasopressin, we employed immunofluorescence methods. We used two of the four parallel series obtained from two females and two males. The sections were incubated sequentially in: (1) 1 % sodium borohydride in TBS at room temperature (RT, approximately 25 °C) for 30 min; (2) 0.05 M TRIS-buffered saline, pH 7.6 (TBS), with 0.3 % Triton X-100 and 4 % normal serum of the species in which the secondary antibody was raised at RT for 1 h; (3) primary antibodies, diluted as indicated in Table 1, in TBS with 0.2 % Triton X-100 and 4 % normal serum of the species in which the secondary antibody was raised for 48 h at 4 °C; (3) fluorescent-labelled secondary antibodies diluted as indicated in Table 1, in TBS, for 90 min at RT; (4) 4',6-diamidino-2-phenylindole (DAPI) 600 nM in TBS for 5 min at room temperature. After each step, the sections were washed in TBS (except between steps (2) and (3)) I would remove the brackets indicating the steps 2 and 3 and would close the brackets opening after "TBS". As follows:

"....TBS (**except between steps 2 and 3**)" The sections were finally rinsed thoroughly in TB and mounted onto gelatinized slides and coverslipped with fluorescence mounting medium (Dako, Glostrup, Denmark).

AQ6

Immunohistochemistry analyses

DAB immunohistochemistry was studied using a microscope Leitz DMRB (Leica AG, Germany) with a digital camera (Leica DFC300 FX), obtaining photomicrographs of specific areas within the sections. The background was subtracted and the resulting images processed with ImageJ (National Institutes of Health, USA) to improve the brightness and contrast, with no further processing. The figures were elaborated with GIMP free software.

Immunofluorescence was analysed with an Olympus FV1000 Confocal Microscope System, mounted on an inverted microscope. Triple scans were made to identify DAPI, Alexa Fluor 488 and Rhodamine Red X/Alexa fluor 546. Excitation wavelengths were 405 nm for DAPI, 488 nm for Alexa Fluor, and 488 and 559 nm for Rhodamine Red X and Alexa 546. Emission wavelengths were 461, 520, 591 and 573, respectively.

ZOptical sections with a **Z** distance between 1.5 and 4 μm of separation were taken at $\times 100$, $\times 200$ and $\times 600$ magnifications through the regions of interest. To minimize channel spillover, the images were sequentially acquired and saved as OIF and TIF files. The stacks obtained were further processed with ImageJ to optimize the brightness and contrast. No manipulation of individual image elements was performed. The figures were elaborated with GIMP software.

Results

The antibodies against OT and OT-specific neurophysin employed in the present work (see Table 1) revealed an identical distribution of cells and fibres (Figs. 1, 2), thus confirming previous studies of antibody specificity (Ben-Barak et al. 1985; Whitnall et al. 1985). Oxytocin and its neurophysin are co-localized at the subcellular level (Belenky et al. 1992) and immunostaining of both peptides is undistinguishable (Butovsky et al. 2006). We will therefore refer jointly to these immunolabellings as OT-like immunoreactivity (OT-ir). In addition, removing the primary antibody resulted in a complete lack of labelling. In the double immunofluorescent labelling experiment, removing the primary antibody for either OT or AVP

resulted also in lack of labelling for the corresponding peptide. Sequential acquisition of the confocal images and posterior examination of labelled elements allowed us to discard crossed immunoreactivity of the antibodies detecting AVP and OT: there were singly (either OT- or AVP-ir) and doubly labelled structures intermingled in many regions of the samples (see Figs. 3, 4, 5 and 6).

Fig. 1

Distribution of oxytocin-like immunoreactivity in the brain of CD1 mice. Semi-schematic camera lucida drawings of specific regions of selected coronal sections through the left hemisphere of a representative case (a female; case M1225). The approximate rostro-caudal level of each section is indicated on a schematic sagittal view of the mouse brain and the distance to bregma in mm is indicated in each section. Immunostained somata are shown as *solid blue circles* and thick and OT-ir fibres are represented as *thick and thin red lines* to reflect their actual appearance. Details of framed areas are shown in Fig. 6. Scale bars 500 μ m

Fig. 2

Details of OT elements in immunoreacted coronal sections of the brain of female mice (anteroposterior level is indicated as distance from bregma in mm). Picture **a** corresponds to an anteroposterior level similar to Fig. 1 e. In the framed area, shown in (**a'**), AC/ADP immunoreactive cells appear closely associated to a blood vessel crossing vertically. **b** A caudal level of the Pa and lateral hypothalamic OT-ir accessory group. The framed area containing the AN somata is illustrated in (**b'**). **c** Section of the medial hypothalamus showing the AN of the DMH. **c'** Corresponds to the framed area of the DMH containing OT-ir cells. **d** The LPB contains highly OT-ir fibres in its ventrolateral portion. The beaded and twisted aspect of these OT-ir fibres is shown in (**d'**). Scale bar in (**a**) (valid for **a–d**), 200 μ m. Scale bar in **a'** (valid for **a'–d'**), 40 μ m

Fig. 3

Simultaneous immunofluorescence for oxytocin-related neurophysin (*red*) and vasopressin (*green*) in two coronal sections through the left preoptic

area of a female mouse (distance from bregma is indicated in mm). In picture **a**, the rostral-most nonapeptidergic cells of the AC/ADP appear between the ventral BSTM and the dorsal MPA. In **b**, a slightly more caudal section shows the rest of AC/ADP immunoreactive somata occupying the dorsal MPA, confined between the ventral BSTM, the BAC and the ADP. **a'**, **b'** Confocal sections through the AC/ADP group show a majority of double-stained cells (*white arrows*) displaying intense OT labelling plus mild AVP labelling. OT immunoreactivity of similar intensity concentrates in the soma periphery and processes, whereas AVP immunoreactivity concentrates in the soma centre and shows variably intensity among cells. **a''**, **b''** Singly OT-ir (*black arrowheads*), single AVP-ir (*white arrowheads*) and doubly labelled fibres (*white arrows*) appear in the strial part of the preoptic area. **a'''** In the periventricular nucleus (Pe), somata show a pattern of double staining, similar to that of AC/ADP (intensely stained for OT, variably stained for AVP). **b'''** ~~The~~At the transition between the AC/ADP and the nonapeptidergic cells of the rostral Pa. ~~At this level of the Pe, there are~~ shows singly labelled AVP-ir cells plus a variety of doubly labelled neurons. In addition to the AC/ADP somata, single OT- or AVP-immunoreactive bipolar cells are clearly visible in the medial border of the BSTMPM (**a**)(**a**), ~~. c A high magnification view of the medial BSTMPM to illustrate singly AVP-ir (white arrowhead) and singly OT-ir (black arrowhead) labelled neurons~~as illustrated at higher magnification in **c** (singly AVP-ir, *white arrowhead*; singly OT-ir, *black arrowhead*). The two low-power images (**a**, **b**) are the sum of two confocal slices, separated 4 μm in the *z* axis, acquired sequentially with a 10 $\times$ objective. The cytoarchitecture is inferred from DAPI staining of the same sections (not shown). *Scale bar* in **a** 100 μm (valid also for **b**). *Scale bar* in **c** 30 μm (valid also for **a'**–**a'''**, **b'**–**b'''**)

Fig. 4

Simultaneous immunolabelling of oxytocin (*red*) and vasopressin (*green*) in three coronal sections of the left paraventricular nucleus (Pa) of a female mouse (anteroposterior level is indicated as distance from bregma in mm). **a**, **a'** The rostral Pa contains double-labelled cells similar to the ones in AC/ADP (*white arrows*), as well as single OT-ir cells (*black arrowheads*) and single AVP-ir cells (*white arrowheads*). The anterior and lateral hypothalamus ~~show also~~ show OT-ir, AVP-ir and double-stained somata at this level. **b**, **b'** In the middle area of the Pa, double-labelled somata are infrequent. AVP-ir cells gather in the lateral subnucleus of the Pa (PaL), while OT-ir somata predominate in ~~theits~~ medial subdivision ~~of the Pa~~ (PaM). **a''**, **b''** Intensely double-stained cells appear in the ventro-medial portion of the Pa and adjacent periventricular region (Pe). **c** The caudal Pa (PaPo) contains only OT-ir somata and some AVP-ir processes. Low-magnification images (**a–c**) are the sum of two confocal slides, separated by 4 μm in the *z* axis, acquired sequentially with a 10 $\times$ objective. The cytoarchitecture is inferred from DAPI staining of the same sections (not shown). Scale bar in **a** 100 μm (also valid for **b**, **c**). Scale bar in **a** 30 μm (also valid for **a/b'''**)

Fig. 5

Simultaneous immunolabelling of oxytocin (*red*) and vasopressin (*green*) in three coronal sections of the left supraoptic nucleus (SON) of a female mouse (anteroposterior level is indicated as distance from bregma in mm). **a** The rostral SON contains mostly OT-ir cells, situated in the dorsomedial region of the nucleus, whereas *fewer* AVP-ir somata are located in the ventrolateral portion of the SON. OT-ir (*black arrowhead*), AVP-ir (*white arrowhead*) as well as double-stained somata (**a'**, *white arrow*) appear in the lateral hypothalamic region at this rostro-caudal level. **b** AVP-ir cells arrange in the ventrolateral and caudal regions of the SON. **b'** Scarce double-labelled somata appear at medio-dorsal locations. **c** The caudal SON contains almost exclusively AVP-ir cells, alike the adjacent hypothalamic area. Scattered OT-ir (*black arrowhead*) and AVP-ir (*white arrowhead*) cells appear in the medial border of the nearby anterior medial amygdala (MeA). **d** At caudal levels of the SON, abundant AVP-ir and few OT-ir processes enter the medial amygdala edge. **d'**, **d''** Some single OT-ir (*black arrowhead*), AVP-ir (*white arrowhead*) and double immunoreactive somata (*white arrow*) appear in the medial portion of the anterior medial amygdala. **e**, **e'** In the retrochiasmatic supraoptic nucleus (SOR), abundant AVP-ir cells (*white arrowheads*) and processes extend along the ventral surface, next to

the optic tract, while few OT-ir somata (*black arrowheads*) and fibres are located immediately dorsal to the AVP-ir elements. Low-magnification images are the sum of two confocal slides, separated 4 μm in the z axis, acquired sequentially with a 10 $\times$ objective. The cytoarchitecture is inferred from DAPI staining of the same sections (not shown). *Scale bar* in **a** 100 μm (also valid for **b–e**). *Scale bar* in **a'** 30 μm (also valid for **b'**, **d'**, **d''**, **e'**). *Scale bar* in **d'''** 5 μm

Fig. 6

Simultaneous immunolabelling of oxytocin (*red*) and vasopressin (*green*) in coronal sections of five selected areas displaying doubly stained processes in the left hemisphere of a female mouse (anteroposterior level is indicated as distance from bregma in mm). Approximate location of the selected areas is framed in Fig. 1 a–c, j. ~~Anteroposterior level is indicated in each area as distance from bregma in mm.~~ **a** In the rostral-most septal area, straight and thick OT-ir processes display also very weak AVP immunoreactivity (*white arrow* in **a'**). Occasional AVP-ir fibre tangles show low OT immunoreactivity near the dorsal taenia tecta (DTT) and septohippocampal nucleus (Shi) (*white arrows* in **a''**). Some fibres show only OT (*black arrowhead*, **a''**) or only AVP (*white arrowhead*, **a'**) immunoreactivity. **b** In the nucleus accumbens (Acb), doubly labelled long processes display different levels of immunoreactivity for OT and AVP (**b'**, **b'''**, *white arrows*), with most of the fibres being highly immunoreactive for both peptides (**b''**, *white arrows*). The medially adjacent ventral diagonal band (VDB) shows an AVP-ir terminal field which continues dorsally into the medial septum (**b**). **c**, **d** Ventrally to the anterior commissure, at its caudal edge, OT-ir processes of different diameters appear in the anterior medial bed nucleus of the stria terminalis (BSTMA) and strial part of the medial preoptic area (StA). Most of these OT-ir processes also show mild AVP immunoreactivity (**c'**, **d'**, *white arrows*). Some highly AVP-ir elements appear intermingled and show no OT immunoreactivity (**c'**, **d'**, *white arrowheads*). **e** In the central amygdala (Ce), long immunoreactive processes of different calibres display AVP and OT immunoreactivity. Most of the fibres are immunoreactive for both OT and AVP (*white arrows*, **e'** **e''**). Intensity of OT and AVP immunoreactivity is variable, with some processes labelled only for OT (*black arrowhead*) or AVP (*white arrowhead*) (**e'**). The cytoarchitecture is inferred from DAPI staining of the same sections (not shown). *Scale bar* in **a** 100 μ m (also valid for **b–e**). *Scale bar* in **a'** (valid for all the *insets*), 10 μ m

Distribution of OT immunoreactivity was indistinguishable between the males and the females analysed, thus suggesting that, in basal

physiological conditions, there is no apparent sexual dimorphism in the distribution of OT. Likewise, and in spite of the sexually dimorphic distribution of AVP (Rood ~~and de Vries~~ et al. ~~2011b~~2013; Otero-Garcia et al. 2014), the distribution of cells and fibres in which AVP + OT are co-localized was similar in the two males and two females analysed.

For the description of the results we will follow the atlas by Paxinos and Franklin (2001). We will first describe and illustrate the distribution of oxytocin-immunoreactive (OT-ir) cell bodies and processes based on our material of indirect (ABC) immunoperoxidase. Then, we will report the co-distribution of OT and AVP using simultaneous immunofluorescence for both peptides.

Distribution of OT immunoreactivity

Our results fit and extend previous anatomical findings on the distribution of OT-related neurophysin in the brain of mice (Castel and Morris 1988). We will first describe the location of OT-ir cell bodies, followed by the distribution of OT-ir neural processes.

Distribution of OT-ir cell bodies

Most OT-ir somata are grouped in four locations: (a) a cell group located next to the anterior commissure, extending into the BST; (b) cell bodies in the paraventricular hypothalamic nucleus (Pa); (c) the supraoptic nucleus (SON); and (d) the medial amygdala (Me). In addition, scattered cells appear in different hypothalamic locations, most of which in the proximity to these cell groups and surrounding the walls of the third ventricle (3V).

The most rostral group of OT-ir cells is located at the boundary between the extended amygdala and the preoptic area, next to the anterior commissure. This cell group was named as the anterior commissural nucleus by Rhodes et al. (1981), or the rostral paraventricular nucleus by Hou-Yu et al. (1986) in their descriptions of the OT-ir cells in the rat. However, using the atlas of the mouse brain by Paxinos and Franklin (2001), this OT-ir cell group seems to be located neither in the bed nucleus of the anterior commissure (BAC) nor in the paraventricular nucleus, but in an ill-defined, unnamed region ventral to the BAC, medial to the bed nucleus of the stria terminalis medial division (BSTM) and lateral to the anterodorsal preoptic nucleus (ADP; Figs. 1 e, 2 a, 3 a, b).

Following our previous description of the distribution of AVP (Otero-Garcia et al. 2014), we name this cell group as the ADP/AC. The OT-ir cells of the ADP/AC form a compact group, which occasionally is in close apposition to large-calibre blood vessels (Fig. 2 a, a'). A close look into the OT-ir immunofluorescence in this region combined with DAPI counterstaining (not shown) allows to perceive that some OT-ir cells extend from the ADP/AC into the posterointermediate part of the medial BST (BSTMPI), notably at rostral levels (Fig. 3 a). Outside the main group, a few cells also extend dorsally within the space between the fornix and the posteromedial BSTM (BSTMPM; Figs. 1 e, 2 a). Also ventrally, scant OT-ir cell bodies spread into the caudal medial preoptic area (MPA) and nucleus (MPO; Figs. 1 f, 2 a, 3 a, b) and in the lateral preoptic area (LPO; Fig. 2 a). Slightly more rostrally, a few OT-ir cell bodies can also be seen around the anterior edge of the third ventricle, next to the ventricular wall, in the periventricular nucleus (Pe; Figs. 1 d, e, 2 a, 3 a) and the ventromedial preoptic area (VMPO; Fig. 1 c).

In the anterior hypothalamus, dense populations of OT-ir cells are located in all the subdivisions of the Pa, with the exception of the lateral Pa (PaL; see Figs. 1, 4), where there are very few OT-ir cells (~~Fig. 1 g, h~~ Figs. 1 g, h, 2 b). Thus, abundant immunoreactive cells are observed in the anterior parvicellular (PaAP) and ventral (PaV) subdivisions, while more disperse cells appear in the medial (PaM) and posterior (PaPo) subdivisions (Fig. 1 i, j). The OT-ir cell group of the Pa appears to extend into the adjacent periventricular nucleus (Pe), where OT-ir cells usually display processes oriented parallel to the ventricular wall (Fig. 1 d–h). In fact, at those anteroposterior levels where few cells are present in the Pa, OT-ir cells are lacking in the adjacent Pe (e.g. at the level of the PaPo; Fig. 1 i, j).

In the SON, there is a cluster of OT-ir cells that show a fainter staining than those in the Pa. Within the SON, OT-ir cells are heterogeneously distributed. Most of the OT-ir neurons are located in the anterior part of the SON where they occupy the dorsal and medial aspects of the nucleus (Fig. 1 g, h). A few OT-ir small cell bodies surpass the rostral border of the SON, impinging into the ventral aspect of the MPA (ventral part of Fig. 3 a, b). The retrochiasmatic division of the supraoptic nucleus (SOR) also contains relatively few moderately stained cell bodies (Fig. 1 j). Here also, OT-ir cells are mainly located in the dorsal (deep) portion of the

nucleus (Fig. 5 e, e').

At the rostral-most levels of the SON, some OT-ir cells seem to have migrated caudolaterally, surpassing the boundaries of the nucleus, to enter the medial region of the rostral aspect of the medial amygdala (Me; Fig. 1 h–j). Thus, within the Me there is a population of OT-ir cells, especially dense in its anterior division (MeA; Fig. 5 d), although a few immunoreactive neurons are also seen in the posterodorsal one (MePD). Most of the cells there are oriented parallel to the optic tract (opt).

Between the Pa and the SON, immunoreactive somata are scattered in the lateral hypothalamic area (LH) and anterior hypothalamic area (AH; Fig. 1 g–j), being more abundant in the strip between the LH and the zona incerta (ZI), lateral to the PaPo (Figs. 1 i, 2 b). A scattered population of OT-ir somata is also observed ventral to the anterior Pa and dorsolateral to the Sch, between the medial preoptic nucleus (MPO) and the ventromedial preoptic nucleus (VMPO; Fig. 1 f). This cell group may correspond to the “mouse accessory nuclei (mAC)” described by (Castel and Morris 1988). Finally, scarce OT-ir cells appear in the ventral portion of the dorsomedial hypothalamic nucleus (DMH) (Figs. 1 k, 2 c–c'). All these scattered cells conform to the so-called nonapeptidergic accessory cells or nuclei (Krisch 1976; Castel and Morris 1988; Rood and de Vries 2011 ~~a~~, ~~b~~, ~~c~~).

Distribution of OT-ir fibres

Immunoreactive OT fibres are largely restricted to the cerebral hemispheres and hypothalamus with fewer and sparser terminal fields in the midbrain and brainstem.

In the cerebral hemispheres, no immunoreactive cells were found in the neocortex and hippocampus. In fact, OT-ir fibres in the cortex are sparse and restricted to the dorsal taenia tecta (DTT), the claustrum (Cl) and dorsal endopiriform cortex (DEn) (Fig. 1 a). Immunoreactive processes in these locations are very thick and show a characteristic twisted morphology. However, the OT-ir innervation of these structures shows a high inter-individual variability in terms of fibre density (from virtually absent to moderately dense) and, in some cases, an apparent asymmetrical distribution, with fibres nearly or completely restricted to a single hemisphere.

From the DTT, OT-ir fibres extend into the rostral tip of the septum. In relation with this, sparse linear fibres are observed in the septohippocampal (SHi) and semilunar nuclei (SL), as well as in the lateral (ventral and lateral divisions; LSV and LSI) and medial (MS) septal nuclei. Also in the subcortex, OT-ir processes are seen in the ventral striatum. Thick, intensely immunoreactive fibres appear in the medial half of the core of nucleus accumbens (AcbC; Fig. 1 a, b), whereas thin, branched, beaded fibres cross the accumbens shell (AcbSh) (Fig. 1 b). Beyond the AcbSh, a few OT-ir fibres enter the medioventral striato-pallidum (mvStP) and the cell bridges of the ventral striatum, but no immunoreactivity is observed in the olfactory tubercle (Fig. 1 b).

The terminal field in the ventral striatum extends caudally into the bed nucleus of the stria terminalis (BST) and innervates many elements of the so-called extended amygdala (see Martínez-García et al. 2012; Olucha-Bordonau et al. 2014). Within the BST, OT-ir innervation is noteworthy in the medial aspect of the anterior BST (BSTA and BSTMA; Fig. 1 c, d). An especially dense field of OT-ir processes is present in the so-called strial part of the preoptic area (StA; Fig. 1 d) where thick, dendrite-like processes are intermingled with thinner, beaded fibres (Figs. 3 a', 6 d, d'). In the posteromedial BSTM (BSTMPM), there is a network of thin fibres intermingled with thick processes that might be dendrites of the OT-ir cells of the nucleus. This network is restricted to the medial half of the BTMPM and concentrates in the cell-poor area located next to the fornix and the BAC (Figs. 1 e, 2 a). Caudally, few long OT-ir fibres appear to cross the posterior edge of the BSTMPM (Fig. 1 f).

The amygdala contains notable OT immunoreactivity confined to the central (Ce) and medial (Me) nuclei. In the Me, OT-ir fibres are concentrated in the anterior subnucleus (MeA) where there is a mixture of thick-calibre, dendrite-like processes, probably associated with the cell bodies of the MeA and/or the adjacent SON and thin axon-like fibres. These fibres and processes are mainly oriented parallel to the optic tract (opt; Fig. 1 i, j). The central amygdala shows a remarkable OT-ir fibre network composed of thick and thin beaded fibres located in all three divisions of the nucleus (medial, CeM; lateral, CeL; capsular CeC; Fig. 1 i, j). This innervation shows a high degree of inter-individual variability concerning its distribution and density. Consequently, in some individuals there is a dense field of fibres in the Ce of both hemispheres; in some

others there is an asymmetrical distribution of OT-ir fibres, which are only visible in one of the hemispheres; in others, no or very few immunoreactive fibres are observed in the Ce.

In the preoptic region, most OT-ir processes are located in the medial preoptic area (MPA). Besides the neural processes of the StA apparently originating from the group of OT-ir cells in the ADP/AC (described above; Fig. 1 d), the preoptic regions also show thick OT-ir processes in the ventral half of the caudal MPA and thinner ones in its dorsal half (Fig. 1 e, f). The rest of the preoptic region, including the lateral preoptic area (LPO) and medial preoptic nucleus (MPO), is virtually devoid of OT-ir fibres, except for the processes derived from the scattered somata of the accessory nuclei (AN) (Fig. 1 f).

At rostral levels of the anterior hypothalamus, abundant OT-ir fibres cross the anterior hypothalamic nucleus on their way to the median eminence (Fig. 1 g). These putative neurosecretory axons cross the lateral hypothalamic area (LH) and the ventrolateral tuberal region (Fig. 1 h–j). Dendrite-like processes arise from the OT-ir somata of the Pa, SON and AN (Fig. 1 g–j). In the medial hypothalamic division, only the dorsomedial hypothalamic nucleus (DMH) contains OT-ir innervation (~~Fig. 1 k~~ Figs. 1k, 2c, c'). Some fibres run through the ventral surface of the ~~medi~~medial tuberal (MTu) and ventral tuberomammillary (VTM) nuclei (Fig. 1 k, l). In the premammillary area, few fibres appear in the lateral half of the ventral premammillary nucleus (PMV) and around the arcuate nucleus (Arc) (Fig. 1 l).

From the hypothalamus, a few OT-ir fibres are seen to cross the boundary with the thalamus. Thus, in the posterior hypothalamic area (PH), thin OT-ir fibres oriented in the dorsoventral direction enter the central medial thalamic nucleus (CM) and provide a sparse innervation of the mediodorsal thalamic nucleus (MD), parafascicular nucleus (PF, around the fasciculus retroflexus), lateral habenula (LHb) and the lateral aspect of the caudal paraventricular thalamic nucleus (PV; Fig. 1 k). This contrasts with the anterior paraventricular thalamus (PVA), which is free of labelling (Figs. 1 a, 2 a).

In the mesencephalon, the density of OT-ir fibres is significantly lower than in the forebrain. The periaqueductal grey (PAG) shows the most noticeable OT-ir innervation. In the rostral PAG, thin OT-ir processes

gather in its dorsolateral portion and around the aqueduct (Fig. 1 l). In more caudal sections of the PAG, OT-ir innervation appears in the dorsolateral column and in the ventral half of the PAG, again being denser near the aqueduct (Fig. 1 m). The innervation of the ventrolateral PAG extends ventrolaterally, with long fibres crossing the deep mesencephalic nucleus (DpMe) and branching into a denser terminal field in the dorsomedial region of the substantia nigra (SN). Scattered fibres appear also in the ventral tegmental area (VTA) and dorsal linear nucleus of the raphe (RLi; Fig. 1 m). Caudally, few OT-ir fibres appear in the dorsal raphe and in two unnamed areas, one between the periolivary region (PO) and the subcoeruleus nucleus (SubC; Fig. 1 n) and the other between the ~~Barrington~~Barrington's nucleus (Bar) and laterodorsal termental area (LDTg; Fig. 1 o). Interestingly, the raphe magnus (RMg) shows considerably denser innervation (Fig. 1 n). The lateral parabrachial (LPB) nucleus contains a small cluster of OT-ir fibres in its ventrolateral half. These OT-ir processes in the LPB display two different morphologies: typical thin fibres with axonal buttons accompany highly immunoreactive wrenched thick fibres (~~Fig. 1 n~~Figs. 1 n, 2 d, d').

Finally, thin OT-ir fibres innervate various areas at the level of the medulla (Fig. 1 p). The nucleus of the solitary tract (Sol) shows OT-ir processes, apparently running rostro-caudally, which extend into the dorsal part of the medullary reticular nucleus (MdD). The dorsal tip of the spinal trigeminal nucleus (Sp5) also contains few immunoreactive fibres, but this innervation does not enter the adjacent cuneate nucleus (Cu). Ventrally, the lateral reticular nucleus (LRT) shows a cluster of OT-ir fibres near the putative location of the noradrenergic cell group A1 (Fig. 1 p).

Co-expression of AVP and OT and the size of nonapeptidergic cells in the brain of mice

We have mapped cell bodies co-expressing both nonapeptides in the brain of male and female CD1 mice, employing double immunofluorescence for AVP and OT and confocal microscopy. We have used these data to analyse the size of the AVP and/or OT-ir cells and to identify doubly labelled cells.

First, using Image J, on the photographs of an animal, we measured manually the length and width of the OT and/or AVP-ir neurons in which the cell nucleus was visible. With these data we calculated the area covered by the cell bodies to directly compare the size of the

nonapeptidergic cells in the different nuclei, including the SO/SOR, the medial amygdala (Me), the anterior (PaAP, PaV, PaL and PaM) and posterior (PaPo) parts of the Pa, the periventricular hypothalamic nucleus (Pe), the AC/ADP and the cells ~~confirming with~~making up the AN at the level of the preoptic area. A one-way ANOVA reveals highly significant differences in cell size between these nuclei ($F_{6,208} = 13.820$; $p < 0.0001$). A Student–Newman–Keuls post hoc analysis of these differences indicates that there are two homogeneous groups of cells (Table 2): the Me, PaPo, AN and SO/SOR are composed of relatively small immunoreactive cells, thus deserving the name “parvocellular”, whereas the other nuclei (anterior Pa, Pe and AC/ADP) display significantly larger cells, so they are “magnocellular”.

Table 2

Summary (as obtained with SPSS software) of the results of the Student–Newman–Keuls post hoc analysis of the data on the cell size of OT- and/or AVP-ir cells in the different nuclei of the brain of mice (common level of significance $\alpha = 0.05$)

	<i>n</i>	Average size (μm^2)	
		<i>Parvocellular</i>	<i>Magnocellular</i>
Me	7	122.71	
PaPo	22	132.46	
AN—preoptic region	37	142.05	
SO/SOR	41	143.90	
Anterior Pa	37		184.72
Pe	29		195.84
AC/ADP	36		203.59
Intra-group significance		0.43	0.363
The results indicate that there are two sets of homogeneous means (<i>parvocellular</i> and <i>magnocellular</i>). The nuclei are ordered according to the average size of the immunoreactive cells. The significance refers to the comparison between the two most distant means within the subgroup			

AQ7

Concerning the distribution of double immunoreactive cells, no differences were appreciated between males and females, and the results obtained in

both sexes are described together. We scanned and analysed quantitatively two series of coronal sections of one female and two males to provide quantitative data on the proportion of all nonapeptide-immunoreactive cells expressing only OT, only AVP or both nonapeptides at immunohistochemically detectable levels (Table 3). We also indicate the percentage of the AVP-ir cells that also express OT and the percentage of the OT-ir cells that are also immunoreactive for AVP. In general, most of the somata were immunoreactive for either OT or AVP exclusively. In most hypothalamic locations, only a minor proportion of nonapeptidergic cells showed double labelling, which is especially true in the SON/SOR and posterior Pa.

Table 3

Distribution of nonapeptidergic cell bodies immunoreactive for OT, AVP or both in different nuclei of the brain of mice

	% Nonapeptide- ir cells expressing only OT	% Nonapeptide- ir cells expressing only AVP	% Nonapeptide cells expressing OT + AVP	% OT cells expressing also AVP	% OT cells expressing also AVP
BSTMPM*	71.4	14.3	14.3	17	50
AC/ADP	58.1	1.6	40.3	41.0	96.2
Pe preoptic	42.2	2.2	55.6	56.8	96.2
Pa anterior	59.2	16.5	24.3	29.1	59.2
Pe anterior	43.9	35.1	21.0	32.4	37.5
Pa intermediate	45.7	45.3	9.0	16.4	16.4
Pe intermediate	51.0	28.6	20.4	28.6	41.7
Pa Posterior	96.0	2.0	2.0	2.1	50.0
Pe Posterior*	66.7	0.0	33.3	33.3	100
SON	25.5	73.1	1.4	5.2	1.9
SOR	34.4	64.1	1.5	4.1	2.3
MeA*	37.5	62.5	0.0	0.0	0.0
MePV*	20.0	60.0	20.0	50.0	25.0

AN	33.6	50.9	15.4	31.5	23.1
For each nucleus ($n = 3$), figures indicate the percentage of nonapeptidergic cells (expressing either OT, AVP or both) that show immunoreactivity for OT only, AVP only or double immunostaining (AVP plus OT). In addition, we indicate the percentage doubly labelled cells among those expressing OT and among those expressing AVP.					
* For the BSTMPM, the PePo and the divisions of the Me, the total number of cells recorded is very low (2–4 cells per animal), so that figures are just approximate

By contrast, in the dorsal preoptic region, the AC/ADP contains a high rate of double-stained cells (see Table 3). In most cases, nonapeptidergic cells in the AC/ADP show high OT-ir together with low (but variable) AVP-ir (Fig. 3 a, b, a', b'), a trait that is also found in the rostral edge of the Pa (Fig. 4 a, a'; Table 3). In these nuclei, the proportion of doubly labelled cells showing this profile of immunoreactivity attains as much as a 40 % in the AC/ADP and more than a 20 % in the rostral Pa, while the large majority of the AVP-ir cells (96 % in the ADP/AC; 60 % in the rostral Pa) are also immunoreactive for OT. The main difference between both cell groups is the presence in the anterior Pa, but not in the AC/ADP, of a remarkable population of AVP-ir cells that are quite intensely stained for AVP but negative for OT (white arrowhead in Fig. 3 b'''). This is clearly the rule in the PaAP and PaV at slightly more caudal levels, where abundant singly labelled cells for OT are intermingled with scattered cells displaying intense AVP immunoreactivity, but negative for OT (Fig. 4 a, a'; nearly a 40 % of the AVP-ir cells).

In fact, in most of the Pa, OT-ir and AVP-ir cells are largely segregated in different subnuclei (as described in rats by Hou-Yu et al. 1986) and double-labelled somata are relatively scarcer than in the rostral Pa and dorsal preoptic region. Thus, in the intermediate portion of the Pa, OT-ir cells occupy its medial subdivision (PaM) and the juxtaventricular compartment (periventricular nucleus, Pe), with few OT-ir somata entering the lateral nucleus (PaL). Here, most AVP-ir cells gather in the dorsolateral portion of the PaL, but a few singly AVP-ir cells and processes appear intermingled with OT-ir ones in the PaM (Fig. 4 b, b'). Similar to anterior levels, at these intermediate ones, there are relatively few doubly labelled cells (about 20 % of the nonapeptidergic neurons; see Table 3), usually located in the ventromedial portion of the PaM, ~~but~~ In contrast to the rostral edge of the Pa and the ADP/AC, here doubly

labelled cells show equal intensity for AVP and OT immunoreactivity (Fig. 3 a'', b''). Finally, in the posterior nucleus of the Pa (PaPo), only OT-ir cell bodies remain, intermingled with some AVP-ir thick processes (Fig. 4c), and doubly labelled cells are very scarce (about a 2 % of the nonapeptidergic cells; see Table 3).

In the periventricular area (Pe), immunoreactive somata gather near the ventricular walls. Relative OT and AVP immunoreactivity in Pe somata depends on the rostro-caudal level and parallels the pattern found in the adjoining AC/ADP or Pa. Thus, double-labelled cells are very frequent in the Pe next to the AC/ADP (Fig. 3 a'''; more than 55 % of the nonapeptidergic cells, ~~see Table 1~~). ~~At the anterior, intermediate and posterior levels, doubly labelled cells are also abundant~~, but relatively scarcer ~~more caudally~~ (see Table 3). As a rule, however, the profile of immunoreactivity on the Pe differs from that in the Pa and AC/ADP, so that doubly labelled cells highly immunoreactive for both OT and AVP appear frequently within and nearby the Pe.

Apparently, the supraoptic nucleus (SON) contains more AVP-ir than OT-ir cells. Oxytocinergic somata are located in the dorsomedial aspect of the SON and are more abundant at rostral levels (Fig. 5 a, b). By contrast, the caudal tip of the nucleus contains mostly AVP-ir cells (Fig. 5 c). In the retrochiasmatic division of the supraoptic nucleus (SOR), OT-ir cells also appear dorsal to the AVP-ir ones and are more abundant at rostral levels (not shown). Throughout the SON, almost all cells were immunoreactive exclusively for either AVP or OT, while a very low proportion of the nonapeptide-immunoreactive somata show double labelling (≈ 1 %; see Table 3) with similar intensity of labelling for both peptides (inset in Fig. 5 b').

As described for OT-immunoperoxidase material, some OT-ir and also some AVP-ir cells, showing monopolar or bipolar morphology, trespass the SON boundaries to enter the medial region of the anterior medial amygdala (MeA; Fig. 5 c, d) and of the posteroventral Me (MePV). In the latter division, doubly labelled cells are also present, but at a low frequency (Fig. 4 d'; about 20 % of the nonapeptidergic cells, although this figure might not be representative given the low number of cells found). Finally, the accessory nuclei (AN) of the hypothalamus display a relatively high proportion (≈ 15 %; see Table 3) of nonapeptidergic cells co-

expressing OT and AVP at immunohistochemically detectable levels, which are present at different anteroposterior levels of the hypothalamus (for example, white arrow in Fig. 4a).

Co-expression of AVP and OT: distribution of doubly labelled fibres

In the examined specimens, double-labelled fibres only appear in some forebrain regions. All thalamic, hypothalamic and mesencephalic areas analysed only display single AVP-ir or OT-ir processes. Therefore, in this section we focus on the areas where we observe co-localization of OT and AVP immunoreactivity.

As we have described above, in the cortical areas of the frontal lobe, OT-ir fibres are restricted to a few cortical structures in the anterior cerebral hemispheres, namely the DTT, claustrum and dorsal endopiriform cortex. Some of these structures also show AVP-ir fibres and, in some instances, fibres are doubly labelled. Figure 6a'' shows a representative case in the boundary between the DTT and SHi, where winding varicose fibres are found to be intensely immunoreactive for AVP and faintly stained for OT (white arrows). Singly labelled fibres for either peptide are also seen (black arrowheads point to a OT+/AVP- fibre).

Within the subcortex, the dorsal lateral septal nucleus (LSD) shows some linear processes that appear intensely OT-ir, but negative for AVP (white arrow in Fig. 6a), whereas others are weakly AVP-ir and negative for OT (white arrowhead, Fig. 6a'). In the so-called medioventral striato-pallidum (mvStP, the region located between the Acb and the VDB, see Otero-Garcia et al. 2014), there is a relatively dense AVP-ir-only terminal field. In addition, the Acb itself shows a remarkable population of nonapeptide-immunoreactive fibres. There, many fibres observed are intensely immunoreactive for both OT and AVP (white arrows, Fig. 6b''), although there are also abundant fibres showing strong OT immunoreactivity but weak AVP signal (white arrows, Fig. 6b'''). Less frequently, highly AVP-ir processes show weak OT immunoreactivity (white arrow, Fig. 6b'). Immediately caudal to the Acb, the anterior portion of the medial bed nucleus of the stria terminalis (BSTMA) and strial part of the medial preoptic area (StA) also contain OT-ir processes of different diameters (Fig. 6c, d). Similarly to the adjacent Acb, the BSTMA and StA display some singly OT-labelled fibres (black arrowheads; Fig. 6c', d'), some fibres singly labelled for AVP (white arrowheads), and also doubly

labelled fibres. Most of the latter show strong OT immunolabelling with variable, but fainter AVP labelling (white arrows, Fig. 6c', d').

In the amygdala, the central nucleus (Ce) contains long immunoreactive processes of different calibres (Fig. 6e). Although there are some singly labelled fibres for OT (black arrowheads in Fig. 6e') or for AVP (white arrowhead in Fig. 6e'), most of the fibres in the Ce are immunoreactive for both OT and AVP (white arrows, Fig. 6e', e''). Most (but not all) of the doubly labelled fibres were intensely immunostained for OT and faintly labelled for AVP, although the reverse situation is also occasionally observed.

In the medial amygdala (Me), immunoreactive processes apparently arising from the rostral SON enter its ventromedial edge. Most of the labelled processes in the Me are singly immunoreactive for AVP (white arrowheads in Fig. 5d'', d'''), few are only immunoreactive for OT (Fig. 5d'', black arrowhead), and only scattered fibres display both signals (Fig. 5d''', white arrow). These observations are consistent with the pattern of labelling of the nearby SON and Me immunoreactive somata, which probably originate the mentioned processes that, in fact, show a dendritic morphology.

Discussion

In the present work, we provide a detailed anatomical description of the distribution of OT-immunoreactive (OT-ir) elements in the brain of mice. Further, we describe a novel finding, namely the co-localization of OT and AVP in cell bodies of the rostral hypothalamus and in fibres located in various limbic areas. In agreement with observations in other rodents (Xu et al. 2010), our data reveal no apparent sexual dimorphism in OT immunoreactivity or its co-localization with AVP. In addition, our measurements of the size of the OT- and/or AVP-ir cell bodies in different nuclei of the brain mice somewhat challenge the view that neurosecretory cells are magnocellular, whereas non-neurosecretory ones are smaller. Thus, one of the classically considered magnocellular neurosecretory cell groups, the SON/SOR, is composed of small cells, whereas the other one is actually large celled (magnocellular). Within the Pa, however, the non-neurosecretory posterior part of the Pa (PaPo; Ross et al. 2009) is parvocellular, whereas the anterior, ~~neurosecretory~~neurosecretory part is composed of large cells (see Table 2). Among those nuclei for which their neurosecretory nature is unclear, some of them are composed of large cells

(AC/ADP) whereas others have relatively smaller cell bodies (accessory nuclei, AN; Table 2).

Distribution of OT-immunoreactive cells

The distribution of OT-ir cells that we have found in mice looks, in general, similar to that reported in other mammals, including macaques (Caffé et al. 1989), tree shrews (Ni et al. 2014), naked mole rats (Rosen et al. 2008; Valesky et al. 2012), voles (Wang et al. 1996), Brandt's voles and long-tailed hamsters (Xu et al. 2010), rats (Buijs et al. 1978) and different species of mice (Hermes et al. 1988; Castel and Morris 1988). Our work provides a detailed description of the distribution and location of OT-immunolabelled cell populations, which was lacking for laboratory mice, currently a species of choice for research in neurobiology of social behaviours.

The distribution of OT-ir cells we have observed supports and refines previous neurodevelopmental studies on the origin of the OT-ir cells in the embryonic neuroepithelium of the so-called supraopto-paraventricular hypothalamus. As discussed below, there is evidence suggesting that cells generated in two distinct zones in this hypothalamic neuroepithelium would follow apparently distinct migratory streams (Garcia-Moreno et al. 2010) to five main locations in the adult forebrain. A portion of the supraopto-paraventricular embryonic neuroepithelium would give rise to neurons in: (1) the Pa and adjacent periventricular hypothalamus; and (2) cell groups in the preoptic hypothalamus, rostral to the Pa, including the periventricular (Pe), medial (MPO) and ventromedial (VMPO) preoptic nuclei, as well as the so-called AC/ADP (located among the preoptic area, the posterior BST and the nucleus of the anterior commissure; see Fig. 2a). A second portion of the embryonic supraopto-paraventricular neuroepithelium would originate the cells in: (3) the SON, including its retrochiasmatic portion; and, (4) the anterior Me adjacent to the anterior SON, from where some cells apparently migrate caudally into the posterior medial amygdala. Finally, (5) cells probably arising in both neuroepithelial regions or in their interface would give rise to the accessory OT-ir nuclei (AN), scattered through the preoptic (see above, point 2), perifornical and lateral hypothalamus, with a denser cell group at the level of the PaPo, just ventral to the zona incerta (Figs. 1 i, 2 b-b').

This is supported by analysis of the migration of cells expressing *otp* (which characterizes the supraopto-paraventricular neuroepithelial region; Wang and Lufkin 2000) and by the use of cell-migration tracing techniques in mouse embryos (Garcia-Moreno et al. 2010). In this respect, null mutants for *otp* in which LacZ is expressed under the *otp* promoter lack labelled cells in the Pa and in the rostrally adjacent preoptic-BST area, whereas the supraoptic region shows plenty of Lac-Z positive cells. Even if the latter cell population does not conform to a well-defined SON, it extends into the Me where a neat cell group is found (at least at postnatal day 1; see Fig. 1 b–j in Wang and Lufkin 2000). This strongly suggests that the nonapeptidergic cells in the BST–preoptic interface derive from the paraventricular rather than the supraoptic embryonic neuroepithelial niche.

Comparative studies also support the developmental relationship between SON and Me OT-ir cells. Thus, like in the mouse (Castel and Morris 1988; this work), in other mammals such as the mole rat (Rosen et al. 2008), Brandt's voles and long-tailed hamsters (Xu et al. 2010) and tree shrews (Ni et al. 2014), there seems to be a continuity between the OT-ir cells in the Me and the anterior SON.

In our material, immunoreactive cells in the SON and SOR showed an apparently weaker immunostaining than those in the Pa. This finding could reflect lower rates of synthesis or higher transport rate of the peptide from supraoptic magnocellular somata. Although most of the OT-ir cells in the SON are likely to be neurosecretory, Hawthorn et al. (1985) performed lesions restricted to the SON in rats and reported a depletion of OT in several central structures such as the striatum, the substantia nigra, the parabrachial nucleus and the hippocampus. Some of these nuclei do not show OT-ir fibres in our material, but those findings strongly suggest the existence of SON OT-ir cells displaying central projections. By contrast, the use of viral vectors for tracing specifically the projection of OT-expressing cells in the SON of rats (Knobloch et al. 2012) suggest that central projections of these cells are restricted to the horizontal limb of the diagonal band nucleus, portions of the Acb shell and maybe of the Ce and hippocampus (see their supplementary figure S2). In any case, there is evidence that OTergic SON cells do show central projections.

In the Pa, OT-ir cells are abundant in all subdivisions except for the lateral

(PaL) and their distribution is very similar to that found in the rat (Condés-Lara et al. 2007). Immunostained somata are particularly abundant in the anterior Pa, which mostly contains neurosecretory cells. However, some of the OT-ir cells in the anterior Pa project centrally, as indicated by tracing studies in voles (Ross et al. 2009) and the presence of branching OT-ir axons in the vicinity of the anterior Pa of the rat (Hatton et al. 1985). This suggests that some OT-ir neurosecretory cells in the anterior Pa may originate axon collaterals to central targets such as the Acb. In fact, recent experiments in rats using viral tracers under the control of the OT promoter have demonstrated that neurosecretory cells (e.g. projecting to the posterior pituitary) in the Pa and AN send collaterals to the Ce and Acb (Knobloch et al. 2012). This would allow the coordination of neurosecretion with modulation of behaviour through central release (Veenema and Neumann 2008). Such coupling of OT neurosecretion with synaptic OT release in central targets is a feasible mechanism that would explain the increase of OT blood levels associated with specific behaviours in humans (for example, maternal and paternal care; Feldman et al. 2010) and non-human mammals (social interactions in cattle youngsters, Yayou et al. 2015), without the need of transport of OT through the blood–brain barrier, a mechanism that has not been demonstrated (Neumann and Landgraf 2012).

By contrast to the anterior Pa, data in voles indicate that OT-ir cells in the dorsocaudal portion of the Pa (PaPo) are not neurosecretory, as they do not uptake systemically injected fluorogold (Ross et al. 2009). This is supported by viral tracing experiments in rats, where OT-expressing cells in the PaPo innervate the nucleus of the solitary tract but do not project to the posterior pituitary (Knobloch et al. 2012).

Besides the described hypothalamic OT-ir cells, we have characterized an additional OT-ir cell group in the so-called AC/ADP. At least in mice, this is a cytoarchitectonically ill-defined cell group, located between the ventral BSTMP and the dorsal MPOA (see Paxinos and Franklin 2001). Some authors studying the rat brain consider it as a particular nucleus of the AN or name it as the anterior commissural nucleus (AC, Rhodes et al. 1981). Others envisage these cells as belonging to the anterior division of the Pa (Swanson and Kuypers 1980; Hou-Yu et al. 1986; Xiao et al. 2005). Wang et al. (1996) described a similar group of OT-ir cells in the BST of voles, ventral to the anterior commissure. According to our results

in the mouse, the AC/ADP constitute a distinct population of OT-ir cells, separated from the anterior Pa, as shown in horizontal sections in the Brattleboro strain rat by Rhodes et al. (1981; see ACN in their Fig. 3) and sagittal sections through the hypothalamus of Sprague–Dawley rats (Hou-Yu et al. 1986; rPVN in their Fig. 6).

Interestingly, some of the OT-ir cells in this region appear densely packed in close association to large blood vessels (Fig. 2 a, b). Similar association between OT-ir cells and blood vessels appears in a region somewhere between the Pa and the *stria medullaris* in rats, which might correspond to the mouse AC/ADP (Fig. 4b in Buijs 1978) and in other regions of the brain (Hou-Yu et al. 1986). Associations between OT-ir cells and the capillary endothelium have been observed at ultrastructural level in the preoptic and paraventricular regions of the hypothalamus in the golden-mantled ground squirrel, *Spermophilus lateralis* (Muchlinski et al. 1988). Altogether, these data suggest that cells of the AC/ADP could contribute to neurosecretion through somato-dendritic release of OT to the perivascular space, even if OT-ir neurons of this region do not project to the pituitary, at least in voles (Ross et al. 2009). This is another neuroendocrine mechanism that could explain the correlation between central OT-mediated behaviours and circulating levels of the neurohormone (see above).

Distribution of OT-immunoreactive processes

The distribution of OT-ir fibres depicted in this work is consistent with the previously reported distribution in rats (Buijs 1978) and mice (Castel and Morris 1988) and does not differ substantially from less detailed descriptions in other mammals like the naked mole rat (Rosen et al. 2008) and the tree shrew (Ni et al. 2014). In all cases, the majority of OT-ir fibres is located in the hypothalamus and corresponds to the neurosecretory axons travelling from the Pa and SON to the pituitary. These OT-ir neurosecretory axons show a characteristic straight, varicose morphology. However, other regions of the brain contain remarkable OT-ir terminal fields with fibres of different morphologies, which strongly suggest the existence of central OTergic circuits.

AQ9

Terminal fields of OT-ir fibres in the cerebral hemispheres are likely related to a role of OTergic systems in motivated, emotional, socio-sexual behaviours. Thus, we have seen a noticeable density of OT-ir fibres in the

medial Acb core and the Acb shell, also found in other rodent species such as rats and voles (Ross et al. 2009). By contrast, the distribution of OT receptors (OTR) shows important inter-species differences associated with variations in social behaviour (Beery et al. 2008; Staes et al. 2014). In rats, OTR binding occurs in the Acb shell (Veinante and Freund-Mercier 1997), while in voles OTR binding fills the entire striatum, including the Acb shell and core (Lim et al. 2004). However, only low-resolution descriptions of OT receptor (OTR) distribution are available for the mouse brain (Insel et al. 1993; Hammock and Levitt 2013). Precise descriptions of OTR binding and presynaptic OT elements in the mouse striatum would be useful, since OT transmission in the Acb is the focus of multiple studies on the neural basis of pair bonding (Young and Wang 2004), social reward mechanisms (Dölen et al. 2013) and addiction to drugs of abuse (Sarnyai and Kovács 1994).

More caudally within the ventral cerebral hemispheres, the BSTA and StA show axon-like OT-ir fibres, similar to those in the adjacent Acb core, intermingled with thick processes. The thick processes could be swollen axons or dendrites from the caudally adjacent AC/ADP immunoreactive cell group. Ross et al. (2009) point to the anterior Pa as the origin of the OT innervation of these basal telencephalic structures in rodents, as in horizontal sections they observed OT-ir fibres linking the PaAP with the Acb, which cross the intervening StA and BSTA.

Another centre showing OT innervation, which is involved in emotional aspects of social behaviour, is the central amygdala (Ce). There, OT-ir fibres also show quite a variable distribution pattern among individuals, abundant in some specimens while almost absent in others. This recalls the distribution of AVP-ir fibres in the same nucleus (Otero-Garcia et al. 2014), an issue that will be discussed below. Our results in mice do not fit those by Knobloch et al. (2012) in rats, as we did not notice differences in the morphology and density of OT-ir fibres in the CeM, CeL and CeC of mice. This likely reflects inter-specific differences, which might be related to the important differences in behaviour between rats and mice (e.g. maternal care, see below).

The nonapeptidergic innervation of the Ce (this work for OT; Otero-Garcia et al. 2014, for AVP) and other regions of the fronto-temporal lobe connected with the amygdala, such as OT-ir fibres in the DTT (this work)

and the AVP-ir ones in the infralimbic and prelimbic cortices (this work and Otero-Garcia et al. 2014), show a high inter-individual variability and an apparent lateralized (asymmetric) distribution, at least in some individuals. This asymmetry may have functional significance. In fact, there is evidence of functional left–right asymmetries in the amygdala and prefrontal cortex of rodents (Sanchez and Dominguez 1995; Banczerowski et al. 2003) and humans (Breiter et al. 1996; Markowitsch 1998; Evans et al. 2014). Therefore, asymmetric, highly variable nonapeptide-containing fibre distribution in the central amygdala, frontal (pre- and infra-limbic) cortex of mice and other structures could reveal a hidden functional lateralization in relation to social interaction, which might represent an ancient evolutionary trait inherited by rodents and primates from their common ancestors.

In the hypothalamus, our results reveal OT-ir axon-like processes in the dorsomedial hypothalamus (Figs. 1 k, 2 c), a key centre for suckling stimuli to elicit OT release inducing milk ejection (Takano et al. 1992). These fibres suggest backward projections from the Pa and/or SON neurosecretory cells to the dorsomedial hypothalamus, which could modulate the OT-mediated milk ejection reflex (Honda and Higuchi 2010a, b) through feedback mechanisms.

The role of nonapeptidergic transmission in the midbrain and hindbrain has been less studied, in comparison with the forebrain. Nonetheless, our results indicate that OT-ir fibres are quite dense in several centres of the midbrain and hindbrain. For instance, a relatively dense OT-ir innervation in the PAG may be involved in OT regulation of opiate-mediated nociception (Yang et al. 2011). Considerable OT-ir innervation is also seen in the substantia nigra and ventral tegmental area. In the mouse, OT can directly activate specific neurons in the VTA (Tang et al. 2014), where OT activity appears to regulate various behaviours in rats, including maternal behaviour (Pedersen et al. 1994; Pedersen 1997), food intake (Mullis et al. 2013) and penile erection (Melis et al. 2007; Succu et al. 2008). In women, OT increases the VTA activation to salient sexual and infant stimuli (Gregory et al. 2015) in nulliparous and postpartum women, respectively. Finally, the raphe magnus shows a specific OT-ir innervation, from which OT can act on serotonergic neurons (Eaton et al. 2012; Mottolese et al. 2014) modulating anxiety (Yoshida et al. 2009) and aggression (Pagani et al. 2015).

Distribution of nonapeptidergic cells co-expressing OT and AVP

Early studies using IHC and ISH techniques suggested that AVP and OT were produced in mutually exclusive sets of neurons (Mohr et al. 1988). However, analyses of the expression of the genes encoding OT and AVP using quantitative RT-PCR suggest that all magnocellular neurosecretory cells in the hypothalamus express both peptides, in some cases at dramatically different levels (Kiyama and Emson 1990; Glasgow et al. 1999). Thus, mRNA ratios in the range of 1:500 for the minor peptide versus the major one are found in neurons that would probably appear as expressing only one of the peptides, using immunohistochemical or in situ hybridization procedures. By contrast, a ratio of about 1:2 is found in those neurons usually considered to co-express both peptides. This reflects a limitation of immunohistochemical or in situ hybridization techniques.

Taking these limitations into account, in most of the Pa and in the SON, OT-ir and AVP-ir cells constitute distinct, partially intermingled cell populations. In this respect, our findings roughly fit the results of the studies by Rhodes et al. (1981) and Hou-Yu et al. (1986) comparing the distribution of OT-ir and AVP-ir cells in the rat Pa. Thus, in the Pa, OT-ir cells concentrate in the PaV, PaM and PaPo, as well as in the adjacent periventricular nucleus. In contrast, AVP-ir cells are mainly found in the PaL, with few cell bodies in the antero-ventral aspect of the nucleus, where they are intermingled with the dense population of OT-ir cells (see Fig. 4).

However, concerning the SON, our findings (Fig. 5) differ from those in the rat (Rhodes et al. 1981; Hou-Yu et al. 1986). Thus, in the mouse, AVP-ir cells constitute the large majority of the nonapeptidergic cells of the nucleus, whereas OT-ir somata are restricted to the dorsomedial SON, especially at rostral levels, and to a few scattered cells in the dorsal portion of the SON (see Fig. 5). By contrast, the rat SON shows a more balanced proportion of OT- and AVP-ir cells, which are intermingled instead of segregated, especially at anterior levels of the nucleus (Rhodes et al. 1981; Hou-Yu et al. 1986). However, our quantitative data reveal a similar, low proportion of doubly labelled cells in the supraoptic nucleus of mice (about 1.5 %, see Table 3) and rats (2–3 % of the neurosecretory population in basal conditions; Xi et al. 1999; Mezey and Kiss 1991).

Interestingly, doubly labelled neurons are relatively frequent at rostral

levels of the Pa, the AC/ADP and the adjoining periventricular nucleus. In fact, a majority of the OT-ir somata in the rostral edge of the PaAP and in the AC/ADP show also weak-to-moderate AVP immunoreactivity. In the AC/ADP, Rood et al. (2013) and Otero-Garcia et al. (2014) described an AVP-ir cell group, different from the sexually dimorphic AVP-ir population of the BSTMP. Our present results suggest that the OT-ir and AVP-ir groups next to the anterior commissure correspond in fact to a single AC/ADP population of cells that are immunolabelled for both peptides. The characteristic immunolabelling of the rostral PaAP and AC/ADP oxytocinergic cells (high OT-ir plus low AVP-ir) reinforces the classification of the AC/ADP as a distinct cell group in the mouse brain.

Other co-localization studies in neuropeptidergic cells of the rat and mouse hypothalamus fit our observations in mice. Thus, Xiao et al. (2005) described a cell group expressing both OT and nitric oxide synthase in the region corresponding to the AC/ADP of the rat brain (which they call the anterior levels of the anterior magnocellular Pa, see their Fig. 6a–c). This fits the co-expression of NADPH diaphorase in the non-dimorphic AVP-ir cell population of the AC/ADP in the mouse (Otero-Garcia et al. 2014). Note also that, like the OT-ir cells we are reporting, some of these diaphorase/AVP-positive cells are associated with blood vessels (see Fig. 2a in Otero-Garcia et al. 2014). Therefore, the AC/ADP apparently contains a cell population characterized by expressing OT, AVP and nitric oxide synthase/NADPH diaphorase.

Besides this cell population expressing high levels of OT and relatively lower levels of AVP, in some locations cells showed comparable levels of immunoreactivity for both OT and AVP. These equally co-labelled cells were mainly located in the Pa near the ventricle and in the Pe. A low proportion of equally co-labelled nonapeptidergic cells also occur in the accessory nuclei and, less frequently, in the SON.

As discussed above, a few OT-ir cells surpass the borders of the SON and enter the medial amygdala. Some of these OT-ir cells in the Me show a co-expression phenotype similar to the AC/ADP and PaAP OT-ir cells (see Fig. 5 d'), namely intense OT immunostaining and weaker AVP immunolabelling. This is relevant because the Me contains high amounts of AVP and OT receptors (Dubois-Dauphin et al. 1996; Hammock and Levitt 2013; Veinante and Freund-Mercier 1997), but lacks important

nonapeptidergic innervation (Rood and de Vries, 2011; Rood et al. 2013; Otero-Garcia et al. 2014). Thus, these cells could be a source of both nonapeptides via somato-dendritic release (Ludwig and Leng 2006). Noteworthy, these cells are located in the main area of convergence of olfactory and vomeronasal projections from the main and accessory olfactory bulbs (Kang et al. 2009; Cádiz-Moretti et al. 2013). Therefore, it is possible that chemosensory information, which in mice is critically involved in socio-sexual behaviours, influences the nonapeptidergic systems at this level of the medial amygdala, a key node of the sociosexual brain network (Newman 1999).

Distribution of OT-, AVP-ir fibres and co-labelled processes

In general, OT-ir processes show a more restricted distribution and lower density than AVP-ir ones (compare Fig. 1 in the present work with Fig. 1 in Otero-Garcia et al. 2014).

Interestingly, our results of double immunolabelling indicate that double OT + AVP fibres are restricted to forebrain areas known to be implicated in motivational and emotional functions, such as the ventral striato-pallidum, the central amygdala and the intervening BST. In the ventral striato-pallidum, the most remarkable nonapeptidergic terminal field is the distinct terminal field of single AVP-ir fibres in the mvStP, a fibre system that is sexually dimorphic and testosterone dependent (Otero-Garcia et al. (2014). In this region, no OT-ir fibres are present. This fits the occurrence of a high density of vasopressin V1a receptor reported in this region in another mice strain (Dubois-Dauphin et al. 1996).

By contrast, the nucleus accumbens (Acb), both its shell and core, contains sparse, thick-calibre fibres intensely immunoreactive for OT, many of which are also moderately AVP-ir (Fig. 6). In fact, most of the nonapeptidergic fibres in the Acb are co-labelled, especially in the medial Acb core. Thus, the OT-ir and AVP-ir processes previously described in the Acb core may actually correspond to the same fibres. The available, low-resolution maps of OT receptor binding in the brain of mice (*Mus musculus*) do not give enough detail on the distribution of OTR in the ventral striatum (Insel et al. 1993; Hammock and Levitt 2013). However, this issue has been studied in other rodents, such as the singing mice of the species *Scotinomys teguina* and *S. xerampelinus* (Campbell et al. 2009)

and the rat (Veinante and Freund-Mercier 1997). Both studies coincide in showing relatively low density of OT binding in the Acb core, but higher density of OTR in the Acb shell. By contrast, AVP binding appears scarce in both Acb shell and core. Thus, if data in these rodents were valid for mice, there would be a clear mismatch among the nonapeptides and their receptors at the level of the Acb. Nonapeptidergic fibres (doubly labelled) would release relatively large amounts of OT and smaller amounts of AVP in both the shell and core. By contrast, receptors, mainly OTR, would be largely restricted to the shell and absent in the core. Receptors for AVP (V1aR) would be absent in the Acb but concentrated in the mvStP, where there is an AVP terminal field arising from a different cell population, thus likely involved in a different function.

Nonetheless, AVP and OT receptors are *somewhat* promiscuous (Manning et al. 2012), so that OT could also act through V1a receptors—although with lower affinity than to OTR. Conversely, AVP could bind OT receptors near the site of release (e.g. in the Acb shell). Ross et al. (2009) analysed the ultrastructure of the OT-ir fibres in the Acb of voles and described large-calibre unmyelinated axons filled with dense core OT-ir vesicles, which did not form synaptic contacts but were apposed to dendrites of striatal neurons. This is strongly suggestive of an important role of volume transmission of nonapeptides in the Acb (Landgraf and Neumann 2004). Taken together, receptor promiscuity and volume transmission of OT and AVP suggest a cross talk between both nonapeptidergic systems (the doubly labelled one in the Acb and the AVP-specific one in the mvStP) at the level of the ventral striato-pallidum.

A similar situation is found in the central and medial amygdaloid nuclei. Co-labelled fibres appear in all subdivisions of the Ce (Fig. 6), whereas the adjoining MePD shows a sexually dimorphic, testosterone-dependent, AVP-specific terminal field (Otero-Garcia et al. 2014). As discussed above, binding experiments in mice are scarce and restricted to low-resolution description of the distribution of binding to AVP V1a receptors (Dubois-Dauphin et al. 1996) and OTR (Insel et al. 1993; Hammock and Levitt 2013). In the rat, Veinante and Freund-Mercier (1997) provided a detailed description of OTR and V1aR binding in the extended amygdala of the rat. If their results could be extrapolated to mice (as suggested by comparison with Dubois-Dauphin et al. 1996), they would again show a partial mismatch between nonapeptidergic fibres and their receptors. Thus,

OTR density is high in the CeL and moderate in the MePD (see their Fig. 10, 11, 12), whereas V1aR binding is low to moderate in the CeM and AStr, but virtually nil in the CeL and MePD. This partially segregated distribution of receptors contrasts with the nonapeptidergic innervation of these structures. The MePD shows a relatively dense terminal field of only AVP-ir and no OT-ir fibres, but expresses high levels of OTR. Similarly, doubly labelled AVP + OT fibres (more labelled for OT- than for AVP-ir) are scattered throughout the Ce (CeL and CeM), whereas V1aR are confined to the CeM (and AStr) and OTR is present in the CeL.

In this respect, Huber et al. (2005) described the local circuits in which OT, acting in the CeL and AVP, acting in the CeM, regulate fear responses in the rat. The likely co-release of OT and AVP at different proportions from the same axons in both divisions of the Ce, suggested by our findings in mice, should be taken into consideration for refining the model of regulation of fear by nonapeptides, based on physiological studies in the rat by Knobloch et al. (2012) and Stoop (2012).

A similar mismatch in the distribution of nonapeptidergic fibres and their receptors is likely occurring in other portions of the cerebral hemispheres within the extended amygdala. Thus, in the BSTMA/StA (Figs. 3 a, 6 c, d) and in the MPOA, OT-ir fibres are scarcer than AVP-ir ones and show a partially segregated distribution. However, like in the rostrally adjacent nucleus accumbens, many of the nonapeptidergic fibres of the BSTA and StA are double labelled. The importance of OT and AVP transmission in the BST and MPOA for regulating socio-sexual functions such as maternal behaviour is discussed below, together with the possible origin of central OT + AVP-ir projections.

By contrast, in the septal region, especially in its ventrolateral portion, OT-ir and AVP-ir fibres (Otero-Garcia et al. 2014, Fig. 1 d) are present with no evidence of double labelling. In mice and rats, the septum contains receptors for AVP (Dubois-Dauphin et al. 1996) and OT (Veinante and Freund-Mercier 1997; Hammock and Levitt 2013). Given that OT and AVP transmission in the septal area plays a key role in social behaviour (Veenema and Neumann 2008; Lukas et al. 2013; Bychowski et al. 2013), the interplay between both nonapeptidergic systems may be an important factor in regulating this behaviour.

Unlike in the cerebral hemispheres, nonapeptidergic fibres in other parts of

the forebrain, midbrain and brainstem are immunoreactive for either OT or AVP. In most places, like in the PAG, OT- and AVP-ir innervations partially overlap, although AVP-ir fibres are more abundant and cover wider areas. In other areas, both peptides show a complementary distribution. Thus, in the thalamus OT-ir fibres innervate the paraventricular thalamic nucleus (PV), whereas AVP-ir innervation is located in the contiguous habenula (Hb) (not shown). As in other parts of the brain, however, volume transmission and receptor promiscuity may allow a cross talk between both nonapeptidergic systems also at these levels (Dubois-Dauphin et al. 1996; Young et al. 1999).

Possible origin of doubly immunoreactive innervation in the cerebral hemispheres

Some similarities are apparent between the distinct nonapeptidergic ~~cell~~ ~~groups~~ ~~nuclei~~ showing OT + AVP-ir double-labelled cells and the innervation of particular areas in the forebrain, such as the Acb and Ce. Most co-labelled fibres in the Acb and Ce are highly OT-ir plus moderately AVP-ir. This pattern reminds the labelling of cell bodies in the AC/ADP and anterior Pa, also highly OT-ir and slightly AVP-ir. Thus, it is tempting to speculate that the immunoreactive cells in the AC/ADP and anterior Pa are the origin of the innervation of the Acb and Ce, plus the intervening portions of the BSTMA/StA. This is supported by previous studies using different experimental approaches. For example, Hawthorn et al. (1985) reported that lesions restricted to the Pa or involving large portions of the hypothalamus including the Pa, SON and AN resulted in OT depletion in the caudate-putamen (striatum) and septum, whereas lesions restricted to the SON had no effect. This suggests an origin of OTergic innervation of the cerebral subcortex in Pa cells and maybe the adjoining AN. More recent neural tracing experiments in rats have shown that tracer injections in a region of the BST (dorsomedial BST; Dong and Swanson 2006), apparently equivalent to the AC/ADP of mice, results in anterograde labelling compatible with the view that it projects to the Acb and most of the Ce. In addition, fluorogold injections in the Acb of voles resulted in retrograde labelling in cells of the Pa, some of which are immunoreactive for OT (Ross et al. 2009). Finally, anterograde tracing in rats using viral vectors which label specifically OT-expressing cells Knobloch et al. (2012) have shown that OTergic cells in the rostral Pa and AN originate fibres in the Acb and Ce.

As a conclusion, our data fit previous findings in the literature suggesting that nonapeptidergic cells in the AC/ADP, rostral Pa (and maybe AN) might give rise to important central projections, among other structures to the Ce and Acb. At least some of them are neurosecretory cells projecting to the pituitary and to these forebrain centres through axonic collaterals (in the rat, Knobloch et al. 2012). Our data strongly suggests that many of these cells projecting to central targets co-express both nonapeptides at immunohistochemically detectable levels.

Nonapeptides and maternal behaviour

The precise location of co-labelled cells and processes bring about some interesting functional considerations. In our preparations, co-expressing phenotypes are mainly found in the AC/ADP, anterior Pa and periventricular hypothalamus. The AC/ADP is located in between the MPOA and the ventral BSTM, an anatomical region that is crucial for the expression of maternal behaviour in the rat (Numan et al. 1988; Numan and Numan 1996; Olazabal et al. 2002). The adjacent anterior paraventricular hypothalamus, but not more caudal portions of the Pa, also seems critical for these responses (Insel and Harbaugh 1989). These authors discussed their findings in the context of the role of OT in maternal behaviour. Since our results indicate the presence of abundant double-labelled OT-AVP neurons in both the AC/ADP (apparently in the so-called BSTv region) and anterior Pa, it is tempting to suggest that these particular nonapeptidergic cell groups and their projections to the Acb and Ce might play a key role for the expression of maternal behaviours in rodents.

More recently, Tsuneoka et al. (2013) explored the role of the MPA and adjacent structures of the brain in the control of maternal behaviour in mice. Using c-fos expression mapping, they showed that pup stimuli activated most of the centres in the preoptic hypothalamus in virgin females, but especially in parturient and postpartum females. When they focused their analysis on the OT (neurophysin I-expressing) cell populations, Tsuneoka et al. (2013) showed a sharp increase of c-fos expression in the OT cells of the AC/ADP (anterior commissural nucleus, using their nomenclature), preoptic periventricular nucleus, the sparse OT-ir cell population of the MPA and in the SON of parturient females (processed 2 h after delivery). This strongly suggests that OT cells in the

preoptic hypothalamus and surrounding areas have an intense activity during delivery, maybe in association with the release of OT to blood during parturition. This might reflect the role of these OT neurons as neuroendocrine cells (e.g. SON), or a double role in neurosecretion and central neurosignalling (see discussion above).

Interestingly, oxytocinergic cells of the AC/ADP were not activated by pup stimuli in virgin females, but showed a specific increase in c-fos expression not only in parturient females, but also in postpartum females that were briefly exposed to foster pups 7 days after delivery. This suggests two possible changes induced in the brain of female mice, as a consequence of pregnancy, parturition and lactation. One possibility is that pup-sensitive cells that did not express OT at histochemically detectable levels in the brain of virgin mice start expressing OT after delivery. Alternatively, pup-insensitive, OT-expressing cells in the brain of virgin females become sensitive to pup stimuli after delivery.

Tsuneoka et al. (2013) also performed ibotenic acid lesions of different portions of the preoptic area. In both virgin and parous females, lesioning the central MPA results in an increased latency to retrieve pups and in pup killing. However, apparently ibotenic lesions did not affect the number of OTergic cells, thus suggesting that these effects of the lesions are not directly dependent on the OTergic neurons, but on non-OTergic cells of this region.

In the model of maternal behaviour proposed by Numan and Woodside (2010), the MPOA-BSTv region regulates the response to pups through their projections to two different functional systems. The projections of MPOA and ventral BST target several structures relevant for maternal behaviour, including to the Acb core and shell, the ventral tegmental area (VTA) and the Ce. On the one hand, projections to the ventral tegmental area (VTA) would facilitate proactive and voluntary maternal responses, which would constitute pup-directed appetitive behaviours. On the other hand, other projections of the MPOA-BSTv would reduce anxiety responses to pups, thus decreasing pup avoidance. These authors also propose a role of OT in facilitating the change from the non-maternal (pup avoidance, no pup-directed behaviours) to the maternal state (pup approach and pup-directed proactive responses) in rats. Our results suggest that nonapeptidergic cells in the ADP/AC and anterior Pa could be responsible

for these effects. Proactive, pup-directed maternal responses might be facilitated not only by preoptic/BSTv OTergic projections to the VTA (Numan and Numan 1996), which increase dopamine neurotransmission in the Acb in response to pup stimuli (Shahrokh et al. 2010), but maybe also by the release of OT/AVP in the Acb shell and core. In fact, it has been shown in voles that activation of oxytocin receptors in the Acb facilitates maternal behaviour (Olazábal and Young 2006).

Furthermore, suppression of pup avoidance by OT might also be facilitated by nonapeptide release in the Ce leading to fear/anxiety down-regulation, as revealed by the detailed electrophysiological and optogenetic studies by Knobloch et al. (2012) in the rat. Again, these key nonapeptidergic fibres might arise from the OT + AVP-expressing cells in the ADP/AC and anterior Pa.

In this way, activation of this key nonapeptidergic cell groups in the ADP/AC and anterior Pa would lead to both facilitating effects of OT on maternal behaviour. Thus, the onset of maternal behaviour might be associated with either specific activation of these cell groups by pup stimuli (Tsuneoka et al. 2013) and/or a change in the expression pattern of nonapeptides in these or adjacent cells and/or of their receptors in their synaptic targets (e.g. Acb, VTA, Ce). Although variations in the expression of nonapeptide receptors during motherhood are well documented (e.g. Caughey et al. 2011), there is also evidence for an increased expression of the nonapeptides in relation to pregnancy and lactation. For instance, in the rat it has been shown that the basal levels of co-localization in SON magnocellular neurons rise from 2 to 3 % up to 17 % in SON somata, and up to 24 % at nerve terminals in the posterior pituitary (Mezey and Kiss 1991) at the second day of lactation, due to an increase in the expression of AVP in OT neurons (see also Jirikowski et al. 1991). In addition, OTergic projections from the Pa to the Ce overexpress the neuropeptide during lactation (Knobloch et al. 2012). Further studies are needed to check whether similar up-regulation of the expression of nonapeptides occurs in the central-projecting cell groups during motherhood in mice.

Conclusions

Our results show that in the brain of mice, there is a population of cells expressing both OT and AVP at immunohistochemically detectable levels.

These cells are concentrated in a preoptic centre just ventromedial to the posterior BST and in the rostral edge of the Pa. The distribution of fibres with a similar immunohistochemical profile, together with tracing studies in other rodents, suggests that these cells might innervate the Acb, Ce and portions of the intervening BST. A review of previous literature indicates that cells in this location are critical for the expression of maternal behaviour (Numan and Numan 1996; Tsuneoka et al. 2013), through their projections onto systems involved in motivation (increased pup-directed responses) and anxiety (decreased pup-elicited avoidance responses). Our data thus suggest that changes in these cells or in their targets in the ventral striatum and central amygdala (e.g. in the expression of nonapeptides or their receptors) might result in the onset of maternal behaviour involving decreased pup avoidance and the expression of pup-directed appetitive responses in rats, as well as maternal aggression in mice and rats. Given the nearly spontaneous pup care shown by females of mice, differences between rats and mice are expected in this neural system (e.g. relative to hormone sensitivity).

Acknowledgments

This work was funded by the Spanish Ministry of Science—FEDER (BFU2013-47688-P), the Junta de Comunidades de Castilla-La Mancha/FEDER (PEIC11-0045-4490) and the Universitat Jaume I. This work is part of the doctoral thesis of Marcos Otero-García, granted by the FPU (Formación de Profesorado Universitario) programme of the Spanish Ministry of Education and Science. The authors gratefully acknowledge Dr. Harold Gainer for his generous gift of antibodies. ~~This paper is part of the doctoral thesis of Marcos Otero-García.~~

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Banczerowski P et al (2003) Lesion of the amygdala on the right and left side suppresses testosterone secretion but only left-sided intervention decreases serum luteinizing hormone level. *J Endocrinol Invest* 26(5):429–434

Beery AK, Lacey EA, Francis DD (2008) Oxytocin and vasopressin receptor distributions in a solitary and a social species of tuco-tuco (*Ctenomys haigi* and *Ctenomys sociabilis*). *J Comp Neurol* 507(6):1847–1859

Belenky M et al (1992) Ultrastructural immunolocalization of rat oxytocin-neurophysin in transgenic mice expressing the rat oxytocin gene. *Brain Res* 583(1–2):279–286

Ben-Barak Y et al (1985) Neurophysin in the hypothalamo-neurohypophyseal system. I. Production and characterization of monoclonal antibodies. *J Neurosci* 5(1):81–97

Bielsky IF et al (2005) The V1a vasopressin receptor is necessary and sufficient for normal social recognition: a gene replacement study. *Neuron* 47(4):503–513

Bosch OJ (2011) Maternal nurturing is dependent on her innate anxiety: the behavioral roles of brain oxytocin and vasopressin. *Horm Behav* 59(2):202–212

Breiter HC et al (1996) Response and habituation of the human amygdala during visual processing of facial expression. *Neuron* 17(5):875–887

Buijs RM (1978) Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. Pathways to the limbic system, medulla oblongata and spinal cord. *Cell Tissue Res* 192(3):423–435

Buijs RM et al (1978) Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. *Cell Tissue Res* 186:423–433

Butovsky E et al (2006) Chronic exposure to Δ^9 -tetrahydrocannabinol downregulates oxytocin and oxytocin-associated neurophysin in specific brain areas. *Mol Cell Neurosci* 31(4):795–804

Bychowski ME, Mena JD, Auger CJ (2013) Vasopressin infusion into the lateral septum of adult male rats rescues progesterone-induced impairment in social recognition. *Neuroscience* 246:52–58

Cádiz-Moretti B, Martínez-García F, Lanuza E (2013) Neural substrate to associate odorants and pheromones: convergence of projections from the main and accessory olfactory bulbs in mice. In: East ML, Dehnhard M (eds) Chemical signals in vertebrates 12. Springer, New York, pp 269–275. doi:10.1007/978-1-4614-5927-9

Caffé AR et al (1989) Vasopressin and oxytocin systems in the brain and upper spinal cord of *Macaca fascicularis*. *J Comp Neurol* 287(3):302–325

Caldwell H, Young 3rd WS (2006) Oxytocin and vasopressin: genetics and behavioral implications. *Handbook of neurochemistry and molecular neurobiology*, pp 573–607. doi:10.1007/978-0-387-30381-9_25

Caldwell HK, Wersinger SR, Young WS 3rd (2008) The role of the vasopressin 1b receptor in aggression and other social behaviours. *Prog Brain Res* 170:65–72

Campbell P, Ophir AG, Phelps SM (2009) Central vasopressin and oxytocin receptor distributions in two species of singing mice. *J Comp Neurol* 516(4):321–333

Carter CS et al (2008) Oxytocin, vasopressin and sociality. *Prog Brain Res* 170:331–336

Castel M, Morris JF (1988) The neurophysin-containing innervation of the forebrain of the mouse. *Neuroscience* 24(3):937–966

Caughey SD et al (2011) Changes in the intensity of maternal aggression and central oxytocin and vasopressin V1a receptors across the peripartum period in the rat. *J Neuroendocrinol* 23(11):1113–1124

Chamero P et al (2007) Identification of protein pheromones that promote aggressive behaviour. *Nature* 450(7171):899–902

Choleris E, Pfaff DW, Kavaliers M (2013) Oxytocin, vasopressin and related peptides in the regulation of behavior. In: Oxytocin, vasopressin and related peptides in the regulation of behavior. pp 379–381.

<http://ebooks.cambridge.org/ref/id/CBO9781139017855>

Condés-Lara M et al (2007) Branched oxytocinergic innervations from the paraventricular hypothalamic nuclei to superficial layers in the spinal cord. *Brain Res* 1160(1):20–29

DeVries GJ et al (1985) The vasopressinergic innervation of the brain in normal and castrated rats. *J Comp Neurol* 233(2):236–254

Dölen G et al (2013) Social reward requires coordinated activity of nucleus accumbens oxytocin and serotonin. *Nature* 501(7466):179–84. <http://www.ncbi.nlm.nih.gov/pubmed/24025838>

Donaldson ZR, Young LJ (2008) Oxytocin, vasopressin, and the neurogenetics of sociality. *Science* 322(5903):900–904

Dong HW, Swanson LW (2006) Projections from bed nuclei of the stria terminalis, dorsomedial nucleus: implications for cerebral hemisphere integration of neuroendocrine, autonomic, and drinking responses. *J Comp Neurol* 494(1):75–107

Dubois-Dauphin M, Barberis C, De Bilbao F (1996) Vasopressin receptors in the mouse (*Mus musculus*) brain: sex-related expression in the medial preoptic area and hypothalamus. *Brain Res* 743(1–2):32–39

Eaton JL et al (2012) Organizational effects of oxytocin on serotonin innervation. *Dev Psychobiol* 54(1):92–97

Egashira N et al (2007) Impaired social interaction and reduced anxiety-related behavior in vasopressin V1a receptor knockout mice. *Behav Brain Res* 178(1):123–127

Evans DW et al. (2014) Social cognition and brain morphology: implications for developmental brain dysfunction. *Brain Imaging Behav*

Feldman R et al (2010) Natural variations in maternal and paternal care are associated with systematic changes in oxytocin following parent-infant contact. *Psychoneuroendocrinology* 35(8):1133–1141. doi:10.1016/j.psyneuen.2010.01.013

Garcia-Moreno F et al (2010) A neuronal migratory pathway crossing from diencephalon to telencephalon populates amygdala nuclei. *Nat Neurosci* 13(6):680–689

~~Glasgow E et al (1999) Single cell reverse transcription-polymerase chain reaction analysis of rat supraoptic magnocellular neurons: Neuropeptide phenotypes and high voltage-gated calcium channel subtypes. Endocrinology 140(11):5391-401.~~
Glasgow E et al (1999) Single cell reverse transcription-polymerase chain reaction analysis of rat supraoptic magnocellular neurons: neuropeptide phenotypes and high voltage-gated calcium channel subtypes. *Endocrinology* 140(11):5391-401.

AQ10

Gregory R et al (2015) Oxytocin increases VTA activation to infant and sexual stimuli in nulliparous and postpartum women. *Horm Behav* 69:82–88

Hammock EAD, Levitt P (2013) Oxytocin receptor ligand binding in embryonic tissue and postnatal brain development of the C57BL/6J mouse. *Front Behav Neurosci* 7:195. doi: 10.3389/fnbeh.2013.00195
~~<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3858721&tool=pmcusercontent&rendertype=abstract>~~

Hatton GI, Cobbett P, Salm AK (1985) Extranuclear axon collaterals of paraventricular neurons in the rat hypothalamus: intracellular staining, immunocytochemistry and electrophysiology. *Brain Res Bull* 14(2):123–132

Hawthorn J, Ang VT, Jenkins JS (1985) Effects of lesions in the hypothalamic paraventricular, supraoptic and suprachiasmatic nuclei on vasopressin and oxytocin in rat brain and spinal cord. *Brain Res* 346(1):51–57

Hermes MLHJ et al (1988) Oxytocinergic innervation of the brain of the garden dormouse (*Eliomys quercinus* L.). *J Comp Neurol* 273:252–262

Honda K, Higuchi T (2010a) Effects of unilateral electrolytic lesion of the dorsomedial nucleus of the hypothalamus on milk-ejection reflex in

the rat. *J Reprod Dev* 56(1):98–102

Honda K, Higuchi T (2010b) Electrical activities of neurones in the dorsomedial hypothalamic nucleus projecting to the supraoptic nucleus during milk-ejection reflex in the rat. *J Reprod Dev* 56(3):336–340

Hou-Yu A et al (1986) Comparative distribution of vasopressin and oxytocin neurons in the rat brain using a double-label procedure. *Neuroendocrinology* 44(2):235–246

Huber D, Veinante P, Stoop R (2005) Vasopressin and oxytocin excite distinct neuronal populations in the central amygdala. *Science* 308(5719):245–248

Insel TR, Harbaugh CR (1989) Lesions of the hypothalamic paraventricular nucleus disrupt the initiation of maternal behavior. *Physiol Behav* 45(5):1033–1041

Insel TR et al (1993) Gonadal steroids have paradoxical effects on brain oxytocin receptors. *J Neuroendocrinol* 5(6):619–628

Isogai Y et al (2011) Molecular organization of vomeronasal chemoreception. *Nature* 478(7368):241–245

Jin D et al (2007) CD38 is critical for social behaviour by regulating oxytocin secretion. *Nature* 446(7131):41–45

Jirikowski GF, Ramalho-Ortigao FJ, Caldwell JD (1991) Transitory coexistence of oxytocin and vasopressin in the hypothalamo neurohypophysial system of parturient rats. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme* 23(10):476–480

Kang N, Baum MJ, Cherry JA (2009) A direct main olfactory bulb projection to the “vomeronasal” amygdala in female mice selectively responds to volatile pheromones from males. *Eur J Neurosci* 29(3):624–634

Kiyama H, Emson PC (1990) Evidence for the co-expression of

oxytocin and vasopressin messenger ribonucleic acids in magnocellular neurosecretory cells: simultaneous demonstration of two neurohypophysin messenger ribonucleic acids by hybridization histochemistry. *J Neuroendocrinol* 2(3):257–259

Knobloch HS et al (2012) Evoked axonal oxytocin release in the central amygdala attenuates fear response. *Neuron* 73(3):553–566.
doi:10.1016/j.neuron.2011.11.030

Krisch B (1976) Immunohistochemical and electron microscopic study of the rat hypothalamic nuclei and cell clusters under various experimental conditions. Possible sites of hormone release. *Cell Tissue Res* 174(1):109–127

Landgraf R, Neumann ID (2004) Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication. *Front Neuroendocrinol* 25(3–4):150–176

Lim MM, Murphy AZ, Young LJ (2004) Ventral striatopallidal oxytocin and vasopressin v1a receptors in the monogamous prairie vole (*Microtus ochrogaster*). *J Comp Neurol* 468(4):555–570

Liu H et al (1994) Synaptic relationship between substance P and the substance P receptor: light and electron microscopic characterization of the mismatch between neuropeptides and their receptors. *Proc Natl Acad Sci USA* 91(3):1009–1013

Ludwig M, Leng G (2006) Dendritic peptide release and peptide-dependent behaviours. *Nat Rev Neurosci* 7(2):126–136

Lukas M et al (2013) Oxytocin mediates rodent social memory within the lateral septum and the medial amygdala depending on the relevance of the social stimulus: male juvenile versus female adult conspecifics. *Psychoneuroendocrinology* 38(6):916–926

Manning M et al (2012) Oxytocin and vasopressin agonists and antagonists as research tools and potential therapeutics. *J Neuroendocrinol* 24(4):609–628

Markowitsch HJ (1998) Differential contribution of right and left amygdala to affective information processing. *Behav Neurol* 11(4):233–244. <http://www.ncbi.nlm.nih.gov/pubmed/11568425>

Martínez-García F et al (2012) Chapter 6—piriform cortex and amygdala. In: GP Charles Watson, George Paxinos, Luis Puelles, Charles Watson, L Puelles (eds) *The mouse nervous system*, pp 140–172. Academic Press, San Diego.
<http://www.sciencedirect.com/science/article/pii/B9780123694973100068>

Mottotese R et al (2014) Switching brain serotonin with oxytocin. *Proceedings of the National Academy of Sciences of the United States of America*, vol 111, issue 23, pp 8637–42.
<http://www.ncbi.nlm.nih.gov/pubmed/24912179>

Melis MR et al (2007) Oxytocin injected into the ventral tegmental area induces penile erection and increases extracellular dopamine in the nucleus accumbens and paraventricular nucleus of the hypothalamus of male rats. *Eur J Neurosci* 26(4):1026–1035

Merighi A et al (1989) Ultrastructural localization of neuropeptides and GABA in rat dorsal horn: a comparison of different immunogold labeling techniques. *J Histochem Cytochem* 37(4):529–540

Meyer-Lindenberg A et al (2011) Oxytocin and vasopressin in the human brain: social neuropeptides for translational medicine. *Nat Rev Neurosci* 12(9):524–538

Mezey E, Kiss JZ (1991) Coexpression of vasopressin and oxytocin in hypothalamic supraoptic neurons of lactating rats. *Endocrinology* 129(4):1814–1820

Mohr E et al (1988) Expression of the vasopressin and oxytocin genes in rats occurs in mutually exclusive sets of hypothalamic neurons. *FEBS Lett* 242(1):144–148

Muchlinski AE, Johnson DJ, Anderson DG (1988) Electron microscope study of the association between hypothalamic blood vessels and oxytocin-like immunoreactive neurons. *Brain Res Bull* 20(2):267–271

Mullis K, Kay K, Williams DL (2013) Oxytocin action in the ventral tegmental area affects sucrose intake. *Brain Res* 1513:85–91

Nephew BC, Bridges RS (2008) Central actions of arginine vasopressin and a V1a receptor antagonist on maternal aggression, maternal behavior, and grooming in lactating rats. *Pharmacol Biochem Behav* 91(1):77–83

Neumann ID, Landgraf R (2012) Balance of brain oxytocin and vasopressin: Implications for anxiety, depression, and social behaviors. *Trends Neurosci* 35(11):649–659.
<http://dx.doi.org/10.1016/j.tins.2012.08.004>

Newman SW (1999) The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. *Ann N Y Acad Sci* 877:242–257

Ni RJ et al (2014) Distribution of vasopressin, oxytocin and vasoactive intestinal polypeptide in the hypothalamus and extrahypothalamic regions of tree shrews. *Neuroscience* 265:124–136

Nishimori K et al (2008) New aspects of oxytocin receptor function revealed by knockout mice: sociosexual behaviour and control of energy balance. *Prog Brain Res* 170:79–90

Nodari F et al (2008) Sulfated steroids as natural ligands of mouse pheromone-sensing neurons. *J Neurosci* 28(25):6407–6418

~~Numan M, Callahan EC (1980) The connections of the medial preoptic region and maternal behavior in the rat. *Physiol Behav* 25(5):653–665~~

AQ11

Numan M, Numan M (1996) A lesion and neuroanatomical tract-tracing analysis of the role of the bed nucleus of the stria terminalis in retrieval behavior and other aspects of maternal responsiveness in rats. *Dev Psychobiol* 29(1):23–51

Numan M, Woodside B (2010) Maternity: neural mechanisms, motivational processes, and physiological adaptations. *Behav Neurosci*

124(6):715–741

Numan M et al (1988) Axon-sparing lesions of the preoptic region and substantia innominata disrupt maternal behavior in rats. *Behav Neurosci* 102(3):381–396

Olazábal DE, Young LJ (2006) Oxytocin receptors in the nucleus accumbens facilitate “spontaneous” maternal behavior in adult female prairie voles. *Neuroscience* 141(2):559–568

Olazabal DE et al (2002) MPOA cytotoxic lesions and maternal behavior in the rat: effects of midpubertal lesions on maternal behavior and the role of ovarian hormones in maturation of MPOA control of maternal behavior. *Horm Behav* 41(2):126–138

Olucha-Bordonau FE et al (2014) Amygdala: structure and function. In: Paxinos G (ed) *The rat nervous system*. Academic Press, London, pp 441–490

Otero-Garcia M et al (2014) Extending the socio-sexual brain: arginine-vasopressin immunoreactive circuits in the telencephalon of mice. *Brain Struct Funct* 219(3):1055–1081

Pagani JH et al (2015) Raphe serotonin neuron-specific oxytocin receptor knockout reduces aggression without affecting anxiety-like behavior in male mice only. *Genes Brain Behav* 14(2):167–176

Paxinos G, Franklin KBJ (2001) *The mouse brain in stereotaxic coordinates*. Academic Press, San Diego

Pedersen CA (1997) Oxytocin control of maternal behavior. Regulation by sex steroids and offspring stimuli. *Ann N Y Acad Sci* 807: 126–145
AQ12

Pedersen CA et al (1994) Oxytocin activates the postpartum onset of rat maternal behavior in the ventral tegmental and medial preoptic areas. *Behav Neurosci* 108(6):1163–1171

Pobbe RLH et al (2012) Oxytocin receptor knockout mice display

deficits in the expression of autism-related behaviors. *Horm Behav* 61(3):436–444. doi:10.1016/j.yhbeh.2011.10.010

Rhodes CH, Morrell JI, Pfaff DW (1981) Immunohistochemical analysis of magnocellular elements in rat hypothalamus: distribution and numbers of cells containing neurophysin, oxytocin, and vasopressin. *J Comp Neurol* 198(1):45–64

Roberts SA et al (2010) Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. *BMC Biol* 8:75

~~Rood BD, de Vries GJ (2011a) Vasopressin innervation of the mouse (Mus musculus) brain and spinal cord. J Comp Neurol~~

Rood BD, De Vries GJ (2011**b**) Vasopressin innervation of the mouse (Mus musculus) brain and spinal cord. *J Comp Neurol* 519(12):2434–2474

~~Rood BD, de Vries GJ (2011b) Vasopressin innervation of the mouse (Mus musculus) brain and spinal cord. J Comp Neurol~~

Rood BD et al (2013) Site of origin of and sex differences in the vasopressin innervation of the mouse (Mus musculus) brain. *J Comp Neurol* 521(10):2321–2358

Rosen GJ et al (2008) Distribution of oxytocin in the brain of a eusocial rodent. *Neuroscience* 155(3):809–817

Ross HE et al (2009) Characterization of the oxytocin system regulating affiliative behavior in female prairie voles. *Neuroscience* 162(4):892–903. doi:10.1016/j.neuroscience.2009.05.055

Sabatier N, Shibuya I, Dayanithi G (2004) Intracellular calcium increase and somatodendritic vasopressin release by vasopressin receptor agonists in the rat supraoptic nucleus: involvement of multiple intracellular transduction signals. *J Neuroendocrinol* 16(3):221–236

Sanchez MA, Dominguez R (1995) Differential-effects of unilateral

lesions in the medial amygdala on spontaneous and induced ovulation. *Brain Res Bull* 38(4):313–317. <Go to ISI>://A1995RV80800002

Sarnyai Z, Kovács GL (1994) Role of oxytocin in the neuroadaptation to drugs of abuse. *Psychoneuroendocrinology* 19(1):85–117

Shahrokh DK et al (2010) Oxytocin-dopamine interactions mediate variations in maternal behavior in the rat. *Endocrinology* 151(5):2276–2286

Shipley MT, Adamek GD (1984) The connections of the mouse olfactory bulb: a study using orthograde and retrograde transport of wheat germ agglutinin conjugated to horseradish peroxidase. *Brain Res Bull* 12:669–688

Staes N et al (2014) Oxytocin and vasopressin receptor gene variation as a proximate base for inter- and intraspecific behavioral differences in bonobos and chimpanzees. *Plos One* 9(11):e113364. doi:10.1371/journal.pone.0113364

Stoop R (2012) Neuromodulation by oxytocin and vasopressin. *Neuron* 76(1):142–159. doi:10.1016/j.neuron.2012.09.025

Succu S et al (2008) Oxytocin induces penile erection when injected into the ventral tegmental area of male rats: role of nitric oxide and cyclic GMP. *Eur J Neurosci* 28(4):813–821. <http://www.ncbi.nlm.nih.gov/pubmed/18671741>

Swanson LW, Kuypers HG (1980) The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. *J Comp Neurol* 194(3):555–570

Takahashi A, Miczek KA (2013) Neurogenetics of aggressive behavior: studies in rodents. *Curr Top Behav Neurosci* 17:3-44.

Takano S et al (1992) Lesion and electrophysiological studies on the hypothalamic afferent pathway of the milk ejection reflex in the rat.

Neuroscience 50(4):877–883

Tang Y et al (2014) Oxytocin activation of neurons in ventral tegmental area and interfascicular nucleus of mouse midbrain.

Neuropharmacology 77:277–284

Telleria-Diaz A, Grinevich VV, Jirikowski GF (2001) Colocalization of vasopressin and oxytocin in hypothalamic magnocellular neurons in water-deprived rats. *Neuropeptides* 35(3–4):162–167

Tobin V, Leng G, Ludwig M (2012) The involvement of actin, calcium channels and exocytosis proteins in somato-dendritic oxytocin and vasopressin release. ~~Front Physiol 3:1–7~~ *Front Physio* 3:261.

[doi:10.3389/fphys.2012.00261](https://doi.org/10.3389/fphys.2012.00261)

Toth I, Neumann ID (2013) Animal models of social avoidance and social fear. *Cell Tissue Res* 354(1):107–118

Trueta C, De-Miguel FF (2012) Extrasynaptic exocytosis and its mechanisms: a source of molecules mediating volume transmission in the nervous system. ~~Front Physiol 3:1–7~~ *Front Physio* 3:319.

[doi:10.3389/fphys.2012.00319](https://doi.org/10.3389/fphys.2012.00319)

AQ13

Tsuneoka Y, Maruyama T, Yoshida S, Nishimori K, Kato T, Numan M, Kuroda KO (2013) Functional, anatomical, and neurochemical differentiation of medial preoptic area subregions in relation to maternal behavior in the mouse. *J Comp Neurol* 521(7):1633–1663

Valesky EM et al (2012) Distribution of oxytocin- and vasopressin-immunoreactive neurons in the brain of the eusocial mole rat (*Fukomys anselli*). *Anat Rec* 295(3):474–480

Veenema AH, Neumann ID (2008) Central vasopressin and oxytocin release: regulation of complex social behaviours. *Prog Brain Res* 170:261–276

Veinante P, Freund-Mercier MJ (1997) Distribution of oxytocin- and vasopressin-binding sites in the rat extended amygdala: a

histoautoradiographic study. *J Comp Neurol* 383(3):305–325

Wang W, Lufkin T (2000) The murine *Otp* homeobox gene plays an essential role in the specification of neuronal cell lineages in the developing hypothalamus. *Dev Biol* 227(2):432–449

Wang Z et al (1996) Immunoreactivity of central vasopressin and oxytocin pathways in microtine rodents: a quantitative comparative study. *J Comp Neurol* 366(4):726–737

Whitnall MH et al (1985) Neurophysin in the hypothalamo-neurohypophysial system. II. Immunocytochemical studies of the ontogeny of oxytocinergic and vasopressinergic neurons. *J Neurosci* 5(1):98–109

Xi D, Kusano K, Gainer H (1999) Quantitative analysis of oxytocin and vasopressin messenger ribonucleic acids in single magnocellular neurons isolated from supraoptic nucleus of rat hypothalamus. *Endocrinology* 140(10):4677–4682

Xiao M et al (2005) The distribution of neural nitric oxide synthase-positive cerebrospinal fluid-contacting neurons in the third ventricular wall of male rats and coexistence with vasopressin or oxytocin. *Brain Res* 1038(2):150–162

Xu L et al (2010) Oxytocin and vasopressin immunoreactive staining in the brains of Brandt's voles (*Lasiopodomys brandtii*) and greater long-tailed hamsters (*Tscherskia triton*). *Neuroscience* 169(3):1235–1247. doi:10.1016/j.neuroscience.2010.05.064

Yang J et al (2011) Oxytocin in the periaqueductal gray participates in pain modulation in the rat by influencing endogenous opiate peptides. *Peptides* 32(6):1255–1261

Yayou K-I, Ito S, Yamamoto N (2015) Relationships between postnatal plasma oxytocin concentrations and social behaviors in cattle. *Anim Sci J* ~~=Nihon chikusan Gakkaiho~~ 86(8):806-813.

Yoshida M et al (2009) Evidence that oxytocin exerts anxiolytic effects

via oxytocin receptor expressed in serotonergic neurons in mice. *J Neurosci* 29(7):2259–2271

Young LJ, Wang Z (2004) The neurobiology of pair bonding. *Nat Neurosci* 7(10):1048–1054

Young LJ et al (1999) Increased affiliative response to vasopressin in mice expressing the V1a receptor from a monogamous vole. *Nature* 400(6746):766–768

Zoli M, Agnati LF (1996) Wiring and volume transmission in the central nervous system: the concept of closed and open synapses. *Prog Neurobiol* 49(4):363–380