MAPPING THE ACTIONS OF PROLACTIN IN THE MOUSE BRAIN

Sexual Dimorphism, Steroid Regulation and the Neuroendocrinology of Maternal Behaviour

Tesis Doctoral en Neurociencias

Hugo Salais López

Dirigida por

Fernando Martínez García **Carmen Agustín Pavón**

Burjassot y Castellón, 2017

VNIVERSITAT DE VALÈNCIA

Programa de Doctorat en Neurociències Bàsiques i Aplicades

UNIVERSITAT JAUME·I

Fernando Martínez García, Catedràtic de Fisiologia de la Unitat Predepartamental de Medicina de la Universitat Jaume I (UJI) de Castelló, i Carmen Agustín Pavón, Professora Ajudant Doctora del Departament de Biol·logia Cel·lular, Biol·logia Funcional y Antropologia Fìsica de la Universitat de València

Certifique

Que ***en Hugo Salais López****, Màster en Neurociències per la Universitat de València, ha realitzat sota la nostra direcció el treball titulat*

"Mapping the Action of Prolactin in the Brain. Sexual Dimorphism, Steroid Regulation and the Neuroendocrinology of Maternal Behaviour"

per a l'obtención del grau de Doctor en Neurociències Bàsiques i Aplicades.

La qual cosa signem a Burjassot, el dia 18 de maig de 2017

Fernando Martínez-García, PhD

Carmen Agustín-Pavón, PhD

VNIVERSITAT DE VALÈNCIA

Programa de Doctorat en Neurociències Bàsiques i Aplicades

UNIVERSITAT JAUME·I

Fernando Martinez-Garcia, Professor of Physiology of the Predepartmental Unit of Medicine at the Universitat Jaume I (UJI), and Carmen Agustín Pavón, Lecturer of the Department of Cell Biology, Functional Biology and Antropology at the Universitat de València (UV)

Hereby certify

That ***Mr. Hugo Salais López****, MSc in Neuroscience at the University of València, has performed, under our supervision the work entitled:*

"Mapping the Action of Prolactin in the Brain. Sexual Dimorphism, Steroid Regulation and the Neuroendocrinology of Maternal Behaviour"

to obtain the PhD in Basic and Applied Neurosciences.

What we sign as required in Burjassot, on May the 18th, 2017

Fernando Martínez-García, PhD

Carmen Agustín-Pavón, PhD

Durante la realización de esta tesis, el autor ha sido beneficiario de una beca del Programa de Formación de Profesorado Universitario del Ministerio de Educación (FPU 12/05472).

Este trabajo se ha enmarcado en los proyectos financiados por:

- Ministerio de Economía y Competitividad-FEDER. *Señales vomeronasales y control amigdalino del comportamiento sociosexual: un modelo experimental de la neurobiología del comportamiento social y sus alteraciones* (BFU2013-47688-P)

- Junta de Comunidades de Castilla-La Mancha/FEDER. *Alteraciones en los sistemas quimiosensoriales en las enfermedades de Alzheimer y Parkinson* (PEIC11-0045-4490)

- Vicerectorado de Investigación de la Universitat Jaume I de Castelló (UJI-B2016-45)

- Generalitat Valenciana. El cerebro maternal de roedores como modelo para el control de la agresividad: papel de la prolactina y la neurotransmisión oxitocinérgica (Prometeo 2016/076)

- Ministerio de Economía y Competitividad. Defensa maternal, refuerzo y olfacción: Neurobiología de las conductas sociales motivadas dependientes de feromonas en ratones (BFU2016-77691-C2-1-P).

AGRADECIMIENTOS

Emprender el camino del doctorado no siempre es tarea fácil. Este espacio está dedicado a todos aquellos que de una manera u otra han contribuido a allanar el camino para mí y han hecho que éste haya sido un periodo muy enriquecedor en mi vida.

En primer lugar, quiero darte las gracias a ti, Ferrán. Por haberme dado cabida en el grupo y haber valorado mis cualidades dentro y fuera del laboratorio. Por haber guiado mi trabajo durante estos casi 7 años y haber hecho posible que llegue hasta aquí. Pero, sobre todo, por haberme enseñado tanto y aún hoy seguir enseñándome.

Si bien llegaste un poco más tarde, todo esto también es extensivo a ti, Carmen. Gracias por tu apoyo, ayuda y consejo durante este tiempo, por enriquecer este trabajo aportando puntos de vista diferentes y, por supuesto, por enseñarme a escribir menos “barroco” ;)

Gracias a ti también, Quique. Aunque no figure en la documentación oficialmente, tú has sido mi tercer director.

Gracias también a Dori, que siempre ha estado ahí ayudando a que todo funcione y avance.

Llegamos ahora a mis compañeros. Gracias a los que vinieron antes que yo y ahora están desperdigados por el mundo. A Marcos, por seguir poniendo el “lol” en “xylol”, tan vigente ahora como cuando nos conocimos. A Ana, mi compi de comportamiento maternal, y por supuesto a Lluis y a Beña. Espero que os vaya muy bien allá donde acabéis.

Gracias también a los que todavía quedan y los que han ido llegando. A Ceci y Sergio, por los ángeles de relojería y las otras 2112 canciones, por presentarme a los gunters, a Battle Pope y a Harry Dresden, y por supuesto por zurrarme con zombies, robots, princesas y gatetes. A María y a Cinta, a vosotras un “grasias” especial con acento (if you know what I mean). A Manoli y David, a quienes espero seguir conociendo dentro y fuera del trabajo. Y por último, pero no menos importante, gracias también al resto de mis compañeros de UJI, tanto los permanentes como los que han pasado temporalmente por ahí, hacéis que la paliza de coche valga la pena.

Gracias a mis amigos Víctor y Héctor, ha sido un privilegio compartir un puesto en “El Consejo” con vosotros todos estos años, y espero que sea por muchos más.

Gracias por supuesto al Dr. Rodríguez al Cuadrado. Siempre dispuesto a ofrecer una cerveza, buena música y asesoramiento legal, esté en Berlín, Granada, Madrid o Costa Rica.

Tampoco puedo olvidarme de mi familia. Os doy las gracias a todos, habéis sido un apoyo muy importante para mí durante este tiempo, en los momentos buenos y en los no tan buenos. Pero si hoy estoy escribiendo esto, es especialmente gracias a mi madre y a mi padre. No sólo habéis tenido un comportamiento parental excelente conmigo, me habéis dado mi educación, un regalo muy valioso gracias al que hoy estoy aquí.

Y, finalmente, guardo un sitio especial para ti, Erica. Ya estabas ahí cuando me embarqué en todo esto y has sido testigo de ello hasta el final. Me has apoyado y aconsejado en los momentos difíciles y me has dado tu cariño cuando más lo he necesitado. Culminar este trabajo y esta etapa no habría sido posible sin tí. Gracias por todo.

"La verdadera ciencia enseña, por encima de todo, a dudar y a ser ignorante"

Miguel de Unamuno

"¡Vamos Smithers, esto no es una ciencia exacta, es neurocirujía!"

C.M. Burns

INDEX

FIGURE AND TABLE LIST

ABBREVIATIONS

PRL	prolactin
PRLR	prolactin receptor
PRLR-l	prolactin receptor, long form
PRLR-s	prolactin receptor, short form
AVP	arginine vasopressin
OXT	oxytocin

10N	dorsal motor nucleus of vagus
12N	hypoglossal nucleus
3V	third ventricle
AAD	anterior amygdaloid area, dorsal part
AAV	anterior amygdaloid area, ventral part
AC	anterior commissural nucleus
aca	anterior commissure, anterior part
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
AHC	anterior hypothalamic area, central part
AHiAL	amygdalohippocampal area, anterolateral part
AHiPM	amygdalohippocampal area, posteromedial part
AHP	anterior hypothalamic area, posterior part
AP	area postrema
APT	anterior pretectal nucleus
Aq	aqueduct (Sylvius)
Arc	arcuate hypothalamic nucleus
AVPe	anteroventral periventricular nucleus
BAC	bed nucleus of the anterior commissure
Bar	Barrington´s nucleus
BIC	nucleus of the brachium of the inferior colliculus
bic	brachium of the inferior colliculus
BLA	basolateral amygdaloid nucleus, anterior part
BLP	basolateral amygdaloid nucleus, posterior part
BLV	basolateral amygdaloid nucleus, ventral part
BMA	basomedial amygdaloid nucleus, anterior part
BMP	basomedial amygdaloid nucleus, posterior part
bp	brachium pontis
BSTIA	bed nucleus of the stria terminalis, intraamygdaloid division
BSTLD	bed nucleus of the stria terminalis, lateral division, dorsal part
BSTLJ	bed nucleus of the stria terminalis, lateral division, juxtacapsular 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
BSTMV	bed nucleus of the stria terminalis, medial division, ventral 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
cc	corpus callosum
CC	central canal
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, anterodorsal part
CeMPV	central amygdaloid nucleus, medial division, posteroventral part

cg	cingulum
CI	caudal interstitial nucleus of the medial longitudinal fasciculum
Cir	circular nucleus
CLi	caudal linear nucleus of the raphe
CP	choroid plexus
cp	cerebral peduncle, basal part
CPu	caudate putamen (striatum)
Cu	cuneate nucleus
CxA	cortex-amygdala transition zone
D3V	dorsal third ventricle
Den	dorsal endopiriform nucleus
Dk	nucleus of Darkschewitsch
DLG	dorsal lateral geniculate nucleus
DLPAG	dorsolateral periaqueductal grey
DM	dorsomedial hypothalamic nucleus
DMPAG	dorsomedial periaqueductal grey
DMTg	dorsomedial tegmental area
DP	dorsal peduncular cortex
DpG	deep grey layer of the superior colliculus
DpMe	deep mesencephalic nucleus
DPO	dorsal periolivary region
DpWh	deep white layer of the superior colliculus
DR	dorsal raphe nucleus
DTgC	dorsal tegmental nucleus, central part
DTT	dorsal taenia tecta
eml	external medullary lamina
EW	Edinger-Westphal nucleus
f	fornix
fi	fimbria of the hippocampus
fmi	forceps minor of the corpus callosum
fr	fasciculus retroflexus
HDB	nucleus of the horizontal limb of the diagonal band
I	intercalated nuclei of the amygdala
ic	internal capsule
IF	interfascicular nucleus
IGL	intergeniculate leaf
IL	infralimbic cortex
InC	interstitial nucleus of Cajal
InCO	intercollicular nucleus
InG	intermediate grey layer of the superior colliculus
InWh	intermediate white layer of the superior colliculus
IP	interpeduncular nucleus
IPAC	interstitial nucleus of the posterior limb of the anterior commissure
IRt	intermediate reticular nucleus
La	lateral amygdaloid nucleus
LA	lateroanterior hypothalamic nucleus
LC	locus coeruleus
Ld	lambdoid septal zone
LD	laterodorsal thalamic nucleus
LDTg	laterodorsal tegmental nucleus
LGP	lateral globus pallidus
LH	lateral hypothalamic area
LHb	lateral habenular nucleus
ll	lateral lemiscus
lo	lateral olfactory tract
LOT	nucleus of the lateral olfactory tract
LP	lateral posterior thalamic nucleus
LPAG	lateral periaqueductal grey
LPBC	lateral parabrachial nucleus, central part
LPBD	lateral parabrachial nucleus, dorsal part
LPBE	lateral parabrachial nucleus, external part
LPBI	lateral parabrachial nucleus, internal part
LPBV	lateral parabrachial nucleus, ventral part
LPMR	lateral posterior thalamic nucleus, mediorostral part
LPO	lateral preoptic area
LRt	lateral reticular nucleus
LSD	lateral septal nucleus, dorsal part
LSI	lateral septal nucleus, intermediate part
LSO	lateral superior olive
LSV	lateral septal nucleus, ventral part
LV	lateral ventricle
MCLH	magnocellular nucleus of the lateral hypothalamus
mcp	middle cerebellar peduncle
MCPC	magnocellular precommissural nucleus
MCPO	magnocellular preoptic nucleus
MdD	medullary reticular nucleus, dorsal part
MdV	medullary reticular nucleus, ventral part
ME	median eminence
MeA	medial amygdaloid nucleus, anterior part
MePD	medial amygdaloid nucleus, posterodorsal part
MePV	medial amygdaloid nucleus, posteroventral part
MGD	medial geniculate nucleus, dorsal part
MGM	medial geniculate nucleus, medial part
MGP	medial globus pallidus
MGV	medial geniculate nucleus, ventral part
MHb	medial habenular nucleus
ml	medial lemniscus

mlf	medial longitudinal fasciculus
MnPO	median preoptic nucleus
MnR	median raphe nucleus
MPA	medial preoptic area
MPB	medial parabrachial nucleus
MPBE	medial parabrachial nucleus, external part
MPO	medial preoptic nucleus
MS	medial septal nucleus
mt	mammilothalamic tract
MTu	medial tuberal nucleus
MZMGV	marginal zone of the medial geniculate
ns	nigrostriatal bundle
opt	optic tract
OPT	olivary pretectal nucleus
OT	nucleus of the optic tract
ox	optic chiasm
PAG	periaqueductal grey
PaLM	paraventricular nucleus, lateral magnocellular part
PaMM	paraventricular nucleus, medial magnocellular part
PaPO	paraventricular nucleus, posterior part
PaV	paraventricular nucleus, ventral part
PCom	nucleus of the posterior commissure
Pe	periventricular hypothalamic nucleus
PeF	perifornical nucleus
PF	parafascicular thalamic nucleus
PH	posterior hypothalamic area
PIL	posterior intralaminar thalamic nucleus
Pir	piriform cortex
PLCo	posterolateral cortical amygdaloid nucleus
PM	paramedian lobule
PMD	premammillary nucleus, dorsal part
PMV	premammillary nucleus, ventral part
PnC	pontine reticular nucleus, caudal part
PnO	pontine reticular nucleus, oral part
PnR	pontine raphe nucleus
PnV	pontine reticular nucleus, ventral part
PoT	posterior thalamic nuclear group, triangular part
PP	peripeduncular nucleus
PPT	posterior pretectal nucleus
PPTg	pedunculopontine tegmental nucleus
Pr5	principal sensory trigeminal nucleus
PSTh	parasubthalamic nucleus
PV	paraventricular thalamic nucleus
PVA	paraventricular thalamic nucleus, anterior part
py	pyramidal tract
R	red nucleus
RCh	retrochiasmatic area
Re	reuniens thalamic nucleus
RMg	raphe magnus nucleus
Rob	raphe obscurus nucleus
rs	rubrospinal tract
Rt	reticular thalamic nucleus
s5	sensory root of the trigeminal nerve
SCh	suprachiasmatic nucleus
SCO	subcommissural organ
scp	superior cerebellar peduncle
SFi	septofimbrial nucleus
SFO	subfornical organ
SG	suprageniculate thalamic nucleus
SHi	septohippocampal nucleus
SI	substantia innominata
SLEA	sublenticular extended amygdala
sm	stria medullaris of the thalamus
SN	substantia nigra
SNC	substantia nigra, pars compacta
SNR	substantia nigra, reticular part
SO	supraoptic nucleus
sol	solitary tract
Sol	nucleus of the solitary tract
SolC	nucleus of the solitary tract, commissural part
SolDL	nucleus of the solitary tract, dorsolateral part
SolG	nucleus of the solitary tract, gelatinous part
SolM	nucleus of the solitary tract, medial part
SolV	nucleus of the solitary tract, ventral part
Sp5	spinal trigeminal nucleus
Spa	subparaventricular zone of the hypothalamus
SPFPC	subparafascicular thalamic nucleus, parvicellular part
SPO	superior paraolivary nucleus
st	stria terminalis
STh	subthalamic nucleus
str	superior thalamic radiation
SubB	subbrachial nucleus
SubCD	subcoeruleus nucleus, dorsal part
SubCV	subcoeruleus nucleus, ventral part
SubG	subgeniculate nucleus
SubI	subincertal nucleus
SuMM	supramammillary nucleus, medial part
TC	tuber cinereum
Te	terete hypothalamic nucleus
Tu	olfactory tubercle
VEn	ventral endopiriform nucleus
VLG	ventral lateral geniculate nucleus

VLGMC	ventral lateral geniculate nucleus, magnocellular part
VLGPC	ventral lateral geniculate nucleus, parvicellular part
VLPAG	ventrolateral periaqueductal grey
VLPO	ventrolateral preoptic 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
VRe	ventral reuniens thalamic nucleus
vsc	ventral spinocerebellar tract
VTA	ventral tegmental area
ZI	zona incerta
ZID	zona incerta, dorsal part
ZIV	zona incerta, ventral part

I

General Introduction

The correct function of the body involves the coordinated action of its two main regulatory systems: the nervous and endocrine systems. For a successful neuroendocrine regulation, both systems have to communicate and interact reciprocally. In this regard, the brain is the main director of endocrine signals in the body (acting through the hypothalamus and pituitary gland), but hormones do also target the brain to regulate central functions and behaviour. Endocrine regulation of the brain is commonly carried out by steroid hormones which, given their lipoid nature (as cholesterol derivatives), can readily access the brain by passively diffusing through the blood-brain barrier (BBB). Conversely, peptide hormones (at least proteins with considerable size) lack the capacity of crossing the BBB and, consequently, have their actions generally restricted to the systemic level, being unable to directly influence behaviour.

Prolactin (PRL) is a remarkable exception to this scenario. Despite its peptide nature, this hormone can be transported into the brain by means of at least one active transport mechanism (Walsh et al. 1987; Mangurian et al. 1992). Furthermore, the PRL receptor (PRLR) is widely expressed by neurons in many brain nuclei (Bakowska and Morrell 1997). As a result, PRL is an important endocrine regulator of the brain, currently known to intervene in multiple processes related to homeostasis, reproduction and behaviour (Freeman et al. 2000), in the female and also in the male brain. Perhaps the most eminent feature of PRL is its key role in maternal physiology. Discovered and best-known for its role in mammary gland development and milk production during lactation in mammals (Riddle et al. 1933a), PRL exerts a complex and coordinated regulation of the brain during motherhood, adjusting physiology for the particular demands of this period and promoting the expression of maternal behaviours to properly manage the offspring.

This work is aimed at better characterizing the central actions of PRL in the mouse brain, focusing on its interaction with other important neuroendocrine regulators and its multifaceted role in the neuroendocrinology of reproduction and motherhood. We will initiate this introduction by characterising the main molecular and endocrine features of PRL and its signalling mechanisms, including the PRL receptor (PRLR), PRL-associated signal transduction pathways and the regulation of PRL signalling in the brain, with special attention to its interaction with gonadal steroids. Then, we will give some insights on the functional identity of PRL, focusing on its paramount role integrating

neurological, physiological and behavioural adaptations to the maternal period. Finally, we will focus on the lactogenic regulation of maternal behaviour.

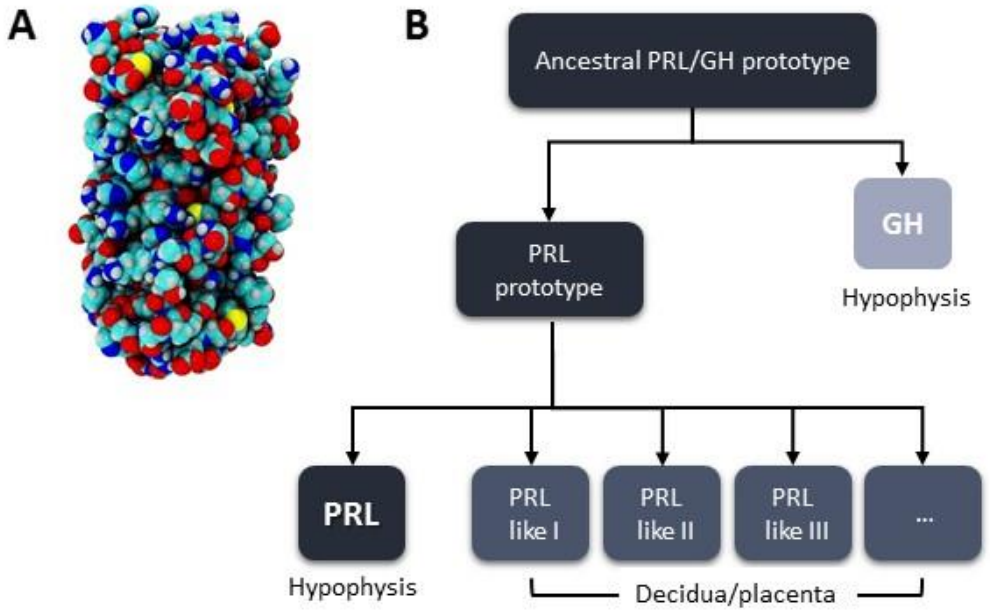


Figure I.1 Prolactin: molecular and phylogenetical features

(A) 3D structural model of Prolactin (PRL). PRL is a polypeptide hormone synthesized and secreted by the lactoroph cells of the adenohypohpysis. The PRL molecule has a molecular weight of 23 kDa and is arranged in a single amino acid chain, folded due to three intramolecular disulphide bonds. The structure of PRL is subject to substantial heterogeneity due to differing glycosylation, phosphorylation and sulfation processes.

(B) PRL has multiple structural homologues in the body, including growth hormone and placental lactogens. PRL, growth hormone and placental lactogens all belong to the group I of the helix-bundle protein hormone/cytokine family. Genes encoding these molecules diverged from a common ancestral gene by gene duplication 400 million years ago (Freeman et al. 2000). Modified and adapted and from Soares et al. (2004).

A. PROLACTIN – GENERAL FEATURES

Prolactin is a polypeptide hormone (Fig I.1A) belonging to the type I helix-bundle protein (cytokine) family (Rand-Weaver et al. 1991), which includes also growth hormone (GH) and other lactogenic molecules such as placental lactogens (Horseman and Yu-Lee 1994). The protein is encoded by a single gene in humans and other primates and by several paralogue genes in rats, mice and other mammals (Soares 2004). The main form of the molecule consists of 197 to 199 amino acids (depending on the species) with a molecular mass of 23 kDa (Shome and Parlow 1977; Kohmoto et al. 1984), but several structural variants exist, generated by alternative splicing, proteolytic cleavage and other posttranslational modifications (Freeman et al. 2000).

Despite the major involvement of PRL in mammalian reproduction and lactation, the evolutionary origin of PRL precedes ostensibly the appearance of mammals (Fig I.1B). The PRL gene seems to have originated in teleost fish (Rand-Weaver et al. 1991; Breves et al. 2014) or even in chondrichthyans (Yamaguchi et al. 2015) by duplication of the ancestral GH gene (Kawauchi and Sower 2006). The primitive function of PRL, however, remains poorly understood. In chondrichthyan and teleost fish, PRL exerts osmorregulatory functions aimed at maintaining plasma homeostasis in response to environmental osmolarity variations (Breves et al. 2014). Alternatively, it is hypothesized that PRL originated to regulate different aspects of the post-mating phase of reproduction, several of them closely related to integumentary and osmolar regulation (Horseman and Gregerson 2013). Thus, somehow, in mammals PRL eventually assumed the role of regulating integumentary glandular development and secretion of milk (Horseman and Gregerson 2013).

In mammals, the major site of PRL synthesis are the lactotrophs , a heterogeneous population of specialized secretory cells located in the ventrolateral portion of the adenohypophysis (Nakane 1970). In addition, PRL and other structural homologues with lactogenic properties are produced in a wide range of tissues and organs. These include the placenta, amnion and decidua (synthesizing the so-called placental lactogens, Soares 2004), the non-pregnant uterus (Walters et al. 1983), male reproductive organs (Marano and Ben-Jonathan 2014), the mammary gland (where PRL is released into milk itself, Grosvenor et al. 1993), lymphocytes and other immuno-competent cells (Gala and Shevach 1994) and, importantly, the brain. In the brain, PRL-producing neuron populations have been localized to date within the hypothalamus of the rat, namely in the paraventricular and supraoptic nuclei (Mejía et al. 1997), whereas in situ hybridisation performed in sheep brain tissue identified PRL expression in the paraventricular and periventricular nuclei, medial preoptic area and bed nucleus of the stria terminalis (Roselli et al. 2008). Conversely, PRL-immunoreactive nerve fibers have been characterised in a variety of mammals within several hypothalamic and extrahypothalamic sites (including the cerebral cortex, hippocampus, amygdala, septum, caudate putamen, cerebellum, brainstem and circumventricular organs, Freeman et al. 2000). Still, little is known so far about the signalling dynamics and functionality of centrally-produced PRL.

Consistent with its functional complexity, PRL has a great number of target tissues. In addition to the mammary gland, PRL binding sites have been detected in the

reproductive system of male and female, hypophysis, heart, lungs, kidney, liver, pancreas, muscle or skin, among others (Freeman et al. 2000). Perhaps, the most important target for PRL action, and the one that will focus the present work, is the brain. In addition to locally-produced PRL, systemic PRL can also be granted access to the brain, bypassing the blood-brain barrier through an active transport mechanism mediated by the choroid plexus (Walsh et al. 1987; Mangurian et al. 1992). Furthermore, PRL might gain access to the SNC through circumventricular organs, specialized regions lacking conventional blood-brain barrier (Ganong 2000).

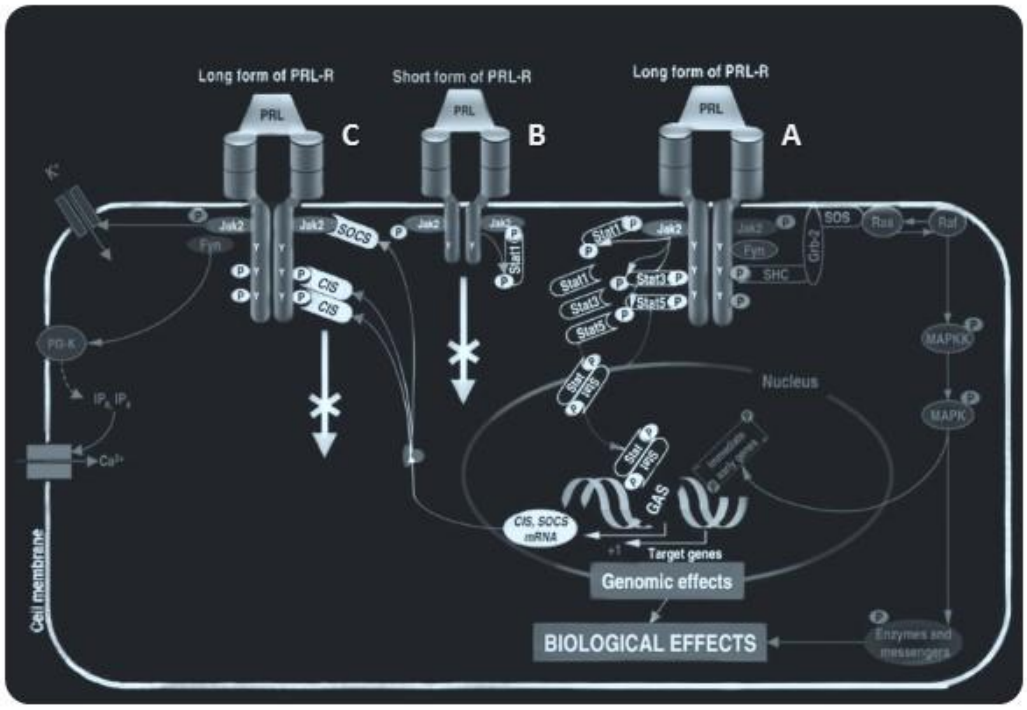


Figure I.2 The prolactin receptor: structural variants and signalling pathways

Prolactin binds to the PRL receptor (PRLR), a single membrane-bound protein of the class 1 cytokine receptor superfamily. The structural variants of the PRLR are highly variable between species and are generally classified as short- or long isoforms, according to the length of their intracellular domain. Modified and adapted from Freeman et al. (2000).

(A) The main signalling pathway associated to the activation of the long form of the PRLR (PRLRl) is the Jak/STAT pathway. After PRL binding and heterodimerization of the PRLRl, the Janus kinase (JaK) docks to the intracellular domain of the PRLRl heterodimer and initiates a chain of transphosphorylation of several STAT (Signal Transducer and Activator of Transcription) proteins. The final step of this pathway involves phosphorylation of STAT5 to pSTAT5 and translocation to the cell nucleus, where it binds to GAS motifs to mediate the biological effects of PRL signalling. The PRLRl is known to trigger alternative signalling pathways, too, such as the MAP kinase pathway.

(B) The short form of the PRLR (PRLRs) is considered to be non-functional, unable to initiate the Jak/STAT and other pathways.

(C) Signalling of the PRLR is subject to a negative feedback control mediated mainly by CIS and SOCS proteins.

B. PROLACTIN RECEPTOR AND SIGNALLING

Prolactin exerts its action through its binding to the PRL receptor (PRLR). The PRLR is a single membrane-bound protein belonging to the class 1 cytokine receptor superfamily (Bazan 1990), with a single extracellular domain plus a transmembrane and an intracellular domain (Freeman et al. 2000). Several PRLR isoforms have been identified to date, generated by different transcription initiation sites and by alternative splicing (Hu and Dufau 1991). Variants of the PRLR have identical extracellular and transmembrane domains, differing only in the composition and length of the intracellular domain (Bole-feysot et al. 1998). These isoforms also occur with high interspecies variability. Rats, for instance, have three total isoforms (with a short, intermediate and long intracellular domain, respectively), whereas mice express one long and three different short isoforms of the PRLR (Bole-feysot et al. 1998). The long forms are considered fully functional, able to initiate the conventional signalling pathways associated to the PRLR, whereas short isoforms of the PRLR are deemed non-functional (Berlanga et al. 1997).

Figure I.2 illustrates the structural variability of the PRLR, as well as the major signalling pathways associated to each PRLR variant. Successful initiation of PRLR signalling involves the binding of PRL to the PRLR and the subsequent dimerization of a second functional PRLR to the hormone-receptor complex (Goffin et al. 1996). Then, signal transduction is initiated by the phosphorylation of intracellular domain-associated Janus kinase 2 (Jak2, Lebrun et al. 1995), which in turn transphosphorylates several docking sites of the intracellular domain of the PRLR (Rui et al. 1992). Upon this event, several signalling cascades can be triggered. The main pathway associated to the PRLR involves the STAT protein family (Signal Transducers and Activators of Transcription) and is termed the Jak/STAT pathway (Ihle et al. 1994). In this pathway, several different members of the STAT family interact with the PRLR after Jak2 phosphorylation, leading ultimately to the phosphorylation of STAT5 (Bole-feysot et al. 1998). Then, pSTAT5 dimerizes and translocates to the nucleus to bind to specific DNA domains, where it acts as a transcription factor, leading to genomic and biological responses of the cell (Bole-feysot et al. 1998). Although Jak/STAT is the major pathway associated to the PRLR, especially in the brain and other sites (Freeman et al. 2000), evidence indicates that alternative signalling events might be triggered by PRLR activation, too. These include activation of the mitogen-activated protein (MAP) kinase pathway (Buckley et al. 1994)

or other kinases (Berlanga et al. 1995; al-Sakkaf et al. 1997) or changes in K^+ (Prevarskaya et al. 1995) or Ca^{2+} (Ratovondrahona et al. 1998) concentrations.

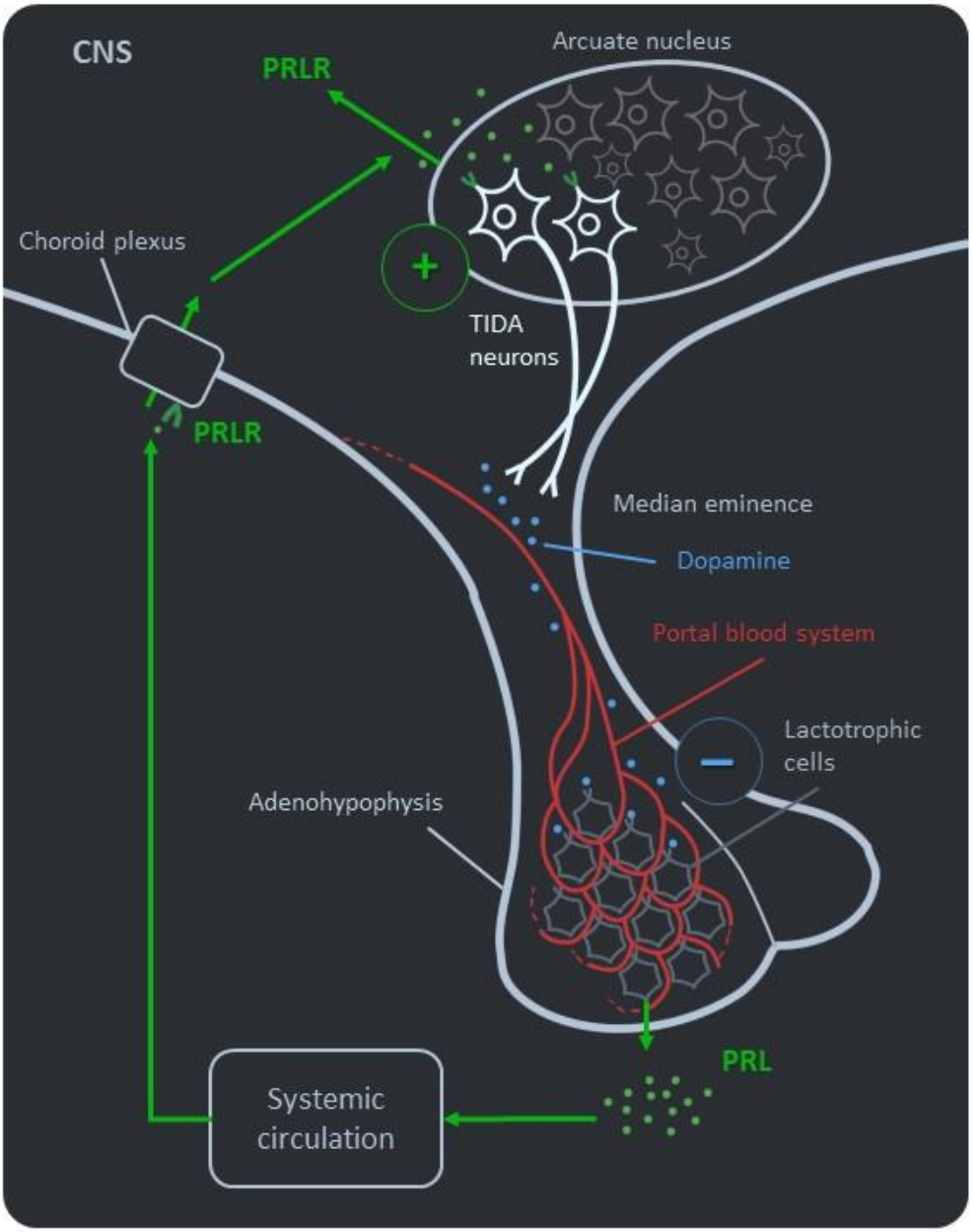


Figure I.3 Feedback regulation of hypophyseal prolactin secretion

The main source of systemic PRL are the lactotrophs of the adenohypophysis. Lactotrophic synthesis and secretion of PRL is regulated by a short-loop negative feedback mechanism dependent on the tuberoinfundibular dopaminergic (TIDA) neurons. Systemically released PRL enters the CNS through the choroid plexi and circumventricular organs and activates the TIDA neurons in the Arcuate nucleus of the hypothalamus. Then, TIDA neurons, the axons of which run through the Median Eminence, release Dopamine into the hypophyseal portal blood system. Dopamine then inhibits PRL secretion of the hypophyseal lactotrophs acting through D2 receptors. Modified and adapted from Grattan et al. (2002).

C. REGULATION OF CENTRAL PROLACTIN SIGNALLING

The multiple and pleiotropic roles of PRL on the central control of brain function, physiology and behaviour require a complex and fine-tuned regulation of PRL signalling within the brain. Mechanisms integrating this regulatory system can be roughly divided into five categories. The first milestone of central regulation of PRL signalling is the secretion of PRL itself. As stated above, major sources of PRL interacting with brain tissue in normal conditions are systemic PRL (Freeman et al. 2000) and brain PRL. Although there is growing evidence of PRL being produced within the boundaries of the brain (DeVito et al. 1987; Paut-Pagano et al. 1993; Marano and Ben-Jonathan 2014), knowledge on the actual dynamics and functional role of this lactogenic element is still scarce. Conversely, the mechanisms underlying feedback regulation of hypophyseal lactotroph cells have been thoroughly characterized. Lactotroph cells have a high degree of spontaneous secretory activity, and are mainly under a tonic inhibitory control mediated by a short-loop negative feedback mechanism carried out by the hypothalamus (Freeman et al. 2000). The main inhibitor of lactotroph function is dopamine (Ben-Jonathan and Hnasko 2001a), which is released to the hypothalamo-hypophyseal portal vascular system by tuberoinfundibular dopaminergic (TIDA) and periventricular-hypophyseal dopaminergic (PHDA) neurons (Goudreau et al. 1992). Dopamine then inhibits secretory function of hypophyseal lactotroph cells acting through D2 receptors (Caron et al. 1978). The short-loop negative feedback regulation mentioned above is established by the reciprocal activation of TIDA and PHDA neuron activity by PRL (DeMaria et al. 1999; Lyons et al. 2012), mediating PRL inhibition of its own secretion. Figure I.3 illustrates the main features of this regulatory mechanism, focusing on TIDA neurons. Activation of PRL secretion is achieved, in turn, by disinhibition, i.e., inhibition of this negative feedback mechanism. The disinhibition of PRL secretion might occur under special physiological situations, for instance during the period of lactation (Andrews et al. 2001b; Anderson et al. 2006).

The second level of central PRL signalling regulation is the access of systemic PRL into the brain. Although PRL is apparently unfit to diffuse through the blood-brain barrier, an active mechanism in the choroid plexus transports it into the brain (Walsh et al. 1987; Mangurian et al. 1992). This active transport was proposed to depend on PRLR, but recent evidence suggests additional mechanisms independent of PRLR (Brown et al. 2015). Assuming the existence of a PRLR-mediated import of PRL into the brain, the regulation of PRLR expression (Mangurian et al. 1992) or of its affinity to PRL (Tabata et

al. 2012) represents additional mechanisms to regulate PRL access to the brain and thus central PRL signalling.

The third level of regulation of PRL signalling pertains to the expression of its receptors in the brain. A comprehensive mapping of the sites of expression of the PRLR is currently available for the female rat brain (Bakowska and Morrell 1997; Bakowska and Morrell 2003). However, there is extensive evidence in the literature indicating a local or global regulation of the expression of PRLR under certain physiological conditions or stimuli, namely the period of lactation (Pi and Grattan 1999), suckling somatosensory stimulation (Pi and Voogt 2001), parturition or even just the presence of pups (Ma et al. 2005a), chronic stress (Faron-Górecka et al. 2014) or the circulating levels of PRL (Muccioli and Di Carlo 1994).

In this context, special attention should be paid to gonadal steroids, which stand among the best-documented modulators of PRLR expression and PRL signalling (Furigo et al. 2014b) in the brain. Thus, for example, in the rat estradiol (and to a lesser extent progesterone) are responsible for the upregulation of PRLR expression (long form) in the female rat during the proestrous and estrous phases of the estrous cycle, late pregnancy and lactation (Sugiyama et al. 1994). This upregulation is also extensive to the choroid plexus, so that access of PRL to the brain might also be enhanced during these periods. Conversely, ovariectomy leads to a dramatic downregulation of PRLR expression that can be partially restored by estradiol administration (Sugiyama et al. 1994; Shamgochian et al. 1995). On the other hand, evidence in the mouse indicates that androgens exert an inhibitory role on hypophyseal PRL secretion (O'Hara et al. 2015). Regarding the close regulatory relationship of gonadal steroids and PRL, one of the main purpose of this work is to further characterise the effect of gonadal steroid regulation on central PRL signalling.

The fourth category of PRL regulation comprises different events associated to posttranslational modifications of the PRLR that provide a means to limit or enhance PRLR-associated signal transduction. Thus, for instance, short isoforms (thought to be non-functional, see section 1.2), are hypothesized to work as dominant negative forms of the receptor, sequestering functional forms by heterodimerization (Lesueur et al. 1991; Berlanga et al. 1997).

Finally, differential regulation downstream the PRLR might also take place to modulate PRL signalling in the brain. In this context, a number of molecular species have been

identified to date that exert an inhibitory action on the Jak/STAT pathway of the PRLR. These are SOCS (suppressors of cytokine signalling), which inhibit Jak kinases (Pezet et al. 1999) and CIS (cytokine-inducible SH2-containing proteins), which compete with STAT proteins for the docking sites of the intracellular domain of the PRLR (Masuhara et al. 1997). SOCS are responsible, for example, for the disinhibition of hypophyseal PRL secretion during lactation (Anderson et al. 2006).

Although there is evidence discretely supporting each of these levels of regulation, we intend to provide a global, integrated view of the changes in PRL signalling taking place in the brain of males and females under certain physiological conditions. To do so, we will focus on analysing the lasts steps downstream the cascade of PRL signalling, by employing the immunohistochemmical detection of phosphorylated STAT5 as a functional marker of PRL-derived signalling (see Section B). This methodology provides an integrated view of all the aforementioned mechanisms. The first goal of the present work is to explore whether basal PRL signalling shows a sexually dimorphic pattern in the brain of the mouse. In addition, we aim at analysing the effects of sexual steroids on the pattern of PRL signalling in the brain of females (oestrogens, progesterone) and males (testosterone). Finally, we focus on the well-known role of PRL in the regulation of the maternal condition.

D. CENTRAL ACTIONS OF PROLACTIN AND THE SHAPING OF THE MATERNAL BRAIN

Prolactin was originally identified for its lactogenic function (Riddle et al. 1933a). Today we know that the functional complexity of this hormone lies far beyond the promotion of mammary gland development and milk production: PRL is synthesized in multiple sites and targets a wide array of tissues, subserving more than 300 different biological functions (Bennett and Morris 1989). Central functions of PRL in non-maternal individuals include, among others, the regulation of different neuropeptidergic systems such as corticotropin-releasing factor (CRF), oxytocin or vasopressin (Aguilera et al. 2008; Donner and Neumann 2009; Blume et al. 2009; Vega et al. 2010; Sirzen-Zelenskaya et al. 2011), of the hypothalamus-pituitary-adrenal (HPA) axis (Fujikawa et al. 1995; Fujikawa et al. 2004), pain sensitivity (Nicoletti et al. 1983), sleep and wakefulness (Roky et al. 1995; Machado et al. 2017), or grooming behaviour (Drago et al. 1983; Drago and Lissandrello 2000). One of the most studied actions of PRL in the central nervous system involves the control of sexual behaviour in females and males (Krüger et

al. 2002; Egli et al. 2010). This control appears to be sexually dimorphic, a matter that will be explored in depth in the first chapter of this work (Study 1).

In mammals and other vertebrates, successful reproduction requires a broad range of adaptations to occur in the mother. In order to meet the demands of motherhood, central homeostatic regulation must be readjusted, lactation must be established correctly and new behaviours (maternal behaviours) must emerge. Most of these physiological and behavioural adaptations originate in changes of the brain during pregnancy, favoured by the endocrine signals of this period. These changes must be initiated before parturition and persist during postpartum periods, thus requiring endocrine agents acting through late pregnancy and lactation. Lactogenic hormones (including PRL) fulfil the former criteria and are good candidates to assume this role. During lactation, females display a sustained state of hyperprolactinaemia, granted by disinhibition of hypophyseal PRL release (Andrews et al. 2001a) in response to suckling stimulation by the pups (Freeman et al. 2000; Cservenák et al. 2010). Consistent with the aforementioned criteria, chronic hyperprolactinaemia during the postpartum period grants the maintenance of lactation (lactogenesis and galactopoiesis, Freeman et al. 2000) and other maternal adaptations (see below). During pregnancy, however, circulating PRL levels are high during early stages but drop during the second half of pregnancy (approximately from day 8-9 in mice and 10 in rats, Soares et al. 2004) until the moment of delivery (Fig I.4). Nevertheless, this suppression of hypophyseal PRL release is compensated by an alternative source of lactogenic signals: coinciding with the drop in hypophyseal PRL, the trophoblast giant cells of the placenta produce the so-called placental lactogens (PLs) (Yamaguchi et al. 1992). These are proteins closely related in sequence to hypophyseal PRL that also bind the PRLR with high affinity (Kelly et al. 1976) and initiate the same signalling pathways (Soares et al. 1998a), thus mimicking several actions of hypophyseal PRL (Bridges and Freemark 1995; Linzer and Fisher 1999). Hence, they are considered functional substitutes of hypophyseal PRL during mid- and late pregnancy. One of the main goals of this work is to characterise the patterns of lactogenic signalling occurring in the brain during the second half of pregnancy in the mouse, as well as to explore the hypophyseal or extrahypophyseal source of this signalling.

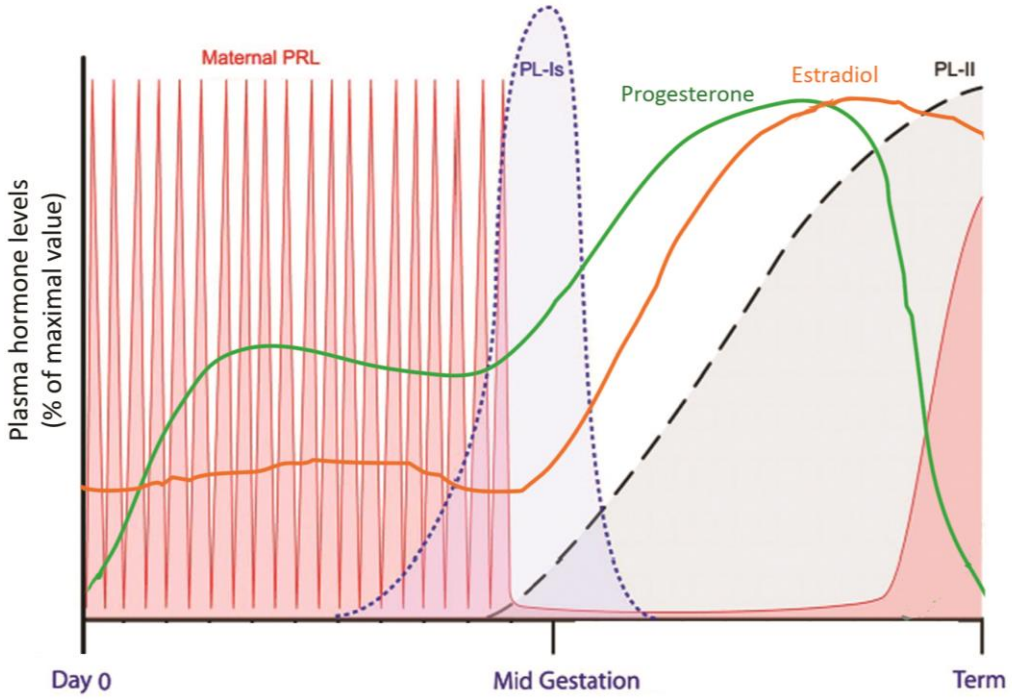


Figure I.4 Profiles of gonadal steroids and lactogenic hormones during pregnancy

The most relevant endocrine signals of pregnancy are gonadal steroids estradiol (orange) and progesterone (green) and PRL (pink). This graph depicts the systemic levels of these hormones during pregnancy in the rat and other rodents. After impregnation, PRL secretion adopts a twice-daily surge profile. Close before mid-gestation, coinciding with the rise in gonadal steroids, the placenta begins to produce placental lactogen I (PL-I, blue), which in turn stimulates the synthesis of placental lactogen II (PL-II, black). Both PRL structural homologues inhibit the production of hypophyseal PRL, which during the second half of pregnancy shows very low circulating levels. During the last day(s) pre-term, as a result of a sudden drop in progesterone and the consequent elevation of the estradiol/progesterone ratio, there is a pre-term surge of hypophyseal PRL. Adapted from Soares et al. (2004).

Consequently, lactogenic hormones (PRL and PL) are considered to initiate and subsequently maintain the neural, physiological and behavioural changes directed to meet the requirements of motherhood, thus deserving the appellative of "the maternal hormones". Regarding maternal physiology, pre- and postnatal development of the offspring is a demanding task requiring a huge energetic investment. In this context, lactogenic input is responsible for the regulation of energy metabolism and increase food intake in order to assure sufficient resources for this task (Augustine et al. 2008). Maternal commitment also requires the proper recognition and identification of the offspring (e.g. distinguishing infants from other conspecifics), which in rodents (macrosmatic animals) is driven by chemosensory signals. To assist this particular

requirement, it is known that lactogenic signals in mice induce neurogenesis in certain brain regions related to the processing of pup-derived chemosignals and the formation of related memories (Shingo et al. 2003; Larsen and Grattan 2010a). During lactation, dams overcome a transient state of anoestrus and become infertile. This process appears to be dependent on suckling-induced stimulation of the pups, which leads to a PRL-mediated inhibition of luteinizing hormone (LH) pulsatile secretion (Araujo-Lopes et al. 2014). Prolactin is also known to modulate oxytocinergic and vasopressinergic circuits during pregnancy and lactation, leading to increased oxytocin release in lactating rats (Parker et al. 1991) and to the upregulation of hypothalamic oxytocin and vasopressin mRNA (Van Tol et al. 1988; Ghosh and Sladek 1995). Furthermore, in order to prepare the dam for a better protection of the offspring, lactogenic hormones are involved in the modulation of the HPA axis and other limbic regions, directed to the attenuation of the stress response and the increase of resilience to stress and anxiety (Torner et al. 2001). A remarkable action of PRL in the brain is the stimulation of maternal behaviour, which indeed constitutes the focus of the last chapter of this thesis. The following section will review in depth the features of this behavioural phenomenon, as well as the available evidence on the involvement of PRL on its regulation.

E. PROLACTIN AND MATERNAL BEHAVIOUR

E.1. INTRODUCTION TO MATERNAL BEHAVIOUR

Parental behaviour is a variety of species-specific social interactions aimed at increasing offspring survival until its reproductive maturity (Numan and Insel 2003). Parental behaviour originated in species where reproduction represents a high energetic investment (homeothermal species) as an evolutionary strategy to ensure this investment and thus increase parental inclusive fitness. The two vertebrate groups that best represent these requirements and, consequently, where parental behaviour has evolved further are mammals and birds. Even though under certain circumstances males may develop parental behaviours (as will be discussed further on this work), in the majority of mammalian species (including the rat and the mouse among other research models), parental behaviour is carried out by the mother. In mammals this is mostly due to the mother being the ultimate responsible for lactation (Kleiman and Malcolm 1981). For the aforementioned reasons, these behaviours are commonly referred to as maternal behaviours. Interestingly, in some species, the dam might share maternal care with external individuals not directly related to the offspring (Solomon and French

1997). This is the case, for instance, of laboratory strains of mice, where non-maternal females reared in the same cage as the mother participate in a communal breeding of the pups. This phenomenon is termed allomaternal behaviour.

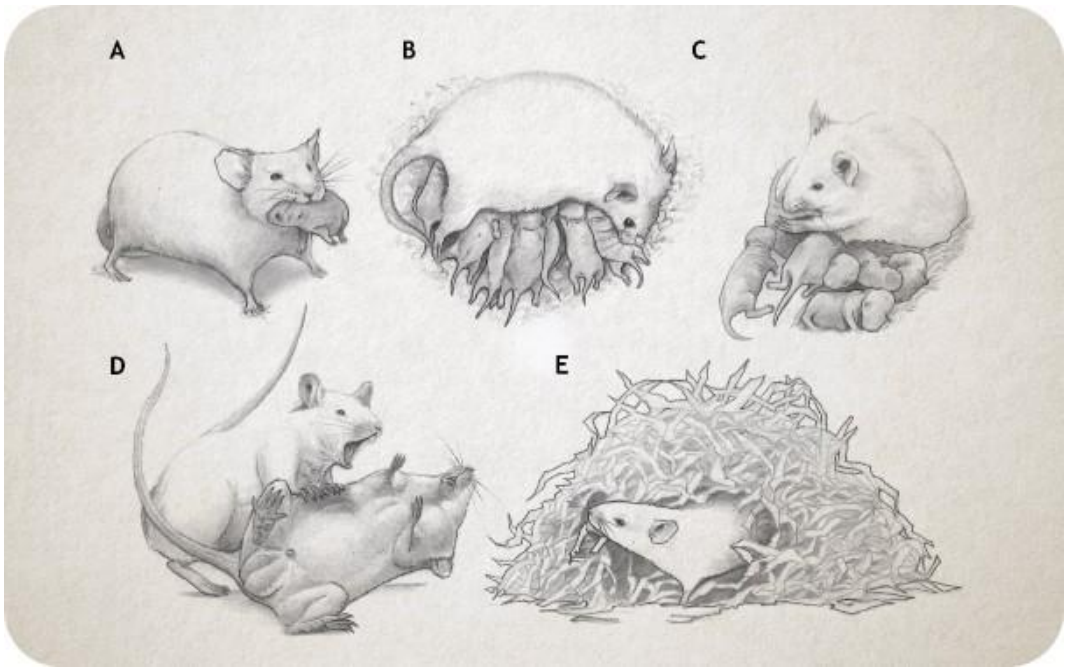


Figure I.5 Components of maternal behaviour in rodents

Maternal behaviours constitute a heterogenous array of behavioural outputs. In rodents, these are classified as pup-directed (upper row) or non-pup-directed (lower row). Pup-directed behaviours include seeking the pups and retrieving them into the nest area (pup retrieval, A), nursing of the pups in different postures (B) and licking/grooming them (C). Maternal behaviours not directed to pups include the territorial defence of the nest site with fierce aggressive outcomes (maternal aggression, D), and the building of a nest site close before term (E).

The expression of maternal behaviours is highly influenced by the degree of development of pups at the moment of birth (Rheingold 1963). In this context, a basic distinction can be set between precocious species (such as sheep and other ungulates), where infants are mobile virtually from the moment of birth, and species that give birth to altricial pups, which are unable to move out of the nest for several days or weeks. In the latter group, which includes mice and rats (research models of interest in this work), but also humans, basic patterns of maternal behaviour can be roughly divided into pup directed and non-pup directed behaviours (Gammie 2005) (Fig I.5). The former include nursing of the pups, crouching over them to provide warmth, retrieving and grouping them into a nest and grooming and licking them, especially their anogenital region to facilitate micturition and defecation reflexes. A recent characterization of pup-directed behaviours has provided a more restrictive classification of maternal responses. Numan and Stolzenberg (2008) differentiated, on the one hand, proactive maternal responses,

including pup-seeking and retrieval behaviour and, on the other hand, as reflexive maternal responses tied to proximal pup stimulation, such as nursing/crouching. On the other hand, non-pup directed maternal behaviours include the building of a nest, placentophagia after parturition and the defence of the nest site. The latter is termed maternal aggression when it is displayed against conspecifics, which commonly represent a threat for pups (engaging, for instance, in infanticidal behaviours) (Lonstein and Gammie 2002).

In addition to ensure immediate survival of the offspring, maternal behaviour has a profound impact on the infants´ long-term physical and psychological development. Thus, the quality and quantity of received maternal care influences some important future phenotypes of the pups , such as cognitive and motor fitness (Rutter et al. 2012), HPA axis reactivity (Meaney 2001; Vaiserman 2015), emotionality (Mehta et al. 2009), the vulnerability to certain affective disorders (Canetti et al. 1997; Repetti et al. 2002; Andersen et al. 2008; Zhang et al. 2013) and even the quality of the future parenting style (Francis et al. 1999; Fleming et al. 2002). The impact on these developmental traits of the received maternal care is commonly inherited through epigenetic mechanisms (Zhang et al. 2013).

E.2. NEURAL SUBSTRATE OF MATERNAL BEHAVIOUR

Adult individuals are engaged in different kind of interactions with conspecifics that collectively constitute their social behaviours. These include sexual, agonistic (territorial aggressions) and affiliative interactions. Social behaviours are under control of the so-called socio-sexual brain network (SBN; Newman, 1999). The SBN is a phylogenetically old and highly conserved neural network (schematically illustrated in Fig I.6 A) composed of several nodes fulfil three main features: 1) they are known to be implicated in the regulation of various forms of social behaviour; 2) they are reciprocally interconnected (to allow for a network-type of activity); and 3) they show abundant neurons expressing gonadal steroid receptors (which allows for a dimorphic expression of social behaviours). Currently, the SBN includes six major nodes: the medial extended amygdala (including the medial amygdala and medial posterior bed nucleus of the stria terminalis), the lateral septum (LS), the medial preoptic area of the hypothalamus (MPA), the anterior (AH) and paraventricular (Pa) nuclei of the hypothalamus, the ventromedial hypothalamic nucleus (VMH) and the midbrain periaqueductal grey (PAG), together with other tegmental motor areas.

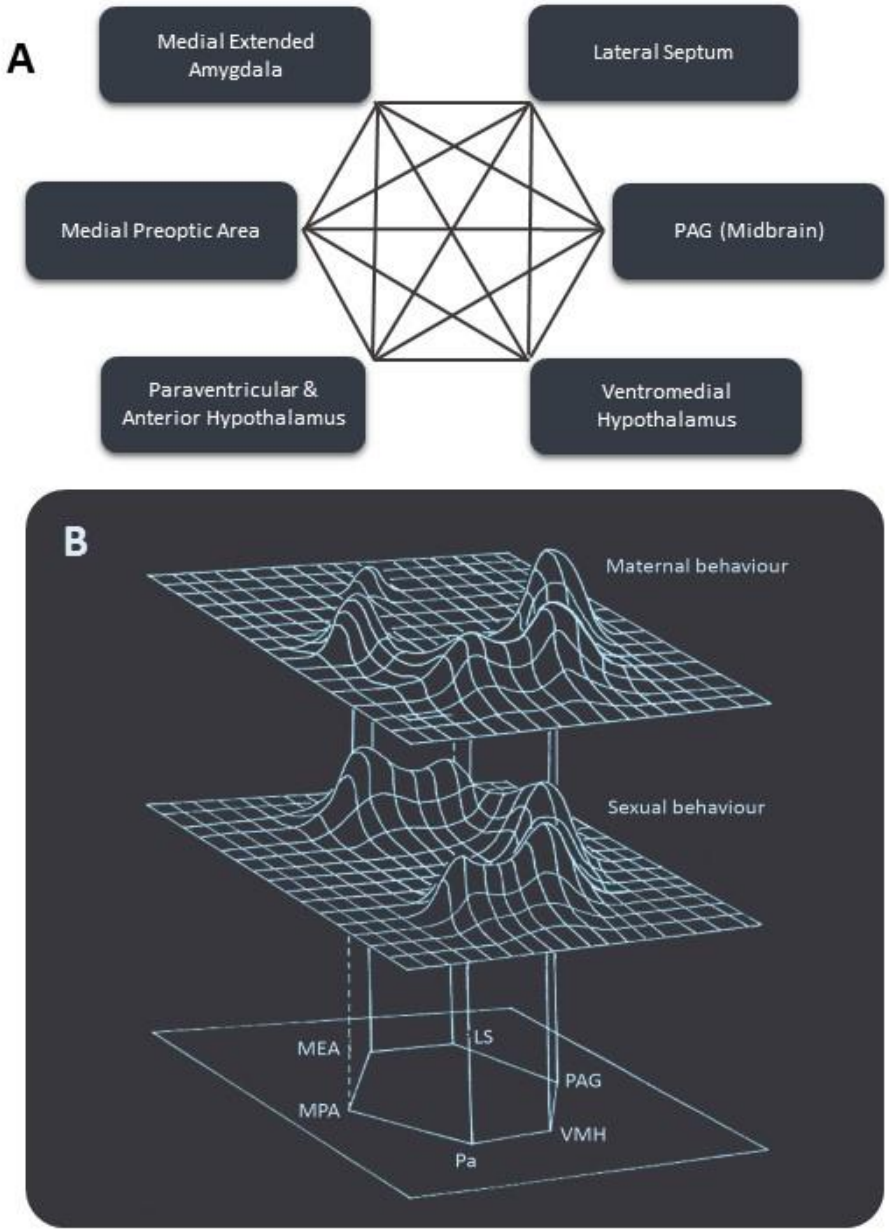


Figure I.6 The Sociosexual Brain Network

As proposed by Newman (1999, figure modified from original paper) and others (Goodson 2005), the Sociosexual Brain Network (SBN) is a functional brain network responsible for the expression of every social and reproductive behaviour (including maternal behaviour).

(A) The SBN is composed of six highly interconnected nodes: the medial extended amygdala, lateral septum, medial preoptic area, paraventricular and anterior hypothalamus, ventromedial hypothalamus and specific midbrain areas (including the periaqueductal grey).

(B) Early gene expression data and other evidences show distinctive activation patterns across the SBN which are specific for each behavioural output (e.g. maternal vs sexual behaviour).

Whereas several other brain nuclei might also participate discretely in the control of specific social behaviours, the SBN is viewed as the core neural circuitry of the social brain, participating in the integration of all social behaviours. Consistent with this idea, the expression of each specific social or reproductive behaviour (including the different subcomponents of maternal behaviour) would be correlated not to the discrete activation of a specific nucleus or a linear circuit, but to distinct relative activation patterns of the whole SBN (Fig I.6 B).

A recurring idea throughout this work is that motherhood requires adaptive changes in the brain of dams, leading to the development of successful maternal behaviours. This changes target the different nuclei of the SBN driving it into a "maternal state", in which the social behaviour repertoire is biased towards maternal behaviours. In other words, under the influence of the endocrine agents discussed above, the socio-sexual brain network turns into *the maternal brain*.

Neural substrate of proactive maternal responses: importance of motivation

As introduced earlier, pup-directed maternal care include passive (or reflexive) and proactive behaviours (Numan and Stolzenberg, 2008). Proactive maternal behaviours are those in which motivation drives the dam towards the pups, and consist of appetitive and consummatory components (Hansen et al. 1991). Thus, proactive maternal responses are initiated by the mother actively seeking the pups (appetitive) and retrieving them to the nest where dams engage in pup licking-grooming (consummatory). Conversely, passive-reflexive responses would be elicited by proximal pup stimuli (e.g. nursing behaviour). The phenomenon of motivation was defined by Pfaff (1982) as an internal process that modifies the way an organism responds to a certain class of external stimulus. Hence, motivation would make the individual become aroused and direct its attention and behaviour towards a specific incentive stimulus. This view of motivated pup-directed maternal responses implies that pups and their stimuli have to possess incentive properties. Indeed, Pereira and Morrell (2011) showed that early postpartum rats significantly prefer pup-associated versus cocaine-associated environments in a conditioned place-preference task. In the same vein, rat dams press a bar at a significantly higher rate than virgin females if rewarded with a pup (Lee et al. 2000), provided they are able to interact with the pup. Altogether, this view requires the neural pathways managing reward and motivation to be incorporated to the functional system controlling maternal behaviour expression.

Interestingly, pup stimuli only acquire incentive properties with pregnancy (or alternatively with a hormonal sensitisation mimicking the hormonal milieu of pregnancy, see below). Whereas primiparous rat dams exhibit full maternal motivation and the full repertoire of maternal behaviours on their first exposure to pups, naïve virgin females (which have not undergone pregnancy) display aversive reactions (neophobia) towards pups (Numan and Insel 2003). Only after repeated exposure of a virgin female rat to pups (for 5-8 days), they become habituated to their presence, approach the pups and, when in contact with them, eventually start expressing maternal care. This so-called maternal sensitisation, however, renders suboptimal maternal behaviour in which, as detailed below, motivation for the pups is relatively low.

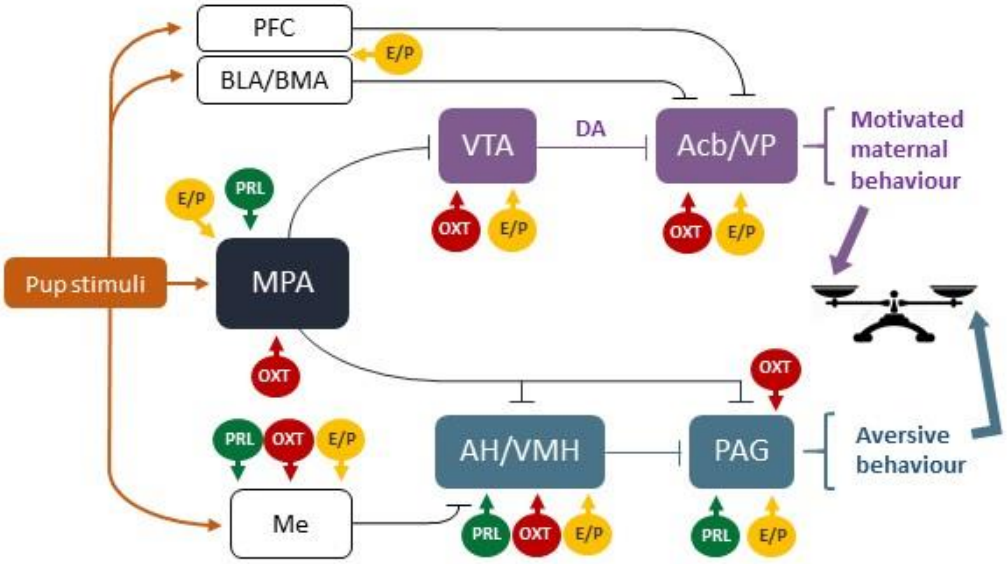


Figure I.7 Neural model for approach-avoidance responses to pups in the rat

Schematic representation of the main neural pathways regulating the response to pups in the female rat. This model features two distinctive neural pathways that integrate aversive and (proactive) maternal responses, respectively. The master control in this model is the medial preoptic area (MPA) of the hypothalamus, which favours either of the two pathways depending on the situation.

Virgin naïve female rats show aversive responses to pups. Pup stimuli reach the MPA, which activates the aversion pathway, composed of the anterior and ventromedial hypothalamic nuclei (AH/VMH) and the midbrain periaqueductal grey (PAG), with the additional input of the medial amygdala (Me).

During pregnancy, however, the MPA activity is modulated by oxytocin (OXT, red), PRL (green) and gonadal steroids (yellow). This leads to the inhibition of the aversive pathway and the activation of the maternal pathway, integrated by the ventral tegmental area (VTA) and its dopaminergic (DA) projection to the nucleus accumbens/ventral pallidum (Acb/VP). Gonadal steroids (Mitra et al. 2003), PRL (Bakowska and Morrell 1997, our results) and OXT (Gimpl and Fahrenholz 2001) exert regulatory actions over all the nodes of these pathways. Modified and adapted from Numan and Woodside (2010).

Numan and Woodside (2010) developed a functional model, based on evidence in rats, that integrates the interaction of brain motivation systems and major SBN nodes to explain the onset of maternal behaviour and the subsequent shift of aversive into proactive (motivated) maternal responses to pups. According to this model (Fig I.7), two mutually exclusive pathways control the final response of the individual towards pups. The avoidance pathway is integrated by the anterior (AH) and ventromedial (VMH) hypothalamic nuclei and those subregions in the periaqueductal grey (PAG) that have been shown to mediate defensive responses (Bandler and Shipley 1994; Risold et al. 1994). Pup-derived chemosensory stimuli would activate the medial amygdala (MeA), which in turn, through its projections to the AH/VMH-PAG circuit, would trigger avoidance and defensive behaviours. Conversely, the pathway for proactive maternal responses is represented by the mesolimbic dopaminergic system connecting the ventral tegmental area (VTA) to the nucleus accumbens (Acc) and the ventral pallidum (VP). This pathway is mainly responsible for assigning incentive properties to pup stimuli (as reviewed above). Numan and Woodside (2010) postulate that DA release in the Acc makes ventral pallidum easily excited by pup stimuli.

In virgin female rats, pup stimuli trigger the activation of the avoidance pathway, whereas the maternal pathway is inhibited. Maternal sensitisation of virgin females, in turn, represents a progressive inhibition of the avoidance pathway, which is eventually out-weighed by attractive tendencies towards pups. However, full activation of the maternal pathway is only reached in rats through the endocrine events of pregnancy (see section I.5.3). The master-control element in this model, responsible for the activation or inhibition of either pathways, is the medial preoptic area (MPA), an integrant of the SBN (see above) classically regarded as the key region for the expression of maternal responsiveness (Numan 1996). In lactating dams exposed to pups, GABAergic (inhibitory) neurons in the MPA express Fos protein, a reliable marker of neuronal activity (Lonstein and De Vries 2000). Thus, these inhibitory neural population would contribute to suppress the avoidance circuitry in primiparous female rats, whereas proactive maternal responses would be activated through direct MPA projections to the mesolimbic dopaminergic system (Numan et al. 2005; Numan and Stolzenberg 2009). Finally, pup-derived chemo- and somatosensory inputs would reach the MPA, Acc and VP from the basolateral (BLA) and basomedial (BMA) nuclei of the amygdala, which in turn receive convergent olfactory and vomeronasal inputs from the MeA and piriform cortex (Pir) (Martinez-Garcia et al. 2012; Cádiz-Moretti et al. 2016).

The whole collection of neural centres and connections involved in the expression of proactive maternal responses is depicted on Figure I.8.

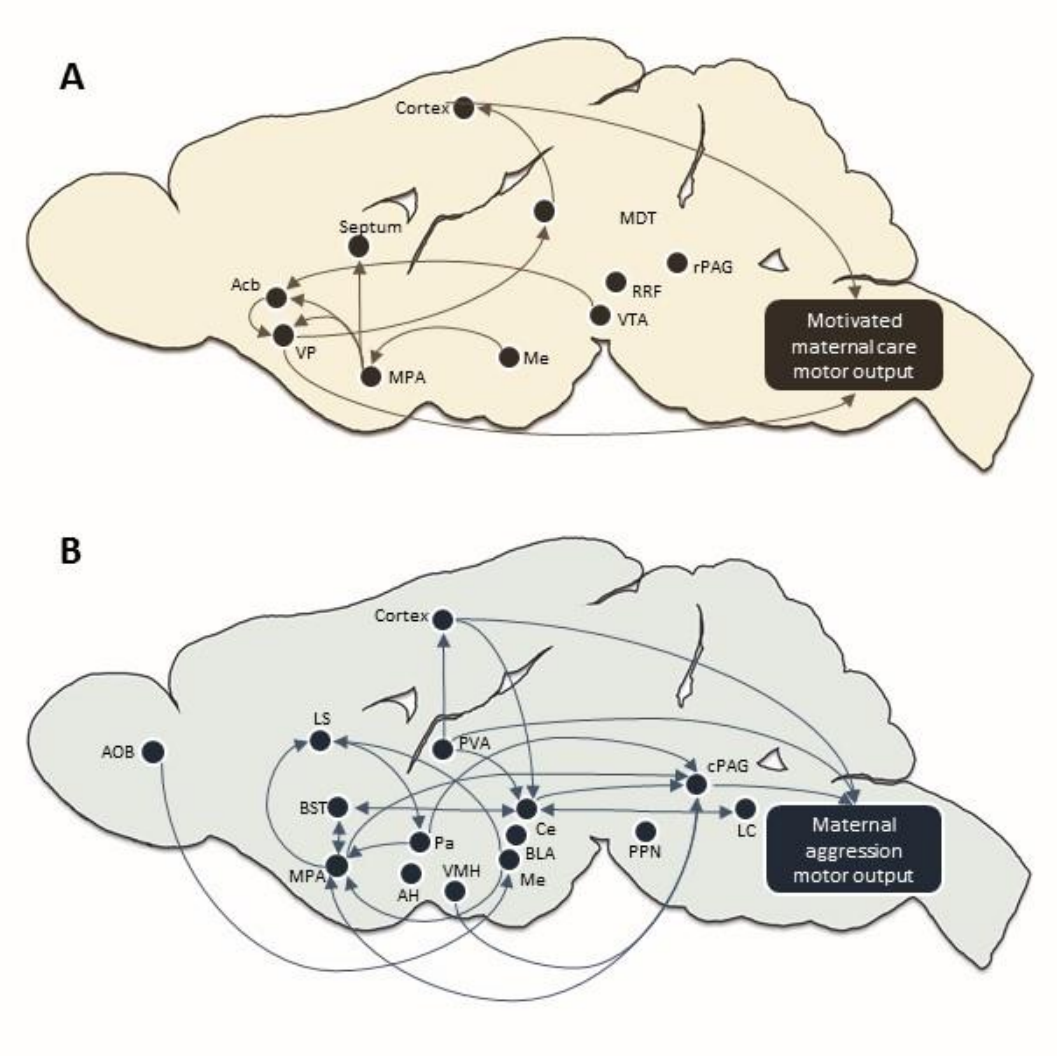


Figure I.8 Brain regions and connections involved in maternal behaviour

Schematic representations of the major brain regions and connections involved in the integration of motivated (proactive) maternal responses (A) and maternal aggression (B). Based on available evidence from lesion, activation and early gene expression studies. Modified and adapted from Gammie (2005).

Neural substrate of maternal aggression

In contrast to maternal care, maternal aggression is not directed to pups. Substantial evidence arising from lesion, genetic and pharmacological manipulation approaches (reviewed in Lonstein and Gammie 2002; Numan and Insel 2003; Gammie 2005) support the view that maternal aggression and pup-care are coordinated by distinct (but overlapping) pathways. Figure I.8 provides an integrated view of the neural centres and pathways that have been implicated in the control of maternal aggression, as compared

to maternal care. Noteworthy centres in this functional system include, for instance, the MeA. The MeA is an integrative centre that presumably conveys chemosensory and non-chemosensory inputs to effector centres for affiliative, defensive or agonistic behaviours (Baum and Bakker 2013; Bergan et al. 2014), including maternal aggression (Unger et al. 2015). Thus, the MeA also represents one of the main regions engaged in the control of both maternal aggression and maternal care. Further nuclei involved in the control of both kinds of maternal behaviour include the LS (Flannelly et al. 1986; Gammie 2005), the Pa (Consiglio and Lucion; Insel and Harbaugh 1989), the VMH (Hansen and Ferreira 1986; Hansen 1989; Lin et al. 2011) and, importantly, the MPA and its continuum with the BST (Numan 1996). It is noteworthy that all these nuclei represent the key nodes of the SBN.

Maternal aggression is partially facilitated by the decrease in anxiety and fearfulness concurrent with the maternal state (Bosch et al. 2005; Bosch and Neumann 2010). Thus, bolder dams are fitter to defend their pups from dangerous threats. It is worth noting that this decrease in anxiety and fearfulness is achieved by the modulation of some of the aforementioned sites, for instance the Pa (as head of the HPA axis, Douglas et al. 2005) or the BST, as well as other non-convergent sites such as the central amygdala (Ce) (Davis and Shi 1999) (which, with the BST, conforms the central extended amygdala).

Nonapeptidergic systems in the regulation of maternal behaviour

Neuropeptides are well-documented modulators of social and reproductive behaviours across vertebrate evolution (Insel and Young 2000). In mammals and other vertebrate taxa, research on this matter has focused primarily on nonapeptides (neuropeptides composed of nine amino acids), which include two members within mammals: arginine-vasopressin (AVP) and oxytocin (OXT). Both OXT and AVP share some common features, including a high structural similarity (differing only in the third and eighth positions of their amino acid sequence) and both are involved in several functions, especially regarding central homeostatic and behavioural regulation (Stoop 2012). From this point, however, this section will focus specifically on OXT, since part of this work has devoted to study specifically the central OXTergic systems.

Even though our primary interest on OXT relates to its central actions, it is worth mentioning that this nonapeptide subserves also important functions as a systemic neurohormone. In this regard, OXT is synthesized by magno- and parvocellular

neurosecretory hypothalamic neurons populations (mainly located in the paraventricular and supraoptic nuclei) and released directly into circulation at the neural lobe of the hypophysis (Ross et al. 2009). Systemically released OXT is involved, among others, in the stimulation of uterine contractions during labour (Blanks and Thornton 2003) or of milk ejection during lactation (Nishimori et al. 1996). In addition to its peripheral roles, OXT is also produced and released in different circuits within the brain, where it behaves rather as a neurotransmitter or neuromodulator (Otero-García et al. 2015). There are five major oxytocinergic populations in the brain, located in: 1) the ventral aspect of the medial posterior BST; 2) adjacent to the former, the area comprising the anterior commissural and anterodorsal nuclei of the preoptic hypothalamus (AC/ADP); 3) the Pa and 4) the SO, intermingled in both cases with neurosecretory neurons; and 5) the medial amygdala (Otero-García et al. 2015). In addition, some scattered OXTergic cells can be also found within circumventricular positions in the preoptic and anterior hypothalamus (Otero-García et al. 2015).

Evidence in the literature firmly supports the idea that brain OXTergic circuits are involved in the regulation of the SBN and thus contribute to the control of different social behaviours, including for instance social recognition (Gur et al. 2014), pair bonding and mother-infant attachment (Numan and Young 2016), agonistic responses (Calcagnoli et al. 2014) and, importantly, maternal behaviour (Bosch and Neumann 2012). First evidence of the involvement of OXT in the control of maternal behaviour was reported by Pedersen and collaborators, who stimulated the expression of spontaneous maternal care in virgin female mice through the intracerebroventricular (ICV) infusion of OXT (Pedersen and Prange 1979; Pedersen et al. 1982). In the same vein, ICV infusions of OXT antiserum or OXT antagonists blocked the onset of maternal care in steroid-primed virgin (Pedersen et al. 1985; Fahrbach et al. 1985, respectively) and postpartum (van Leengoed et al. 1987) female rats. Considering maternal aggression, OXT appears to exert regime- and region-dependent effects on the expression of this trait by rat dams. For example, acute ICV infusion of OXT had no effect on maternal aggression of Wistar rat dams (Neumann et al. 2001), whereas chronic infusion did increase indicators of maternal aggression (Bosch and Neumann 2012). In addition, heightened aggression in high anxiety-bred (HAB) Wistar rats was found to be directly related to increased OXT release in the Pa and Ce (Bosch et al. 2004; Bosch et al. 2005). Altogether, the brain OXTergic system becomes enhanced during pregnancy and postpartum leading to local release of OXT in several brain regions, which contributes to

a fine-tuned control of both maternal care and maternal aggression (Bosch and Neumann 2012).

Consistent with the former, it is important to highlight the actions of OXT in the medial preoptic area (MPA) as the master control of maternal responsiveness (Numan and Numan 1996; Numan and Insel 2003). According to Numan and Woodside's model (2010) for voluntary proactive maternal responses (but see also Fig I.7), OXT plays an instrumental role in the priming of the MPA that leads to the inhibition of pup-aversion pathways and the activation of the maternal pathways. Supporting this hypothesis, OXT receptor expression is induced in this area during pregnancy (Champagne et al. 2001), and OXT action on the MPA or on the mesolimbic dopaminergic pathway also increases the expression of maternal care in rat dams (Pedersen et al. 1994). However, the exact origin of this particular OXTergic input to the MPA and maternal pathway is only partially known. There is evidence of OXTergic innervation of the accumbens nucleus (Acb) arising from the Pa (Knobloch et al. 2012), but the contribution of other OXT population has not yet been examined. One possibility could be that these projections stemmed partially from the AC/ADP region, too. Interestingly, the AC/ADP OXTergic population is located exactly between the ventral BST and the MPA, the key region for maternal responsiveness in the rat (Numan and Numan 1996) and mouse (Tsuneoka et al. 2013). In addition, several lesion studies affecting this area (Numan et al. 2005) or fibres of passage between the ventral BST and MPA including the AC/ADP (Numan et al. 1990) effectively disrupted maternal care. Hence, the AC/ADP OXTergic cell population becomes an interesting target in the study of the central control of maternal behaviours, a target that we intend to cover in the present work.

E.3. ROLE OF PROLACTIN IN MATERNAL BEHAVIOUR REGULATION: A COMPARATIVE VIEW

Earlier on this introduction, we proposed that the neural substrate underlying maternal behaviour integration is the sociosexual brain network (SBN). In order to acquire a maternal profile and allow for the expression of maternal behaviours, the SBN had to undergo a process of maternal priming. The ultimate purpose of this is to ensure the transient expression of maternal behaviours specifically during the postpartum period (or alternatively under the constant interaction with pups). Hence, the critical factors mediating this priming correspond to specific signals of this scenario, mainly: pup-derived cues (sensory factors) and hormonal signals of pregnancy and lactation (endocrine factors) (Fig I.9). Importantly, we will see how PRL, our hormone of interest,

plays an instrumental role among the latter. Additionally, the experience of previous maternal events (experiential factors) might play an additional role, facilitating role, too.

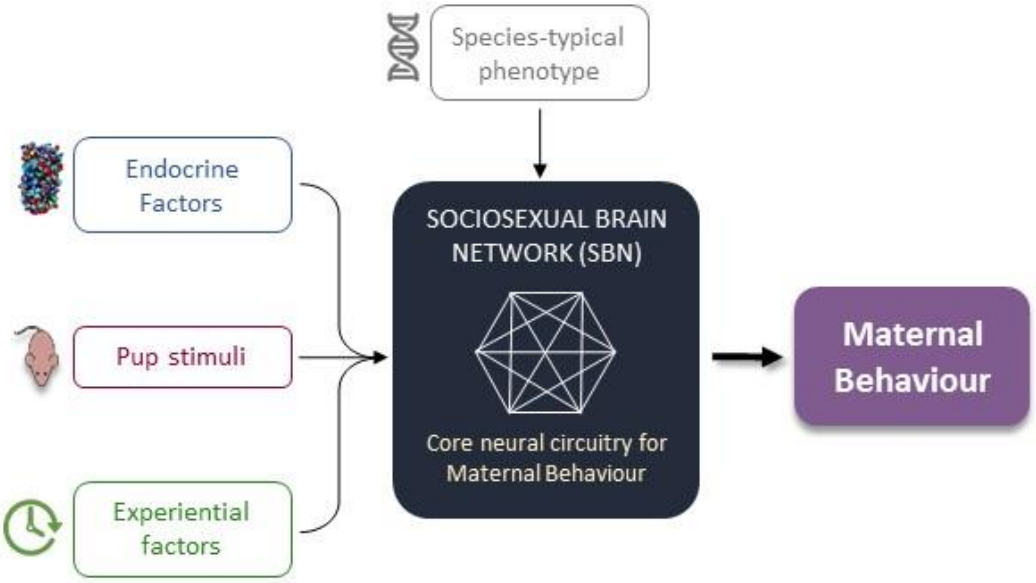


Figure I.9 Main factors influencing the onset of maternal behaviour

Conceptual model representing the possible factors influencing the onset of maternal behaviours. Duing the maternal period, a common, phylogenetically conserved core neural system (the Sociosexual Brain Network, SBN) is influenced by different types of factors to enable the expression of maternal behaviours. These factors include: endocrine signals of pregnancy and lactation (e.g. PRL or gonadal steroids), pup stimulation, previous epigenetic changes or previous maternal experiences (both considered experiential factors). These lead to the development of the maternal brain from the SBN and to the onset of maternal behaviours. Modified and adapted from Numan and Insel (2003).

In Numan and Insel's (2003) view, inasmuch as the SBN represented the phylogenetically conserved core that provides the common features of maternal behaviour expression across mammalian evolution, the aforementioned factors driving the process of maternal priming convey a framework for the emergence of interspecies differences with adaptive significance in the onset of maternal responsiveness. In other words, how and when maternal behaviours are expressed in distinct mammalian species is largely dependent on the differential action of critical regulatory factors operating on a common neural system, rather than on the divergence of this neural system. Whereas the former section analysed the available knowledge on the neural substrate for maternal behaviours, this section will focus on how the above mentioned factors, with a particular focus on PRL, interact in different species to regulate maternal behaviour

expression. This variable action of PRL and other factors is well exemplified by the cases of the rat and the mouse.

As we advanced in the previous section, in the rat, pup-avoiding virgin females become spontaneously maternal when undergoing pregnancy. This behavioural conversion is mediated by the priming of the MPA, which in consequence favours the activation of the maternal pathways over aversion pathways in response to pup-derived sensory input (see Fig I.7). Consistent experimental evidence indicates that the major element driving this maternal priming in the MPA is the hormonal milieu of pregnancy (Fig I.4), specifically PRL and/or placental lactogens acting on top of the proper background of gonadal steroid (estradiol and progesterone) levels. Hence, gonadectomised virgin female rats treated with estradiol and progesterone in a specific profile mimicking levels of late pregnancy showed significantly shortened latencies (1-2 days) to show maternal care towards foster pups (Bridges et al. 1985). In turn, these latencies were brought back to high levels (6-7 days) when experimental females were hypophysectomised (Bridges et al. 1985) or underwent a pharmacological disruption of hypophyseal PRL release with the dopaminergic D2 receptor agonist bromocriptine (Bridges and Ronsheim 1990). Then, short latencies where restored again in PRL-disrupted animals by the grafting of PRL-secreting hypophyseal implants (Bridges et al. 1985) or by systemic treatment with exogenous PRL (Bridges and Ronsheim 1990). On a follow-up study, Bridges and collaborators obtained the same facilitating effect on the onset of allomaternal behaviour by directly infusing PRL (Bridges et al. 1990a) or placental lactogen (Bridges and Freemark 1995; Bridges et al. 1996) into the MPA of gonadectomised, estrogen-primed, bromocriptine-treated female rats. Altogether, this evidence strongly supports the hypothesis that in the rat, the onset of maternal behaviour is driven primarily by PRL and/or placental lactogens, acting in a synergistic fashion with gonadal steroids in the MPA and possibly other nuclei, too (Bridges et al. 1999). The sensory stimulation derived from the pups, by contrast, plays a role in the rat in the maintenance (rather than the onset) of maternal behaviours during lactation, especially the somatosensory stimulation of the nipples and ventral trunk region (Numan and Insel 2003).

In contrast to the rat, virgin female laboratory mice seem not neophobic towards pups, but instead show short latencies to express maternal care (Stolzenberg and Rissman 2011; Martín-Sánchez et al. 2015b). Since these animals have not been subject to endocrine signals of pregnancy, one would think that in the mouse, hormones play an

accessory (at most) role in the onset of maternal behaviour, whereas other factors such as pup-derived sensory stimulation might be more important (Svare et al. 1982). However, additional evidence suggests a more critical role of PRL than previously thought. First, expression of maternal behaviour in virgin females is restricted to maternal care, but it does not include maternal aggression, which is exclusively shown by pregnant and lactating females (Mann et al. 1984; Martín-Sánchez et al. 2015a). Furthermore, regarding maternal care, PRLR-knockout mice display dramatic deficits in this behaviour (Lucas et al. 1998a). Hence, in the context of Numan and Woodside's model (Fig I.7), virgin female mice might show no aversion responses towards pup-derived sensory inputs, but not necessarily a constitutive activation of the MPA and of the pathway for voluntary proactive maternal responses. Precisely at this point is where PRL might exert a more critical role in the onset of motivated maternal behaviours, in a similar (but not so evident) fashion as in the rat. This regulatory action of PRL could be exerted directly on the MPA, on other nuclei within this neural system or indirectly through the modulation of other effectors such as oxytocinergic pathways (see section 1.5.2). One of the aims of the present work will be to better contextualize this possible action of PRL in the management of maternal care and maternal aggression, with a particular focus on its actions onto the brain OXTergic cells in the preoptic region.

F. AIMS OF THIS WORK

The present doctoral thesis intends to gain a deeper insight on the actions of PRL and derived lactogenic hormones in the mouse brain. First, we aim at characterising the patterns of PRL signalling in the brains of female and male mice under different physiological and reproductive conditions. In addition, we want to analyse how these patterns are shaped by different endocrine factors. Finally, we also aim at exploring the role of central lactogenic signalling in female mice in the context of maternal behaviour regulation. The specific aims of this work are:

I. To describe the basic patterns of PRL signalling in the brain of female and male mice and to analyse putative dimorphic differences in this respect.

II. To analyse the specific role of male (testosterone) and female (estradiol and progesterone) gonadal steroids in the regulation of these patterns.

III. To examine the changes in the basal patterns of PRL signalling in the brain of female mice occurring during pregnancy and lactation.

IV. To evaluate the contribution of hypophyseal PRL in contrast to alternative lactogenic sources, to the shaping of lactogenic signalling patterns in the female brain during the period of pregnancy.

V. To investigate the action of lactogenic signals onto a specific population of the OXTergic circuits of the maternal brain in lactating dams.

VI. To develop a new behavioural model, based on the proactive maternal response of pup retrieval, for the measurement of maternal motivation in the female mouse. This test will be used to explore differences in maternal motivation between dams and virgin females, as well as the effect of prolonged pup contact on maternal motivation of virgin females.

VII. To explore the possible role of lactogenic signalling in the preoptic area and of the OXTergic cells in the same region on the expression of two maternal behaviours: motivated pup retrieval and maternal aggression.

1

Sexual Dimorphism and Gonadal Steroid Influence in Brain Prolactin Responsiveness

1.1. RATIONALE AND AIMS

The key role of prolactin in maternal physiology has traditionally biased neuroendocrinological studies towards its effects in the brain of females, whereas the dynamics of this hormone in the male brain has drawn less attention. For instance, the distribution of the PRL receptor (PRLR) has been documented for the female (Bakowska and Morrell 1997; Bakowska and Morrell 2003) but not the male rodent brain. In the same vein, the regulatory relationships between PRL and female gonadal steroids (estradiol and progesterone) have been characterised at different levels (Sugiyama et al. 1994; Torner et al. 1999; Furigo et al. 2014a), but the interactions of PRL with testosterone in males are only marginally understood (Gill-Sharma 2009; O'Hara et al. 2015). Male PRL has received some attention in the clinical field, since pathologies such as lactotroph adenomas (Capozzi et al. 2015) and some antipsychotic treatments that target dopamine D2 receptors (Riecher-Rössler et al. 2009) and thus affect PRL feedback mechanism (Besnard et al. 2014) lead to hyperprolactinaemia. In turn, dysregulation of PRL release in men yields different forms of sexual dysfunction as well as other clinical conditions (Rastrelli et al. 2015). In this regard, it is already known for some decades that PRL is directly involved in the control of male copulatory behavior in rats (Drago et al. 1981), mice (Bartke et al. 1987) and other rodent models (Shrenker and Bartke 1987). In sum, prolactin has also documented roles in male physiology with a presumable central component (Bartke et al. 1987)(Bartke et al. 1987)(Bartke et al. 1987) and could also share other central functions that to date have been described only in females.

The experiments carried out in the present study aim to provide a comprehensive description of the distribution of PRL-sensitive cells in the brain of the mouse. For this purpose, we compared the signaling associated to PRL in the brains of male and female mice, identifying sexually dimorphic differences in the distribution and levels of this signaling. In addition, we sought to gain a deeper insight on the role of gonadal steroids in the shaping of the central responsiveness to PRL in females (estradiol and progesterone) and males (testosterone). To achieve these goals, we used the immunohistochemical detection of the phosphorylated form of signal transducer and activator of transcription 5 (pSTAT5). As introduced before (see section I.2), the STAT5 protein plays a pivotal role in the main signalling pathway associated to the PRLR, the Jak/STAT pathway. Specifically, STAT5 is activated through phosphorylation (pSTAT5) in the last step downstream this pathway and translocates to the cell nucleus to mediate

the biological effects of PRL signalling (Fig I.2). Hence, the immunohistochemical detection of pSTAT5 provides an integrative functional measure of the major component of PRL signalling in the brain.

We first characterised the basic distribution patterns of pSTAT5-ir in males and females. In order to overcome the variability due to the estrous cycle (thus anticipating the possible influence of gonadal steroids on brain PRL signaling; Sugiyama et al. 1994; Furigo et al. 2014; our own observations), we conducted this study using ovariectomized females treated with estradiol and progesterone (steroid-primed). The male group, by contrast, was gonadally intact. To ensure high and homogenous levels of circulating PRL, both experimental groups received injections of a high dose of exogenous PRL before perfusion. This allowed mapping the cells of the brain of both females and males that respond to PRL, thus revealing possible cases of sexual dimorphism in this feature.

Furthermore, we compared the levels of pSTAT5-ir between the steroid-primed ovariectomized female mice mentioned above (treated with estradiol and progesterone) with other ovariectomized females treated either with estradiol alone or with vehicle (oil, control group). Concerning males, we explored the contribution of testosterone to brain PRL responsiveness by comparing pSTAT5-ir levels of our previous sample of males (gonadally intact) with long-term castrated males (which lacked testosterone).

The main goal of this study is to set the neuroanatomical basis for our understanding of the modulatory action of PRL and other lactogens in the brain of males and females. This will set the foundations for studying the influence of PRL in the behaviour of males and females, which will be analysed in the general discussion.

1.2. MATERIAL AND METHODS

1.2.1. ANIMALS

For the present study, n=30 mice of the CD1 strain (Charles River Laboratories, France) were used, 18 females and 12 males, aging between 8 and 24 weeks. These animals were housed in polypropylene cages (145 mm wide, 465 mm long and 215 mm high; Panlab) under controlled temperature (24 ± 2 ºC) and lighting conditions (12h:12h; lights ON at 8 am), with ad libitum access to food and water. Males were housed individually if

intact or in groups (4 to 6 animals per group) if castrated, whereas females were group-housed (4 to 6 animals per group). Animals were treated throughout according to the European Union Council Directive of June 3rd, 2010 (6106/1/10 REV1) and procedures were approved by the Committee of Ethics on Animal Experimentation of the University of Valencia, where the experiments were performed.

1.2.2. EXPERIMENTAL DESIGN

For the purpose of this study, experimental females underwent ovariectomy and a subsequent gonadal steroid replacement schedule. After ovariectomy (see section 1.2.3), females were randomly distributed in 3 experimental groups: (1) group OO received vehicle (oil); (2) group EO received estradiol plus vehicle; and (3) group EP received estradiol plus progesterone as treatment.

Males, in turn, were randomly assigned to two experimental groups: (1) Intact males, which were left undisturbed; and (2) Castrated males, which underwent orchidectomy (section 1.2.3). Both groups received sham subcutaneous implants equivalent to females, filled with vehicle.

After 7 days of hormonal treatment (females) or sham treatment (males), both males and females received a PRL supplement in form of a single i.p. injection of ovine PRL (Brown et al. 2010). An additional control group of gonadally intact males (n=3) was processed without receiving any PRL injections. This allowed evaluating the basal levels of PRL signalling in the brain of adult males, for which there are no details in the literature. For further details on the gonadal replacement treatment and/or the administration of PRL, the reader is referred to section 1.2.4. Forty five minutes after PRL supplementation (during the peak of PRL signalling and STAT5 phosphorylation in response to a PRL challenge, Brown et al. 2010), animals were transcardially perfused. Then, brain tissue was extracted and processed (section 1.2.5) for the immunohistochemmical detection of pSTAT5 (section 1.2.6). The resulting patterns of pSTAT5 immunoreactivity (pSTAT5-ir) were analysed and compared qualitatively and quantitatively (sections 1.2.7 and 1.2.8). For the first part of this study, we compared the patterns of intact males and females of group EP in search for sexually dimorphic differences. Second, we evaluated the effect of estradiol and progesterone on PRL signalling in the female brain by comparing the patterns of pSTAT5-ir of experimental

females (groups OO, EO and EP). Finally, we explored the effect of testosterone on PRL signalling in the male brain by comparing the patterns of pSTAT5-ir of experimental males (groups Intact and Castrated).

1.2.3. OVARIECTOMY AND ORCHIDECTOMY

Experimental females underwent ovariectomy at 10 weeks of age, whereas males of Castrated group were orchidectomized at approximately 12 weeks of age. Both surgical procedures were conducted under i.p. ketamine (Imalgene 500, Merial, Toulouse, France, 75mg/kg) and medetomidine (Domtor 1mg/ml, Esteve, Barcelona, Spain, 1 mg/kg). Animals also received butorphanol tartrate 1% (Torbugesic, Fort Dodge, Girona, Spain, 20 µl s.c.) for pain control and sedation. Ovariectomy was performed through two incisions at both sides of the lumbar region of the back, whereas orchidectomy was performed via a single midline incision on the scrotal sac. After surgery, i.p. atipamezol hydrochloride (Antisedan, Pfizer, New York, USA, 1 mg/kg) was administered to reverse anaesthesia and facilitate awakening and restoration. Animals were left at least 7 days of recovery.

1.2.4. HORMONE TREATMENTS

As anticipated before, experimental females received either vehicle (oil, group OO), vehicle plus estradiol (group EO) or estradiol plus progesterone (group EP), as part of the gonadal steroid replacement schedule. In accordance with the experimental induction of the estrous cycle (Rissman et al. 1997), estradiol was administered on a slow-release profile during 7 days, by means of the subcutaneous placement of silastic tubing implants (Dow Corning Corporation) filled with 20 µg/ml β-estradiol (Sigma, St Louis, MO, USA) diluted in sunflower oil. Silastic tubing had an inner diameter of 1.67 mm and an outer diameter of 2.41 mm, and implants were cut to a length of 20 mm.

By contrast, progesterone (Sigma, St Louis, MO, USA) was administered to females acutely in a 500 µg subcutaneous injection, diluted in sunflower oil, in the morning of the seventh day of estradiol (or vehicle) treatment. Three hours after progesterone administration, females received a 5 mg/kg i.p. dose of ovine PRL (Sigma, St Louis, MO, USA) and perfused 45 minutes later (Brown et al. 2010). The administration of PRL granted homogeneous supraphysiological levels of circulating PRL, thus allowing evaluation of steroid influence on PRL signalling excluding the documented effects of

estradiol on hypophyseal PRL release (Maeda et al. 1996). Males, in turn, received equivalent subcutaneous implants filled with vehicle during seven days. During the morning of the seventh day, they were administered ovine PRL (as with females) and perfused 45 minutes later.

1.2.5.TISSUE COLLECTION AND HISTOLOGICAL PROCESSING

Animals were given an overdose of sodium pentobarbital (Vetoquinol, Madrid, Spain) and were transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. Brains were carefully extracted and post-fixed overnight through immersion in the same fixative, then cryoprotected by immersion in 30% sucrose in 0.01M PB until they sank (2-3 days), indicating the complete penetration of the cryoprotectant in the tissue. Then, brains were cut using a freezing microtome (Microm HM-450, Walldorf, Germany) in five parallel series of 40 µm thick coronal sections and series stored in PB-30% sucrose at -20ºC.

1.2.6.IMMUNOHISTOCHEMISTRY FOR pSTAT5

Immunohistochemistry was conducted in free-floating sections under light shaking at room temperature (25^{o}C) unless otherwise stated. Immunohistochemistry protocol was adapted from Brown et al. (2010; 2011). Tissue sections were thoroughly rinsed between stages for at least three 10-min washes in TRIS-buffered saline, 0.05M, pH 7.6 (TBS). After thawing, sections underwent an initial antigen retrieval step, consisting in 2 sequential 6 minutes incubations in 0.01 M TRIS buffer (TB), pH 10 at 85^{o}C, and brought quickly to room temperature in between. Tissue was then incubated in (a) 1% hydrogen peroxide (H_2O_2) for 30 minutes, for endogenous peroxidase inhibition, (b) 2% BSA, 2% goat serum and 0.3% Triton X-100 in TBS for 1h, in order to block unspecific labelling, (c) rabbit anti-pSTAT5 primary antibody (pSTAT5 Tyr694; Cell Signalling Technology, Beverly, MA) diluted 1:500 in TBS plus Triton X-100 0.1% for 72 h at 4ºC, (d) biotinylated goat anti-rabbit IgG (Vector Laboratories, Peterborough, UK) 1:200 in TBS for 90 minutes and (e) avidin-biotin-peroxidase complex (ABC Elite kit; Vector Laboratories) in TBS for 90 minutes. Peroxidase label was developed using 0.005% 3-3'-, diaminobenzidine (Sigma) and 0.01% H_2O_2 in TB pH 7.6 for about 15 minutes, obtaining thereby a brown nuclear staining. Sections were rinsed in TB and mounted onto gelatinized slides, dehydrated in alcohols, cleared with xylene and coverslipped with Entellan.

1.2.7. ANALYSIS OF pSTAT5 IMMUNOREACTIVITY

We analysed tissue preparations using an optical microscope Leitz DMRB (Leica AG, Germany). For the qualitative mapping of the corresponding patterns of pSTAT5-ir distribution in intact males and ovariectomized, steroid-treated females, we registered the presence or absence of pSTAT5-ir in every single brain site for each specimen of the respective groups. Regarding the quantitative assessment of the levels of PRL signalling in the respective experimental groups, we analysed the density of cells showing pSTAT5 immunoreactivity (pSTAT5-ir) in selected brain sites showing pSTAT5 expression in both females and males. To do so, we designed representative frames of the chosen nuclei (Fig 1.1) using the stereotaxic atlas of Paxinos and Franklin (2004), and we obtained photomicrographs of these frames in both hemispheres using a digital camera (Leica DFC495) attached to the same microscope. Image processing and analysis was conducted on Image J. Briefly, we subtracted background light and converted the RGB colour image to greyscale by selecting the green channel. Then, we binarised the greyscale image setting the 75% of the mode of the histogram as a threshold, thus including every pixel below this threshold as positively labelled. We filtered smaller noise particles by an additional processing consisting of the following Image J commands: “fill holes”; “open” (3 iterations) and “watershed”. Particles were additionally filtered by area (larger than 70 μm2, corresponding to an approximate diameter of 9.4 μm) and finally counted automatically by Image J. We calculated the mean (interhemispheric) density of pSTAT5-imunoreactive cell nuclei for each specimen by dividing the mean value of these counts for both hemispheres by the area of the respective frame.

1.2.8. STATISTICAL ANALYSIS

Statistical analysis of the resulting data was performed on the SPSS software package. After checking for normality (Kolmogorov-Smirnov test with Lilliefors’ correction) and homogeneity of variances (Levene's test), we performed three different statistical comparisons. First, we compared levels of pSTAT5-ir density between Intact males and EP females, in search for sexually dimorphic differences of quantitative nature. Only part of the samples of part of the brain nuclei fulfilled the criteria for a parametric analysis, in case of which they were subject to an independent t-test. The samples of the remaining nuclei were subject to a non-parametric Kruskal-Wallis ANOVA. Second, we compared all

three groups of experimental females (OO; EO and EP) in order to examine possible effects of gonadal steroids in PRL signalling. In this case, the samples were not fit for a parametric analysis, so we ran a non-parametric Kruskal-Wallis ANOVA for each of the surveyed nuclei. Since this comparison included more than two groups, we used Dunnett´s post-hoc method for multiple comparisons to further analyse significant differences revealed by the Kruskal-Wallis ANOVA test. Last, we checked for the effect of orchidectomy on male pSTAT5-ir levels by comparing both groups of males, Intact and Castrated. Once again, the samples of part of the nuclei were subject to a parametric independent t-test and the rest to a non-parametric Kruskal-Wallis ANOVA. For each statistical test, we applied a significance level of 0.05.

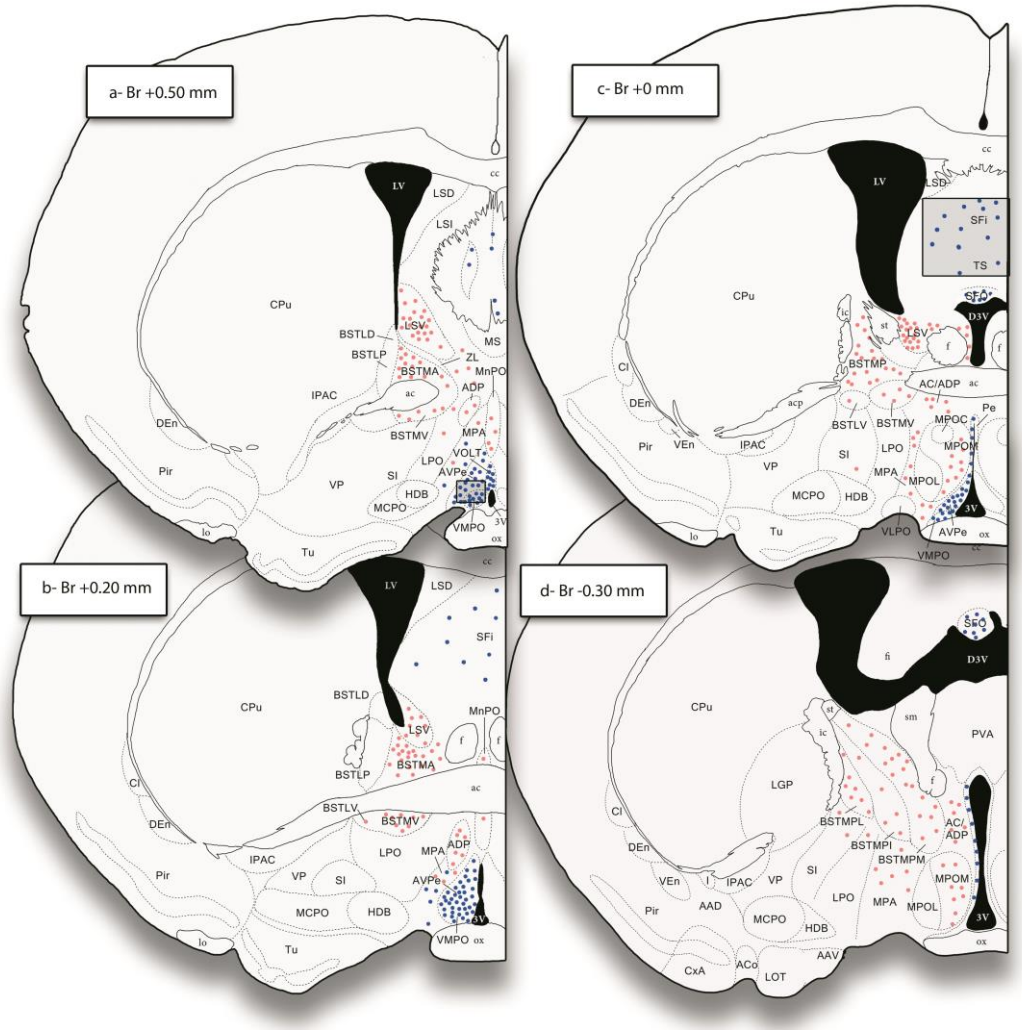
1.3. RESULTS

1.3.1. MAPPING OF pSTAT5 IMMUNOREACTIVITY IN THE FEMALE AND MALE MOUSE BRAIN

In this section, we compare the distribution of pSTAT5-ir in the brains of ovariectomized, steroid-primed and PRL supplemented female mice with gonadally intact, PRL supplemented male mice. Immunohistochemistry for pSTAT5 in the examined tissue rendered a defined staining restricted to the cell nucleus. For this and the following descriptions, we will adhere to the neuroanatomical terminology proposed by Paxinos and Franklin (2004, see abbreviation list).

A Patterns of pSTAT5 immunoreactivity in the brain of ovariectomized, steroid-primed virgin female mice

Our sample of ovariectomized, steroid-primed females displayed a common pattern of pSTAT5-ir with little interindividual variation. This pattern is depicted in Figure 1.1, which shows the common labelling for males and females (dark blue) and labelling exclusively present in females (red). Immunoreactivity for pSTAT5 in females was widespread in the basal telencephalon and hypothalamus, but was also found within thalamic, midbrain and brainstem structures. Remarkably, the choroid plexus appeared positively labelled in the whole sample of females (not shown) This is likely related to their role in PRL transport to the brain (Mangurian et al. 1992; but see Brown et al. 2015).


a- Br +0.50 mm
c- Br +0 mm
b- Br +0.20 mm
d- Br -0.30 mm
LV
LSD
LSI
CPu
BSTLD
BSTLP
BSTMA
LSV
MS
ZL
MnPO
IPAC
ac
ADP
DEn
BSTMV
MPA
VOLT
LPO
Pir
VP
SI
AVPe
HDB
MCPO
VMPO
3V
ox
lo
Tu
cc
SFi
TS
SFO
D3V
st
f
Cl
BSTMP
AC/ADP
acp
Pe
BSTLV
MPOC
Pir
VEn
IPAC
MPOM
MPOL
VLPO
fi
sm
PVA
LGP
BSTMPL
AC/
ADP
BSTMPI
BSTMPM
AAD
AAV
CxA
ACo
LOT

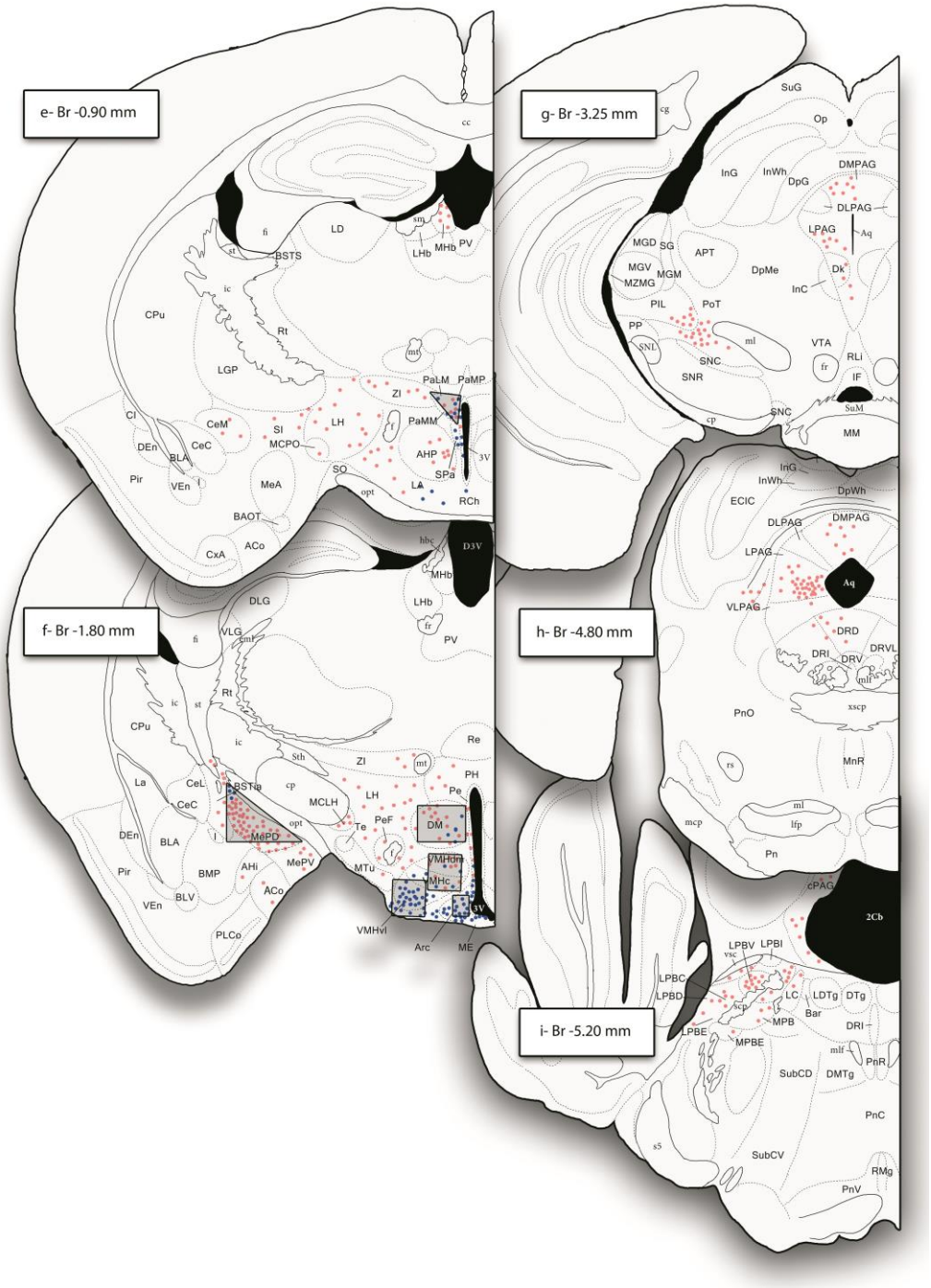


Figure 1.1 Mapping of pSTAT5 immunoreactivity in the brains of male and female mice

Representative camera lucida drawings of coronal sections of the mouse brain showing the distribution patterns of pSTAT5-ir in female and male mice. Pink dots represent pSTAT5 expression exclusive of ovariectomized, steroid-primed females. Dark blue dots encode overlapping expression of pSTAT5-ir in both the female and male specimens. Each dot represents approximately 4 cell nuclei labelled for pSTAT5. Shaded areas illustrate the counting frames designed for the AVPe/VMPO region (Fig. 1.1a), the Pa (Fig. 1.1e), and the Arc, VMHvl, VMHc and VMHdm, DM and MePD (all frames in Fig. 1.1f) as part of the quantitative analysis performed in this work (see below). Approximate distance to bregma is enclosed for each section.

Telencephalon

Within the telencephalon, pSTAT5-ir was detected in the amygdaloid region, the bed nucleus of the stria terminalis (BST) and the lateral septum (LS). In the amygdala, the medial posterodorsal nucleus (MePD) contained a very rich population of labelled cells which occupied the whole area of this nucleus (Fig 1.1f, 1.2AI). Furthermore, the medial central (CeM, Fig 1.1e) and the basomedial (BMA, not shown) nuclei of the amygdala, the anterior nucleus of the cortical amygdala (ACo), the intramygdaloid BST (BSTia) and the amygdalohippocampal area (AHi) showed scarce labelling in some of the specimens (Fig 1.1f). In the BST, pSTAT5-ir was observed in the medial anterior and medioventral nuclei (BSTMA and BSTMV, respectively, Figs 1.1a and b) and in the medial posterior divisions of the nucleus (BSTMPM; BSTMPI and to a lesser extent, BSTMPL, Figs 1.1c and d). Finally, pSTAT5-ir was moderately present in the septal region, especially in the ventral and intermediate aspects of the lateral septum (LSV and LSI, Figs 1.1a and b) and in the area comprising the triangular septal nucleus (TS) and the septofimbrial nucleus (SFi, Figs 1.1a-c and 1.2BI), in addition to the subfornical organ (SFO, Fig 1.1c and d).

Diencephalon

In the hypothalamus, pSTAT5-ir was present in all of the major rostrocaudal divisions, with densest labelling found within juxtaventricular sites, but some labelling also present in more lateral compartments. In the preoptic hypothalamus (Figs 1.1a-d and 1.2CI), labelling was most prominent in the vascular organ of the lamina terminalis (VOLT, Fig 1.1a), the anteroventral periventricular (AVPe, Figs 1.1a-c), the ventromedial preoptic (VMPO, Figs. 1.1a-c) and the periventricular (Pe, Figs 1.1c and d) nuclei. In addition, other sites displayed scattered pSTAT5-ir, namely the medial preoptic area (MPA, Figs 1.1a-d), the medial (but not central or lateral) division of the medial preoptic nucleus (MPOM, Figs 1.1c and d), the lateral preoptic nucleus (LPO, Figs 1.1a-d), or the area comprising the anterior commissural nucleus and the anterodorsal preoptic nucleus of the hypothalamus (AC/ADP, Figs 1.1b-d).

In the anterior hypothalamus (Fig 1.1e and 1.2DI), the paraventricular nucleus (Pa) displayed pSTAT5-ir principally in its ventral (PaV) and medial parvocellular (PaMP), but also in the medial and lateral magnocellular populations (PaMM and PaLM, respectively) and the posterior aspect of the nucleus (PaPO). Further pSTAT5-ir was observed in some

specimens in the lateral hypothalamic region (LH) and, exceptionally, in the subparaventricular nucleus (SPa) and in the anterior hypothalamic area (AHA).

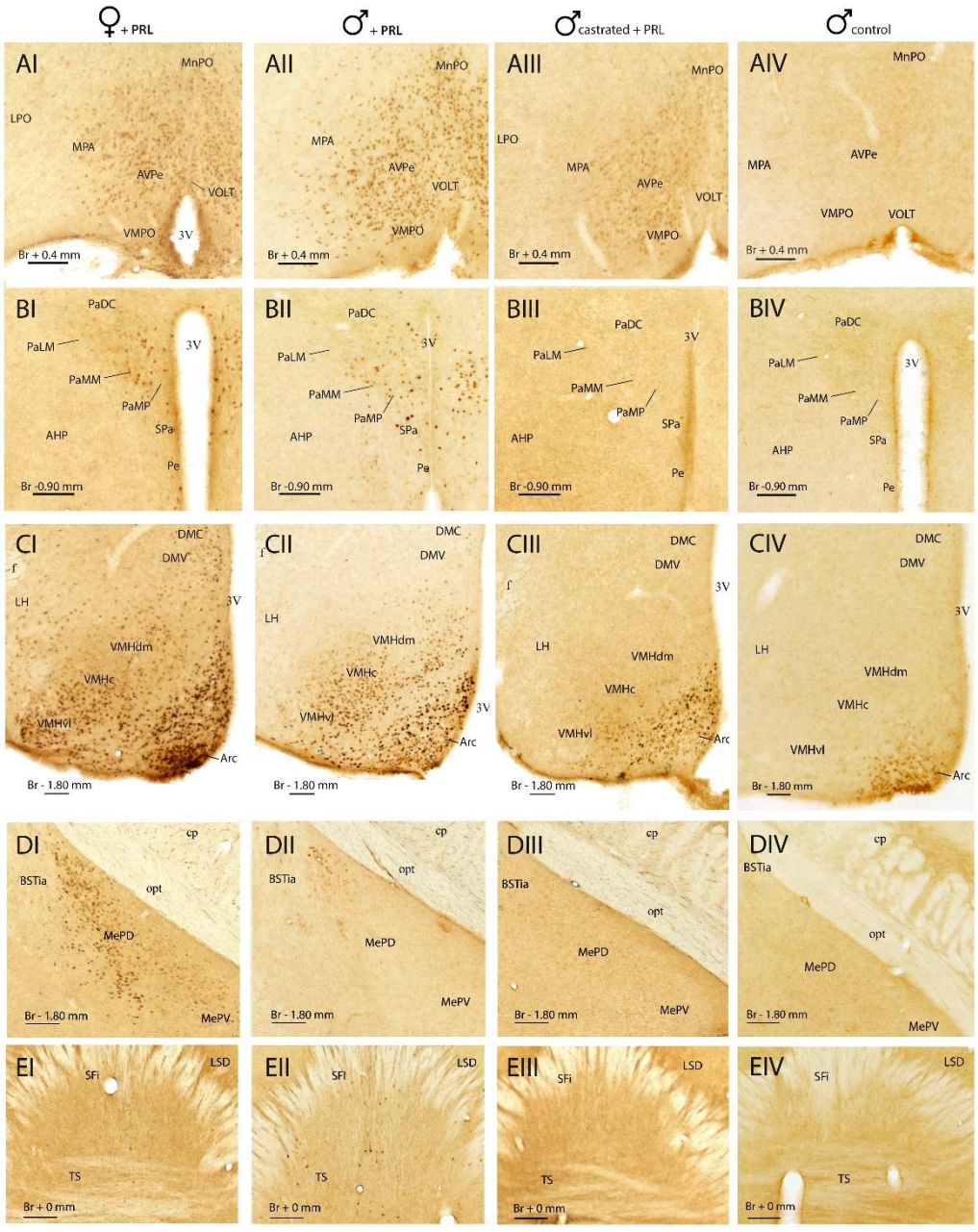


Figure 1.2 Representative examples of pSTAT5 immunoreactivity in the brain of female and male mice

Photomicrographs illustrating pSTAT5 labelling in representative brain sections in an ovariectomized, steroid-primed female supplemented with PRL (I), an intact, PRL-supplemented male (II), a castrated, PRL supplemented male (III) and a intact control male, not supplemented with PRL (IV). Sections correspond to the preoptic hypothalamus (A), the paraventricular region in the anterior hypothalamus (B), the tuberal hypothalamus (C), the caudal septum (D) and the medial posterior amygdala (E). The approximate distance to bregma is indicated in each section. Scale bars correspond to 100 μm.

In the tuberal region of the hypothalamus (Fig. 1.1f and 1.2EI), the retrochiasmatic region (RCh) displayed scattered labelling, whereas the arcuate nucleus (Arc) showed high levels of pSTAT5-ir, with some immunoreactive cell nuclei overextending into the median eminence (ME). In addition, pSTAT5-ir was present in the dorsomedial (DM) and ventromedial (VMH) hypothalamic nuclei. Within the latter, pSTAT5-ir density was higher in the ventrolateral division (VMHvl) but also moderately present in the central and dorsomedial divisions (VMHc; VMHdm). Finally, the medial tuberal nucleus (MTu), the perifornical nucleus (PeF), the magnocellular lateral hypothalamic nucleus (MCLH) and the posterior hypothalamic region (PH) appeared exceptionally labelled for pSTAT5 in some specimens of the aforementioned groups.

The thalamic region, in turn, was generally devoid of pSTAT5-ir, with the exception the posterior intralaminar complex (PIL, Fig 1.1g). Other thalamic sites displayed pSTAT5-ir anecdotally: the zona incerta (ZI, Figs 1.1e and f), the medial habenula (MHb, Fig 1.1e), the precommissural nucleus (PrC, not shown) and the subparafascicular nucleus (SPF, not shown).

Midbrain and brainstem

In the midbrain and brainstem regions, pSTAT5-ir was noticeable mainly in the lateral (LPAG) and dorsomedial (DMPAG) divisions of the periaqueductal grey (Figs 1.1g and h), as well as in the dorsal raphe nucleus (DR, Fig 1.1h). Finally, pSTAT5-ir was also present in the parabrachial nucleus (PB), both in its medial (MPB) and lateral (LPB) aspects, and in the locus coeruleus (LC) (Fig 1.1i).

B Patterns of pSTAT5 immunoreactivity in the brain of male mice

Immunolabelling for pSTAT5 in the brain of gonadally-intact male mice was only present after exogenous PRL administration, whereas non-supplemented male mice virtually lacked any immunoreactivity in the brain, except for the arcuate nucleus (Arc, Fig 1.2 EIV) and scarce labelled cells in the Pe (not shown). Still, PRL-supplemented male mice showed a more restricted distribution of pSTAT5-ir than ovariectomized, steroid-primed females. Importantly, the male pattern was a fraction of that of females. In other words, no brain structure displayed pSTAT5-ir exclusively in males. Immunolabelling for pSTAT5 in the brain of gonadally-intact male mice was virtually restricted to the hypothalamus,

with labelled cells also present in a few telencephalic structures (Figs 1.1 and 1.2). In contrast to females, no pSTAT5-ir was found within thalamic, midbrain or brainstem structures. In the telencephalon, pSTAT5-ir was observed mainly in the septum, specifically in the TS/SFi area (Figs 1.1 a-c and 1.2BIV). Furthermore, the SFO (Figs 1.1 c and d) also contained some immunoreactive cells. In the amygdala, the MePD displayed scarce pSTAT5-ir, with very few immunolabelled cells restricted to the upper corner of the nucleus, in close contact to the BSTia (Figs 1.1f and 1.2AIV). This distribution contrasts strongly with that of female mice, where the whole nucleus shows densely labelled pSTAT5-immunoreactive cells.

In the hypothalamus, pSTAT5-ir was present in the preoptic, anterior and tuberal regions. In the preoptic region (Figs 1.1a-d and 1.2CIV), pSTAT5-ir was concentrated in the juxtaventricular nuclei, namely the VOLT, the AVPe, the VMPO and the Pe. As in females, the adjoining MPA also displayed a few immunolabelled cell nuclei (Figs. 1.1a-d). In the anterior hypothalamus, pSTAT5-ir was once again restricted to juxtaventricular structures, namely the Pa and the Pe (Fig. 1.1e and 1.2DIV). In the Pa, labelling was limited to the medial aspect of the nucleus, mainly to the ventral (PaV), medial magnocellular (PaMM) and medial parvocellular Pa (PaMP) subdivisions, with very few labelling observed in the lateral aspect of the nucleus (PaLM) or the SPa. In the tuberal hypothalamus (Fig. 1.1f and 1.2EIV), the Arc displayed abundant pSTAT5-ir, with some immunolabelled cells displaced into the ME. Moreover, the RCh displayed some scattered immunolabelling as well as the DM (in some males). Finally, pSTAT5-ir was observed in the VMH, with higher levels in the VMHvl and sparse labelling in the VMHc and VMHdm.

1.3.2. QUANTITATIVE ANALYSIS OF pSTAT5-IR DENSITY IN IMMUNOREACTIVE AREAS OF THE FEMALE AND MALE MOUSE BRAIN

To check for additional, quantitative cases of sexual dimorphism in PRL-derived signalling, we analysed the density of pSTAT5-ir in the main nuclei showing pSTAT5-ir in intact males and ovariectomized, steroid-primed females. We designed counting frames for the following brain nuclei: the AVPe/VMPO region, the Pa, the Arc, the DM, the VMHvl, the central and dorsomedial VMH (VMHc), the MePD and the SFi (anatomical location of frames included in Fig 1.1).

Then, we assessed the mean interhemispheric density of pSTAT5-ir and performed separate statistical tests on each of the analysed nuclei. When data fulfilled the criteria for a parametric statistical analysis (the AVPe, the Arc, the Pa and the VMHvl), we performed t tests for independent samples. For the remaining nuclei (the DM, the VMHc, the MePD and the SFi), we performed a non-parametric Mann-Whitney test. The results of these analyses are illustrated on Figure 1.3.

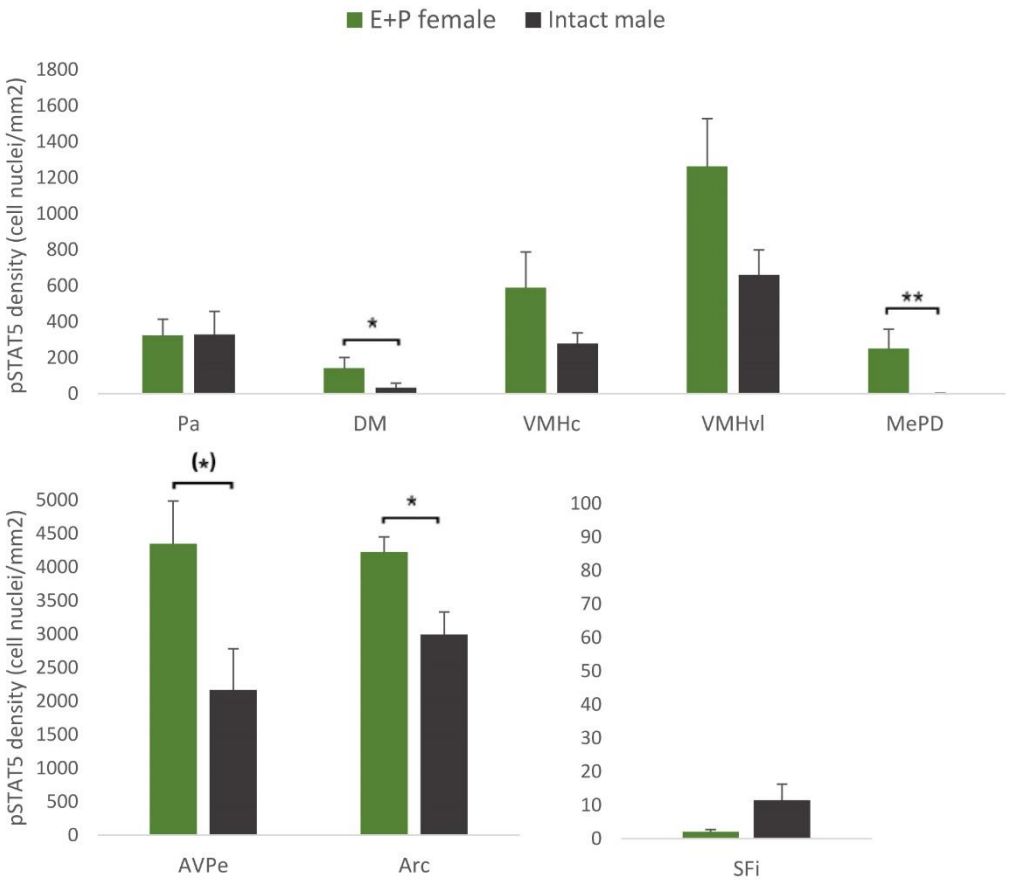


Figure 1.3 Quantitative analysis of pSTAT5 immunoreactivity in selected brain regions of female and male mice

Assessment of pSTAT5-ir density (pSTAT5-positive cell nuclei/mm2) in the major brain regions with expression of pSTAT5-ir in ovariectomized, steroid-primed virgin female mice and intact male mice, both PRL-supplemented. Bar histograms show mean interhemispheric pSTAT5-ir density ± SEM in gonadally-intact, vehicle-treated males (n=6; black) and ovariectomized females treated with estradiol plus progesterone (n=6; green). Counting frames for each of the analysed nuclei are included in Fig. 2.1. Statistical analysis was applied independently to each brain region (independent t-test for parametric data or Mann-Whitney test for non-parametric data, see Results). *$P \leq 0.05$; **$P \leq 0.01$; (*) $P \geq 0.06$.

Females showed a general trend towards higher levels of pSTAT5-ir than males. This effect only reached statistical significance in the Arc ($t(10)=3.043$, $p=0.012$), the DM ($U=32.0$; $p=0.026$) and the MePD ($U=30.0$; $p=0.004$), although the AVPe/VMPO also showed a strong trend towards higher pSTAT5-ir density in females ($t(9)=2.183$; $p=0.057$). Additional examples of the distribution of pSTAT5-ir in the analysed nuclei of males and ovariectomized, steroid-primed females are shown on Figure 1.2.

1.3.3. EFFECT OF PROGESTERONE AND ESTRADIOL ON BRAIN PROLACTIN SIGNALLING IN THE FEMALE MOUSE BRAIN

To further explore the putative role of gonadal steroids in modulating central PRL signalling in the female brain, we compared our previous sample of ovariectomized virgin females treated with estradiol and progesterone (group EP) with two additional groups of ovariectomized virgin females: vehicle-treated (group OO) and estradiol-treated (group EO). After a general inspection of the patterns of pSTAT5-ir in these groups, we designed a new set of counting frames including up to 12 brain regions involved in the management of social and reproductive behaviours and in the control of PRL release (Newman 1999; Ben-Jonathan and Hnasko 2001b; Gammie 2005), which at first glance displayed potential differences in pSTAT5-ir density due to the steroid treatment. These counting frames are depicted in Figure 1.4 and include the following brain sites: the LSV, BSTMPM, CeM and MePD in the telencephalon; the AVPe, AC/ADP, MPO, Pa, Arc and VMHvl in the hypothalamus; the PIL in the thalamus and the LPAG in the midbrain.

Separate non-parametric one-way Kruskal-Wallis ANOVA tests for the density of pSTAT5-ir in each brain region revealed significant differences in pSTAT5-ir density between OO, EO and EP groups in 5 out of the 12 analysed nuclei (Fig. 1.6). Discrete inter-group differences were further explored with Dunnett´s post-hoc method for multiple comparisons, which revealed significant effects generally consisting of an important increase in pSTAT5-ir density affecting one or both groups treated with estradiol (EO and EP) as compared to the vehicle group (OO). All of the telencephalic nuclei included in this analysis showed significant differences in pSTAT5-ir density except for the CeM ($p=0.246$). In the MePD ($\chi 2$ $(2)=9.748$; $p=0.001$), the EP group displayed significantly higher pSTAT5-ir density than the OO group ($p=0.007$), whereas differences between the EO and OO group did not reach significance. In the BST region, we found a

trend towards inter-group differences in the BSTMPM ($\chi 2$ (2)=5.558; p=0.062). In the septal region, we found a significant effect in the LSV ($\chi 2$ (2)=11.263; p=0.004), where pSTAT5-ir density of the EO and EP groups are again significantly higher than the OO group (p=0.004 and p=0.038, respectively).

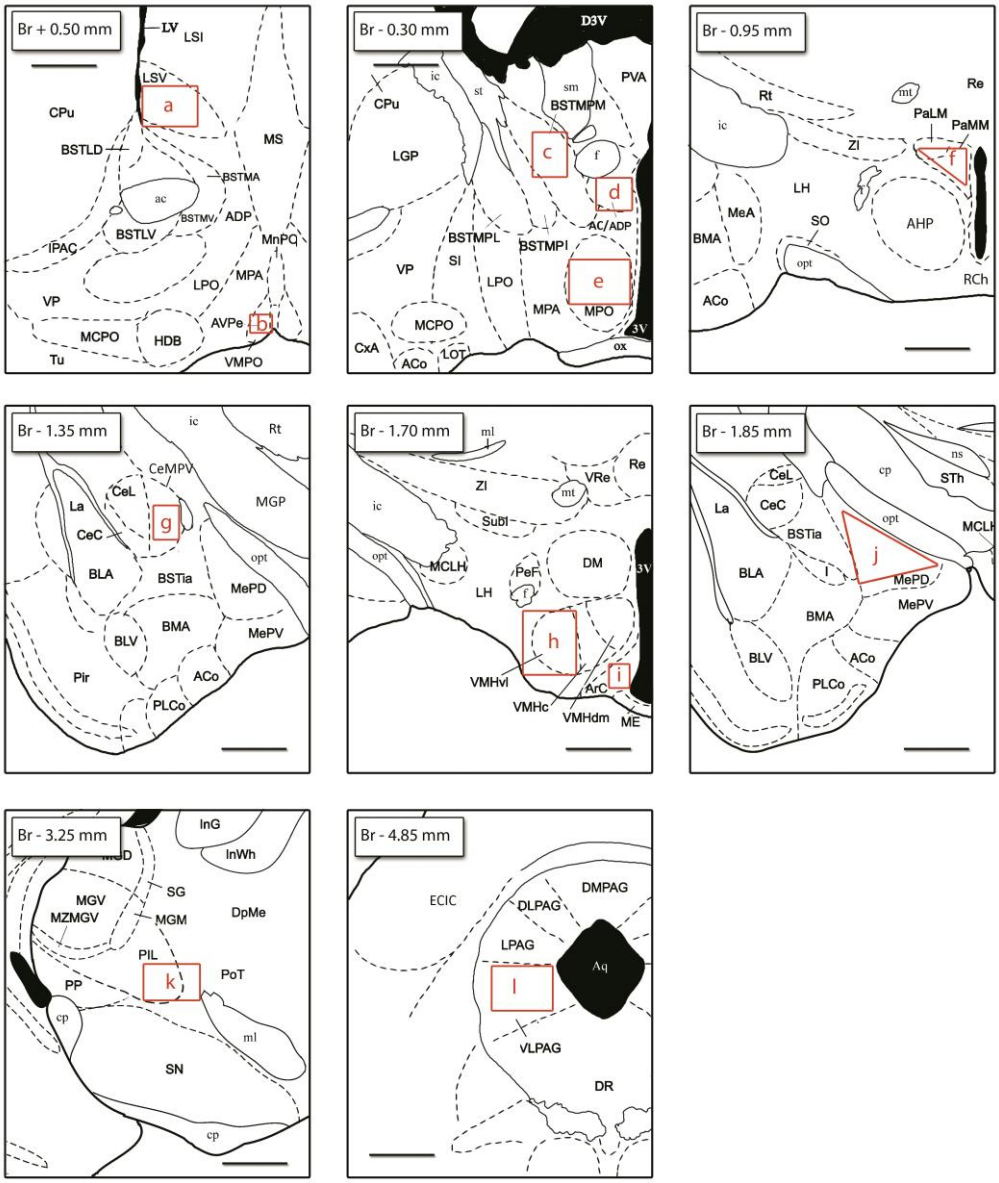


Figure 1.4 Anatomical location of counting frames for ovariectomized, steroid-primed females

Semi-schematic camera lucida drawings of selected coronal sections featuring the counting frames (red) chosen for pSTAT5-ir quantification in ovariectomized, steroid-primed female mice (groups OO; EO and EP; see Fig. 2.5). Frames include: a) the LSV; b) the AVPe; c) the BSTMPM; d) the AC/ADP; e) the MPO; f) the Pa; g) the CeM; h) the VMHvl and ventrolateral adjoining area; i) the Arc; j) the MePD; k) PIL and adjoining posterior thalamic region and l) the VLPAG. Approximate distance to bregma enclosed for each section. Scale bars: 500 μm.

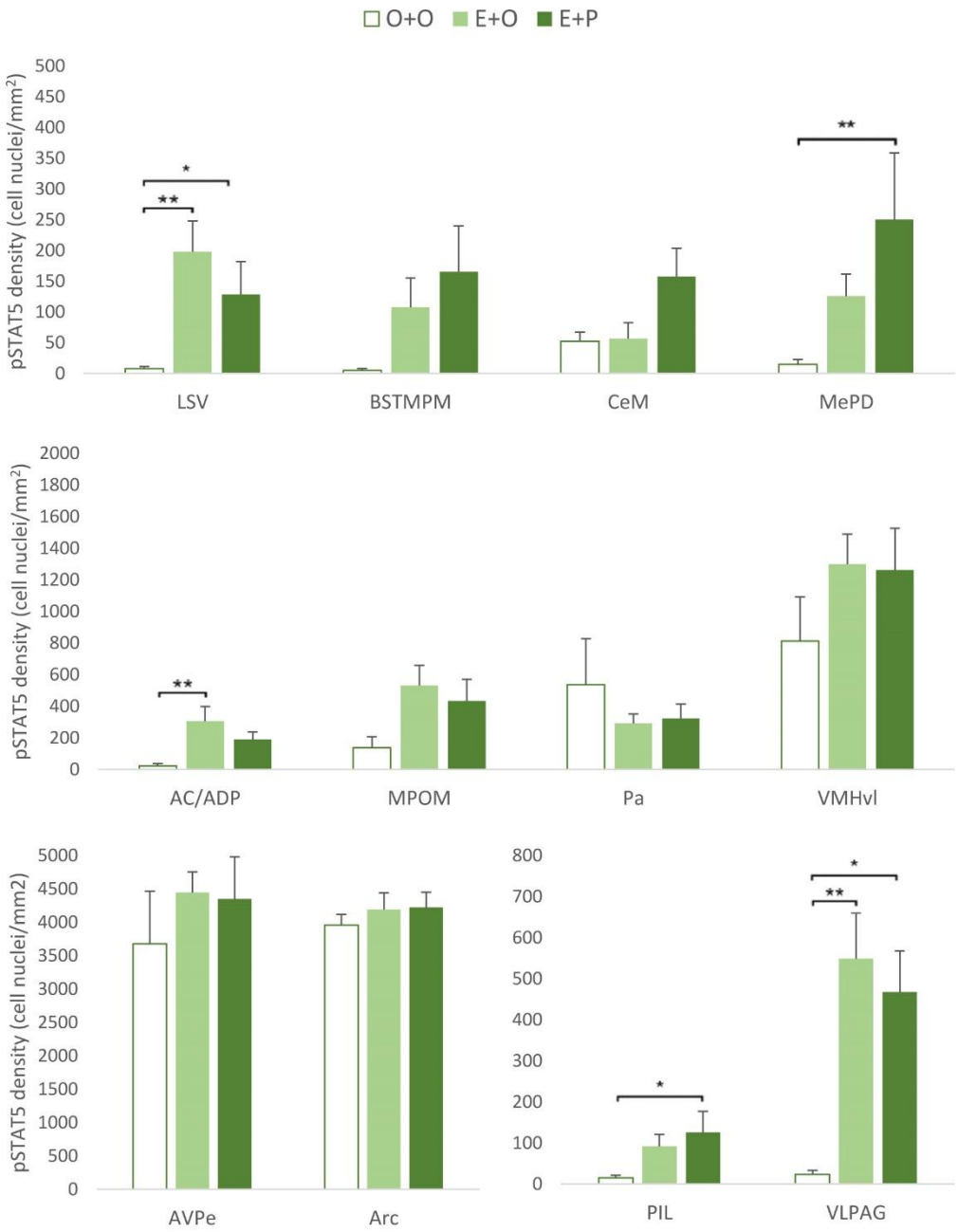


Figure 1.5 Gonadal steroid influence in pSTAT5 immunoreactivity of the female mouse brain

Assessment of pSTAT5-ir density (pSTAT5-positive cell nuclei / mm2) in three groups of ovariectomized virgin female mice with different gonadal steroid treatments, within 12 brain regions involved in the management of social and reproductive behaviours and in the control of PRL release (Newman 1999; Ben-Jonathan and Hnasko 2001b; Gammie 2005). Bar histograms show mean interhemisferic pSTAT5-ir density ± SEM in ovariectomized virgin female mice treated with: vehicle (group OO; white; n=6), estradiol (group EO; light green; n=6) or estradiol + progesterone (group EP; dark green; n=6). Non-parametric Kruskal-Wallis ANOVA with Dunnett´s post-hoc pairwise comparisons was applied separately to each brain region. *$p \leq 0.05$; **$p \leq 0.01$.

In the preoptic hypothalamus, a significant effect of the treatment was observed in the AC/ADP ($\chi 2$ (2)=9.182; p=0.010) but not in the AVPe/VMPO (p=0.806) and MPOM (p=0.08). In the AC/ADP, post-hoc analysis determined that the EO group had significantly higher pSTAT5-ir density than the control (p=0.01) whereas differences between the control and EP group are only marginally significant (p=0.07). No significant effects of steroids were found in the remaining hypothalamic nuclei (anterior hypothalamus, Arc and VMH) (p>0.4).

Finally, the thalamic PIL displayed significant inter-group differences ($\chi 2$ (2) =8.510; p=0.014), as well as the midbrain VLPAG ($\chi 2$ (2) =10.900; p=0.004) with significantly increased levels of pSTAT5-ir relative to control in the EP group for the PIL (p= 0.015) and in both the EO (p=0.007) and the EP (p=0.024) groups for the VLPAG.

1.3.4. EFFECT OF TESTOSTERONE WITHDRAWAL IN PROLACTIN SIGNALLING IN THE MALE MOUSE BRAIN

Finally, we explored the putative role of testosterone in the regulation of PRL-derived signalling in the male brain. We compared pSTAT5-ir density between our previous group of intact males and a sample of long-term castrated males (Fig 1.6). For this comparison, we returned to the original set of frames, comprising those brain regions with pSTAT5-ir in male specimens. This frames are depicted on Figure 1.1 and include the SFi and MePD in the telencephalon and the AVPe, Pa, Arc; VMHvl, VMHc and DM in the hypothalamus. In this case, only the Arc and AVPe fulfilled the criteria for parametric analysis. Hence, we performed an independent t-test for these two nuclei and a non-parametric Mann-Whitney test for the rest. The results of this statistical comparison are illustrated on Figure 1.6 and reveal a significant effect of testosterone withdrawal in diminishing the levels of pSTAT5-ir in the Pa (U= 33.00; p=0.004), in both the ventrolateral and central /dorsomedial VMH (U= 30.00, p=0.015 for both frames) and in the SFi (U= 26.00, p=0.05). Figure 1.2 provides some photographic examples of the appearance of pSTAT5-ir of intact and castrated males in the analysed sites.

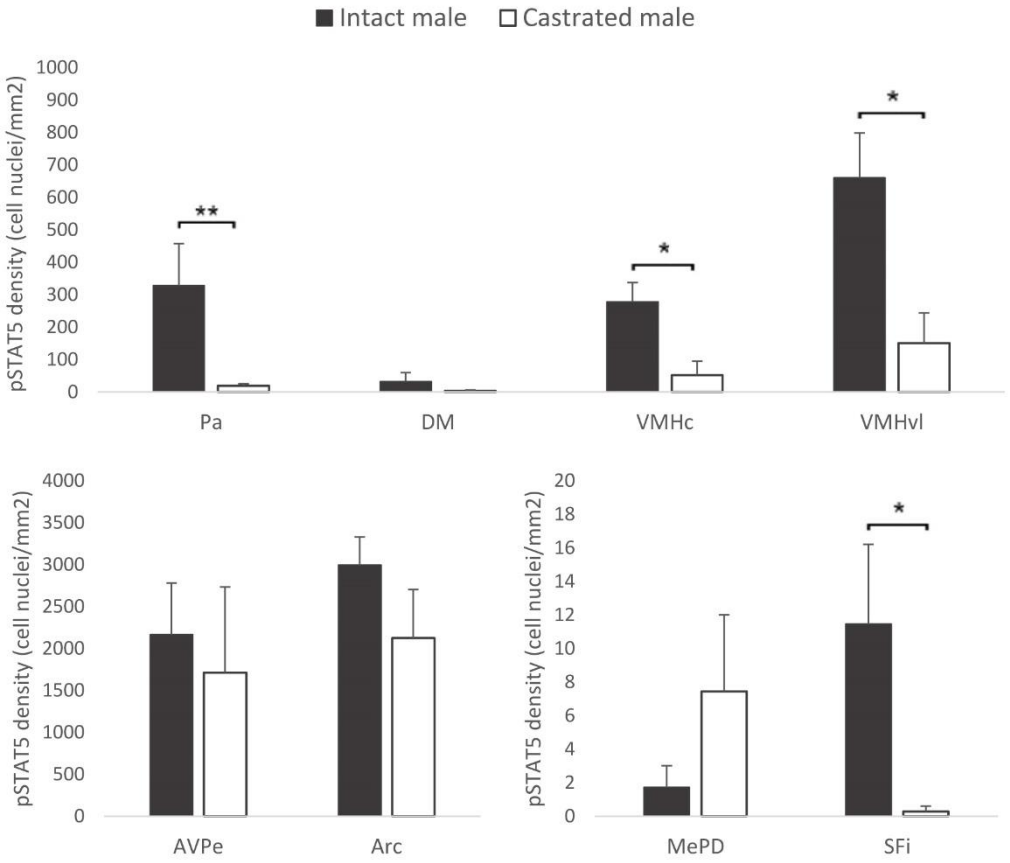


Figure 1.6 Effect of testosterone withdrawal in pSTAT5 immunoreactivity of the male mouse brain

Assessment of pSTAT5-ir density (pSTAT5-positive cell nuclei/mm2) in gonadally-intact male mice and castrated male mice within the major brain regions showing pSTAT5 expression in the male mouse brain. Bar histograms show mean interhemispheric pSTAT5-ir density ± SEM in intact, PRL-supplemented males (n=6; black) and castrated, PRL-supplemented males (n=6; white). Counting frames for each of the analysed nuclei are shown in Fig. 1.1. Statistical analysis was applied independently to each brain region (independent t-test for parametric data or Mann-Whitney test for non-parametric data). *P ≤ 0.05; **P ≤ 0.01.

1.4. DISCUSSION

The present study examined the distribution of PRL-derived signalling in the female and male CD1 mouse brain. We compared the central patterns of pSTAT5-ir in ovariectomized, steroid-primed female mice and gonadally intact male mice, both administered exogenous PRL. We evaluated pSTAT5-ir levels in qualitative terms (distribution in the brain) and quantitative terms (assessment of pSTAT5-ir density in selected brain nuclei). As expected, we found more restricted patterns (Fig 1.1) and lower levels (Fig 1.3) of pSTAT5-ir in males than in females, with several regions showing

sexually dimorphic expression of pSTAT5-ir. Following this descriptive analysis, we evaluated the contribution of the gonadal steroids estradiol and progesterone to PRL signalling in the female brain. For this task, we conducted a hormonal substitution experiment on ovariectomized virgin female mice, comparing pSTAT5-ir density after vehicle, estradiol-vehicle and estradiol-progesterone treatment, respectively. We found a generalised enhancement of PRL-derived signalling after estradiol treatment, which was locally modulated by progesterone in different ways (Fig 1.5). In males, we evaluated the contribution of testosterone to central PRL signalling by comparing pSTAT5-ir density in castrated versus intact males. We found that, in a number of nuclei, PRL-derived signalling decreased significantly after testosterone withdrawal (Fig 1.6). In this section, we will first comment on the observed sexually dimorphic differences in distribution and density of pSTAT5-ir. Then, we will discuss the role of estradiol and progesterone in the regulation of central PRL signalling in female mice. Finally, we will focus on the male brain to discuss on the role of testosterone as a putative regulator of central PRL signalling and on the mutual regulatory relationship of PRL and testosterone.

1.4.1. ON THE METHOD: pSTAT5 IMMUNODETECTION ON PROLACTIN-SUPPLEMENTED MICE

In this experiment, distribution and levels of PRL responsiveness were assessed in brain tissue of gonadally intact males and of ovariectomized, steroid-primed female mice. In order to avoid any bias due to variable or low circulating PRL levels, both experimental females and males were supplemented with a standard dose of exogenous PRL. This supplementation allowed for a controlled increase of circulating PRL, granting homogenous supraphysiological levels and access of the hormone to the brain and therefore a maximal and (relatively) constant brain response on the experimental animals.

The methodological approach chosen to evaluate central PRL responsiveness in this experiment (and the main strategy of choice throughout the present work) is the immunohistochemical detection of pSTAT5. This protein (pSTAT5) is the phosphorylated (active) form of Signal Transducer and Activator of Transcription 5 (STAT5), a key element in the Jak/STAT signalling pathway of the long form of the PRLR (PRLR-l) that, once phosphorylated, will translocate to the cell nucleus to mediate the biological effects of PRL signalling (Fig I.2). The specific detection of pSTAT5 results in an

advantageous approach to assess PRL actions in the brain, in comparison to other histological strategies like, for instance, PRLR immunohistochemistry (IHC) or in situ hybridisation (ISH). Both of these alternative strategies are limited to assess the distribution of the mRNA (ISH) or the protein (IHC) of the PRLR, but they do not take into account putative posttranslational or posttranscriptional (ISH) modifications modulating the receptor's activity or sensitivity. On the contrary, pSTAT5 immunodetection provides a downstream functional measure for PRL-derived signalling, identifying the signalling effectively occurring in the tissue in response to the binding of PRL to its receptor.

However, this methodology is not without limitations. First, it does not show signalling independent of STAT5 phosphorylation (Buonfiglio et al. 2015) such as that occurring through other signalling pathways associated to the PRLR-l (Fig I.2) or through the short PRLR isoforms (PRLR-s) (Goupille et al. 1997; Binart et al. 2010). Regarding the former, it is indeed unavoidable to miss certain amount of PRL-derived signalling through alternative pathways of the PRLR-l, but, remarkably, the Jak/STAT pathway is generally considered the main signalling pathways of the PRLR-l (for more details, see section I.2). Concerning the PRLR-s, evidence suggests that these variants are non-functional or do not relate to the major endocrine actions of PRL (Lesueur et al. 1991). Instead, other studies conclude that the PRLR-s represent a dominant negative form of the receptor, which by dimerization inhibits the functional PRLR-l (Berlanga et al. 1997). Furthermore, expression levels of the PRLR-s remain unchanged during the estrous cycle and are similar in virgin, pregnant and lactating female rats (Sugiyama et al. 1994; Nogami et al. 2007). This supports the view that the PRLR-s variants are not directly involved in reproduction. This is one of the reasons why we (and others) focus in the signalling cascade of the long form of the PRLR for analysing the putative role of PRL in maternal physiology and behaviour.

A further limitation of pSTAT5 detection, however, is the potential crosstalk between signalling associated to PRL and growth hormone (GH) or leptin. All three hormones bind to receptors of the type-I cytokine receptor superfamily and appear to relate to STAT5, among other pathways (Bennett et al. 2005; Gong et al. 2007). Nevertheless, our material belonging to male mice not supplemented with exogenous PRL (Fig 1. IV) is devoid of pSTAT5-ir (except for the Arc and Pe), indicating that most pSTAT5-ir is attributable to PRL supplementation. This has also been shown for virgin female mice (Brown et al. 2010). Altogether, pSTAT5 immunostaining can be considered a reliable

marker for the activation of the PRLR-l, which in turn represents the major component of lactogenic signalling in the brain.

1.4.2. SEXUALLY DIMORPHIC PROLACTIN SIGNALLING IN THE MOUSE BRAIN

Prolactin signalling in the female mouse brain

Herein, we report novel findings of the patterns of PRL-derived signalling in the brains of PRL-supplemented CD1 virgin females, which were ovariectomized and treated with estradiol and progesterone. This model was chosen at the expense of freely-cycling virgin females, based on previous observations that the latter display substantial rates of interindividual variability in the extension and intensity of the brain patterns of pSTAT5-ir (see Chapter 2). Ovariectomized, steroid-primed virgins, on the contrary, are a valid model to emulate the proestrous/estrous phase of the estrous cycle (Akinci and Johnston 1993; Rissman et al. 1997), and provided relatively homogenous pSTAT5-ir patterns. For a more extensive description of the variable patterns of pSTAT5-ir found in the brains of freely-cycling virgins, the reader is referred to chapter 2 of the present work.

Immunoreactivity for pSTAT5 in ovariectomized, steroid-primed females treated with estradiol and progesterone was extensive in the amygdala (medial and central nuclei), basal telencephalon (lateral septum and BST), hypothalamus (preoptic, anterior and tuberomammilary regions) and, to a lesser extent, to discrete thalamic (habenula), midbrain (PAG) and brainstem (raphe nuclear complex and lateral parabrachial nucleus) structures (Fig 1.1). Importantly, this pattern shows a substantial overlap with the circuitry integrating the Sociosexual Brain Network (SBN) and other associated nuclei. The SBN is the neural network involved in the expression of social and reproductive behaviours (see Introduction and Newman 1999). This observation suggests a strong connection between PRL and female sociosexual behaviour regulation, which will be thoroughly analysed in the following chapters of this work, especially regarding maternal behaviour.

The distribution of pSTAT5-ir in the brain of the female mouse was first published by Brown et al. (2010), who analysed central pSTAT5-ir after PRL supplementation in virgin females of the inbred strain C57BL/6J. Remarkably, the pattern of pSTAT5-ir they observed was relatively reduced as compared to the one reported in the present work,

carried out in ovariectomized, steroid-primed CD1 females also with exogenous administration of PRL. This is possibly due to differences in gonadal steroid input, as our females were primed with estradiol and progesterone (modelling proestrous/estrous) and females of the cited work were in diestrous. Moreover, these observations could also underlie interstrain differences between CD1 (outbred) and C57/BL6J (inbred) animals, mirroring documented behavioural interstrain differences in, for instance, allomaternal behaviour expression (Parmigiani et al. 1999). Importantly, our pSTAT5-ir pattern fits the distribution of the mRNA for the PRLR much closer than the pattern of pSTAT5-ir found in C57BL/6J mice (Brown et al. 2010). This suggests either a mismatch in the brain of C57BL/6J mice between the distribution of the mRNA and the receptor protein (likely due to posttranscriptional, posttranslational or other regulatory mechanisms) or some kind of inhibition of central PRL signalling, as compared to the CD1 strain.

Prolactin signalling in the male mouse brain

Our study reveals a clear sexual dimorphism in PRL-derived signalling in the mouse brain upon exogenous PRL administration, in favour of females. Female mice display richer and more extensive patterns of pSTAT5-ir than males, an expected outcome regarding the close link of PRL with female reproduction. The analysis of the socio-sexual brain of males and females of different rodents and other mammals has revealed several cases of sexual dimorphism, which would be related to the dimorphic expression of many social behaviours, such as aggression and sexual behaviour (Bayless and Shah 2016). In most occasions these sexual dimorphism favours males. For instance, the medial preoptic area shows a larger nucleus an higher cell density in the brain of males than in females (He et al. 2013; Tsuneoka et al. 2017). A similar case has been reported for the posterodorsal division of the medial amygdala (Morris et al. 2008). In addition, all the centres of the socio-sexual brain show a characteristic innervation by arginine-vasopressin (AVP) fibres that, again, is denser in males than in females (Rood et al. 2013; Otero-Garcia et al. 2014).

Our findings represent an additional case to the short list of female-biased sexually dimorphic traits. As we will discuss in the next chapter, this is likely related to the close relationship of PRL to maternal behaviour and physiology. In fact, whereas the distribution of PRL-derived signalling in the female mouse brain has focused substantial

research, this work is, to our knowledge, the first descriptive analysis of the action of PRL in the male mouse brain. Our results indicate that the male mouse brain is indeed sensitive to PRL, despite the lower connection of this hormone to male physiology and behaviour. However, male mice displayed lower levels of PRL signalling in terms of extension and density of the pSTAT5-ir patterns, as compared to ovariectomized, steroid-primed virgin females. Moreover, patterns of pSTAT5-ir in males were a fraction of those of female mice. In other words, we found no single brain structure labelled in males but not in females.

Structures in the male brain positively labelled for pSTAT5-ir are located mainly in the hypothalamus (the AVPe and VMPO, MPA, Pe, Pa, Arc, DM and VMH) and, to a lesser extent, in the telencephalon (SFi in the septum and MePD in the amygdala), with no additional pSTAT5-ir detected in any of the remaining brain divisions (Fig 1.1). In contrast to females, most of the major nodes of the SBN (the lateral septum, BST, medial amygdala or PAG, see Fig I.6) are unresponsive to PRL in males, even under high circulating levels of the hormone (e.g. exogenous administration). This indicates, as advanced before, a lower implication of PRL in the regulation of sociosexual behaviour in males, as compared to females, even though it does not exclude a minor role in some male-specific behaviours (see General discussion). According to our quantitative analysis (Fig 1.3), pSTAT5-ir density was significantly lower in males than in females in part of the nuclei with expression of pSTAT5 in both sexes (the AVPe/VMPO, Arc, DM and MePD). No significant difference on pSTAT5-ir density was found in the rest of the analysed nuclei.

Furthermore, it is worth noting the brain of male mice lacking systemic PRL supplementation is virtually devoid of pSTAT5-ir, except for the Arc (Fig. 1.2) and some cells in the anterior periventricular hypothalamus (not shown). These results suggest, in the first place, that PRL is indeed the source of all the signalling we report, whereas any other endocrine agents signalling through the Jak/STAT pathway, such as growth hormone (Furigo et al. 2016), do not contribute substantially to STAT5 phosphorylation in the brain. In addition, it indicates that in basal conditions PRL signalling in the male brain is very low, probably due to low circulating levels of the hormone (Sinha et al. 1977; Guillou et al. 2015). In fact, the presumed location of the TIDA neurons (as revealed by tyrosine hydroxylase immunohistochemistry), the dorsal aspect of the Arc (Li et al. 1999), displays scarce pSTAT5-ir in the control male mouse (not supplemented

with PRL, Fig 1.2 IV), supporting the view of very low or even null circulating PRL levels in these male mice. Despite this observations, basal levels of circulating PRL might not always be negligible, as Sinha et al (1977) reported periodic serum PRL peaks during the dark period in male mice of the C3H/St strain.

In line with these observations, PRL would influence the brain of males especially under some physiological conditions in which there is substantially increased hypophyseal or extra-hypophyseal PRL release. This might happen in relation with stress (Torner et al. 2004), as well as after copulatory behaviour (Kamel et al. 1977).

1.4.3. PROLACTIN SIGNALLING IN THE FEMALE MOUSE BRAIN: REGULATION BY ESTRADIOL AND PROGESTERONE

Progesterone and estradiol are well-documented modulators of PRL signalling. For instance, they upregulate PRLR expression during proestrous and oestrous (Sugiyama et al. 1994) and increase PRLR mRNA levels in pituitary lactotrophs (Scully et al. 1997) and, consequently, circulating PRL levels (Freeman et al. 2000). In this work, we intended to provide an integrative view of how the gonadal steroids ultimately contribute to shape the central patterns of PRL signalling. For this purpose, we conducted a hormonal substitution experiment on ovariectomized virgin female mice in which we assessed the effect of controlled doses of estradiol (EO) or estradiol plus progesterone (EP) on the central levels of pSTAT5-ir induced by administration of exogenous PRL (Fig 1.5). The results of this analysis revealed a general trend towards an increase of pSTAT5-ir density after estradiol treatment (with or without progesterone). This trend reached significance in the MePD, the LSV, the AC/ADP, the thalamic PIL and the VLPAG. Of these, only the LSV and the VLPAG display a significant increase in both the EO and EP groups, whereas the increase found in the MePD and the PIL groups reached significance only in the EP group and that found in the AC/ADP only in the EO group.

Altogether, this reflects a rather complex regulation of PRL signalling by gonadal steroids. Our results suggest that indeed, estradiol exerts a positive modulation of lactogenic signalling. On top of it, progesterone might display either a synergistic action (pSTAT5-ir density in the MePD and PIL only increases significantly when both gonadal steroids are administered together), no significant effect (in the LSV and the VLPAG the significant increase is maintained in both estradiol-treated groups regardless of progesterone) or even a possible antagonizing effect (the AC/ADP experiments a

significant increase in the EO group, which is not paralleled by the EP group). This duality of progesterone fits previous findings in the literature. On the one hand, progesterone has been shown to act synergistically with estradiol, potentiating the positive regulatory effects of the former on, for instance, hypophyseal PRL release and PRLR-l expression (Sugiyama et al. 1994). On the other hand, some studies have reported a progesterone-mediated downregulation of PRLR in the MPA during late pregnancy in the rat, which temporarily blocks the onset of maternal behaviour until progesterone levels drop close to parturition (Bridges and Hays 2005). Altogether, our findings and previous evidence in the literature suggest that estradiol-progesterone interaction on central PRL action is dual and region-dependent.

Surprisingly, our results do not fit completely the colocalisation of PRLR (Bakowska and Morrell 1997) and estrogen receptor α (ERα) with pSTAT5-ir, as published by Furigo et al. (2014a). According to this study, several sites where we have found no apparent differences in pSTAT5-ir density related to gonadal steroid treatment do express abundant ERα receptors co-localising with pSTAT5-ir, e.g. the AVPe, the medial MPO, the Arc or the VMHvl. Aside from some possibly relevant methodological differences (different PRL dosage and exposition time, for instance), this apparently contradictory results might have different explanations. On the one hand, it is possible that PRL signalling were dissociated from gonadal steroid regulation in some regions, depending on the function of PRL in those regions. For example, in the Arc, it is likely that levels of lactogenic signalling respond directly to circulating PRL levels, as the primary function of this nucleus is the feedback control of PRL secretion. Consistent with this, estradiol and progesterone are known to modulate this function through regulation of the expression of angiotensin II receptors in the arcuate nucleus (Donadio et al. 2006). On the other hand, estradiol regulation of PRL signalling might not always have a positive valence. According to the existing literature, estradiol regulates the expression of Suppressor of Cytokine Signalling (SOCS) protein family, responsible for downregulating signalling through the PRLR. For example, estradiol enhances the activity of SOCS3 promoter in the mouse (Matthews et al. 2005), and chronic estradiol treatment increases the expression of mRNA for SOCS3 and cytokine-inducible SH2-containing protein (CIS) in the rat (Anderson et al. 2008), both of which decrease PRL signalling (Krebs and Hilton 2001). Thus, the final levels of lactogenic signalling we report might result of the balance of opposed regulatory elements and might not simply reflect the direct crosstalk of PRL

with ER-expressing cells. In sum, central lactogenic signalling is influenced by circulating levels of estradiol and progesterone in a dual, complex and anatomically heterogeneous fashion.

1.4.4. PROLACTIN AND TESTOSTERONE: MUTUAL NEUROENDOCRINE REGULATION

One of the main outcomes of the present work is the identification of a putative regulatory role of testosterone in PRL signalling in the male mouse brain. Long term testosterone ablation through orchidectomy led to a significant decrease in pSTAT5-ir density in the SFi, the Pa and both the central/dorsomedial and lateral VMH (Fig 1.6). This indicates a positive regulatory role of testosterone on PRL signalling in those brain regions. The actual mechanism of this positive regulation of testosterone on PRL signalling is not clear, but it is not likely related to the control of systemic PRL levels or the access of PRL to the brain. First, this effect cannot be attributed to an increased release of hypophyseal PRL, since our experimental males were supplemented with exogenous PRL. Moreover, testosterone has documented inhibitory effects on hypophyseal PRL release in male rats (Gill-Sharma 2009) and mice (O'Hara et al. 2015). Therefore, in spite that testosterone appears to inhibit hypophyseal PRL release it nevertheless enhances neural responsiveness to PRL in certain brain regions. This effect is also not likely explained by a facilitated access of systemic PRL to the brain, as our results reveal that the positive effect of castration on pSTAT5-ir is not generalised but rather specific and restricted to discrete brain sites. Hence, our findings point to a regulation of PRL receptor expression or the modulation of PRL signalling downstream the PRL receptor in specific neural populations. Further research is required to elucidate the specific action of testosterone on PRL signalling in male mice.

An additional question of interest is the identity of the actual biological modulator of PRL signalling we report here. Testosterone can be locally metabolized into two different neuroactive steroids: it can be reduced to dihydrotestosterone (Melcangi et al. 2015), which binds to androgen receptors, or aromatised to estradiol (Naftolin et al. 1975) that then binds to estrogen receptors. Our results make it very unlikely that this regulation occurs through the aromatisation of testosterone to estradiol. Although all the nuclei where PRL signalling is affected by castration express at least one form of estrogen receptor (Mitra et al. 2003), none of the examined brain nuclei displays aromatase

activity in the male mouse brain (Stanić et al. 2014). Moreover, none of these nuclei is affected by estradiol treatment in our ovariectomized, steroid-primed females (Fig 1.4). In favour of a direct action of testosterone or of its reduced metabolite, all of the examined nuclei express androgen receptors, with the Pa and VMH showing very high levels (Stanić et al. 2014). Hence, the effect of testosterone on PRL signalling that we report here is likely conveyed through the androgen receptor and involves testosterone itself or a derivate androgen, but not estradiol.

In addition to the reported regulation of testosterone on central PRL signalling, our data, together with evidence in the literature, advance a reciprocal regulatory action of PRL on the hypothalamus-pituitary-gonadal (HPG) axis and ultimately on testosterone release. According to the literature, PRL appears to upregulate the HPG axis and testosterone secretion in males. For instance, Bartke et al. (1987) reported increases in FSH and LH in hyperprolactinaemic male mice, and Chandrashekar and Bartke (1988) observed that exogenous PRL infusions stimulate the increase in serum testosterone in male rats, whereas immunological disruption of endogenous prolactin eliminated this effect. This regulatory action of PRL on the HPG axis likely occurs through the kisspeptin system. Kisspeptin plays an essential role in reproduction, as it regulates gonadotropin-releasing hormone (GnRH) expressing neurons in the brain (Dungan et al. 2006), which constitute the key element of the HPG axis. Therefore, the expression of PRL receptors by kisspeptin neurons (Kokay et al. 2011) allows for a direct role of PRL on HPG axis control and testosterone secretion. In this context, our results provide support for the hypothesis that the brain kisspeptin system is subject to PRL regulation in male mice, too, since our sample of male mice displayed substantial pSTAT5-ir in the hypothalamic nuclei containing kisspeptin-positive neurons (Semaan et al. 2013), the Arc and the AVPe (Figs 1.1a-c, 1.1f, 1.2a and 1.2c). Nevertheless, this possibility requires being experimentally proven by assessing the coexpression of kisspeptin and pSTAT5 immunoreactivity in the aforementioned nuclei.

Altogether, our results and previously published evidence in mice and rats suggest that PRL and testosterone have complex and reciprocal regulatory actions. On the one hand, testosterone inhibits hypophyseal PRL release in male mice (O'Hara et al. 2015), but it also appears to enhance PRL signalling in some brain nuclei (Fig 1.6). On the other hand, PRL might act onto hypothalamic kisspeptin neurons to ultimately stimulate the release of testosterone by the testes.

1.4.5.CONCLUSIONS

The study of the actions of PRL in the brain is traditionally biased towards females, because of the paramount role of PRL on female reproduction and motherhood. Here, we report a comparative study of the patterns of PRL responsiveness in the female and male brains. Our findings indicate that adult female and male mice systemically supplemented with PRL display sexually dimorphic brain patterns of pSTAT5-ir as a result of central PRL-derived signalling. In qualitative terms, female mice display a relatively extensive pSTAT5-ir pattern encompassing most nodes of the socio-sexual brain network. In contrast, males show a reduced pattern of pSTAT5-ir with scarce labelling in the socio-sexual brain circuitry. Additionally, a quantitative analysis of brain nuclei with common pSTAT5 expression in both males and females revealed higher pSTAT5-ir density in females in most of the analysed nuclei. This indicates that PRL has a sexually dimorphic role in the regulation of social behaviours.

Furthermore, we report novel evidence of the regulatory roles of estradiol and progesterone, on the one hand, and testosterone, on the other, in the shaping of the central responsiveness to PRL in females and males, respectively. In females, estradiol exerts a generalised stimulation of PRL signalling, whereas progesterone displays regional differences, acting either synergistically with estradiol, neutrally or antagonizing its effect. In males, testosterone exerts a positive regulatory effect on the responsiveness to PRL of a number of brain centres, as seen by the significant decrease in pSTAT5-ir density in castrated male mice, in comparison to intact males. Despite the relatively reduced responsiveness to PRL of the male brain, our findings reflect that PRL may play relevant roles in male physiology and behaviour that have been understudied.

2

Lactogenic Responsiveness of the Mouse Brain throughout the Female Reproductive Cycle

2.1. RATIONALE AND AIMS

During pregnancy and lactation, the brain undergoes a series of adaptations, changes in the regulation of physiology and behaviour, which allow the female to better face the challenges of motherhood. One of the major agents setting up these maternal adaptations in the brain is PRL, rightfully termed "the maternal hormone". In this study, we sought to explore the impact of PRL on the female brain during the critical periods of pregnancy and lactation. Using the immunohistochemmical detection of pSTAT5 as a measure of central lactogenic signalling, we characterised the distribution of pSTAT5-ir in the brains of late-pregnant females, lactating dams and virgin, freely-cycling females as controls of the basal state. During the second half of pregnancy, evidence suggests that hypophyseal PRL expression is impaired and other non-hypophyseal lactogenic signals operate instead (Soares et al. 1998b; Soares 2004), e.g. the so-called placental lactogens (PLs). These are PRL homologues of placental origin that signal through the PRLR (Soares et al. 1998b) and are known to share several of the functions of PRL, for example the promotion of maternal behaviours (Bridges et al. 1996). Hence, we also aimed at identifying the contribution of non-hypophyseal lactogenic sources like PLs to the modulation of brain function during the period of late pregnancy. To do so, we included an additional group of late-pregnant females in the analysis, where hypophyseal PRL release was pharmacologically depressed by means of bromocriptine (see section 2.2.3).

2.2. MATERIAL AND METHODS

2.2.1. ANIMALS

For the present study, a total of n= 32 adult female CD1 mice (Charles River Laboratories, France) were used, aging between 8-12 weeks. Animals were housed in polypropylene plastic cages (145mm wide, 465 mm long and 215 mm high; Panlab), in groups of 4 to 6 animals, with *ad libitum* access to food and water and under controlled temperature (24 ± 2 ºC) and lighting conditions (12h:12h; lights ON at 8 am). Animals were treated throughout according to the European Union Council Directive of June 3rd, 2010 (6106/1/10 REV1) and procedures were approved by the Committee of Ethics on Animal Experimentation of the University of Valencia, where the experiments were performed.

2.2.2. EXPERIMENTAL DESIGN

For this experiment, n=27 animals were randomly assigned to a total of 4 groups: (1) Virgins (n=7); (2) late-pregnant females (n=6); (3) bromocriptine-treated, late-pregnant females (n=8), and (4) lactating dams (n=6). In order to obtain a more natural image, albeit more variable, of the appearance of PRL signalling in the virgin female brain, for the Virgin group we chose to use freely-cycling virgin females, where neither gonadal steroid levels were controlled experimentally nor estrous cycle was restricted to a particular phase. Regarding groups 2, 3 and 4, mating was ensured by pairing these females with a stud male for a period of 4 days (the mean length of a full estrous cycle), counting the first day as pregnancy day 1 (PD1). Following impregnation, females were housed in pairs until the day of perfusion. Females of Late-pregnant (2) and Bromocriptine late-pregnant (3) groups were perfused on presumed pregnancy day (PD) 18, but the exact pregnancy day (PD14-18) was assessed post-mortem evaluating the developmental stage of the respective foetuses through anatomical signs such as the differentiation of the eyelids, tongue, whiskers or fingers. References for this evaluation can be found at www.emouseatlas.org. In addition, late-pregnant females of group 3 underwent a pharmacological treatment with bromocriptine that abolished virtually any release of hypophyseal PRL (for further details, see section 2.2.3). Females of Lactating group (4) were perfused on postpartum day (PPD) 6. After perfusion of the experimental females, brain tissue was collected and processed for pSTAT5 immunohistochemistry (section 2.2.4), and the resulting immunoreactivity patterns analysed qualitatively and quantitatively (section 2.2.5).

2.2.3. PHARMACOLOGICAL INHIBITION OF HYPOPHYSEAL PROLACTIN

Bromocriptine is a dopamine D2 receptor agonist that selectively targets pituitary lactotrophs, emulating the dopaminergic inhibition exerted by the Tuberoinfindibular (TIDA) and Periventricular-hypohyseal (PHDA) Dopaminergic neurons upon hypophyseal PRL release (see section I.3 and Fig I.3). Consequently, bromocriptine treatment leads to the virtual inhibition of hypophyseal PRL discharge. Treatment for Bromocriptine group (3) consisted in two sequential subcutaneous (s.c.) injections of 100 µg of bromocriptine (2-Bromo-α-ergocriptine, Sigma-Aldrich, St. Louis, USA) diluted in 10% ethanol, 19 and 2 hours prior to perfusion (Sjoeholm et al. 2011). This resulted in an approximate dose of

2 mg/kg of body weight per injection. A similar bromocriptine treatment schedule drastically decreased pSTAT5-ir in the brain of a sample (n=5) of freely cycling virgin female mice (Table 2.1), leaving only some faint, residual labelling. This bromocriptine treatment produced a mean 90.34 % reduction in the analysed brain nuclei. This demonstrates an effective inhibition of hypophyseal release of PRL by the bromocriptine treatment we have applied.

Mean pSTAT5-ir density	VIRGIN (n=7)	VIRGIN + BRC (n=5)	REDUCTION (%)
AC/ADP	189.44	14.56	92.31
Arc	4223.72	260.87	93.82
AVPe	4349.54	343.75	92.10
BSTMPM	1042.86	42.19	95.96
CeM	425.00	0	100
LSV	225.54	21.40	90.51
MePD	250.57	30.70	87.75
MPO	433.57	88.19	79.66
Pa	323.02	73.98	77.10
PIL	157.82	0	100
VLPAG	468.04	89.04	80.98
VMHvl	1262.63	77.38	93.87
Mean reduction percentage			90.34

Table 2.1 Bromocriptine drastically reduces brain pSTAT5-ir density on virgin female mice

Two s.c. 100 μg bromocriptine injections, 19 and two hours prior to perfusion, produced reductions of pSTAT5-ir in the analysed nuclei of the virgin female mouse brain ranging from 77.10% to 100%, with a mean reduction of 90.34 %.

2.2.4. TISSUE COLLECTION, HISTOLOGICAL PROCESSING AND pSTAT5 IMMUNOHISTOCHEMISTRY

Animals were perfused transcardially under sodium pentobarbital anaesthesia (Dolethal; Vetoquinol, Madrid, Spain). Then, brain tissue was extracted and processed for immunohistochemistry. For a complete description of this process, see Section 1.2.5. As for the immunohistochemmical detection of pSTAT5, we applied the same protocol described in section 1.2.6. Briefly, tissue underwent the following steps: (a) an antigen retrieval step under 0.01 M TRIS buffer (TB), pH 10 at 85^{o}C; (b) an endogenous peroxidase inhibition under 1% hydrogen peroxide in TB-saline (TBS); (c) a one hour

blockade of unspecific labelling under 2% BSA, 2% goat serum and 0.3% Triton X-100 in TBS; (d) a 72 hour incubation in primary antibody (Rabbit anti-pSTAT5 pSTAT5 Tyr694; Cell Signalling Technology, Beverly, MA) diluted 1:500 in 0.1% Triton X-100 TBS; (e) a 90 minute incubation in secondary antibody (biotinylated goat anti-rabbit IgG , Vector Laboratories, Peterborough, UK) 1:200 in TBS and (f) a 90 minute incubation in avidin-biotin-peroxidase complex (ABC Elite kit; Vector Laboratories) in TBS. A permanent, brown nuclear staining was obtained through development with 0.005% 3-3'-, diaminobenzidine (Sigma). Finally, sections were mounted onto gelatinized slides, dehydrated in alcohols, cleared with xylene and coverslipped with Entellan.

2.2.5. ANALYSIS OF pSTAT5 IMMUNOREACTIVITY

Both the qualitative and quantitative assessments of pSTAT5-ir in this study were conducted following the same methodology as previously described (section 1.2.7). For the qualitative mapping of the corresponding patterns of pSTAT5-ir distribution in freely-cycling virgins, late-pregnant females and lactating dams, we registered the presence or absence of pSTAT5-ir in every single brain site for each specimen. For the quantitative assessment of the levels of PRL signalling, we analysed the density of cells showing pSTAT5 immunoreactivity (pSTAT5-ir) in a selection of 13 nuclei belonging to the socio-sexual brain and/or involved in the regulation of maternal behaviours. After designing the respective counting frames and obtaining the matching photomicrographs, we conducted the same image analysis in the Image J software as described in our previous study (section 1.2.7). Finally, we calculated the mean (interhemispheric) density of pSTAT5-imunoreactive cell nuclei for each specimen by dividing the mean value of these counts for both hemispheres by the area of the respective frame.

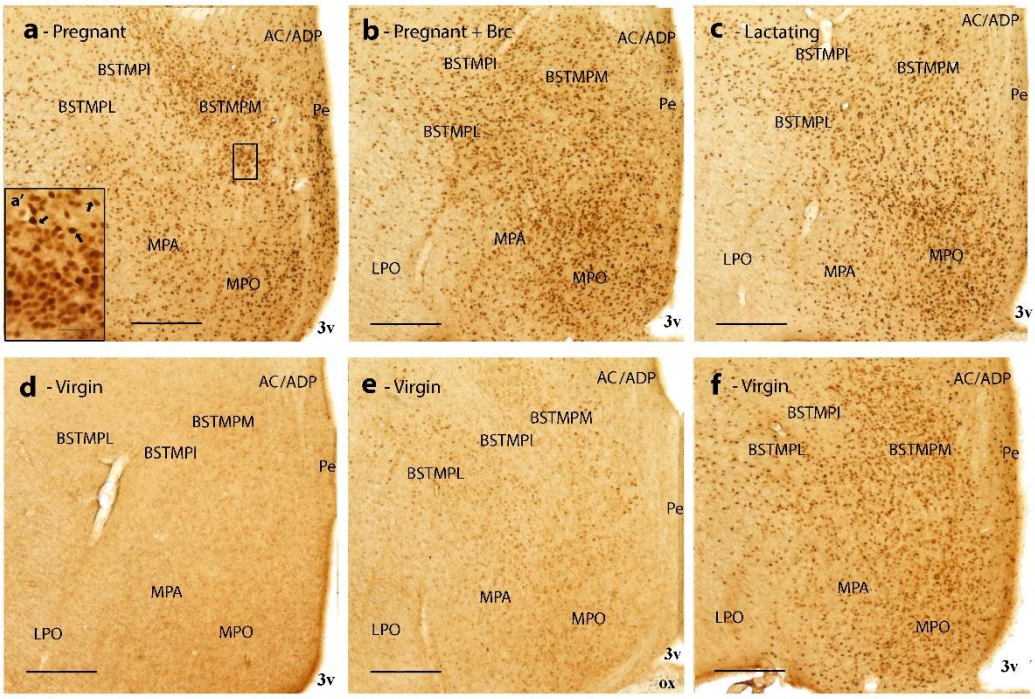


Figure 2.1 Appearance of pSTAT5 immunoreactivity in the brains of late-pregnant, lactating and virgin female mice

Photomicrographs showing the pattern of pSTAT5 immunoreactivity of the BST-MPA region in a) a postconception day 14-pregnant female; b) a day 14 pregnant-female treated with bromocriptine; c) a postpartum day 6 lactating dam and three different virgin females with different staining density (d-f). Immunohistochemistry for pSTAT5 resulted in a specific nuclear labelling (arrows on inset a'). The pattern of pSTAT5-ir was constant among late-pregnant and lactating females (a-c), with a high number of tightly packed immunostained cells in the BST-MPA continuum. Conversely, the pattern of pSTAT5 immunostaining in virgin females showed a remarkable inter-individual quantitative variability, ranging from barely no staining (d), a moderate staining density (e), to an exceptionally high density similar to that in late-pregnant females and dams (f). Every section is approximately 0.25 mm posterior to Bregma. Scale bars: 250 μm; 50 μm (inset).

2.2.6. STATISTICAL ANALYSIS

Statistical analysis was conducted using IBM SPSS Statistics 22 software package. After checking for normality (Kolmogorov-Smirnov test with Lilliefors' correction) and homogeneity of variance (Levene's test), we performed a separate one-way ANOVA test on each of the analysed brain regions to assess inter-group differences (Virgin, Late-pregnant and Lactating groups) on the mean pSTAT5-ir density. We performed an additional set of one-way ANOVAs for the respective brain regions comparing the Late-pregnant and Bromocriptine groups, in search of significant differences attributed to bromocriptine treatment. Statistically significant differences ($p \leq 0.05$) were further explored by means of post-hoc pairwise comparisons with Bonferroni's correction.

2.3. RESULTS

2.3.1. DISTRIBUTION OF pSTAT5 IMMUNOREACTIVITY IN THE BRAINS OF VIRGIN, PREGNANT AND LACTATING MICE

As stated in the previous study, immunohistochemistry for pSTAT5 produced a specific and discrete immunoreactivity restricted to the cell nucleus. We found apparent differences in the patterns of pSTAT5-ir between late-pregnant and lactating females, on the one hand, and virgin females, on the other. Whereas pregnant and lactating females showed a homogenous pattern of pSTAT5-ir distribution with minor inter-individual qualitative differences, virgin females displayed a variable degree of immunolabelling. In this section, we focus on describing the pattern of pSTAT-5-ir in the brain of pregnant/lactating females. Then, we briefly describe the variable immunostaining observed in virgin females (Fig 2.1), which in general terms matches the previous mapping conducted in ovariectomized, steroid-primed virgin female mice (see Study 1).

A Immunoreactivity for pSTAT5 in the brain of pregnant/lactating female mice

A close analysis of the immunoreactivity for pSTAT5 in these females indicates no apparent qualitative differences between both groups, with very few exceptions (see below). Therefore, we first describe the common pattern of distribution of pSTAT5-ir in both experimental groups under the common pattern shared by late-pregnant and lactating females.

Importantly, the choroid plexus showed some degree of immunostaining in every animal, regardless of physiological state (not shown). In addition, in pregnant/lactating females, quite abundant cell labelling was present in the cerebral hemispheres (septum, amygdala, extended amygdala and, to a lesser extent, cortex), diencephalon (mainly in the hypothalamus but also in some thalamic and pretectal nuclei), midbrain (periaqueductal grey and laterodorsal tegmentum) and hindbrain.

Telencephalon

Immunolabeling for pSTAT5 in this major brain division was present mainly in subpallial structures, but appeared also in a small number of pallial areas. Within the *pallial telencephalon*, neither the neocortical region nor the hippocampal formation showed pSTAT5-ir. As for the olfactory cortical areas, the olfactory bulbs were also devoid of

pSTAT5-ir, whereas immunolabelled cells were present in the dorsal taenia tecta (DTT, Fig 2.2a). Furthermore, several nuclei in the cortical amygdala contained pSTAT5-ir, namely the nucleus of the lateral olfactory tract (LOT, Fig 2.2e), the anterior cortical amygdaloid nucleus (ACo, Figs 2.2e and f), the basomedial amygdaloid nucleus (BMA and BMP, Figs 2.2g and h), the ventral basolateral amygdaloid nucleus (BLV, Fig 2.2f) and the amygdalohippocampal area (AHi, Fig 2.2h). It is noteworthy that the pallial telencephalon is one of the few brain sites where the pSTAT5-ir pattern of our pregnant and lactating females showed a certain degree of variability. This variability affects mainly the piriform cortex (Pir), where pSTAT5 immunoreactivity was observed in just one animal (a bromocriptine-treated pregnant female; see Fig 2.2d) and the cortical amygdala. Here, inter-individual variability did not correlate with the physiological status of our experimental groups, as it was observed across untreated pregnant, bromocriptine-treated pregnant and lactating females. Hence, 7 out of 18 animals displayed pSTAT5-ir in the ACo, 6 in the LOT, 7 in the BLV and only 3 animals in the BMP and AHi. In all these cases, immunoreactive cell bodies were sparse and faintly stained. By contrast, the BMA showed pSTAT5-ir in all the animals.

In the *subpallial telencephalon*, pSTAT5-ir was abundant in the septum, the extended amygdala (EA) and the anterodorsal amygdaloid area (AAD). Within the EA, both the central and medial EA displayed immunolabelling. Thus, pSTAT5 was present in the medial and central nuclei of the amygdala, in different subnuclei of the bed nucleus of the stria terminalis (BST), as well as in the sublenticular *substantia innominata* (SI, Fig 2.4a) and the interstitial nucleus of the posterior limb of the anterior commissure (IPAC, Figs 2.2c-e).

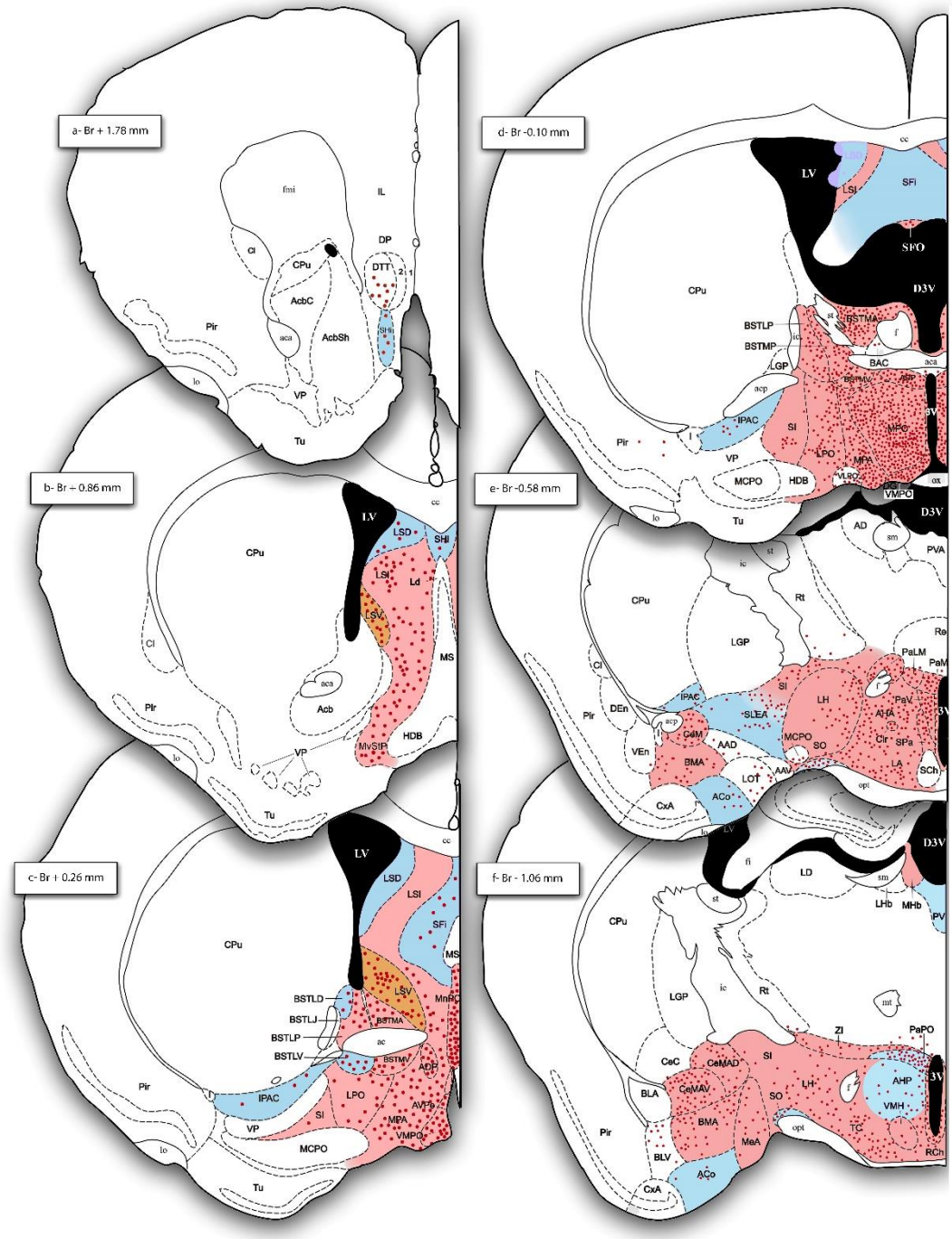
The medial extended amygdala (MEA) displayed pSTAT5-ir in all of its nuclei. Within the amygdala proper, pSTAT5-ir was moderately dense in the anterior (MeA, Fig 2.3b), posteroventral (MePV, Fig 2.3c) and, especially, the posterodorsal (MePD, Fig 2.3c) nuclei of the medial amygdala, as well as in the intramygdaloid division of the BST (BSTIA, Fig 2.3c). In addition, a high density of immunolabelled cells was observed in the medial (BSTMPM), lateral (BSTMPL) and (with a lower density) intermediate (BSTMPI) nuclei of the posteromedial BST (Figs 2.1 a-c and 2.2d).

As for the central extended amygdala (CEA), within the central amygdaloid nucleus, pSTAT5-ir was almost restricted to the medial division of the Ce (CeM, Figs 2.3b and 2.3c), although a small number of cells also appeared in the lateral Ce (CeL, Fig 2.3c).

Within the BST, most of the nuclei in the anterior and lateral BST displayed abundant labelling: the medial anterior (BSTMA, Fig 2.3a), medial ventral (BSTMV, Fig 2.3g) and lateral nuclei of the BST (ventral BSTL, posterior BSTL, dorsal BSTL, Figs 2.2c and d), with the exception of the juxtacapsular nucleus (BSTLJ, Fig 2.2c).

Within the basal cerebral hemispheres, we observed pSTAT5-ir cells in a portion of the ventral striato-pallidum located between the diagonal band nucleus and the shell of the nucleus accumbens, named as medioventral striato-pallidum (Otero-Garcia et al. 2014) because of its relationship with structures of the ventral striatum and pallidum, or ventral septal area (Rood and De Vries 2011), given its apparent continuity with the lateral septum (see Fig 2.2b).

In the septum, we found pSTAT5-ir within the different nuclei of its lateral division (LSV, LSI, and LSD, Fig 2.2b and 2.3a) and in the septo-hypothalamic nucleus (SHy, not shown). Some structures in the medial and posterior septal regions contained pSTAT5-ir, too, such as the septohipocampal (SHi, Fig 2.2b) and septofimbrial (SFi, Fig 2.3a) nuclei and the bed nucleus of the anterior commissure (BAC, Fig 2.2d). Finally, pSTAT5 immunostaining was found in the subfornical organ (SFO, Fig 2.2d).


a- Br + 1.78 mm
b- Br + 0.86 mm
c- Br + 0.26 mm
d- Br -0.10 mm
e- Br -0.58 mm
f- Br - 1.06 mm
fmi
IL
DP
Cl
CPu
DTT
AcbC
Pir
aca
AcbSh
SHi
lo
VP
Tu
cc
LV
LSD
LSI
Ld
LSV
MS
Acb
HDB
MvSt
BSTLD
BSTLJ
BSTLP
BSTLV
BSTMA
BSTMV
ADP
IPAC
LPO
SI
MPA
AVPo
VMPO
MCPO
SFi
MnRC
LGP
BSTLP
BSTMP
BAC
acp
MPO
VLPO
MPA
ox
HDB
D3V
SPO
AD
sm
PVA
Rt
Re
PaLM
PaM
DEn
SLEA
LH
PaV
AI-fA
GdM
AAD
VEn
BMA
MCPO
SO
Cir
SPa
LA
SCh
LOT
AAV
ACo
CxA
opt
fi
LD
LHb
MHb
PV
ZI
PaPO
CeC
CeMAD
CeMAV
BLA
AHP
VMH
TC
MeA
BLV
RCh

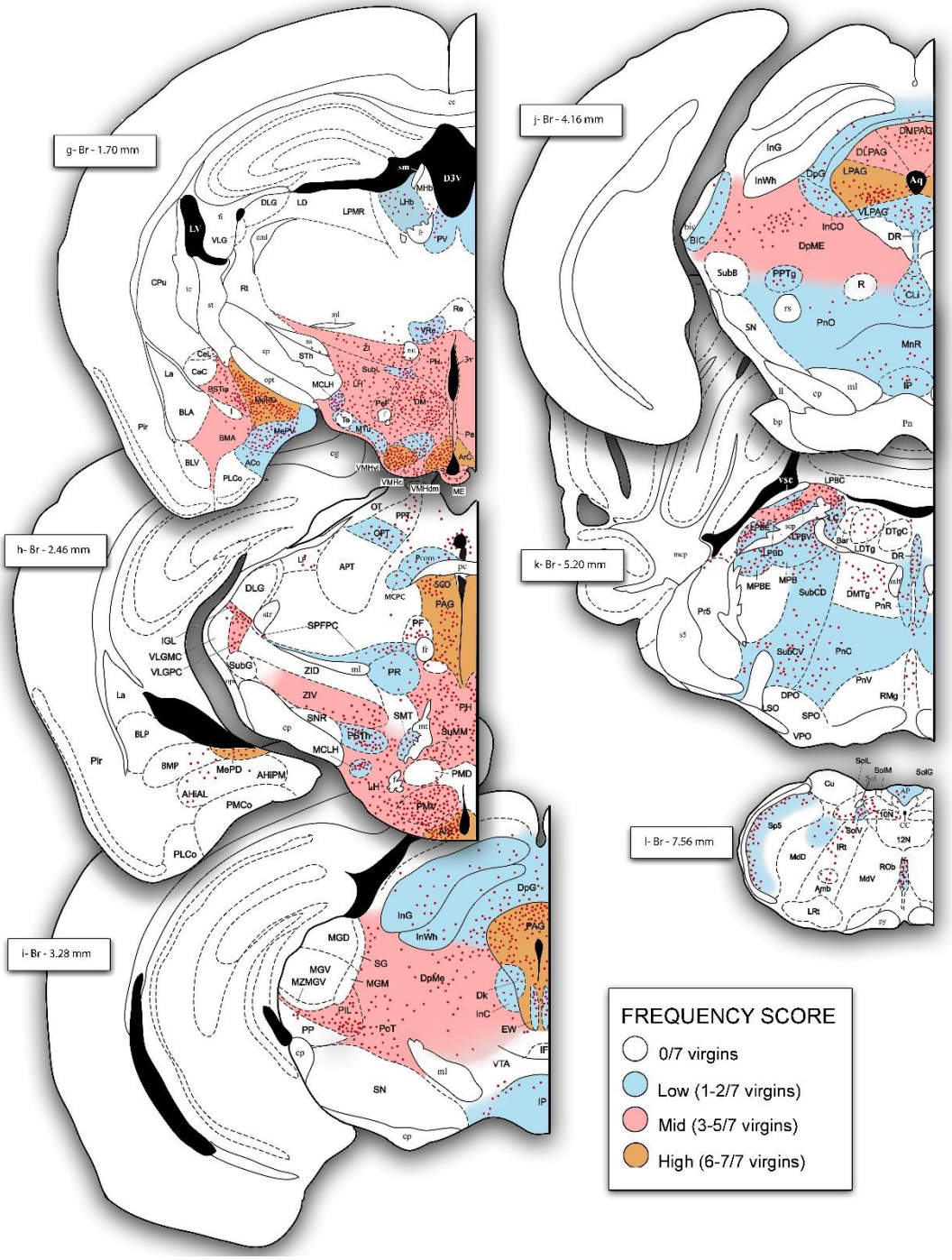


Figure 2.2 Distribution of pSTAT5 immunoreactivity in the brains of late-pregnant, lactating and virgin female mice

Semi-schematic camera-lucida drawings of coronal sections through the brain of a postconception day 14-pregnant female (specimen M1392) treated with bromocriptine before perfusion (see text). Each red dot represents 3-4 immunoreactive cells. Since the distribution and density of immunostained cells was similar in other late-pregnant and lactating females, this figure illustrates the canonical pattern of pSTAT5-ir in the brain of both groups of females. The background colours in the sections encode a frequency score for the presence of pSTAT5 immunostained cells in the brain of virgin females: no pSTAT5-ir in any virgin female (white); pSTAT5-ir in 1 to 2 specimens (blue); pSTAT5 in 3 to 5 specimens (pink); pSTAT5-ir in 6 to 7 animals (orange). Approximate distance to Bregma is indicated for each section.

Diencephalon

Immunoreactivity for pSTAT5 was abundant in many structures of the diencephalon of late pregnant and lactating females, including hypothalamic, thalamic and pretectal nuclei. Apparent pSTAT5-ir was observed in the four major rostro-caudal divisions of the hypothalamus, as well as in the three medio-lateral compartments (periventricular, medial and lateral). Figure 2.4 illustrates the appearance of the pSTAT5-ir pattern in representative examples of the aforementioned sites.

The preoptic region (Fig 2.1, Fig 2.4a) displayed high levels of pSTAT5 expression in the structures surrounding the third ventricle, namely in the anteroventral periventricular (AVPe), ventromedial preoptic (VMPO) and median preoptic (MnPO) nuclei, as well as the periventricular nucleus (Pe) and the vascular organ of the *lamina terminalis* (VOLT, not shown). The medial region of the preoptic hypothalamus displayed widespread pSTAT-ir, too. Labelling was present in the medial preoptic area (MPA) and in every division of the medial preoptic nucleus (MPO, Figs 2.1a-c), as well as in dorsal preoptic structures such as the nucleus of the anterior commissure (AC, Figs 2.1a-c) or the anterodorsal preoptic nucleus (ADP). Finally, within the lateral compartment, the bed nucleus of the stria medullaris (SM, not shown), the lateral preoptic (LPO) and the ventrolateral preoptic (VLPO, not shown) areas displayed scattered pSTAT5 positive cells.

In the anterior hypothalamus (Figs 3.2e-f and 2.4b), the most outstanding pSTAT5-ir cell groups corresponded to the paraventricular (Pa) and supraoptic nuclei (SO, including its retrochiasmatic region, SOR), with a high density of immunostained neurons. Within the Pa complex, labelled cells were observed not only in its magnocellular division (medial, PaMM; lateral, PaLM), but also in the remaining subnuclei, PaAP, PaDC (not shown), PaV (Fig 2.2e) and PaPo, (Fig 2.2f). Other nuclei containing magnocellular neurosecretory neurons, such as the suprachasmatic nucleus, were remarkably devoid of pSTAT5 immunostaining. Additional sparser populations of pSTAT5-positive cells were present in the subparaventricular nucleus (SPa) and Pe within the juxtaventricular compartment, through the anterior hypothalamic area (AHA, AHC and AHP, Fig. 2.2e-f), lateroanterior (LA, Fig 2.2e) and circular (Cir, Fig 2.2e) nuclei of the medial compartment, and in the lateral hypothalamic area (LH).

In the tuberal hypothalamus (Fig 2.4c), the highest density of pSTAT5-ir cells was observed in the arcuate nucleus (Arc), presumably including the TIDA neurons (Yip et al.

2012). Remarkably, a few cells extended from this cluster into the median eminence (ME, Fig 2.2g). In the medial compartment, dense groups of labelled cells were observed in the dorsomedial (DM) and the ventromedial nuclei (VMH). Within the VMH, the highest levels of pSTAT5-ir corresponded to the ventrolateral subdivision (VMHvl), while the central (VMHc) and dorsomedial VMH subnuclei (VMHdm) showed only scattered and faintly stained cells (Fig 2.4c). Interestingly, the cluster of immunostained nuclei in the VMHvl is not confined to the boundaries of the VMH but extends further laterally into the ventromedial aspect of the lateral hypothalamic area (see Fig 2.4c). The rest of the LH displayed a moderate-to-low density and intensity of immunolabeling. Other centres showing pSTAT5-ir in the tuberal hypothalamus include the subincertal (SubI, Fig 2.2g), perifornical (PeF) and the medial tuberal (MTu) nuclei, as well as the magnocellular (MCLH) and parasubthalamic (PSTh, Fig 2.4e) divisions of the lateral hypothalamic area.

In the premammillary hypothalamus (Fig 2.4e), a dense population of pSTAT5-ir cells was observed in the ventral premammillary nucleus (PMV). In the dorsal premammillary nucleus (PMD), pSTAT5-ir was absent in lactating females and present only in half of the animals in the pregnant groups. Finally, the medial supramammillary nucleus (SuMM), the submammillothalamic nucleus (SMT) and the posterior hypothalamic area (PH, Figs 2.2h and 2.4e) displayed scattered immunostaining.

Immunoreactivity for pSTAT5 was observed in several of the nuclear groups of the thalamus. Labelling was present in the ventral thalamus, in some nuclei of the midline dorsal thalamus and habenular complex, in several visual and auditory thalamic nuclei and in the posterior-intralaminar thalamic complex.

In the prethalamus, labelling was present in the zona incerta (ZI, Figs 2.2f-h) and the reticular thalamic nucleus (Rt, Figs 2.2e and f). The Rt showed a variable staining frequency in our sample: labelling was only observed in approximately 50% of the late-pregnant females examined, in which staining was relatively weak and sparse as compared to other regions of the brain.

In the midline thalamus, a few cells were visible in the ventral reuniens nucleus (VRe, Fig 2.2g) and a larger population of pSTAT5 immunoreactive cells was seen in the paraventricular nucleus (PV, Fig 2.2g). Next to the PV, some pSTAT5-ir cells were found within the caudalmost levels of the habenular complex, which represents a notable

exception to the generally stable pattern of pSTAT5-ir. First, staining in the medial (MHb) and lateral (LHb) habenula was not present in lactating females (where the habenular complex is completely blank), but only in some pregnant females, either untreated or treated with bromocriptine. In these animals, labelling in the habenula was variable and heterogenous: the MHb appeared only occasionally stained, whereas the LHb was labelled in 50% of the animals (see Fig 2.4d for a case where both subdivisions are positively labelled and Fig 2.2g for a different case).

Several nuclei of the visual thalamus displayed pSTAT5 immunolabelling. Within the lateral geniculate complex, labelled cells were seen especially in the intergeniculate leaflet (IGL, Fig 2.2h), but also in the ventral geniculate nucleus (VLG), almost restricted to its parvocellular subdivision (VLGPC, Fig 2.2h). Furthermore, it is noteworthy that a small number of immunolabelled cells was found in an unnamed location near to the dorsal division of the medial geniculate nucleus (Fig 2.2i).

However, the largest populations of immunostained cells appeared in several nuclei of the posterior thalamus, including the lateral posterior (LP, Fig 2.2h), parafascicular (PF) and subparafascicular (SPF, with its parvocellular part, SPFPC) nuclei (Fig 2.2h), the retroethmoid nucleus (REth, not shown), the prerubral field (PR, Fig 2.2h), the posterior intralaminar complex (PIL) and the triangular part of the posterior thalamus (PoT). These last two nuclei (PIL and PoT) form a dense and remarkable population of immunolabelled cells in the posterior thalamus, in which a few cells seem to be displaced caudoventrally into an unnamed area just dorsal to the substantia nigra pars compacta (see Figs 2.2i and 2.4f).

The pretectum comprises the limit between the thalamus and the midbrain (Martinez-Ferre and Martinez 2012). There, structures immunolabelled for pSTAT5 included the posterior (PPT) and olivary (OPT) pretectal nuclei (both immunostained in half of the pregnant and lactating animals, see Fig. 2.2h), the precommissural nucleus (PrC, not shown), flanking the posterior commissure and the nucleus of the posterior commissure (PCom), including its magnocellular division (MCPC). Exceptionally, the subcommissural organ (SCO, Fig 2.2h), unlike the rest of the reviewed circumventricular organs, appeared devoid of pSTAT5 immunoreactivity.

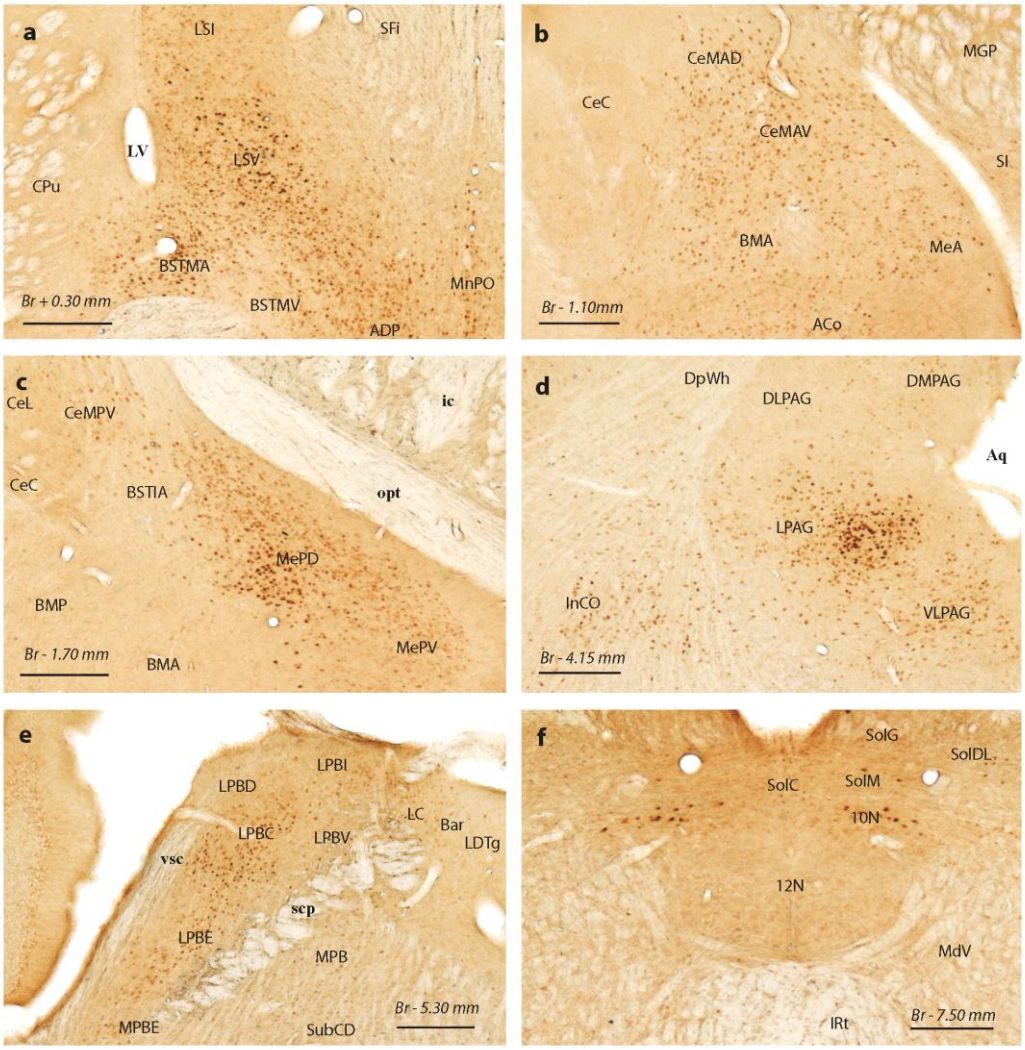


Figure 2.3 Representative examples of pSTAT5 immunoreactivity brain of late-pregnant and lactating female mice I

Photomicrographs showing the pSTAT5-ir pattern in representative telencephalic, midbrain and brainstem regions of pregnant and lactating mice. Sections include the lateral septum and anterior BST (a); the central anterior amygdala (b), the central and medial posterior amygdala (c); the periaqueductal grey (d); the parabrachial complex (e) and the nucleus of the solitary tract and surrounding structures (f). Sections b and f correspond to a postconception day 14 pregnant female (specimen M1435), sections a and d to a bromocriptine-treated, day 14 pregnant female (specimen M1392) and sections c and f to a postpartum day 6 lactating dam (specimen M13111). The approximate distance to Bregma is indicated in each section. Scale bars: 250 μm.

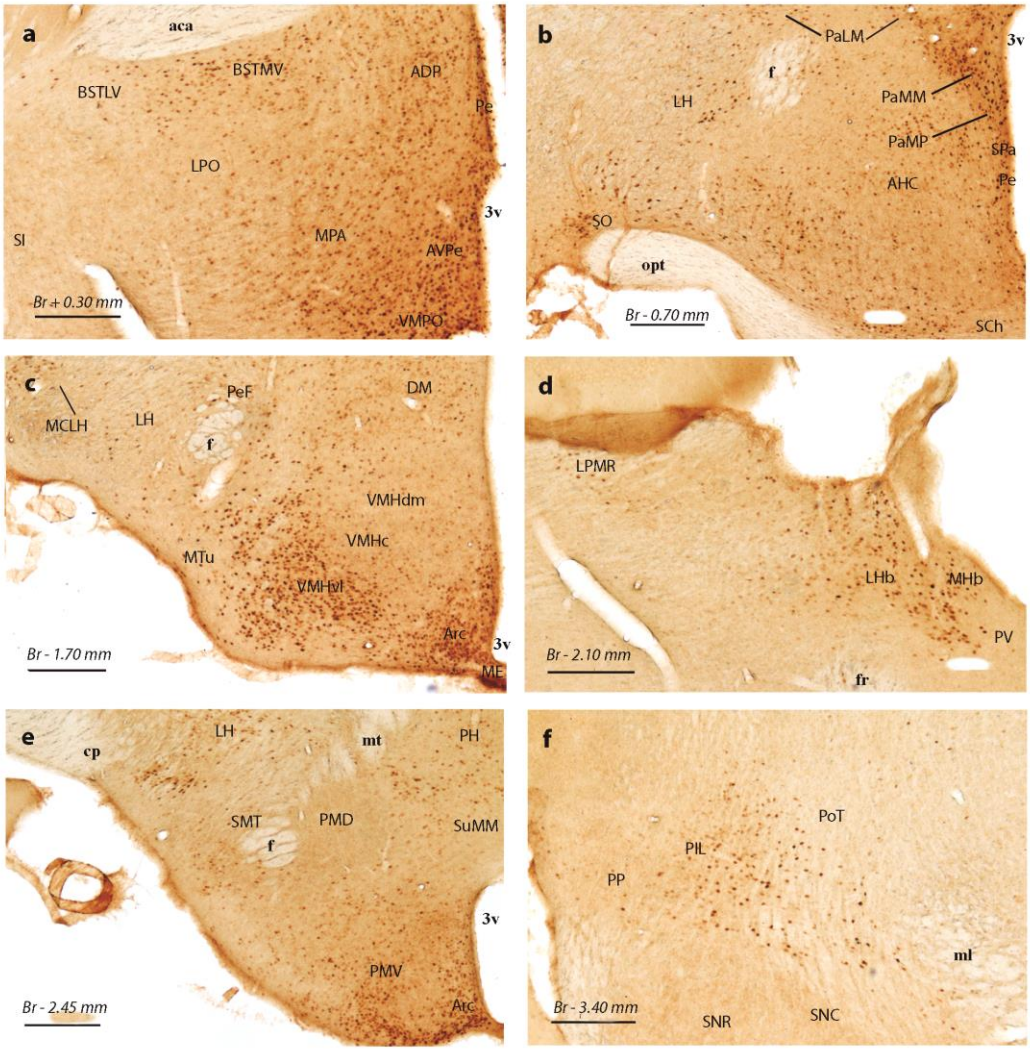


Figure 2.4 Representative examples of pSTAT5 immunoreactivity in the brain of late-pregnant and lactating female mice II

Photomicrographs showing the pSTAT5-ir pattern in representative diencephalic regions of pregnant and lactating mice. Sections include the preoptic hypothalamus (a), the paraventricular and supraoptic nuclei in the anterior hypothalamus (b), the arcuate and ventromedial nuclei in the tuberal hypothalamus (c), the habenular complex of the thalamus (d), the premammillary hypothalamic region (e) and the posterior thalamic region (f). Sections a and b correspond to a postconception day 14-pregnant female (specimen M1435), sections c and d to a bromocriptine-treated, day 15-pregnant female (specimen M1392) and sections e and f to a postpartum day 6 lactating dam (specimen M13111).The approximate distance to Bregma is indicated in each section. Scale bars: 250 μm.

Midbrain and brainstem

In the midbrain, lactotroph-derived signal transduction was observed mainly in the periaqueductal grey (PAG), but also in the adjoining lateral tegmentum and, further dorsally, in the superior colliculus (see Fig 2.3d). The PAG displayed a heterogeneous pSTAT5-ir. The most prominent labelling corresponded to the lateral PAG (LPAG). Secondly, the ventrolateral (LPAG) and dorsomedial (DMPAG) columns showed moderate immunoreactive cells. Finally, the dorsolateral PAG (DLPAG) displayed sparse immunoreactive cells. The Edinger-Westphal nucleus (EW, Fig 2.2i), embedded between the LPAG and the oculomotor nuclei, was also positively labelled for pSTAT5, as were the nucleus of Darkschewitsch (Dk) and the interstitial nucleus of Cajal (InC) (both located lateral to the anterior periaqueductal grey, PAG, see Fig 2.2i).

Dorsal to the PAG, some immunostained cells appeared in the deep (DpG and DpWh, the latter seen in Fig 2.3d) and intermediate (InG and InWh, Fig 2.2i) layers of the superior colliculus and in the intercollicular nucleus (InCO, Fig 2.4d). Within the tegmentum, pSTAT5-it was present in the deep mesencephalic area (DpMe, Fig 2.3d), the nucleus of the brachium of the inferior colliculus (BIC, Fig 2.2j) and the interpeduncular nucleus (IP, Fig 2.2i).

In the rhombencephalon, pSTAT5-ir was remarkably absent in the cortex and deep nuclei of the cerebellum. By contrast, the pons showed a complex pattern of pSTAT5-ir. In the pontine tegmental region, pSTAT5-immunoreactive cells appeared in the pedunculopontine tegmental nucleus (PPTg, Fig 2.2j), the dorsomedial tegmental nucleus (DMTg, Fig 2.2k), the laterodorsal tegmental nucleus (LDTg, Fig 2.3e) and in Barrington´s nucleus (Bar, Fig 2.3e), which displayed a small number of weakly stained cells. Scattered immunostained neurons appeared in the oral (PnO, Fig 2.2j) and caudal parts (PnC, Fig 2.2k) of the pontine reticular formation. Furthermore, the *locus coeruleus* (LC) and the parabrachial complex (PB) displayed relatively abundant immunostaining (Fig 2.3e). Within the parabrachial complex, the highest labelling density was seen in the central (LPBC) and external (LPBE) parts of the lateral parabrachial nucleus (LPB), with the rest of this nucleus (LPB) and the medial parabrachial nucleus (MPB) containing a lower density of immunoreactive cells.

The raphe nuclear complex displayed ample pSTAT5-ir. At rostral levels, the median (MnR), dorsal (DR) and the caudal linear (CLi) nuclei of the raphe were positively stained for pSTAT5 (Fig 2.2j). Caudally, pSTAT5-ir was still observed in the DR, in the pontine

nucleus (PnR, Fig 2.2k) and in the raphe magnus (RMg, Fig 2.2k). Finally, the raphe obscurus (ROb) in the caudalmost region of the hindbrain (Fig 2.2l) also displayed pSTAT5 immunostained cells.

As for the reticular nuclei, pSTAT5-ir appears in the the subcoerulear nucleus, ventral and dorsal divisions (SubCV and SubCD, respectively, the latter seen in Fig 2.3e). More caudally, sparse immunoreactive cells appear in the dorsal and ventral medullary (MdD, MdV, the latter seen in Fig 2.3f) and intermediate (IRt) reticular nuclei, in the ambiguus nucleus (Amb) and, laterally, along the spinal trigeminal nucleus (SP5), in close contact with the spinal trigeminal tract (see Fig 2.2l). Finally, like other circumventricular sites, the area postrema (AP) and the adjoining nuclei, i.e. nucleus of the solitary tract (Sol) and dorsal motor nucleus of the vagus (10N), showed prominent pSTAT5-ir, as seen in Figure 2.3f.

B Immunoreactivity for pSTAT5 in the brain of freely-cycling virgin female mice

As a rule, virgin females showed less labelling than pregnant and lactating ones. In fact, the patterns of pSTAT5-ir observed in virgin females were always a fraction of the one described for pregnant/lactating females, so that no brain centre was labelled for pSTAT5 in virgins but not in pregnant/lactating females. However, virgin females showed a substantial inter-individual variability in the presence of pSTAT5 immunolabelled cells in several brain regions. This variability contrasts with the homogenous patterns previously described for pregnant/lactating females, and even for ovariectomized, steroid-primed virgins (Study 1). We have illustrated this variability by establishing a frequency score for each brain site, according to the proportion of virgin females of our sample (n=7) showing pSTAT5 immunoreactive cells in that site. This score is color-coded in Figure 2.2 and comprises four categories: no labelling in any virgin female (clear background), low frequency of pSTAT5-ir (1-2 animals, blue background); c) intermediate frequency of pSTAT5-ir (3-5 animals, pink background); d) high frequency of pSTAT5-ir (6-7 animals; orange). We also provide an additional example of the qualitative differences in pSTAT5-ir that our female sample displayed in the BST-MPA region (Fig. 2.1).

According to our results, only two nuclei in the whole brain showed labelling consistently in every virgin female of our sample: the Arc (Figs 2.4c and c') and the

MePD (Figs 2.4e and e'). Three more brain centres displayed pSTAT5-ir in most animals, namely the LSV (Figs 2.4d and d'), the ventrolateral VMH (Fig 2.4c and c') and the rostral and ventrolateral PAG (Figs 2.4f and f'). By contrast, the majority of the analysed nuclei showed labelling in 3-5 of the 7 animals. Finally, some brain regions labelled in pregnant/lactating groups were totally devoid of pSTAT5-ir in the whole sample of virgin females. These include portions of the allocortex (DTT; EnD), and specific nuclei of the amygdala (AAD-AAV; LOT; BLV), basal telencephalon (BAC), thalamus or pretectum (Rt; LHb; PPT; LP; PF) and dorsal and pontine tegmental nuclei (Bar; LDTg; DMTg and RMg). This pattern matches in general terms the distribution of pSAT5-ir in ovariectomized, steroid-primed virgin females (Study 1). Even though the variability of the current sample is higher, those brain nuclei more intensely and more consistently labelled (the Arc; MePD; LSV or the LPAG) coincide with those nuclei that showed more abundant labelling among ovariectomized, steroid-primed virgins.

2.3.2. QUANTITATIVE ANALYSIS OF pSTAT5 IMMUNOREACTIVITY IN SELETED BRAIN NUCLEI OF VIRGN, PREGNANT AND LACTATING MICE

We assessed the density of pSTAT5 immunoreactive cell nuclei in 13 brain regions chosen according to their relevance in the context of maternal behaviour regulation. These regions comprise several nodes of the sociosexual brain: the LSV, the MePD, BSTMPM, AC/ADP, MPO, VMHvl and adjoining tuberal region (hypothalamic aggression locus) and LPAG (Newman 1999). We also included the magnocellular neurosecretory nuclei (Pa and SO) and other sites that are also involved in the regulation of maternal behaviours (CeM, AVPe and PIL; Bosch and Neumann 2010; Scott et al. 2015) or related aspects of motherhood (Cservenák et al. 2013), despite not being part of the sociosexual brain network. Finally, we also analysed the Arc, given its role in the feedback control of hypophyseal PRL release (Ben-Jonathan and Hnasko 2001a; Sapsford et al. 2012). The exact frames applied for each of these nuclei are depicted in Figure 2.5.

To explore the variation of pSTAT5-ir density between virgin, pregnant and lactating groups, we performed a separate one-way ANOVA on each of the sampled nuclei. The results of this analysis are summarized in Figure 2.6 and reveal statistically significant inter-group differences in pSTAT5-ir density in most of these brain regions. The main effects revealed by ANOVA were further explored using post hoc Bonferroni multiple comparisons. The results of this post hoc analysis indicate that virgins generally showed significantly less pSTAT5-ir density than one or more of the remaining groups.

The ANOVA of the density of pSTAT5-ir revealed significant differences between groups within the nuclei of the extended amygdala, namely the CeM ($F_{2,15}$=22.071, p<0.001), MePD ($F_{2,15}$=8.016, p=0.004), and BSTMPM ($F_{2,15}$=6.243, p=0.012) and in the septal region (LSV; $F_{2,15}$=5.219, p=0.019) (Fig 2.6a). Post-hoc comparisons indicate that, in the CeM, pSTAT5-ir density was significantly higher in pregnant than in virgin females (p<0.001) or lactating dams (p=0.001). On the other hand, the density of immunoreactive cells in lactating dams was similar to that of virgins (p=0.174). Concerning the MePD, labelling density was significantly higher in pregnant females than in virgin females (p=0.005) and in lactating dams compared to virgin females (p=0.037), whereas both pregnant females and lactating dams displayed very similar labelling density (p=0.949). Regarding the BSTMPM, labelling density in pregnant females was significantly higher than in virgins (p=0.012), but similar to that of lactating dams (p=0.836). On the other hand, although the average density of pSTAT5 immunoreactive cells was higher in dams than in virgin females, this difference did not reach significance (p=0.091). Finally, the LSV showed a significant increase in pSTAT5-ir density in the brain of pregnant females as compared to that of virgin females (p=0.027), but pregnant and lactating females showed identical levels (p=1), whereas the apparent differences between virgin and lactating groups were not significant (p=0.093).

In the preoptic hypothalamus (Fig 2.6b), significant differences in labelling density were observed in the AC/ADP ($F_{2,15}$=5.141, p=0.020) and MPO ($F_{2,15}$=8.345, p=0.004), but not in the AVPe ($F_{2,15}$=0.522; p=0.605). In the AC/ADP, post-hoc comparisons indicate that virgin females had significantly lower pSTAT5-ir density than lactating dams (p=0.035), as well as a trend to lower pSTAT5-ir than pregnant females (p=0.067). By contrast, pregnant and lactating dams showed identical density of immunolabelled cells (p=1). For the MPO, one of the key nodes of the maternal brain, lactating dams showed significantly higher pSTAT5-ir density than virgin females (p=0.003), whereas the rest of the comparisons did not reach significance (p≈0.25 for both comparisons).

In the anterior hypothalamus (Fig 2.6b), the paraventricular ($F_{2,15}$=10.099, p=0.002) and supraoptic nuclei ($F_{2,15}$=52.827, p<0.001) showed significant inter-group differences. In the Pa, both pregnant and lactating groups showed similar densities of pSTAT5-ir (p=0.715), which are significantly higher than in virgin females (p=0.002 and p=0.02, respectively).

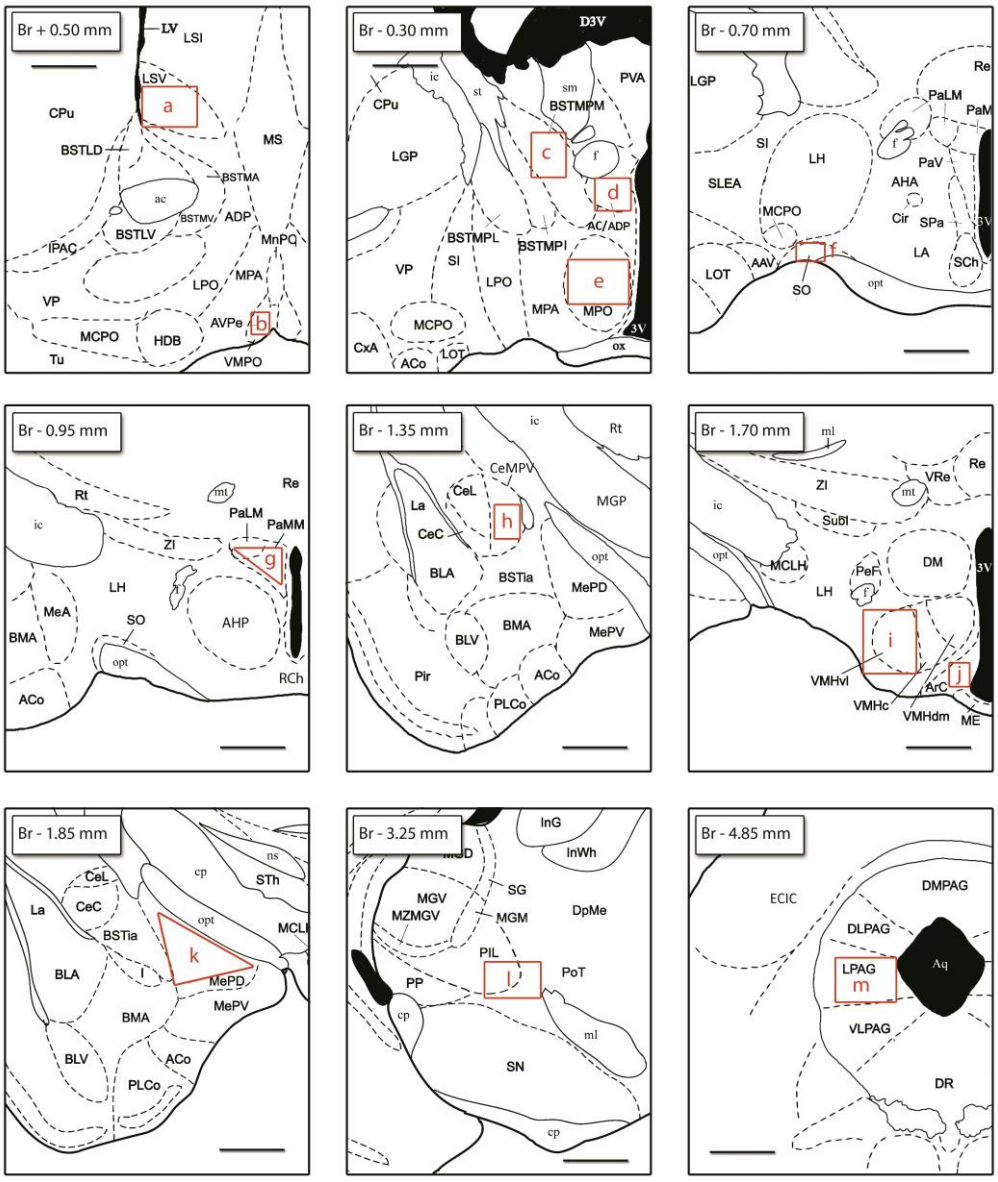


Figure 2.5 Anatomical location of pSTAT5-ir counting frames

Semi-schematic camera lucida drawings of selected coronal sections featuring the counting frames (red) chosen for the quantitative assessment of pSTAT5-ir: a) LSV; b) AVPe; c) BSTMPM; d) AC/ADP; e) MPO; f) SO; g) Pa; h) CeM; i) VMHvl and ventrolateral adjoining area; j) Arc; k) MePD; l) PIL and adjoining posterior thalamic region; m) VLPAG. Approximate distance to Bregma enclosed in each section. Scale bars: 500 μm.

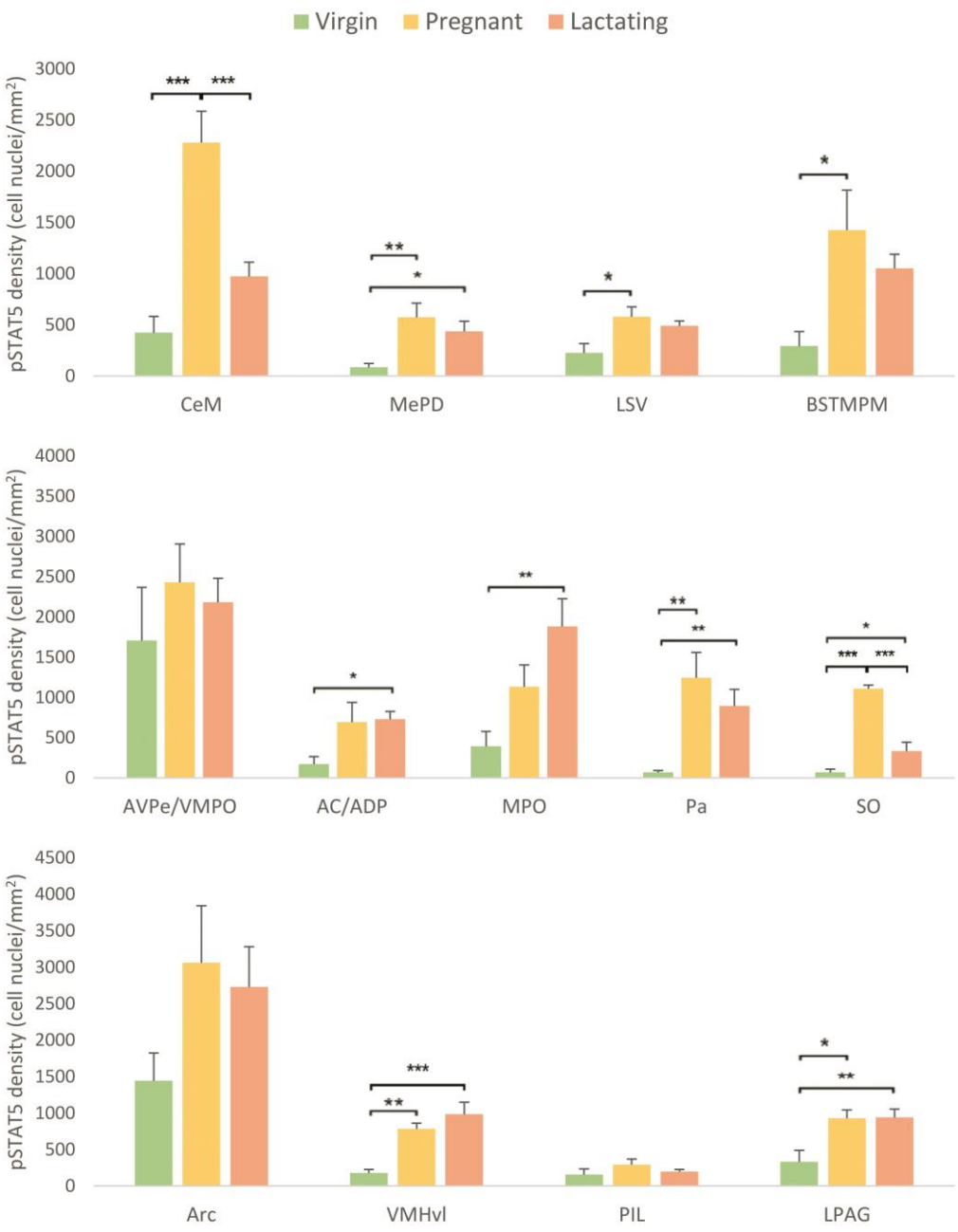


Figure 2.6 Quantification of pSTAT5-ir density in selected brain regions of virgin, late-pregnant and lactating female mice

Assessment of pSTAT5-ir density (pSTAT5-positive cell nuclei / mm²) in 13 brain regions of relevance in the context of maternal behaviours (Newman 1999; Gammie 2005). Bar histograms show mean interhemisferic pSTAT5-ir density ± SEM in virgin female mice (n=7; green); PD 14-18 late-pregnant mice (n= 5; yellow) and PPD6 lactating dams (n=6; orange). Statistical analysis (one way ANOVA with Bonferroni post-hoc comparisons) was applied independently to each brain region. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

In the SO, pregnant females displayed significantly higher pSTAT5-ir density than virgin females and lactating dams ($p<0.001$), whereas lactating dams also displayed significantly higher levels than virgin females ($p=0.049$).

In the tuberal hypothalamus (Fig 2.6c), the VMHvl and laterally adjoining area showed significant inter-group differences in pSTAT5-ir density ($F_{2,15}=16.673$, $p<0.001$). Thus, virgin females displayed significantly lower labelling density than pregnant females ($p=0.004$) and lactating dams ($p<0.001$), whereas pregnant females and lactating dams showed similar labelling density ($p=0.687$). Conversely, the ANOVA revealed no significant differences in labelling density in the sampled area of the arcuate nucleus ($F_{2,15=}2.502$; $p=0.115$).

Similarly, no group effect was observed in the PIL within the posterior thalamus ($F_{3,22}=1.194$, $p=0.335$; see Fig 2.6c). Finally, in the periaqueductal grey (LPAG) the ANOVA revealed a significant group effect ($F_{2,15}=7.475$, $p=0.006$). The post hoc analysis indicates that pregnant and lactating groups showed significantly higher labelling density than virgins ($p=0.029$ and $p=0.012$, respectively) and identical between each other ($p=1$).

Summarizing, this quantitative analysis reveals that in most of the analysed brain regions pSTAT5-ir density (as a measure of lactogenic activity) increased significantly during pregnancy and/or lactation. Furthermore, levels of pSTAT5-ir in the analysed structures do not differ between late-pregnancy and lactation, with the exception of the CeM and the SO, where labelling density significantly decreases from late-pregnancy to lactation.

2.3.3. INHIBITION OF HYPOPHYSEAL PROLACTIN RELEASE DURING LATE PREGNANCY: EFFECT ON BRAIN pSTAT5 IMMUNOREACTIVITY

In order to evaluate any possible effect of impairment of hypophyseal PRL release on the levels of central lactogenic signalling, we compared pSTAT5-ir density between our sample of late-pregnant females and an additional group of late-pregnant females, treated with bromocriptine to suppress hypophyseal PRL release. After checking for normal distribution and variance homogeneity, we performed a t-test for independent samples for each of the analysed nuclei. T-tests revealed no significant differences between treated and non-treated pregnant females in any case (Fig 2.7). P-values were always equal or higher than 0.2, with the single exception of the CeM, where a trend towards significance was found ($p=0.064$). Therefore, our results confirm no significant effect of bromocriptine treatment in pSTAT5-ir density in late-pregnant females.

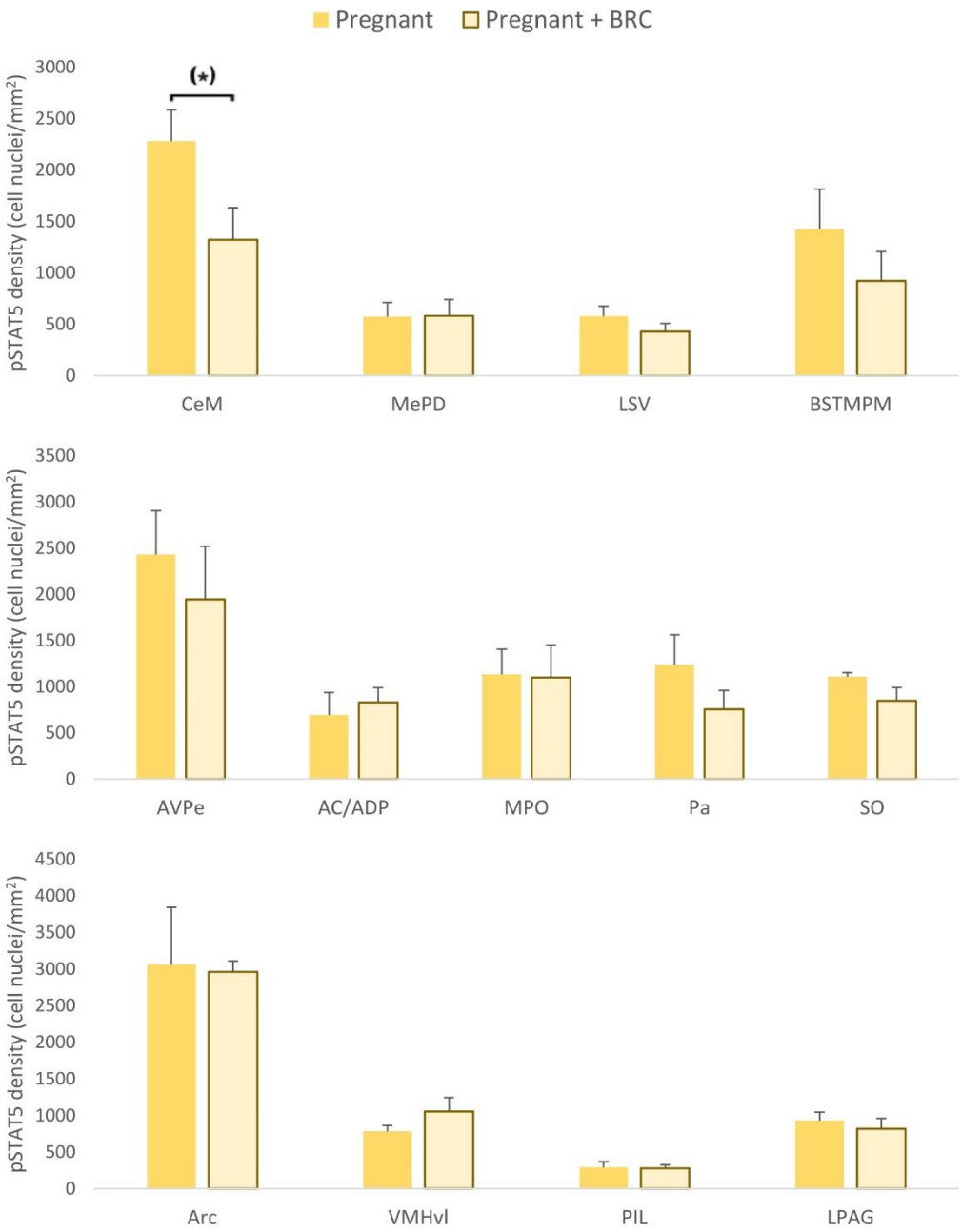


Figure 2.7 Quantification of pSTAT5-ir density in selected brain regions of late-pregnant female mice: effect of bromocriptine

Comparison of pSTAT5-ir density (pSTAT5-positive cell nuclei / mm²) in the former 13 brain regions between PD 14-17 pregnant female mice (n=5; dark yellow) and PD14-17 pregnant female mice treated with two 100 μg s.c. doses of bromocriptine, 19 and 2 hours prior to perfusion, in order to depress hypophyseal PRL release (n=8; light yellow). Bar histograms show mean interhemisferic pSTAT5-ir density ± SEM. Statistical analysis (one way ANOVA with Bonferroni post-hoc comparisons) was applied independently to each brain region. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

2.4. DISCUSSION

In the present study, we have characterised the variation of lactogenic signalling in the brain throughout the reproductive cycle of the female mouse, namely the basal state (virgins), late-pregnancy and lactation. This work is, to our knowledge, the first functional analysis of the central lactogenic activity in in mice including the critical period of pregnancy. In qualitative terms, we have found a common and widespread pattern of pSTAT5-ir (as a measure of lactogenic signalling) in pregnant and lactating females that starkly contrasts with the variable but relatively reduced patterns found in virgin females. In quantitative terms, we have found that lactogenic signalling strongly increases during pregnancy and/or lactation as compared to the basal condition (virgin females). This apparent increase, in addition, is not produced by PRL of hypophyseal origin, but rather by other available lactogenic sources, as suggested by the evidence that bromocriptine treatment has no significant effect in reducing the levels of pSTAT5-ir of late-pregnant females.

In this section, we will focus on discussing our findings relative to pregnancy and lactation. We will first briefly review our data on freely-cycling virgin female mice and compare them with our previous findings on virgins (Study 1). Then, we will review how lactogenic signalling changes with pregnancy and lactation in the analysed regions and hint some possible functional implications of these findings. Finally, we will discuss which lactogenic agents may be responsible for those changes.

2.4.1. PROLACTIN SIGNALLING IN THE BRAIN OF FREELY-CYCLING VIRGIN FEMALE MICE

The distribution of pSTAT5-ir in the brain of the female mouse has been thoroughly examined in the previous chapter of this work, using ovariectomized, steroid-primed virgin females supplemented with exogenous PRL as experimental model. In this study, however, we employed a more naturalistic approach and included a group of freely-cycling virgin females (lacking any kind of PRL supplementation) as a comparative control of the basal PRL responsiveness in females. Consequently, we obtained a sample of pSTAT5-ir patterns with a relatively high variability. Most of the patterns in this sample were relatively reduced, but a small number of animals exceptionally displayed high levels of pSTAT5-ir resulting in rich patterns.

As pointed by our previous findings that gonadal steroid regulate levels of PRL signalling in several brain regions (Study1) and those of others (Sugiyama et al. 1994; Scully et al. 1997), the most feasible source to explain the variable levels of pSTAT5-ir in our sample are gonadal steroids. Circulating PRL has been shown to be under the influence of other physiological factors such as age (Parkening et al. 1982), circadian rhythms (Sinha et al. 1977) or stress (Gala 1990). This source of variability was minimized by using females of the same age (2 to 3 months), which were perfused during the same time window (11:00 to 13:00 in a 12:12 light-dark cycle, lights ON at 8:00; see Materials and Methods) and group-housed to avoid stress derived from social isolation.

Despite the aforementioned variability, freely-cycling virgins display similar patterns to our previous description (Study 1) of pSTAT5-ir in the brain of ovariectomized, steroid-primed females. For example, those nuclei with higher labelling prevalence and density among freely-cycling virgins (e.g. the LSV or MePD, Fig 2.2) are those presenting higher labelling density among ovariectomized, steroid-primed virgins (see Study 1, Figs 1.1 and 1.5). Similarly, some brain regions lacking pSTAT5-ir in ovariectomized, steroid-primed virgins might present labelling among freely-cycling virgins (e.g. the LSD in the septum, the lateral BST or several midbrain and brainstem regions), but this labelling tends to be scarce and present only exceptionally in few individuals. In sum, our current and previous data on central PRL signalling in virgin female mice suggests that PRL can exert a broad degree of activity within the brain. Depending on gonadal steroid regulation and other intrinsic and extrinsic factors, PRL signalling in the female brain can range from a virtual absence to moderately high levels.

2.4.2. SIGNIFICANCE OF LACTOGENIC SIGNALLING IN THE BRAIN DURING PREGNANCY AND LACTATION

Pregnant and lactating mice displayed a rich, widespread and constant pattern of lactogenic responsiveness with minor variations throughout the whole brain. Levels of lactogenic signalling were highest among hypothalamic and telencephalic nuclei, but also apparent in thalamic, midbrain and brainstem regions.

Remarkably, as was brought into attention before (see Section 1.4.1), one of the limitations of the use of pSTAT5 as a functional marker of central PRL-derived signalling is its possible crosstalk with signalling of leptin and growth hormone (Furigo et al. 2016), which also involves the Jak/STAT pathway. In this context, it is important to discuss on

the specificity of the patterns of lactogenic responsiveness obtained in pregnant and lactating females, although the high levels of PLs likely account for most (if not all) of the pSTAT5 immunolabelling observed. Regarding leptin, STAT3 is the main STAT member associated with the leptin receptor (Ladyman et al. 2012), whereas STAT5 phosphorylation associated with leptin signalling has been reported only in the arcuate nucleus (Gong et al. 2007; Mütze et al. 2007) and has not been replicated in all studies (Vaisse et al. 1996). As for GH, regions of the brain showing high levels of expression of GH receptors and responsiveness to GH (detected by pSTAT5-ir; Furigo et al., 2016), such as the hippocampus and dentate gyrus (Burton et al. 1992) or layers 2, 3, 5 and especially layer 6 of the cerebral cortex (Lobie et al. 1993), show no pSTAT5 labelling in any of our mice, neither in virgin nor in pregnant or lactating females. This suggests that GH signalling in the brain is taking place through alternative pathways, e.g. those involving Src kinases (Waters 2015). Moreover, Brown and collaborators (2011) demonstrated, by means of the pharmacological suppression of hypophyseal PRL release with bromocriptine, that pSTAT5-ir in lactating dams is completely attributable to PRL signalling. In sum, the reliability of pSTAT5 immunostaining as a functional marker for the activation of the PRLR-l is extensive to the brain of pregnant and lactating females.

Despite the variability in pSTAT5-ir found in our sample of virgin females, our results confirm, both at a qualitative (Fig 2.2) and quantitative (Fig 2.6) level, that pregnancy and lactation are associated with a significant increase in the lactogenic signalling in the brain. According to our results, 10 out of 13 total brain nuclei experimented a significant increase in pSTAT5-ir density during pregnancy and/or lactation as compared to basal levels among virgins.

Exceptions to this outcome among the structures we have analysed are the AVPe (Fig 2.6b), the Arc and the thalamic PIL (Fig 2.6c), with comparable pSTAT5-ir density between virgins and pregnant/lactating females. These findings do not fit previous data (Brown et al. 2011) reporting an increase in pSTAT5-ir during lactation and after exogenous PRL administration in the AVPe and Arc of C57 animals (PIL was not analysed in the cited work). Remarkably, Brown et al. (2011) found no associated increase in PRLR expression in these nuclei in lactating dams, but were able to induce a comparable increase in pSTAT5-ir in virgin females by administering exogenous PRL. Therefore, heightened levels of circulating PRL occurring during the lactation period were likely

causing the increase in pSTAT5-ir of C57 dams found in this work. These data suggest that the lack of inter-group differences in our animals would be due to virgins of the CD1 strain having relatively high (as compared to C57) basal levels of circulating PRL. Supporting this view, the AVPe and the Arc are located next to circumventricular organs (the VOLT and the ME, respectively), thus having privileged access to systemic PRL (Ganong 2000).

As for the region we refer to as PIL, the cell cluster we have quantified corresponds to a discrete population that does not exactly match the PIL proper, but rather extends from the medial border of this nucleus into the adjoining region of the posterior thalamus (Fig 2.2i). This work is the first description of such population, clearly defined by pSTAT5 immunostaining (Fig 2.3l). This region is known to be a relay station for ascending somatosensory information of the ventral region of the body, which allows suckling stimulation to trigger the release of PRL during lactation (Cservenák et al. 2013). Therefore, it is tempting to suggest a role of pSTAT5-ir in this region in feedback regulation of suckling-induced PRL release through a central modulation of sensitivity to suckling stimulation. In this context, however, our results revealing comparable levels of pSTAT5-ir in virgin and late-pregnant females and in lactating dams are surprising. The functional meaning of this finding is unclear, but maybe could be related to the peculiar maternal behaviour of mice (as compared to rats), where virgin females display nearly spontaneous maternal care (Stolzenberg and Rissman 2011; Martín-Sánchez et al. 2015b).

Focusing on the rest of the analysed nuclei (10), these can be grouped in different categories according to the time course of the observed increase in pSTAT5-ir. Importantly, 8 of them displayed a statistically significant increase in pSTAT5-ir density already during pregnancy (CeM; MePD; LSV; BSTMPM; Pa; SO; VMHvl and LPAG). This suggests that the maternal adaptations of the brain mediated by lactogenic agents occur before parturition and, in most cases, are maintained subsequently during lactation. Supporting this view, it has been shown that mRNA expression of the long form of the PRLR (which signals through the JaK/STAT5 pathway) is markedly increased in the brain of female rats at mid- and late gestation, and this elevated mRNA levels are maintained during the lactation period (Sugiyama et al. 1994). Remarkably, in two of these nuclei, the CeM and SO (Fig 2.6), pSTAT5-ir density peaked during pregnancy and decreased after parturition, so that, in the case of the CeM, levels of pSTAT5-ir were comparable in

dams and virgin females. This evidence further stresses the importance of gestation in the development of the maternal brain, since in the CeM and the SO pregnancy rather than postpartum seems the critical period of lactogenic action, when maternal adaptations would be taking place.

Finally, the two remaining nuclei, the AC/ADP and the MPO, underwent a moderate increase during pregnancy, but this rise only reached statistical significance during lactation (Fig 2.6b). Hence, the critical window of lactogenic input in the AC/ADP and MPO appears to be the period of lactation. This suggests a specific role of these nuclei in the maintenance of maternal behaviours during lactation (see Tsuneoka et al. 2013; Bridges 2015).

Indeed, the most feasible agents to relate to this process are gonadal steroids, estradiol and progesterone. On the one hand, it is well-known that these hormones play a critical role in the physiology of pregnancy and parturition (Fig I.4). On the other hand, our previous work (Study 1) shows how pSTAT5-ir is under regulatory control of gonadal steroids. Still, some of the nuclei analysed in the present study display a significant increase in pSTAT5-ir density associated with pregnancy and/or lactation, which is not paralleled by virgin, ovariectomized and steroid-primed females (Study 1). These nuclei include the CeM, BSTMPM, MPO, Pa, SO and VMHvl, which show enhanced lactogenic signalling specific to pregnancy and lactation, but not by estradiol or estradiol plus progesterone treatment. This suggests that in these nuclei, the increase in pSTAT5-ir during pregnancy is not related solely to the high levels of estradiol or progesterone during mid-to-late pregnancy, but rather to a more complex and pregnancy-specific background of gonadal steroid levels, with increasing estradiol and decreasing progesterone (Fig I.4). Alternatively, other factors specific to pregnancy and lactation, e.g. high levels of circulating placental lactogens (gestation) or PRL (lactation), could be contributing to shape this scenario.

Altogether, the findings of this study reinforce the major role of lactogenic hormones in adapting the female physiology and behaviour to the demands of motherhood (Grattan et al. 2001). But, most importantly, our results lend strong support to the hypothesis that the critical period for the development of these maternal adaptations is pregnancy, as the measured increases in lactogenic signalling occur prepartum in most of the analysed structures. These adaptations include, for instance, the lactogenic modulation of the release of oxytocin and vasopressin by the magnocellular neurosecretory cells in

the Pa and SO (Parker et al. 1991; Ghosh and Sladek 1995; Sirzen-Zelenskaya et al. 2011), the attenuation of stress response at the level of the Pa (Torner et al. 2002), the promotion of hyperphagia (Sauvé and Woodside 2000; Augustine et al. 2008) or the induction of neurogenesis in olfactive areas (Shingo et al. 2003; Larsen and Grattan 2010b)to mediate the recognition of the future offspring (albeit not mediated by the Jak/STAT pathway in this case, Torner 2016). But importantly, one of the major outcomes of this process is the prepartum development of maternal behaviours (Slotnick et al. 1973; Lonstein and Gammie 2002; Gammie 2005; Brunton and Russell 2008). The implication of PRL and other lactogenic agents in the regulation of maternal behaviours will be treated thoroughly in the following chapters of this work (see General discussion).

2.4.3. SOURCE OF BRAIN LACTOGENIC ACTIVITY DURING LATE PREGNANCY: EFFECT OF HYPOPHYSEAL PROLACTIN INHIBITION

An additional issue in the context of the lactogenic development of the maternal brain is the actual origin and identity of the intervening lactogenic agents. As introduced before, the pituitary gland is not the only source of PRL in the body (Freeman et al. 2000). Likewise, PRL is not the only available lactogenic molecule, as it coexists with an array of related peptides with variable degrees of structural homology and common binding and signalling features (Soares et al. 1998b; Soares 2004). It is well-established that hypophyseal PRL plays an instrumental role during lactation, as, in response to the suckling stimulus of the pups, high amounts of this hormone are released from the pituitary and lead to milk production (lactogenesis) and ejection (galactopoiesis) (Bintarningsih et al. 1958; Freeman et al. 2000). This process is granted by a disruption of the negative feedback control of PRL on its own secretion, which leads to a general state of hyperprolactinaemia (Anderson et al. 2006) and an increased access and signalling of hypophyseal PRL to the brain during lactation (Brown et al. 2011). Conversely, during pregnancy the release of hypophyseal PRL appears to be depressed, coinciding with high levels of lactogenic signals of placental origin, the so-called “placental lactogens” (Robertson and Friesen 1981; Kishi et al. 1991; PLs, Soares 2004).

In his study, we aimed at providing direct evidence of the actual role of hypophyseal PRL during the period of pregnancy. For this purpose, we inhibited hypophyseal PRL release in a sample of late-pregnant females by means of bromocriptine. First, bromocriptine administration did not result in any interruption or alteration of pregnancy in the

experimental females, every pregnancy was brought correctly to term. Most importantly, bromocriptine treatment resulted in no significant decrease in pSTAT5-ir density in the analysed structures, as compared to non-treated pregnant females (Fig. 3.7). Hence, these findings provide for the first time a functional confirmation of the suppression of lactogenic input to the brain from a hypophyseal source. Administration of this treatment ensured that most, if not virtually all the pSTAT5-ir found in the brain during late pregnancy is due to non-hypophyseal sources. Arguably, the alternative lactogenic agents operating during pregnancy are most likely PLs. Placental lactogens are known to access the brain (Bridges and Lupini 1991), to bind the long form of the PRLR (Bridges et al. 1996) and even to stimulate maternal behaviour when infused intracerebrally (Bridges and Freemark 1995). However, it is also possible that central neurons producing PRL might also be recruited during pregnancy (Paut-Pagano et al. 1993; Grattan and Kokay 2008). In fact, there is evidence in the rat suggesting that this is the case for the regulation of the stress response and in response to suckling in the Pa and MPA (Torner et al. 2004). Altogether, our findings strongly support the idea that the tuning of the socio-sexual brain for motherhood is initiated before parturition mostly by non-hypophyseal lactogenic sources, likely placental lactogens, and maintained subsequently during lactation by high levels of hypophyseal PRL, as suggested by the inhibitory effect of bromocriptine in the presence of neuronal pSTAT5 in lactating mice (Brown et al. 2011).

2.4.4. CONCLUSIONS

This work explores the physiological variation of PRL-like signalling in the brain of female mice through different reproductive stages. Virgin female mice show variable but generally low pSTAT5-ir. This immunoreactivity shows a dramatic increase with pregnancy, resulting in a widespread pattern that remains mostly unaltered during lactation. Inhibition of hypophyseal PRL release in pregnant females by means of bromocriptine treatment has no effect on the levels of PRL-like signalling, thus suggesting that placental lactogens (or centrally produced PRL) are responsible for the greater part of the observed lactogenic signalling. Our work provides evidence that the maternal brain is being extensively shaped by lactogenic agents during pregnancy, prior to delivery and lactation, including regions involved in the management of maternal behaviours.

3

Maternal Behaviour, Prolactin and Oxytocin – Analysis of Specific Maternal Behaviours and pSTAT5/Oxytocin Expression in the AC/ADP

3.1. RATIONALE AND AIMS

As introduced before, the expression of mammalian maternal behaviour depends mainly on the regulatory action of two different types of factors (Fig. I.9). On the one hand, hormones of pregnancy and lactation, including gonadal steroids and, importantly, PRL, provide a large-scale modulation of the brain into a maternal state, preceding the birth of pups and during their early postnatal development. On the other hand, pup-derived stimuli access the maternal brain as a direct consequence of pup exposure to trigger and modulate the expression of maternal behaviour. The relative contribution of each kind of factor differs among species and is also different for each component of maternal behaviour (see section I.5.2).

Concerning pup-directed maternal care, studies conducted in the rat reveal that pup-derived stimuli elicit radically different reactions in virgin females and dams. As introduced before, virgins show avoidance or aversion towards pups (Fleming and Luebke 1981), whereas in dams, pup stimuli have powerful reinforcing properties, leading to the expression of proactive maternal responses (Hauser and Gandelman 1985; Fleming et al. 1989; Lee et al. 2000; Mattson et al. 2001). Therefore, rat dams, but not virgins, are fully motivated for pups, as a result of the endocrine input of pregnancy. Conversely, virgin females might only show partial motivation towards pups after prolonged maternal sensitisation (Seip and Morrell 2008). The question remains whether this scenario is translatable to the mouse, considering the evident differences with the rat in the onset of pup-directed maternal behaviour. In other words, it is still not known if full maternal motivation in female mice can be reached only through pup-derived stimulation, as suggested by the nearly-spontaneous allomaternal care observed in mice (Martín-Sánchez et al. 2015b), or if, as for maternal aggression, a previous endocrine priming is necessary for its expression.

The first aim of the present study is to assess the relative contribution of pregnancy and pup-derived stimuli to the development of maternal motivation and the expression of motivated (proactive) maternal responses in female mice. For this purpose, we have developed a new behavioural test (based on the pup retrieval paradigm), termed "*motivated pup retrieval test*" (see section 3.2.1), which allows to assess maternal motivation in a more specific manner than conventional pup retrieval tests. In order to determine whether pup-derived stimulation is enough to induce fully motivated maternal care or, in contrast, endocrine agents are increasing motivation for pups, we

have compared the performance in the *motivated pup retrieval test* of three different experimental groups of female mice: lactating dams, godmothers and pup-naïve virgins. Godmothers are virgin females subject to a process of maternal sensitisation by cohabitating with a (non-experimental) maternal female, which grants continuous access and interaction with pups from the moment of their birth (Martín-Sánchez et al. 2015b; Martín-Sánchez et al. 2015a). Lactating dams are exposed to both endocrine signals of pregnancy and lactation and pup-derived stimuli, whereas godmothers are exposed only to continuous infant-derived sensory stimulation. Conversely, virgin females gain access to pups exclusively during behavioural testing and hence will serve as negative controls.

The nonapeptide oxytocin (OXT) plays an instrumental role as a neuromodulator of maternal behaviours, both maternal care (including proactive maternal responses, see Fig I.9) and maternal aggression (Bosch and Neumann 2012). The second aim of this study will be to analyse how both long-term pup exposure and PRL (as a major hormone of pregnancy and lactation) might modulate maternal behaviours by acting upon central OXTergic circuits. Specifically, we will focus our attention in a single population of OXT neurons, located in the anterior commissural/anterodorsal preoptic region of the hypothalamus (AC/ADP) (Otero-García et al. 2015). This OXT neuron cluster occupies a privileged location in the medial preoptic (MPA) region of the hypothalamus and MPA-ventral BST continuum, the well-established key region for the expression of maternal behaviour (Numan et al. 1988; Morgan et al. 1999; Pereira and Morrell 2009; Tsuneoka et al. 2013). Hence, we will analyse the effect of PRL onto OXT-expressing neurons in our three experimental groups and we will also explore possible correlations between PRL/OXT expression and coexpression in the AC/ADP and motivated pup retrieval scores. Thus, we will search for possible indications on the involvement of PRL and OXT in the AC/ADP region in the regulation of maternal motivation.

The contribution of pregnancy and sensory stimuli to the expression of maternal aggression has been already advanced in the work of the former member of our lab, Ana Martín-Sánchez (Martín-Sánchez et al. 2015b; Martín-Sánchez et al. 2015a). According to her findings, virgin female mice become easily sensitised through pup contact to express allomaternal care. Conversely, these females do not become aggressive towards other conspecifics even if they become maternal towards pups: only lactating female mice express maternal aggression. This evidence strongly suggests that maternal

aggression is heavily dependent on endocrine factors whereas maternal care might be hormone-independent in mice. In the present work, we will also explore the immunohistochemmical expression of OXT and pSTAT5 in the AC/ADP of this additional experimental sample of lactating dams, godmothers and virgin female mice tested for maternal aggression. Likewise, we will also search for possible correlations between scores of maternal aggression and AC/ADP expression of OXT and pSTAT5. With this approach, we intend to gain a deeper insight on the role of the AC/ADP and its interaction with PRL and pup-derived stimuli in the regulation of different components of maternal behaviour.

3.2. MATERIAL AND METHODS

3.2.1. ANIMALS, EXPERIMENTAL DESIGN AND BEHAVIOURAL TESTING

Maternal motivation

In this experiment, a total of n=66 female mice of the CD1 strain (Janvier, France) were used. Of these, 26 animals arrived on pregnancy day 10 to our animal facility, together with 40 virgin females of the same age (10 weeks). At the moment of arrival females were pair-housed in polypropylene cages (145 mm wide, 465 mm long and 215 mm high; Panlab) under controlled temperature (24 ± 2 ºC) and lighting conditions (12h:12h; lights ON at 8 am), with *ad libitum* access to food and water. Animals were treated throughout according to the European Union Council Directive of June 3rd, 2010 (6106/1/10 REV1) and procedures were approved by the Committee of Ethics on Animal Experimentation of the Jaume I University of Castellón, where the experiments were performed.

Experimental groups (lactating dams, godmothers and virgins, Fig 3.1) were arranged at the moment of arrival of the animals: 10 pregnant females were randomly assigned to the lactating dam group and 10 virgin females were randomly assigned to each of the Godmother and Virgin groups, respectively. Of the remaining pregnant females (16), 10 were used as non-experimental, accompanying females of experimental godmothers, and the remaining six served as pup-donor mothers for the behavioural testing of experimental virgin females. As for the remaining virgin females (10), these were used as non-experimental accompanying females of experimental dams. The whole housing

design is illustrated on Figure 3.1 A. The morning after parturition, litters were culled down to 8 pups to ensure homogeneous interaction with pups, as some aspects of maternal behaviour are influenced by litter size (Maestripieri and Alleva 1991). One of the pregnant females was removed from the experiment due to problems during labour. This left a total sample size of n=9 lactating dams, n=10 godmothers and n=10 virgins.

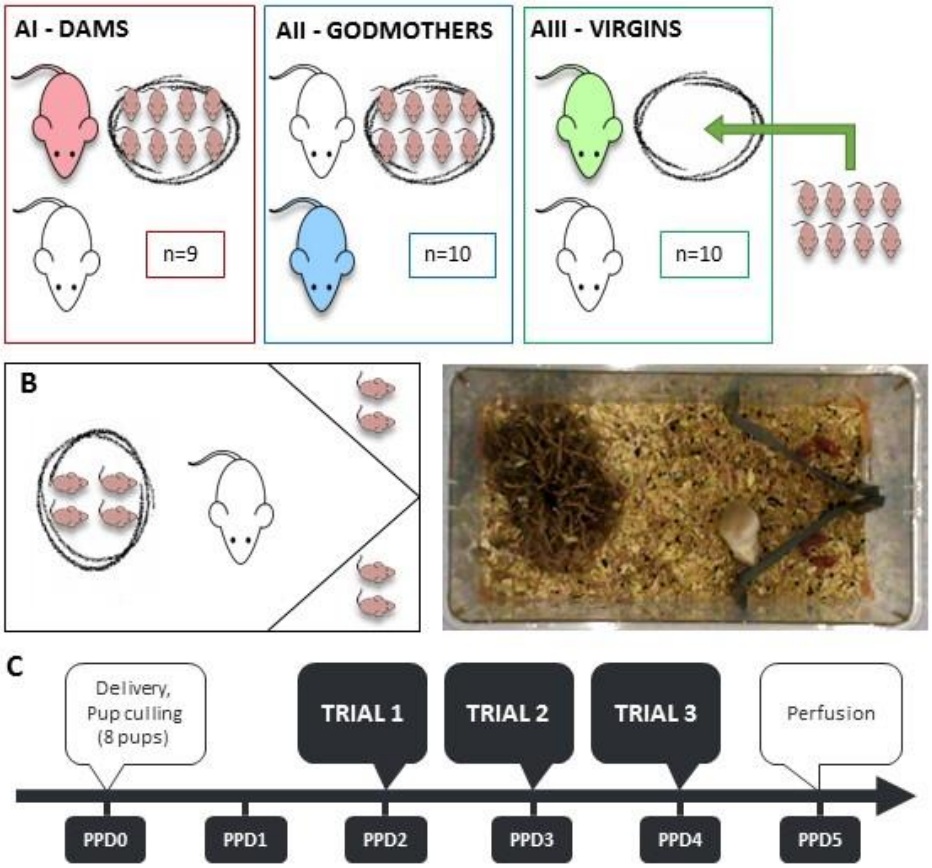


Figure 3.1 Experimental design for the motivated pup retrieval test

(AI-AIII) Housing conditions for the females of each experimental group. (AI) Dams (n=9, red) were housed together with a non-experimental accompanying female, in order to balance the design. (AII) Godmothers (n=19, blue) were housed together with a non-experimental dam, which provided the pups. (AIII) Virgins (n=10, green) were housed together with a non-experimental virgin and lacked any pups in their home cages.

(B) At the time of the test, experimental and accompanying females were removed from their home cage and the following setup was prepared (left): barriers were placed in the distal corners of the home cage and pups were placed as shown (4 pups remained in the nest and 2 pups were placed on each of the distal corners past the barriers). Right: screen capture of a video recording of a motivated pup retrieval test.

(C) Experimental timeline. The day of parturition was considered postpartum day (PPD) 0. Later on PPD0, litters were culled down to 8 pups each. Each experimental female underwent three consecutive trials in the morning of PPD2, PPD3 and PPD4, respectively. On PPD5, approximately 24 hours after the last trial, experimental animals were perfused.

The experimental design consisted of a modified pup retrieval paradigm, in which experimental females were forced to retrieve the pups off the nest site by climbing a 10 cm high wire-mesh barrier. The actual height of the barrier to provide the proper difficulty to the task, as well as the duration of the behavioural recording had been validated on a previous pilot test (data not shown). At the beginning of each trial, experimental and accompanying females were both briefly removed from their home cages in order to arrange the experimental settings (Fig. 3.1B). First, two barriers were placed on each of the distal (related to the nest site's position) corners of the home-cage. Then, with the help of a spoon, 2 pups were carefully placed on each of the distal corners, separated by the barriers. The remaining 4 pups were left on the nest site, in order to prevent the experimental females from building a new nest site on one of the distal corners during the test. Finally, the experimental female was placed back again in the home cage and behavioural recording started.

Starting on the morning of PPD2, experimental animals underwent three daily 10 minute trials daily until PPD4 (Fig 3.1C). Since behavioural testing was performed on the animals' home cage and it was of relevance for the experiment to collect data on the first exposure of the animal to the experimental conditions, no previous habituation was performed. After these trials, on the morning PPD5, experimental animals were transcardially perfused and brain tissue collected (see section 3.2.3).

Video recordings of the behavioural trials were analysed by an observer blind to the experimental conditions. The behavioural measures registered during this analysis included: latency to the first contact with pups, latency to retrieve each of the 4 displaced pups, total time of contact with pups and number of barrier crossings by the experimental animal.

Maternal aggression

The behavioural analysis of this experiment was performed by Ana Martín-Sánchez, former member of our lab, and published elsewhere (Martín-Sánchez et al. 2015a). Briefly, n=27 adult female mice of the CD1 strain were randomly assigned to three different experimental groups: lactating dams (n=9), godmothers (n=9) and virgins (n=9). Each female was housed with another non-experimental female (for details, see Martín-Sánchez et al. 2015a; 2015b) but just allowed configuring balanced dyads for the three

experimental groups: experimental dams were housed with accompanying females in the same way than experimental godmothers were housed with accompanying dams. Experimental females were subject to maternal aggression tests (based on a resident-intruder paradigm), where they were confronted to an intact male and a castrated male (as negative controls) in two consecutive days, in a counterbalanced fashion. For the purpose of the current study, we will focus on the data collected during confrontation with intact males. Maternal aggression tests had a duration of 5 minutes and were performed daily on postpartum days (PPD) 3 to 5, coinciding with the peak of maternal aggression in dams. Tests were video-recorded and evaluated by a person blind to the experimental conditions. Scores of attack and refusal behaviours were collected using SMART 2.5 event-recording software (Panlab, Barcelona, Spain). In the current analysis, we focus on the following measures of aggressive behaviour: latency to first attack, total duration of attacks, number of attacks and mean attack duration.

3.2.2. TISSUE COLLECTION AND HISTOLOGICAL PROCESSING

From this point, the same experimental procedures were performed for both the maternal aggression and the maternal motivation samples. Animals were transcardially perfused with saline and then 4% paraformaldehyde, brain tissue was collected, postfixed in the same fixative, cryoprotected and cut in 4 parallel series of 40 µm-thick frontal sections using a freezing microtome, to be stored at -20ºC. For further details on these procedures, the reader is referred to Section 1.2.5.

3.2.3. DOUBLE-LABEL IMMUNOFLUORESCENCE FOR pSTAT5 AND OXYTOCIN

One of the four parallel series of each animal was used to perform a double-label immunofluorescence for pSTAT5 and OXT. Immunofluorescence was conducted in free-floating sections under light shaking at room temperature (25^{o}C) unless otherwise stated. Tissue sections were thoroughly rinsed between stages for at least three 10-min washes in TRIS-buffered saline, 0.05M, pH 7.6 (TBS). After thawing and before antibody incubation, sections underwent: a) two sequential antigen retrieval steps, consisting of a 20-minute incubation in 1% hydrogen peroxide (H_2O_2) and 1-2% sodium hydroxide (NaOH) in water, pH>13, and a 10 minute incubation in glycine 0.3% and sodium dodecylsulfate (SDS) 0.03% in TBS; b) a 30 minute incubation in 1% sodium borohydride ($NaBH_4$) to block tissue autofluorescence; c) a 60 minute incubation in 4% normal goat serum and 0.03% Triton-X100 in TBS to block unspecific labelling. After these steps,

tissue sections were incubated for 72 hours at 4ºC in both primary antibodies simultaneously: rabbit anti-pSTAT5 monoclonal antibody (pSTAT5 Tyr694; Cell Signalling Technology, Beverly, MA) diluted 1:500 and mouse anti-oxytocin monoclonal antibody (Dr. Harold Gainer, NIH Cat#PS38) diluted 1:200. After incubation in primary antibody and rinsing, tissue was incubated for 90 minutes in a mixture of both secondary fluorescent antibodies: Alexa Fluor 488-conjugated goat anti rabbit IgG (Jackson ImmunoResearch, 111-545-003) diluted 1:250 and Rhodamine Red-X-conjugated goat anti mouse IgG (Life Technologies, Oregon, USA) diluted 1:250. Prior to mounting, sections were counterstained for 45 seconds in DAPI (600 nM 4', 6-diamino-2-fenilindol; Thermo Scientific, IL, USA) to allow for cytoarchitectural analysis. Finally, sections were rinsed, mounted in gelatine-coated slides and coverslipped with the fluorescent mounting medium Fluorsave (Calbiochem, USA).

3.2.4. ANALYSIS OF HISTOLOGICAL PREPARATIONS

Immunofluorescence was analysed using a Leica TCS SP8 confocal system (Leica AG, Germany), mounted on an inverted microscope. Triple scans for DAPI, Alexa Fluor 488 (conjugated to pSTAT5) and Rhodamine Red X (conjugated to OXT) were performed. Excitation wavelengths were 405 nm for DAPI, 488 nm for Alexa Fluor 488 and 559 nm for Rhodamine Red-X, whereas emission wavelengths included 461, 520 and 591 nm respectively. Stacks of sequential Z-sections of the AC/ADP region (sampling frame illustrated on Fig. 3.2) of both hemispheres of each experimental animal were obtained with a 20x magnification and 4-µm separation between neighbour optical sections. After sampling, confocal stacks were reviewed by an observer blind to the experimental groups and a few of them were discarded due to technical shortcomings relative to the microscope sampling.

Histological variables of interest were quantified employing different procedures, each adapted to the nature and limitations of the given variable. First, OXT immunoreactive cell number was counted manually by a blind observer. In this counting protocol, the AC/ADP OXT cell population was identified and cells were counted for each of the stack levels, avoiding to count single cells present in more than one level. Then, mean interhempispheric values were calculated for each animal.

Concerning pSTAT5, the total density of pSTAT5-immunoreactive cell nuclei was counted automatically using ImageJ software. Using a smaller sampling frame (Fig. 3.2), a single

8-bit image of the green channel of the stack was obtained by flattening the different Z-sections of the stack. This image underwent a smooth median filter (2 pixel radius) to prevent that the granulated disposition of pSTAT5 imunofluorescence impaired subsequent steps of the protocol. Then, the image was binarised using the 200% of the median grey value of the image's histogram as a threshold. After binarising, the image was further processed using the following ImageJ commands: Fill holes; Open; Watershed. Particle number was obtained excluding particles smaller than 25 square microns in area (corresponding approximately to 8 microns in diameter), and the final density value was obtained by dividing this count by the sampling frame area, and expressed in number of cells/square millimetre. Then, mean values for both hemispheres were calculated for each animal.

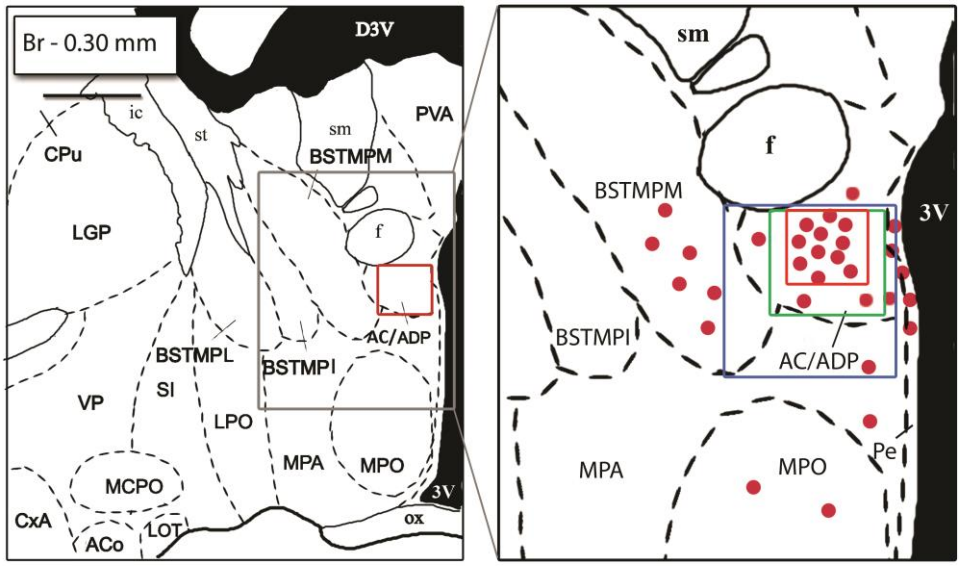


Figure 3.2 Anatomical location of the sampling frames for the AC/ADP region

Confocal stacks of the AC/ADP region were sampled at the approximate level of Bregma – 0.30 mm (blue frame). For the quantification of pSTAT5 density in the AC/ADP region, we employed a frame of 290 μm height and 270 μm width (equivalent to the one used in chapter 1), which covered most of the AC/ADP region (green frame). To analyse the expression of pSTAT5 among OXT positive and OXT negative neuron populations, we employed a smaller frame (220 μm x 220 μm, red frame) centred on the OXT population (red dots).

An additional histological analysis was performed to estimate the relative input of PRL over OXT-positive and OXT-negative neuron populations of the AC/ADP. For this purpose, an additional manual counting of pSTAT5-positive, OXT-positive, double-positive and double-negative neurons (Fig 3.8) was performed by an observer blind to

the experimental conditions of the stacks. Importantly, neurons were identified and differentiated from glial cells using the DAPI staining of the stack (blue channel), according to the size of the nucleus, the appearance and condensation of the chromatin and the presence and size of the nucleoli. Once these counts were obtained, the mean interhemispheric percentage of pSTAT5 immunoreactivity was calculated for both OXT-positive and OXT-negative cell populations.

3.2.5.STATISTICAL ANALYSIS

All of the following statistical procedures were performed using IBM SPSS Statistics 22 software package.

Behavioural data

Methodological details on the statistical treatment of maternal aggression data can be found in Martín-Sánchez et al. (2015a). Regarding motivated pup retrieval, we first searched for possible differences between experimental groups in the access and potential interaction with pups, by analysing the following variables: latency to first contact with pups, total time of pup contact and number of barrier crossings. Since data for these variables were not normally distributed, we performed separate Kruskal Wallis ANOVAs for each trial to search for differences between experimental groups. Then, we performed log rank (Kaplan-Meier) tests in search for significant differences in the survival distributions of the latencies to the retrieval of the first and the fourth pup of each of the three trials. Finally, we were interested in assessing the learning or improvement of the experimental groups in the task. Since latencies were not normally distributed among our experimental groups, we ran separate Wilcoxon signed-rank tests for matched pairs. To do so, we considered the latencies of each experimental animal to retrieve the first pup and compared the distributions of these latencies during the first trial with those in the second and third trials, respectively.

Histological data

To analyse the expression of OXT and pSTAT5 in the AC/ADP of our experimental samples, we performed separate 1-way ANOVAs with Bonferroni post-hoc comparisons for each sample for both the number of OXT-immunoreactive neurons and the density of pSTAT5-immunoreactive cells. To compare the relative input of pSTAT5 over OXTergic and non-OXTergic cell populations of the AC/ADP, we first searched for significant effects of the experimental group and significant differences in the expression of pSTAT5

between both populations. Thus, we performed two-way mixed ANOVAs (independent factor experimental group; repeated measures factor cell population) for each experimental sample. Following this comparison, we looked further for significant differences between experimental groups by performing separate one-way ANOVAs (with experimental group as independent factor) with Bonferroni post-hoc comparisons for each sample and each cell population.

Correlational analysis

Finally, we tested the association of our behavioural and histological variables in a correlational analysis. For the sample tested for maternal aggression, the following behavioural parameters were included: latency to first attack, total number of attacks, total attack duration and mean attack duration. For the sample tested for motivated pup retrieval, we included the latencies to retrieve the first and fourth pups during the third trial and the total number of pups retrieved throughout all three trials of the experiment. Regarding histological measures, we included number of OXT-immunoreactive neurons, density of pSTAT5-ir, the percentage of OXT positive neurons showing pSTAT5 immunoreactivity (pSTAT5-ir) and the percentage of non-OXT cells showing pSTAT5-ir. First, distribution of all variables was analysed in search for normality. Normally distributed variables were tested using a Pearson's correlation. Variables that did not present a normal distribution were logarithmically transformed to apply Pearson's correlation. If after transformation the variable was not distributed normally, a non-parametric Spearman correlation was applied. For both experimental samples (motivated pup retrieval and maternal aggression), correlations were tested for each experimental group separately. We considered the possibility of analysing all the females of each experiment together as a common, global sample. However, the correlations obtained in this global analysis might be spurious or misleading because only some of the experimental groups actually expressed the behaviours measured: only dams expressed aggressive behaviour, whereas only dams and godmothers retrieved pups. Hence, behavioural scores for Virgins (retrieval) and Virgins and Godmothers (maternal aggression), are categorically null or censored. Therefore, we restricted the correlational analysis within those groups that did actually perform the respective behaviour.

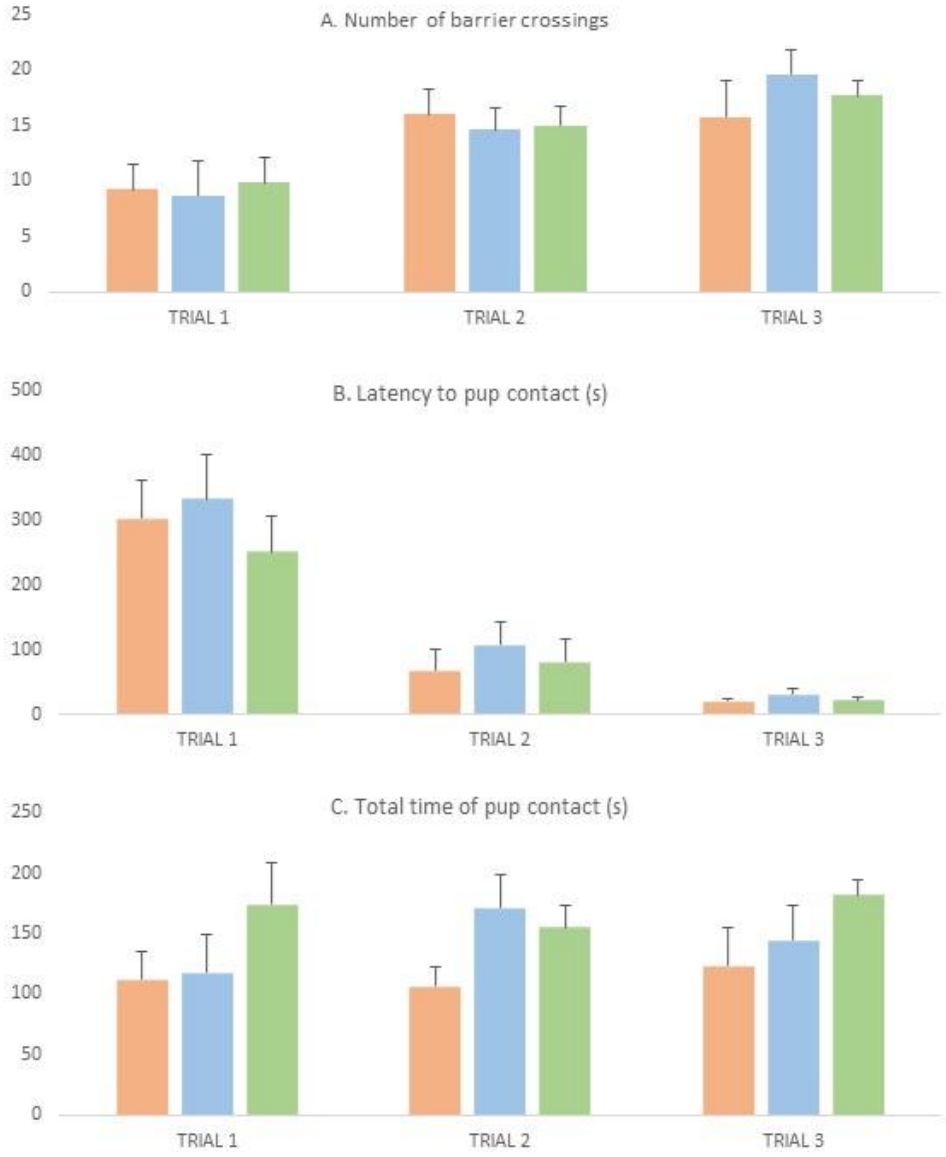


Figure 3.3 Dams, Godmothers and Virgins show equivalent contact with pups in the motivated pup retrieval test

Bar histograms show mean + SEM values of: A) the number of barrier crossings into the compartment where pups were placed; B) the latency to establish the first contact with a pup; and C) the total time of contact with pups. One-way ANOVAs of these measures revealed no statistically significant differences among Dams (n=9), Godmothers (n=10) or Virgins (n=10) in neither of the three consecutive trials, indicating equivalent access and interaction with pups.

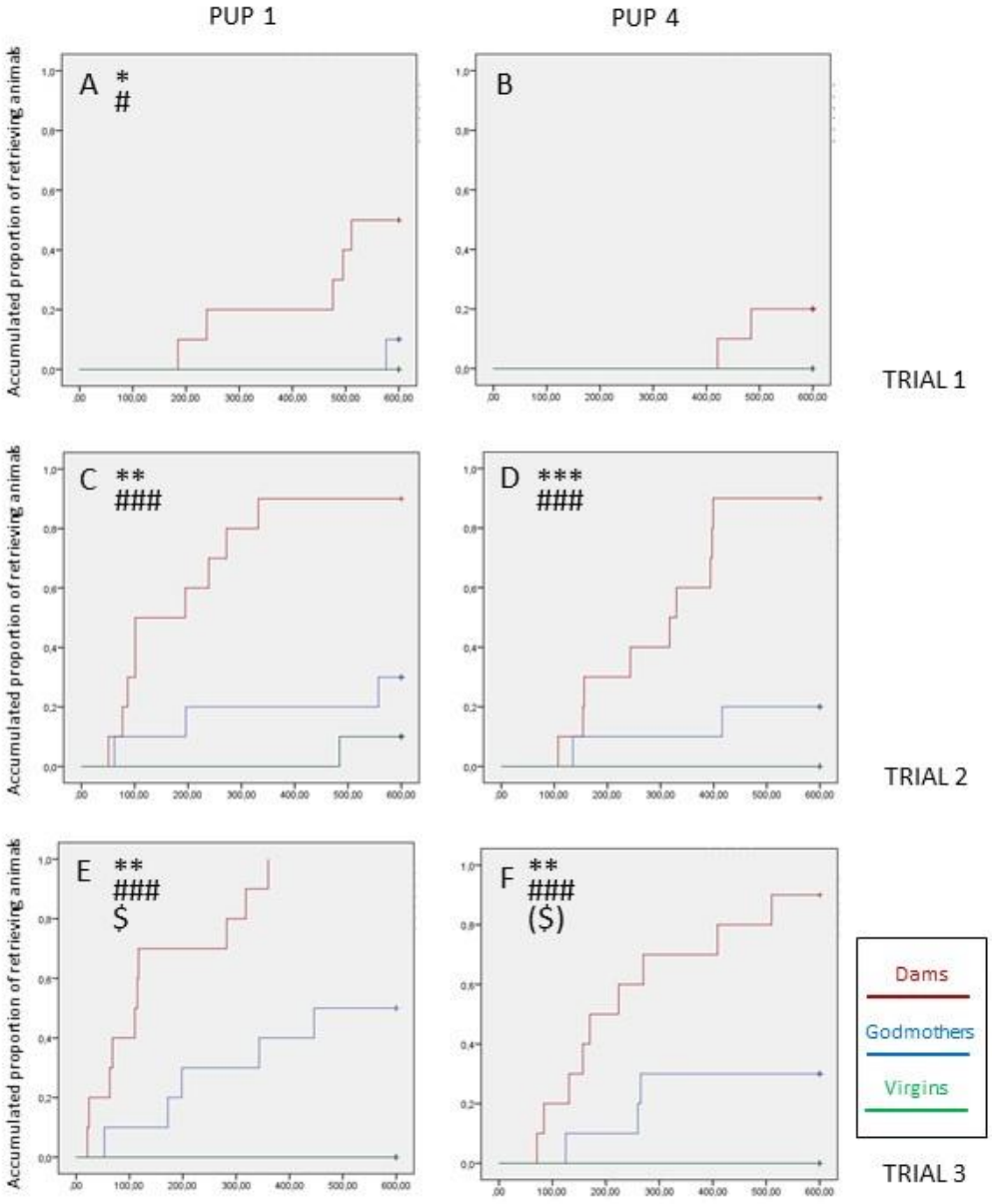


Figure 3.4 Dams, Godmothers and Virgins show distinct performance in the motivated pup retrieval test

Survival plots indicating the accumulated proportion of Dams (red line), Godmothers (blue line) and Virgins (green line) successfully retrieving the first pup (left column) and fourth pup (right column) in each of the three 10 minute trials (rows). Hatched lines in the plots indicate censored cases in the analysis, i.e. animals that did not complete the retrieval of that specific pup. Log-rank (Kaplan Meier) analysis of the aforementioned trials and pups revealed significant differences between dams and godmothers (*) and dams and virgins (#) already during the first trial (A and B). These differences became more marked during the second trial (C and D) and persisted during the third trial (E and F). During the third trial, additional significant differences in retrieval appeared between godmothers and virgins ($) in the first-pup retrieval (E), of which only a non-significant trend persisted in the fourth-pup retrieval (($) $p=0.067$). (Log-rank test; *$p<0.05$; **$p<0.01$; ***$p<0.001$).

3.3. RESULTS

3.3.1. MOTIVATED PUP RETRIEVAL EXPERIMENT

Behavioural Analysis

First, we checked for possible differences in the time that the experimental animals interacted with the pups behind the barriers. Kruskal Wallis tests revealed no significant differences during any of the three trials among experimental groups in the latency (p=0.610; p=0.306; and p=0.670 for each trial, respectively) or the total time in contact with pups (p=0.322; p=0.116; and p=0.245 for each trial, respectively) or the number of barrier crossings (p=0.804; p=0.772; and p=0.397 for each trial, respectively) (Fig. 3.3). These results indicate that all three groups had equivalent access and interaction with pups.

Then, we ran separate survival log-rank tests (Kaplan-Meier) to explore differences in the distributions of the retrieval latencies for the first and the fourth pups in each trial between dams, godmothers and virgins (Fig. 3.4). During the first trial (PPD2), the survival distributions of the latency to retrieve the first pup significantly differed between dams and godmothers ($\chi^2(2)=4.194$, p=0.041) as well as between dams and virgins ($\chi^2(2)=6.389$, p=0.011), but not between godmothers and virgins ($\chi^2(2)=1.0$p=0.317). Regarding retrieval of the fourth pup, however, no significant differences between experimental groups were found (p=0.146). During the second trial (PPD3), survival distributions of the latencies to first and fourth pup retrieval were both significantly different between dams and godmothers ($\chi^2(2)=8.198$, p=0.004 for pup 1; $\chi^2(2)=10.715$, p=0.001 for pup 4) and between dams and virgins ($\chi^2(2)=15.764$, p<0.001 for pup 1; $\chi^2(2)=16.989$, p<0.001 for pup 4), but once again no differences were found between godmothers and virgins (p=0.265 and p=0.146, for pup 1 and 4 respectively). Finally, during the third trial (PPD4), all three groups differed significantly in their first and fourth pup retrieval survival distributions. Dams showed statistically significant differences with godmothers ($\chi^2(2)=9.591$, p=0.002 for pup 1; $\chi^2(2)=6.981$, p=0.008 for pup 4) and virgins ($\chi^2(2)=21.837$, p<0.001 for pup 1; $\chi^2(2)=16.989$, p<0.001 for pup 4). For the first time, godmothers and virgins differed significantly in the latency to first pup retrieval ($\chi^2(2)=6.389$, p=0.011) and the analysis rendered a trend toward significance in the retrieval of the fourth pup ($\chi^2(2)=3.353$, p=0.067). In the godmother group, 50% (Fig 3.4 E) of the females retrieved the first pup, whereas 30% completed the whole task

retrieving the fourth pup (Fig 3.4 F). Conversely, none of the naïve virgin females retrieved any pup in the whole trial.

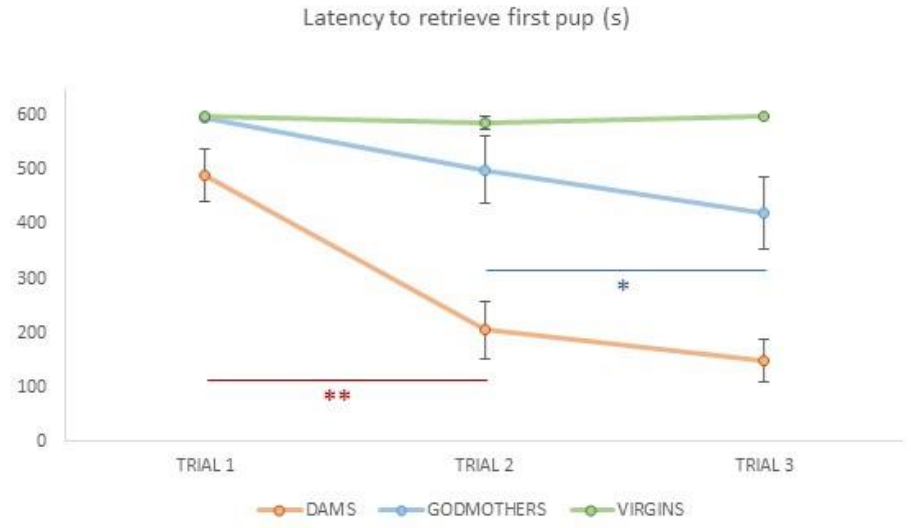


Figure 3.5 Dams, Godmothers and Virgins show different improvement profiles in the motivated pup retrieval test

Mean + SEM latencies to retrieve the first pup in Dams (red line), Godmothers (blue line) and Virgins (green line) during all three trials of the experiment. In order to evaluate the improvement of the experimental groups in the motivated pup retrieval test, we compared for each group the latency to first-pup retrieval during each trial with the latency displayed during the previous trial, using separate Wilcoxon signed-rank tests for matched pairs. According to this analysis, dams showed a significant decrease in the retrieval latency already in trial 2, whereas godmothers started to show significant differences later on trial 3, indicating a slower improvement as compared to dams. By contrast, virgins virtually did not perform any retrieval. (Wilcoxon test; *$p<0.05$; **$p<0.01$).

Finally, we assessed the improvement of each experimental group in the motivated pup retrieval task by comparing first-pup retrieval latencies between trials 1 and 2, and 1 and 3, by means of a Wilcoxon signed-rank test for matched pairs (Fig. 3.5). When matching trials 1 and 2, only dams differed significantly in their latencies to retrieve the first pup ($Z=-2.666$, $p=0.008$), whereas godmothers ($Z=-1.604$, $p=0.109$) and virgins ($Z=-1.000$ $p=0.317$) did not. On the other hand, when comparing trials 1 and 3, dams ($Z=-2.803$, $p=0.005$) and godmothers ($Z=-2.023$, $p=0.043$), did show statistically significant differences in their retrieval latencies, whereas virgins did not ($Z=0$, $p=1.0$). These findings indicate that in a three trial motivated pup retrieval paradigm, dams rapidly improve in the task of retrieving pups (already during trial 2), whereas godmothers display a slower and weaker improvement (measurable only in trial 3) and virgins show no improvement at all.

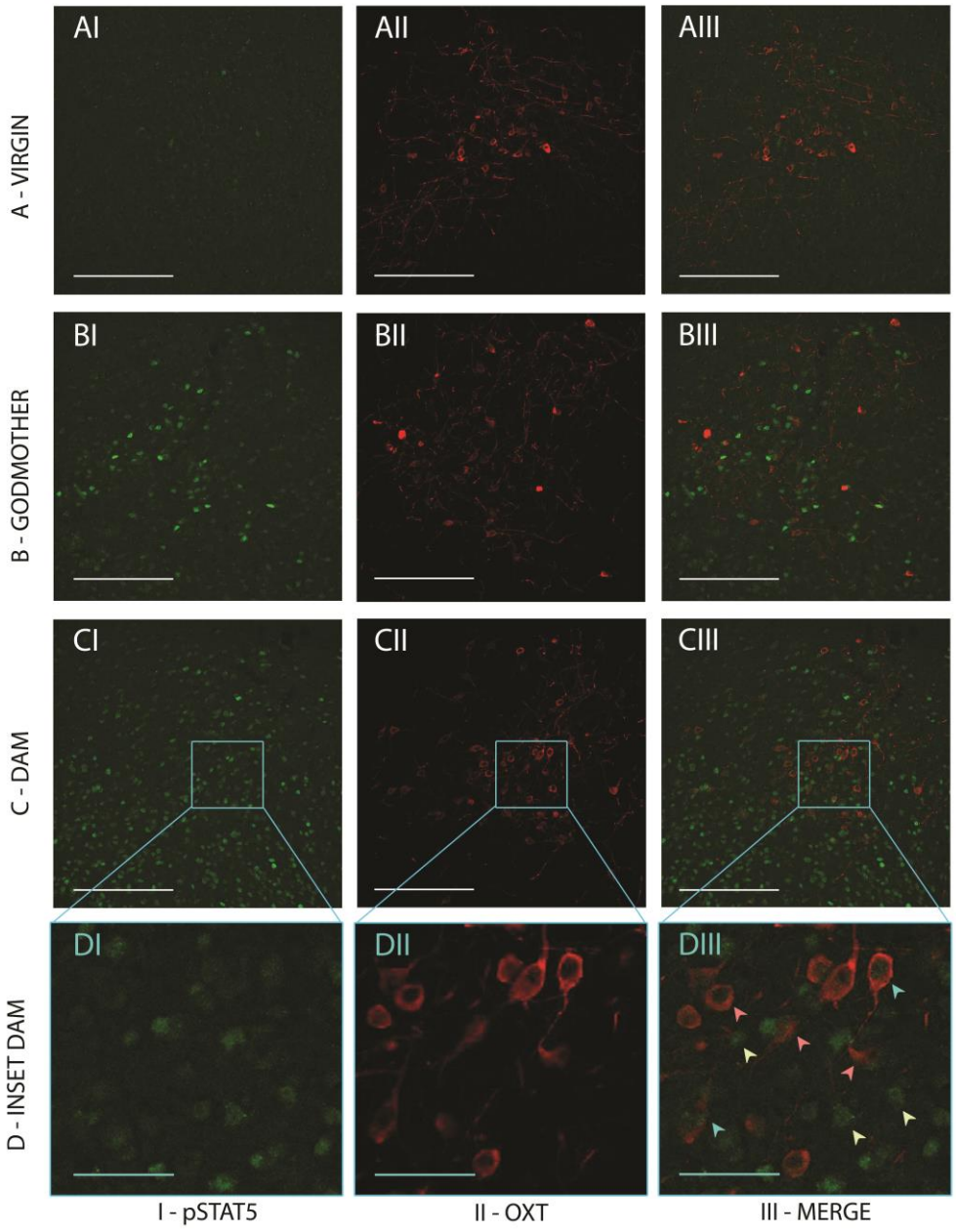


Figure 3.6 Representative confocal images of pSTAT5 and Oxytocin immunofluorescence in the AC/ADP of Virgins, Godmothers and Dams

Representative z-projections of whole confocal stacks of the AC/ADP region of a virgin (row A), godmother (row B) and dam (row C) specimen, tested for motivated pup retrieval. Column I shows pSTAT5-ir (green channel), which is located mainly in the cell nucleus but also in the cytoplasm. Column II shows OXT-immunoreactive somata (red channel). Column 3 shows the merge of green and red channels. The lowermost row (D) shows a magnified inset (blue boxes on row C) of a single z-level of the confocal stack for green (DI), red (DII) and merge (DIII) channels, respectively. Light green arrowheads in the merge inset (DIII) indicate cells immunoreactive only for pSTAT5 (green), pink arrowheads indicate somata immunoreactive only for OXT (red), and blue arrowheads point to double-labelled cells for pSTAT5 (green) and OXT (red). Approximate distance to bregma of the samples is -0.30 mm (see Fig 3.2). Scale bars represent 200 μm for A, B and C and 50 μm for D.

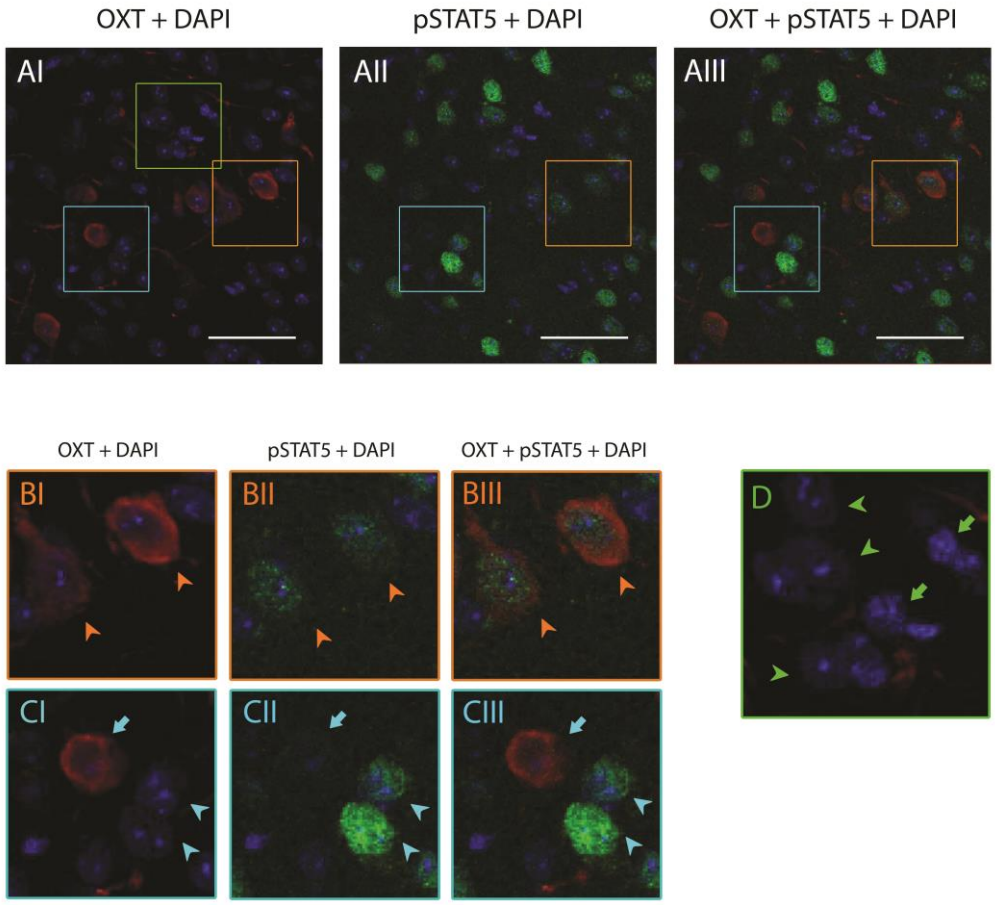


Figure 3.7 Representative confocal images of pSTAT5 immunoreactivity over OXT-positive and OXT-negative neurons in the AC/ADP

Representative extracts from a single z-level of a confocal stack of a specimen tested for maternal aggression. These extracts illustrate four different quantified neuron phenotypes: 1) pSTAT5-positive, OXT-negative neurons; 2) pSTAT5-negative, OXT-positive neurons; 3) double positive neurons for OXT and pSTAT5; and 4) double negative neurons for OXT and pSTAT5.

(A) These neuron phenotypes were identified using all three colour channels: red for OXT-ir, green for pSTAT5-ir and blue for DAPI nuclear staining. AI depicts the composite of red (OXT) and blue (DAPI) channels. AII depicts the composite of green (pSTAT5) and blue (DAPI) channels. AIII depicts the triple composite (OXT, pSTAT5 and DAPI). Colored frames correspond to insets of B, C and D, showing all four neuron phenotypes. Scale bars: 50 μm.

(B) Detail insets for double positive neurons for OXT and pSTAT5 (orange arrowheads) in the OXT+DAPI (BI), pSTAT5+DAPI (BII) and triple OXT+pSTAT5+DAPI (BIII) composite channels.

(C) Detail insets for OXT-negative, pSTAT5-positive (blue arrowheads) and for OXT-positive, pSTAT5-negative (blue arrows) neuron phenotypes) in the OXT+DAPI (CI), pSTAT5+DAPI (CII) and triple OXT+pSTAT5+DAPI (CIII) composite channels.

(D) Detail inset featuring double negative neurons (green arrowheads). Neurons were differentiated from glial cells (green arrows) using the DAPI channel. In the image, neurons show big nuclei with dispersed euchromatine and evident nucleoli, whereas glial cells show sall nuclei with condensed heterochromatine.

Immunoreactivity for Oxytocin and pSTAT5

In this part of the study, we sampled the number of OXT-immunoreactive neurons and density of pSTAT5-immunoreactive neurons at a specific anatomical level of the AC/ADP (Fig 3.2). Figure 3.6 depicts an extract of the confocal stacks obtained during the sampling. One-way ANOVA revealed no statistically significant effect of experimental group in the number of OXT-immmunoreactive neurons (Fig 3.8 A), but found significant differences between experimental groups ($F(2, 25)=4.819$, $p=0.017$) in the density of pSTAT5-ir (Fig 3.8 B). According to Bonferroni post-hoc comparisons, dams differed significantly from virgins ($p=0.015$), whereas godmothers differed neither from dams ($p=0.213$) nor from virgins ($p=0.734$).

Then, we compared the percentage of pSTAT5 immunoreactive neurons amongst the OXT positive population of the AC/ADP and the surrounding OXT negative cells of this region. Descriptive examples of the appearance of pSTAT5 and OXT double immunofluorescence in the AC/ADP of dams, godmothers and virgins are illustrated in Figure 3.7. White arrowheads point to pSTAT5-positive/OXT-positive cells, white arrows point to pSTAT5-positive/OXT-negative cells and yellow arrowheads signal pSTAT5-negative/OT-positive neurons.

We performed a two-way mixed ANOVA in search of significant differences between experimental groups and neuron populations (OXT positive and OXT negative populations, respectively) (Fig 3.8 C and D). We found differences in percentage of pSTAT5 expression between populations only by trend ($F(1)=3.422$, $p=0.082$), whereas there was no significant effect of the experimental group ($F(2)=0.320$, $p=0.730$) or the interaction of both factors ($F(2)=0.908$, $p=0.422$). Afterwards, we performed separate one-way ANOVAs for each population, testing for a possible effect of the experimental group that had not been identified in the previous analysis. One-way ANOVA reported a weak trend to significance in the percentage of pSTAT5 expression in OXT negative neurons ($F(2,17)=2.742$, $p=0.093$) (Fig 3.8 C), whereas no effect was found regarding OXT positive neurons ($F(2,17)=0.018$, $p=0.982$) (Fig 3.8 D).

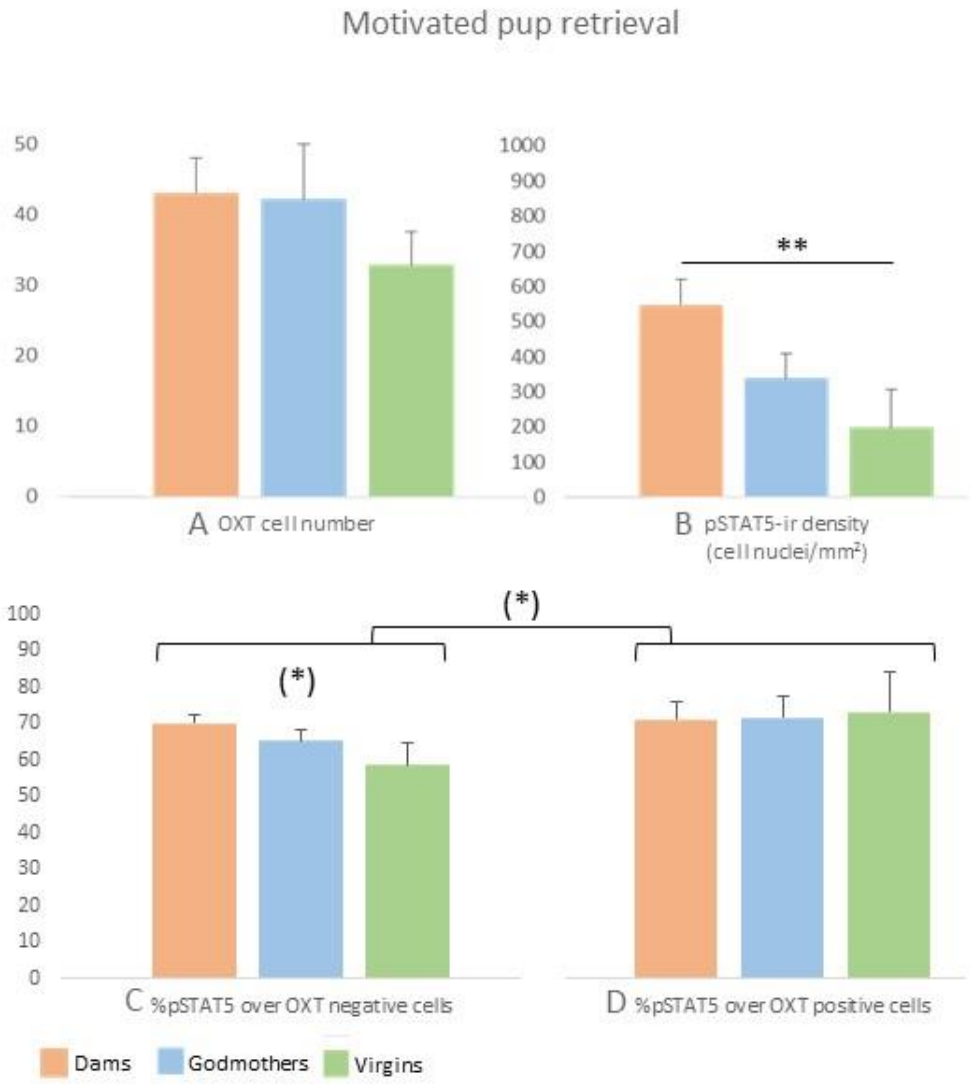


Figure 3.8 Quantification of pSTAT5-ir density and OXT-immunoreactive cell number in the AC/ADP of female mice tested for motivated pup retrieval

Bar histograms including Dams (red), Godmothers (blue) and Virgins (green) show mean + SEM values for: (A) number of oxytocin-immunoreactive neurons; (B) density of pSTAT5-immunoreactive cell nuclei; (C) percentage of pSTAT5 expression on OXT-negative neurons of the AC/ADP; and (D) percentage of pSTAT5 expression on OXT-positive neurons of the AC/ADP. Statistical analysis in panels A and B was performed using one-way ANOVAs with Bonferroni post-hoc comparisons. In panels C and D, differences between experimental groups and neuron population were first assessed by a two-way mixed ANOVA model. Then, differences between experimental groups were further contrasted by separate one-way ANOVAs. (*$p<0.05$; **$p<0.01$).

Correlational Analysis

We confronted the latencies to the retrieval of the first and fourth pup of trial 3, as well as the total number of retrieved pups, to the number of OXT-expressing neurons, pSTAT5-ir density and percentage of pSTAT5 expression over OXT positive and OXT negative neurons of the AC/ADP, respectively (Fig. 3.10 D and Table 3.1). In this case, as explained before (Section 3.2.5), we explored these correlations within dams and godmothers separately, but not virgins, which did not retrieve at all. We found significant associations of retrieval with the number of OXT neurons of the AC/ADP, but not with pSTAT5-ir density. Both dams (Fig 3.10 B, Table 3.2 B) and godmothers (Fig 3.10 C, Table 3.2 C) showed substantial negative correlations between the total number of retrieved pups and the number of OXT neurons (r=-0.69; p=0.036 for dams and r=-0.73; p=0.018 for godmothers, using Spearman's correlation). This indicates that females with greater numbers of OXT-expressing neurons in the AC/ADP retrieve less pups in this test.

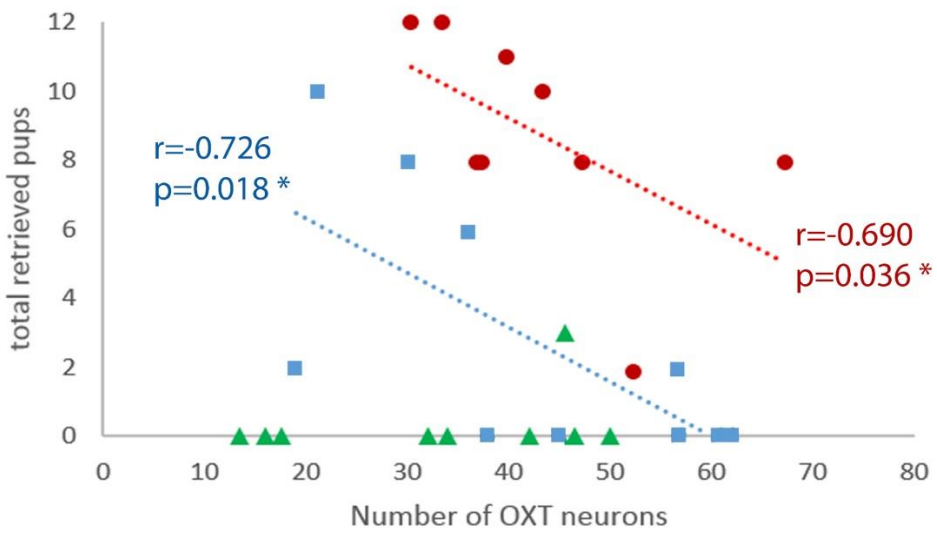


Figure 3.9 Significant correlations between motivated pup retrieval and histological parameters of the AC/ADP

Scatterplot showing the distribution of motivated pup retrieval scores (total number of retrieved pups across the experiment) and number of OXT neurons in the AC/ADP in dams (red circles), godmothers (blue squares) and virgins (green triangles). When experimental females were arranged together as a global sample, Spearman's non-parametric method (r) detected no significant correlation between both parameters (black). When analysed separately, however, both dams (red) and godmothers (blue), but not virgins, showed significant correlations between these parameters. For each of these, correlation coefficients, p-values and regression line are provided.

A-DAMS	OXT cell number	pSTAT5 density	% pSTAT5 over OXT neurons	% pSTAT5 over OXT-negative neurons
Latency T1P1	r= 0.529 p= 0.143	r= - 0.229 p= 0.553	r= - 0.028 p= 0.948	r= 0.300 p= 0.470
Latency T1P4	r= 0.548 p= 0.127	r= - 0.550 p= 0.125	r= - 0.250 p= 0.550	r= 0.577 p= 0.134
Latency T3P1	r= 0.150 p= 0.700	r= - 0.377 p= 0.318	r= 0.084 p= 0.843	r= - 0.190 p= 0.651
Latency T3P4	R= 0.098 p= 0.801	R= - 0.180 p= 0.642	R= 0.224 p= 0.593	R= - 0.028 p= 0.947
Total pups retrieved	r= - 0.699 p= 0.036*	r= 0.356 p= 0.348	r= - 0.116 p= 0.784	r= - 0.358 p= 0.385

B-GODMOTHERS	OXT cell number	pSTAT5 density	% pSTAT5 over OXT neurons	% pSTAT5 over OXT-negative neurons
Latency T1P1	r= 0.407 p= 0.243	r= - 0.466 p= 0.175	r= - 0.204 p= 0.661	r= - 0.618 p= 0.139
Latency T1P4	- (No retrieval)	- (No retrieval)	- (No retrieval)	- (No retrieval)
Latency T3P1	r= 0.668 p= 0.035*	r= 0 p= 1.0	r= - 0.394 p= 0.382	r= 0.477 p= 0.279
Latency T3P4	R= 0.605 p= 0.064(*)	R= - 0.095 p= 0.793	R= - 0.605 p= 0.150	R= 0.676 p= 0.095
Total pups retrieved	r= - 0.726 p= 0.018*	r= - 0.013 p= 0.972	r= 0.394 p= 0.382	r= - 0.477 p= 0.279

Table 3.1 Correlations between motivated pup retrieval and AC/ADP OXT and pSTAT5 expression

Summary of the explored correlations between behavioural variables of motivated pup retrieval (latencies to first and fourth pup retrieval on each trial and total number of retrieved pups) and histological variables of OXT and pSTAT5 expression measured in the AC/ADP (number of OXT neurons, pSTAT5-ir density, percentage of pSTAT5 expression over OXT neurons and percentage of pSTAT5 expression over OXT negative neurons). (A) Correlations obtained for dams separately. (B) Correlations obtained for godmothers separately. For each correlation, the correlation coefficient (R, r) as well as the statistical significance (p) are listed. Boxes in white background represent parametric correlations, performed with Pearson's method (R), whereas grey-shaded boxes indicate non-parametric analyses performed with Spearman's method (r). . Virgins were not analysed separately because they did not retrieve. Significant correlations and non-significant trends are highlighted with a red frame and a pink frame, respectively. *$p<0.05$; (*) $p\leq0.08$.

3.3.2. MATERNAL AGGRESSION EXPERIMENT

Behavioural Analysis

Results of experimental dams, godmothers and virgins tested in the resident-intruder paradigm against intact males can be found in the original publication of our lab (Martín-Sánchez et al. 2015a). Summarizing, significant differences between experimental groups were found in the latency to attack the intact male intruder ($\chi^2(2)=9.8$, p=0.008 on a log-rank test) and the total time spent attacking the intact male intruder (p=0.002 on a randomisation test). Post-hoc analysis revealed a significantly lower latency to attack in dams as compared to godmothers (p=0.045) and virgins (p=0.005), and significant higher attack duration in dams with respect to godmothers (p=0.001) and virgins (p<0.001). Conversely, godmothers and virgins did not differ significantly in either of both measures (p=0.47 and p=0.215, respectively).

Immunoreactivity for pSTAT5 and oxytocin

As in the previous experiment (3.3.1), we quantified the number of OXT-immunoreactive neurons and the density of pSTAT5-ir in a specific anatomical level (Fig 3.2) of the AC/ADP of female mice tested for maternal aggression. One-way ANOVA revealed a slight trend to an effect of experimental group regarding the number of OXT immunoreactive neurons sampled (F (2,20)=3.006, p=0.072) (Fig 3.9 A). On the other hand, it found significant differences between experimental groups in the density of pSTAT5-ir (F(2,17)=15.93, p<0.001) (Fig 3.9 B). Bonferroni post-hoc analysis for pSTAT5-ir density indicated that density of pSTAT5 immunoreactive cell nuclei differed significantly between dams and virgins (p=0.001) and between dams and godmothers (p<0.001), but not between godmothers and virgins (p=1.0).

Regarding percentages of pSTAT5 expression among OXT-positive and OXT-negative neurons (Fig 3.9 C, D) two-way mixed ANOVAs for experimental group and population revealed a strong significant effect of population (F(1)=31.171, p<0.001), indicating that percentages of pSTAT5 expression significantly differed between OXT positive and OXT negative neurons. Conversely, no significant effect of the experimental group (F(2)=0.856, p=0.442) or the interaction of both factors (F(2)=2.287, p=0.132) was found. Subsequent one-way ANOVA found significant differences between dams, godmothers and virgins in the percentage of pSTAT5-ir among OXT negative neurons of the AC/ADP (F(2,17)=6.369, p=0.009) (Fig 3.9 C), but not in the percentage among OXT positive

neurons (F(2,17)=0.097, p=0.908) (Fig 3.9 D). Bonferroni post-hoc comparisons for the OXT negative population indicated that the percentage of pSTAT5 in OXT negative neurons of dams significantly differed from that of virgins (p=0.008), whereas the expression in godmothers did not differ from dams (p=0.198) or from virgins (p=0.332).

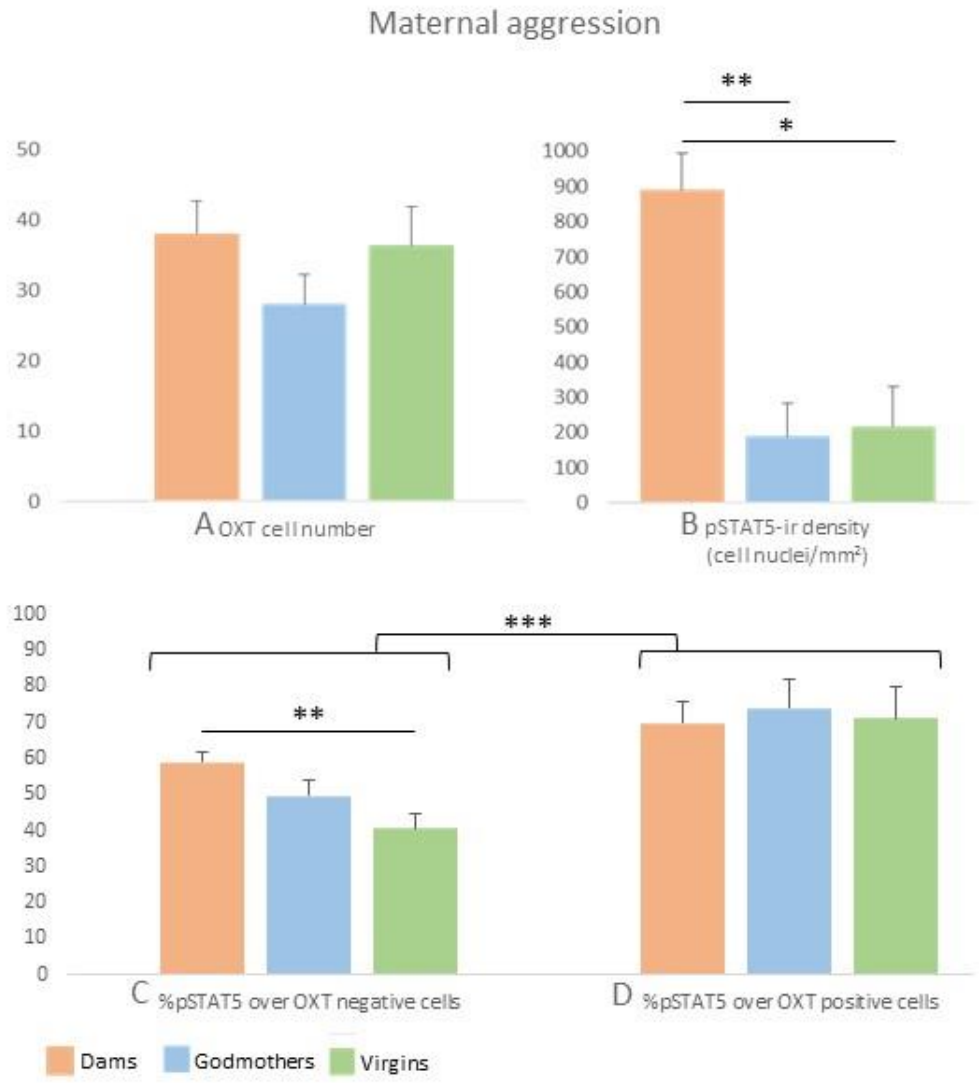


Figure 3.10 Quantification of pSTAT5-ir density and OXT-immunoreactive cell number in the AC/ADP of female mice tested for maternal aggression

Bar histograms including dams (red), godmothers (blue) and virgins (green) show mean + SEM values for: (A) number of oxytocin-immunoreactive neurons; (B) density of pSTAT5-immunoreactive cell nuclei; (C) percentage of pSTAT5 expression on OXT-negative neurons of the AC/ADP; and (D) percentage of pSTAT5 expression on OXT-positive neurons of the AC/ADP. Statistical analysis in panels A and B was performed using one-way ANOVAs with Bonferroni post-hoc comparisons. In panels C and D, differences between experimental groups and neuron population were first assessed by a two-way mixed ANOVA model. Then, differences between experimental groups were further contrasted by separate one-way ANOVAs. (*$p<0.05$; **$p<0.01$).

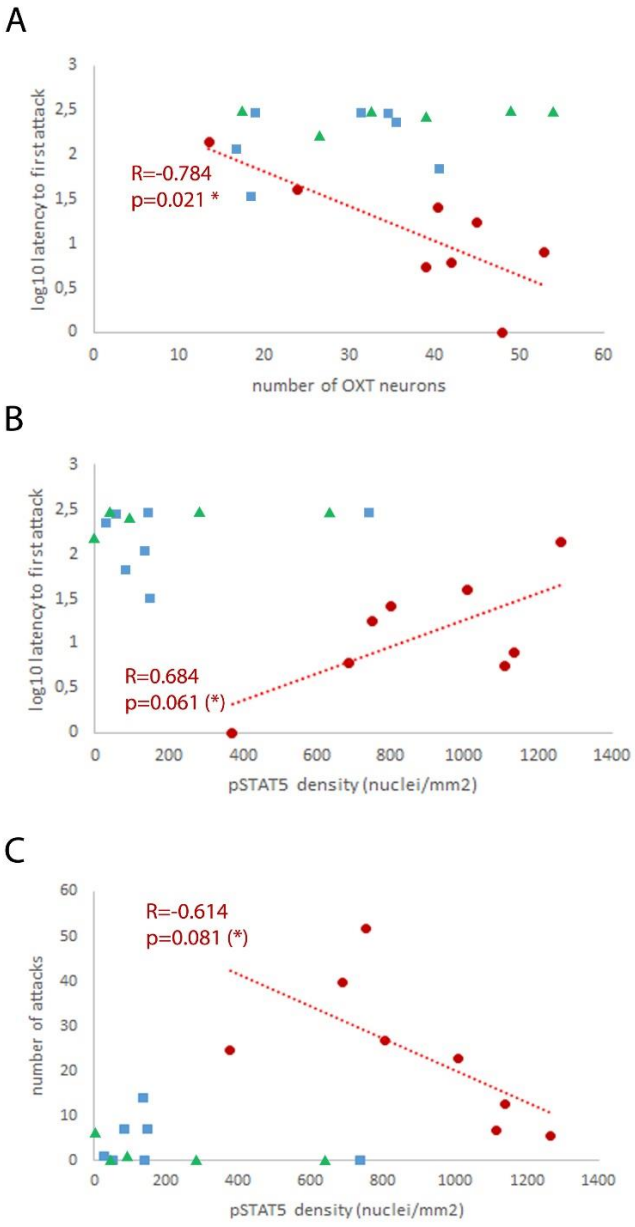


Figure 3.11 Significant correlations between maternal aggression and histological parameters of the AC/ADP

Scatterplots showing the distribution of behavioural measures of maternal aggression and histological variables of OXT and pSTAT5 expression in the AC/ADP, for dams (red circles), godmothers (blue squares) and virgins (green triangles). Correlations according to Pearson's parametric method (R) or Spearman's non-parametric method (r) are listed in each scatterplot, with correlation coefficients (R; r), p-values and respective regression lines provided. Correlations were performed for the global sample (resulting from arranging all three experimental groups together, black) and for Dams (red), the only group showing aggressive behaviour. A) Significant negative correlation in dams between the logarithm (base 10) of the latency to the first attack and the number of OXT neurons. B) Non-significant trend to positive correlation in dams between the logarithm (base 10) of the latency to the first attack and the density of pSTAT5 immunoreactive cell nuclei in the AC/ADP. C) Non-significant trend to negative correlation in dams between the number of attacks and the density of pSTAT5 immunoreactive cell nuclei in the AC/ADP.

DAMS	OXT cell number	pSTAT5 density	% pSTAT5 over OXT neurons	% pSTAT5 over OXT-negative neurons
Log_{10} (latency to first attack)	R= - 0.784 p=0.021*	R= 0.684 P= 0.061(*)	R= 0.440 p= 0.275	R= 0.579 p= 0.133
Total attack duration	R= 0.434 p= 0.282	R= - 0.614 p= 0.106	R= 0.004 p= 0.993	R= - 0.503 p= 0.204
Number of attacks	R= 0.377 p= 0.357	R= - 0.649 p= 0.081(*)	R= 0.066 p= 0.876	R= - 0.428 p= 0.290

Table 3.2 Correlations between maternal aggression and AC/ADP OXT and pSTAT5 expression

Summary of the explored correlations between behavioural variables of maternal aggression (logarithm of the latency to first attack, total attack duration and number of attacks) and histological variables of OXT and pSTAT5 expression measured in the AC/ADP (number of OXT neurons, pSTAT5-ir density, percentage of pSTAT5 expression over OXT neurons and percentage of pSTAT5 expression over OXT-negative neurons) in the dam group. For each correlation, the correlation coefficient (R) as well as the statistical significance (p) are listed. All the depicted correlations were performed using Pearson's parametric method. Neither godmothers nor virgins were analysed separately, as they did not display any aggressive behaviour. Significant correlations and non-significant trends are highlighted with a red frame and a pink frame, respectively. *$p<0.05$; (*) $p\leq0.081$.

Correlational Analysis

We searched for significant correlations between behavioural parameters of maternal aggression (logarithm of the latency to first attacks, total attack duration and number of attacks) and pSTAT5 and OXT expression in the AC/ADP. Since dams were the only group displaying aggressive behaviour at any rate, we restricted our analysis to this experimental group (Fig. 3.10 and Table 3.2). First, using Pearson's correlation we found a robust and significant negative association between OXT-expressing neurons of the AC/ADP and the logarithm of the latency to first attack of dams (R=-0.784; p=0.021; Fig 3.10 A) (but not with number of attacks or total attack time). This finding indicates that dams with increasing number of OXT neurons attack quicker. Furthermore, using Pearson's method we found a strong trend to significance between pSTAT5-ir density and the logarithm of the latency to first attack (R=0.684; p=0.061; Fig 3.10 B) and a weaker trend between pSTAT5-ir density and the number of attacks (R=-0.614; p=0.081; Fig 3.10 C). The sign of both correlations indicates that dams with higher pSTAT5-ir densities are indeed less prone to attack male intruders.

3.4. DISCUSSION

Maternal behaviours involve both an increased motivation for pups, which acquire strong reinforcing properties for dams, as well as the display of aggressive reactions for nest defence. Herein, we discuss the implications of our findings on the possible roles that PRL might play on the neural substrate of such behaviours, particularly on the modulation of AC/ADP neurons of the OXT system. First, we discuss the validity of our new test of pup retrieval to assess maternal motivation for pups. Second, we analyse the changes in the expression of OXT and in PRL responsiveness in the hypothalamic AC/ADP, resulting of prolonged pup exposure as well as pregnancy and lactation. Finally, we explore possible associations of different maternal behaviours (maternal aggression and motivated pup retrieval) with OXT expression and PRL responsiveness in the AC/ADP, in search of hints on the involvement of this nucleus in maternal behaviour regulation.

3.4.1. MOTIVATED PUP RETRIEVAL: A NOVEL BEHAVIOURAL DESIGN FOR THE STUDY OF MATERNAL MOTIVATION

In the classical view of Berridge and Robinson (1998), motivational processes are characterized by three main elements: “liking”, “wanting” and “learning”. Liking is an affective, hedonic reaction (pleasure or reward) to a reinforcer, in our paradigm pups. The first experiences with a given reinforcer elicit learning processes of different kinds (conditioned stimulus association, stimulus-response association). Wanting describes how attention is directed towards a given stimulus that possesses incentive salience (directional aspects of motivation) and how resulting behaviour is energized (activational aspects of motivation) to reach (or avoid) the given reinforcer (Salamone et al. 2016) . The learning processes associated with the first experiences with a reinforcer, in turn, lead to a strong wanting for it and its related stimuli. This wanting represents motivation associated to a reward. In line with this paradigm, maternal motivation can be defined as a sustained state where pups and all the stimuli associated with them acquire incentive salience and elicit proactive maternal responses (i.e. involving directional and activational processes) in the female (Olazábal et al. 2013).

Among the multiple components of maternal behaviour, pup retrieval is commonly regarded as the canonical example of a proactive, pup-directed (motivated) maternal behaviour (Champagne et al. 2004; Gammie 2005; Numan and Woodside 2010). Hence,

conventional pup retrieval tests are usually chosen to assess levels of maternal motivation in rodents (Pedersen et al. 2006; Bayerl et al. 2014). Following this rationale, there have been several attempts to characterise the acquisition of maternal motivation in different models of maternal sensitisation in rodents. In the rat, for instance, virgin females that initially avoid pups are able to develop some degree of maternal motivation after continuous pup exposure (during 8-13 days), but this level of motivation towards pups is low as compared to that of dams (Seip and Morrell 2008). In mice, conversely, the lack of an initial pup aversion among virgin females leads to an apparently different scenario. As our lab (Martín-Sánchez et al. 2015b) and others (Stolzenberg and Rissman 2011) have found, different models of pup-sensitised virgin females (including our godmothers) do not differ significantly from dams when facing a conventional pup retrieval test, they retrieve pups as quickly as lactating dams. This finding might lead to the erroneous impression that pup-sensitised virgins show an equivalent motivation for pups to that of dams, since this classical test of pup retrieval requires no especial effort, that is, does not regard activational aspects of motivation. Motivational processes influence decision making by balancing effort and reward of a given incentive stimulus (Salamone et al. 2016). Hence, proactive maternal behaviours might be easily expressed under lower levels of maternal motivation if the context is not challenging enough for the individual to reach the incentive stimulus. In other words, pup retrieval tests performed in the home cage under standard conditions might require very low motivation thus not allowing discrimination between dams and virgin female mice.

For this reason, we have developed a novel variant of the pup retrieval paradigm in which pup retrieval must be performed under a challenging situation, requiring a high motivation given the strong effort of bringing pups back to the nest. Our design, termed "*motivated pup retrieval*", consists of a pup retrieval assay where experimental animals must overcome an obstacle (climb a 10-cm high wire-mesh wall or barrier) and climb it back with the pups. The design of this test is adapted from effort-related decision making paradigms, which assess motivation by confronting the experimental animals with the choice of a high effort/high reward stimulus and a low effort/low reward stimulus (Yohn et al. 2016).

Our results confirm the validity of our novel design for the measurement of motivation for pups. Previous designs have employed alternative strategies in which access to pups

also posed a challenge, for instance facing a T-maze (a new, anxiogenic environment) (Bridges et al. 1972; Stern and Mackinnon 1976; Mackinnon and Stern 1977; Mayer et al. 1979). These strategies involve not just motivation but fear or anxiety that the female has to overcome. Since this emotional responses are also altered in dams, as compared to virgin females (Neumann et al. 2000), these tests do not properly separate the maternal motivation from fearfulness or anxiety. By contrast, the barriers in our test do not elicit any anxiety or even aversive response, as indicated by the number of barrier crossings performed by our experimental animals (Fig 3.3A) which is similar in all three groups of females. On the other hand, our results support the conclusion by Martín-Sánchez et al. (2015b) indicating that pups themselves are not anxiogenic or aversive for virgin females. In fact, even if the females have to cross the barriers to contact the pups, latency to pup contact and total contact time are similar among all of our experimental groups (Fig. 3.3B and C). Therefore, differences among the females must pertain to motivation.

As expected, dams displayed significantly higher levels of pup retrieval than pup-naïve or sensitized virgins (godmothers; Figs. 3.4 and 3.5), which virtually did not retrieve pups in the first tests. This finding evidences that continuous pup-derived sensory stimulation alone is not able to induce maternal motivation in comparable levels to dams, in contrast to what was previously thought (Stolzenberg and Rissman 2011; Martín-Sánchez et al. 2015b). Thus, neuroendocrine inputs of pregnancy and lactation (likely including PRL) are indispensable for the development of full maternal motivation. Even if dams displayed a significant improvement in motivated pup retrieval through the experiment (Fig 3.5), this might not reflect an increase in motivation but a procedural learning: in the first trial, latency to pup retrieval is longer probably due to the time it takes to look for and find a solution to the challenge of carrying pups through the barrier. Once they find the solution, dams show no further improvement (trials 2 and 3; Fig. 3.5), indicating full maternal motivation.

Furthermore, although godmothers and pup-naïve virgins initially displayed no pup retrieval (Figs 3.4 and 3.5), godmothers improved progressively in subsequent trials and by trial 3 displayed consistent pup retrieval, thus significantly differing from pup-naïve virgins (Fig. 3.4). This finding has two important implications. First, it indicates that prolonged pup exposure is also able to induce certain motivation for pups, although not as effectively as its synergistic action with endocrine signals of pregnancy and lactation.

Second, this gradual acquisition of motivation for pups reflects a process of maternal sensitisation in virgin female mice. Therefore, even if virgins show quasi-spontaneous maternal behaviour, they are still subject to a sensitisation process in which pup stimuli alone are able to increase motivation for pups.

Summarizing, we have developed and validated a novel design for the study of maternal motivation based on the pup retrieval test and the effort-based decision making paradigm. This design has revealed clear differences in maternal motivation between dams and pup-sensitised virgin females (godmothers). This group, in turn, experiments a progressive increase in maternal motivation as a result of a continuous pup exposure, leading to consistent but not fully maternal levels of motivation for pups. Altogether, even if both pup stimuli and endocrine signals of motherhood contribute to the onset and maintenance of maternal motivation, neuroendocrine adaptations of the maternal brain are instrumental for the timely development of full maternal motivation.

3.4.2. OXYTOCIN EXPRESSION AND PROLACTIN RESPONSIVENESS OF THE AC/ADP: CHANGES DURING LACTATION

Given the prominent role of OXT in maternal physiology and behaviour (Bosch and Neumann 2012; Kim and Strathearn 2016), the neural system in charge of the production and release of this nonapeptide is a primary target to undergo maternal adaptations. Among the different populations integrating the central OXTergic pathways, the OXT neuron population of the AC/ADP (Otero-García et al. 2015) is a normally underscored but interesting object of study, given its privileged position in the BSTv/MPA region of the preoptic hypothalamus (key site in maternal behaviour regulation, Terkel et al. 1979; Numan et al. 1998; Consiglio and Bridges 2009). In addition, as shown in Study 2, this is one of the regions in which pSTAT5-ir is increased during pregnancy and lactation. For the above stated reasons, in the present work we conducted an immunohistochemical analysis of OXT and pSTAT5 expression in the AC/ADP region of both of our experimental samples (tested for maternal aggression and for motivated pup retrieval, respectively). In this section, we will focus on commenting changes in AC/ADP OXT neurons and PRL responsiveness in lactating dams, reflecting possible hints of maternal adaptations of this brain region. Conversely, the effects of pup exposure on OXT and PRL signalling in the AC/ADP of godmothers will be discussed below (section 3.4.4).

Concerning OXT, we found no significant changes in the number of OXT-expressing neurons in the AC/ADP of lactating dams, godmothers or pup-naïve virgins of neither of both experimental samples (Fig 3.7B). This replicates previous data of our lab (Martin-Sanchez 2016) excluding the possibility of new OXT neurons appearing during motherhood, or that previously existing neurons of the AC/ADP began to express OXT. Rather than changes in the size of the OXT population, it is reasonable to expect more subtle changes in the activity of OXT neurons associated to lactation (Sirzen-Zelenskaya et al. 2011; Kim and Strathearn 2016). A possible outcome of such changes could be an increase in OXT production, but this cannot be properly measured with the methodology employed in this work.

Regarding PRL signalling in the AC/ADP, our data show a significant increase in pSTAT5 expression during lactation in both experimental samples (maternal aggression and motivated pup retrieval), as compared to pup-naïve virgin females and godmothers (Fig. 3.7A). This observation is in line with our previous findings (chapter 2 of this work) of heightened PRL-derived signalling in the AC/ADP region during lactation, in the context of an increased PRL input of the whole BSTv/MPA area during motherhood. However, it is worth mentioning that lactating dams of the two experimental samples show significant differences in the density of pSTAT5 expression, with dams tested for aggression showing significantly greater density ($p=0.019$ on a t-test for independent samples, data not illustrated in Fig 3.7A) than dams tested for motivated pup retrieval (Fig. 3.7A). The reason for this difference is unknown, because the observed difference appears to be specific of the groups of lactating dams and not shared by all three experimental groups. Furthermore, it is worth reminding that experimental females of both experiments shared equivalent ages (10-11 weeks), housing conditions, number of pups per litter (8) and were sacrificed with only one day difference (PPD5 and 6 for motivated pup retrieval and maternal aggression, respectively). However, it should also be noted that behavioural testing was carried out by different experimenters at different time points. Anyhow, this leads to pose the question of whether behavioural testing (i.e. resident-intruder test versus maternal motivation test) had a differential influence on PRL signalling in dams. Given the well-known role of PRL in the stress response (Neill 1970; Torner et al. 2001), the stressing nature of aggression tests might be at the base of the observed differences in pSTAT5-ir levels in dams, coincidentally the only group in which testing elicited aggression. Further research is required to address this particular issue.

In a functional study using c-Fos expression mapping, Tsuneoka and collaborators (2013) showed a substantial increase in c-Fos expression in response to pup interaction in a pup retrieval test among the OXT neurons of the AC/ADP in postpartum females, as compared to virgin females. This strongly suggests that, because of pregnancy and parturition, the AC/ADP OXT neurons increase their responsiveness to pups, implying a possible participation in maternal behaviours. There are two possible mechanisms mediating this change on AC/ADP OXT cells. On the one hand, pup-sensitive cells that did not express OXT at histochemically detectable levels in the brain of virgin mice could start expressing OXT after delivery. Our results contradict this possibility, as we found no significant differences in AC/ADP OXT cell number between virgins (either pup sensitised or not) and lactating dams in neither of both experimental samples (Fig 3.8 A and 3.9 A). Alternatively, pup-insensitive, OXT-expressing cells in the brain of virgin females could become pup-responsive, which is more likely in the light of our data. In this case, the increase in pup responsiveness of these cells could be conveyed either through endocrine stimulation (likely including PRL) or, alternatively, through non-endocrine mechanisms, where pup-derived sensory stimulation would play a greater role.

Given these possibility, we tested the hypothesis that PRL might be at the base of the maternal increase in pup responsiveness specifically of AC/ADP OXT cells. To explore this, we characterised the specific targets of the input of PRL on the AC/ADP by analysing the percentage of pSTAT5 expression over total OXT-positive neurons and over total OXT-negative neurons in the AC/ADP of virgins, godmothers and lactating dams. Even though our analysis revealed an important percentage of OXT-responsive neurons in the AC/ADP (roughly a 70% of the total OXT neurons), we found no significant changes in this percentage between lactating dams and the remaining groups of virgin females. However, PRL input over OXT-negative neurons in the AC/ADP did increase significantly during lactation, as compared to virgins (Fig 3.8). This increase was highly significant in our maternal aggression sample ($p<0.01$, Fig 3.8 A), whereas the sample tested for motivated pup retrieval showed a weak trend to significance ($p=0.093$, Fig 3.8 B). Our data indicate that PRL input over OXT neurons of the AC/ADP is similar in virgin and postpartum females, but OXT-negative neurons surrounding this OXT population do receive a significantly increased PRL input during postpartum. Therefore, the increased sensitivity of OXT neurons to pup stimuli in postpartum, reported by Tsuneoka et al. (2013) is likely not due to a direct action of PRL over OXT neurons.

This finding leaves an open scenario with multiple, non-mutually exclussive possibilities to explain the maternal increase in pup responsiveness of the AC/ADP OXT population (shown by Tsuneoka et al. 2013). On the one hand, a modulatory role of PRL at other levels of the circuit would still be plausible. In fact, PRL could modulate AC/ADP OXT neurons indirectly through adjacent interneurons, which according to our data could receive an increased PRL input. Alternatively, PRL could also modulate other nodes of the circuit, either upstream or downstream the AC/ADP. Figure 3.12 summarizes some of the possible sites of action of PRL to mediate this process.

In this sense, some afferents from key regions of the SBN that project to the AC/ADP and in which we found significant increase of pSTAT5-ir likely contribute substantially to the process, too. For instance, neuroanatomical tract-tracing studies indicate an afferent connection between the medial amygdala, a classic region receiving and integrating chemosensory stimuli, to the AC/ADP region (Pardo-Bellver et al. 2012). The medial amygdala can convey inputs of pup-derived chemical stimuli over the AC/ADP which, along pup vocalizations (D'Amato et al. 2005), are key for the females to identify their pups. Moreover, Scott et al. (2015) identified a sexually-dimorphic dopaminergic neuron population in the AVPV region of the preoptic hypothalamus (AVPe) that, according to their model, delivers pup-derived stimuli (although it is not specified which and from where) to different OXT neurons to promote maternal care. Even though the authors focus on analysing the projection of these dopaminergic cells to the Pa, their results indicate that these do also project to the AC/ADP region, resulting in another candidate to convey pup-derived sensory inputs to the AC/ADP OXT neurons. Remarkably, PRL could also have a role modulating these inputs, as supported by our findings in Study 2 that the MePD and AVPe display a substantial response to PRL during pregnancy and lactation. These possibilities are summarized in a speculative model in Fig 3.XX, but, anyhow, this issue requires further exploration, of the connectivity and functional responses of the AC/ADP during the maternal period.

3.4.3. PROLACTIN RESPONSIVENESS OF THE AC/ADP REGION: EFFECT OF PUP EXPOSURE

This study is, to our knowledge, the first evaluation of the effect of maternal sensitisation on PRL-derived signalling in the female rodent brain. Our experimental design included a group of "godmothers", virgin females cohabitating with dams and pups. As a reminder, godmothers display: (1) pup-directed allomaternal care in virtually

equivalent levels to dams (Martín-Sánchez et al. 2015b); (2) significantly lower motivation for pups than dams (our results); (3) no maternal-like aggression towards intruders (Martín-Sánchez et al. 2015a). This model constitutes an opportunity to study not only the behavioural consequences of prolonged pup exposure, but also possible neuroendocrine effects in the female brain underlying maternal sensitisation.

Two main, not mutually exclusive options stand out when considering the neural mechanisms directing the process of maternal sensitisation. First, continuous pup exposure could activate endocrine mechanisms (such as the release of PRL in response to pup stimulation of the ventral trunk area Cservenák et al. 2013), that would contribute to maintenance of lactation and maternal behaviours (Garland and Svare 1988). As exemplified here, PRL is a major candidate to mediate such mechanisms. Alternatively, pup stimulation could also trigger maternal sensitisation through non-endocrine mechanisms, e.g. the action of pup-derived stimuli exclusively through neural sensory pathways. In this regard, our data indicate that, at least in the AC/ADP region, five days of continuous pup exposure to pups did not trigger any significant change in the level of PRL responsiveness in godmothers (Fig. 3.7). Nevertheless, this does not exclude the possibility that other relevant brain regions exhibit different profiles in response to persistent pup stimulation. In fact, female mice bearing a deletion of the PRLR gene showed significant deficits in pup retrieval as compared to wild-type females (Lucas et al. 1998a), suggesting a putative PRL-mediated mechanism intervening in the process of maternal sensitisation. In addition, in species with biparental care, paternal behaviour is associated with PRL-associated endocrine changes elicited by pup interaction and with prior paternal experiences (Brown et al. 1995; Ziegler and Snowdown 2000). Therefore, it is feasible to expect a similar mechanism at the base of allomaternal care in virgin female mice. Altogether, a systematic analysis of pSTAT5 expression throughout the brain is warranted to detect possible critical areas of PRL action in the process of maternal sensitisation.

3.4.4. ROLE OF THE AC/ADP IN MATERNAL BEHAVIOUR: CORRELATIONAL ANALYSIS

For the final stage of this study, we confronted our behavioural data on motivated pup retrieval and maternal aggression with our histological data on OXT expression and PRL-derived signalling on the AC/ADP. In doing so, we sought to find associations hinting

possible roles of the AC/ADP region in the regulation of maternal behaviours within the experimental groups that did express these behaviours.

Maternal motivation

According to our findings, the number of AC/ADP OXT-expressing neurons correlated negatively with the total number of retrieved pups in both the dam and the godmother group. In the first place, these findings lend further support to the effect of maternal sensitisation on motivation reported in this study. As illustrated in Figure 3.11, the slope of the regression lines is similar for godmothers and dams, with lower levels of retrieval in godmothers as compared to dams. In addition, these correlations indicate that the greater the OXT neuron population of the AC/ADP, the lower motivated pup retrieval performance. This is at first surprising in the view that OXT is well-known to promote maternal care rather than hinder it (Bosch and Neumann 2012). Nevertheless, this finding can be better understood in the context of anxiety. The release of OXT in different brain sites like the Ce or the Pa has been attributed anxiolytic properties (Blume et al. 2008; Knobloch et al. 2012). On the other hand, anxiety may exert complex and seemingly paradoxical effects on maternal behaviour expression, as reported by the observation of female rats selectively bred for high (HAB) or low (LAB) anxiety (Bosch et al. 2005). For instance, HAB rats, which show high innate anxiety persisting through the maternal period, do also display a highly protective parenting style, including high levels of maternal care and comparatively low latencies in pup retrieval tests (Bosch 2011). Thus, in the context of our results, if OXT release from the AC/ADP OXT were related to the regulation of stress and anxiety, as is the case for OXT released in the Ce and Pa, it is feasible that the number of AC/ADP OXT neurons correlate negatively with pup retrieval performance. In other words, dams and godmothers with less OXT-expressing neurons in the AC/ADP would display higher levels of anxiety and would retrieve pups even more eagerly in the motivated pup retrieval test, reflecting a more protective parenting style. Anyhow, this result suggests that the AC/ADP OXT neuron population would not be directly involved in mediating an increase in maternal motivation, but likely linked to maternal care through its effects on anxiety.

Regarding PRL, we found no direct evidence to link changes in the input of PRL on the AC/ADP to the expression of motivated pup retrieval. Neither pSTAT5-ir in the whole AC/ADP region nor percentage of pSTAT5 expression in OXT-positive or OXT-negative neurons of the AC/ADP correlated significantly with motivated pup retrieval (Table 3.2).

This suggests that there is no direct involvement of PRL action on the AC/ADP in the regulation of maternal motivation.

Maternal aggression

In the present study, we found additional correlations linking OXT and maternal aggression. The number of AC/ADP OXT-expressing neurons is negatively correlated with the logarithm of attack latency to intruder males in dams. Hence, lactating dams with greater number of OXT-expressing cells in the AC/ADP tend to have quicker displays of maternal aggression. This evidence is coherent with previous findings of our lab (Martín-Sánchez et al. 2016) positively correlating maternal aggression with OXT expression in the AC/ADP. It is worth noting, nevertheless, that this study found significant correlations with other measures of maternal aggression, i.e. number of attacks and total attack duration, which we did not replicate directly. This might be due to slight differences in the sampling of the AC/ADP in both studies but in any case reveals coherent correlations linking higher levels of maternal aggression with bigger AC/ADP OXT neuron populations. Importantly, other measures of OXTergic activity have been also linked positively to maternal aggression. For instance, it has been reported how, in postpartum dams, maternal aggression displays are coincident with increased release of OXT in the central amygdala (Ce) and the hypothalamic paraventricular nucleus (Pa) (Bosch et al. 2005). The release of OXT in these sites, in turn, has been attributed anxiolytic properties, as commented above. In turn, anxiety has been negatively correlated to maternal aggression in mice, so that lower levels of anxiety are linked to higher maternal aggression (Maestripieri and D'Amato 1991; Maestripieri et al. 1991; Parmigiani et al. 1999). Altogether, higher OXT activity and release in the Ce and Pa would lead to a decrease in anxiety and to an enhancing effect on maternal aggression. Our findings regarding AC/ADP OXT neurons suggest, in turn, that this OXT population might also be involved in the release of OXT in the Ce and Pa and its anxiolytic, aggression-promoting effect. Further analysis of the OXT terminals of the AC/ADP and Pa of our specimens will contribute to test this hypothesis.

Regarding PRL, we found no direct evidence to consistently link changes in the input of PRL on the AC/ADP to the regulation of maternal aggression. However, we did find two moderate but non-significant correlations among dams. According to them, pSTAT5-ir in the AC/ADP correlated positively with the latency to first attack (on a logarithmic scale) and negatively with the number of attacks. These trends indicate a possible negative

association between PRL action in the AC/ADP and maternal aggression: the higher levels of PRL-derived signalling, the lower aggression. The functional significance for this association is unknown, but it could possibly involve the modulation of OXT release by AC/ADP neurons to exert a fine-tuned inhibitory control over the expression of maternal aggression. More evidence is needed to test this hypothesis.

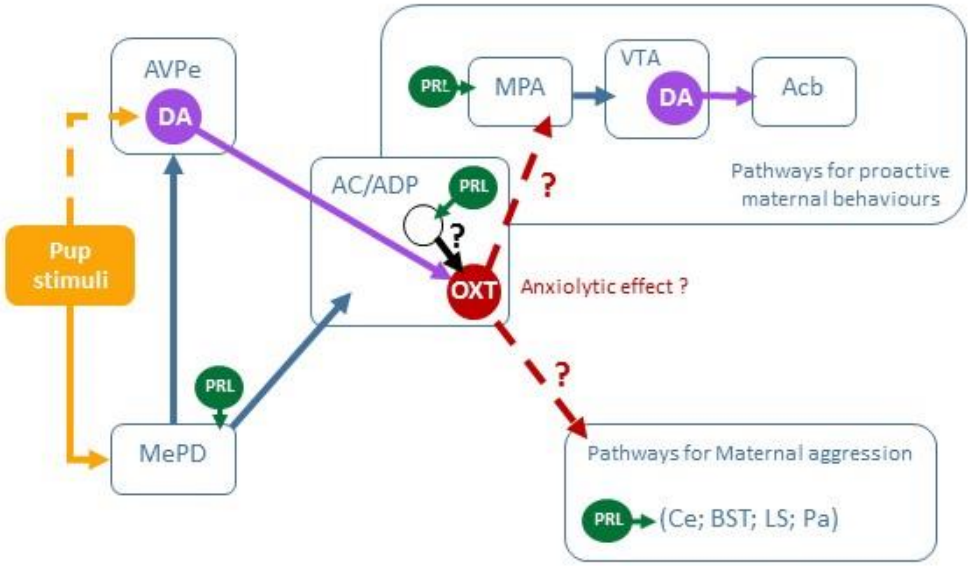


Figure 3.12 Possible role of AC/ADP oxytocinergic neurons in maternal behaviour regulation

Speculative model integrating evidence from this and previous work on the putative roles of AC/ADP OXT neurons in maternal behaviour regulation. According to Tsuneoka et al. (2013), AC/ADP OXT neurons (red) become pup responsive during the postpartum period. This process could be mediated by the indirect input of PRL (green) on other related neurons of the AC/ADP (black), according to our evidence. Alternatively, it could also be mediated by the input of PRL or on other nuclei upstream the AC/ADP conveying pup-derived stimuli, or a direct action of these nuclei. Putative nuclei relaying pup-derived stimuli to the AC/ADP include the MePD (Pardo-Bellver et al. 2012) or the AVPe, which contains a sexually-dimorphic dopaminergic pathway (purple) responsible for this particular function (Scott et al. 2015). In turn, AC/ADP OXT neurons could possibly project to other nuclei or pathways involved in proactive (motivated) maternal behaviour (mesolimbic dopaminergic pathway) or maternal aggression (Ce, Pa, LS and/or BST, Davis and Shi 1999; Bosch and Neumann 2012), releasing OXT to exert an anxiolytic effect.

Role of AC/ADP in the maternal period

The correlational analysis of both experiments (motivated pup retrieval and maternal aggression) does not support a direct role of AC/ADP OXT neurons in maternal behaviour. However, they do suggest a direct action on stress and anxiety regulation, as discussed above, which in turn would influence both maternal care and aggression. To further contrast this hypothesis, it would be advisable to analyse the projections of AC/ADP OXT neurons to other brain sites that participate in the control of stress and anxiety, for instance the Pa (Blume et al. 2008), the septum (Numan and Insel 2003), the anterodorsal BST or the Ce (Davis and Shi 1999). Hence, it would be of interest to explore putative functional and/or anatomical connections between the AC/ADP and these centres. In this regard, the OXT neurons of the AC/ADP, located just rostral from those of the Pa, have been proposed to build an anatomical and functional continuum with the latter, based on evidence of common developmental origin (Otero-García et al. 2015).

Altogether, our findings and those of others reflect the high complexity of maternal behaviour regulation. The different components of maternal behaviour (maternal care and aggression) are integrated by different (Fig I.8) but partially overlapping (the SBN, Fig I.6) neural substrates. These, in turn, might be differentially modulated by other neural responses, e.g. stress and anxiety, as proposed here.

3.4.5. CONCLUSIONS

In this chapter, we have analysed the involvement of the OXTergic neuron population of the AC/ADP in the regulation of maternal aggression and motivated maternal care. We have also tested the interaction of PRL with this OXT population in Dams, Godmothers (subject to maternal sensitisation) and naïve Virgins. On the behavioural side, and in addition to maternal aggression (discussed here, Martin-Sánchez et al. 2015a), we have found that the acquisition of full maternal motivation requires the action of neuroendocrine signals of motherhood. However, maternal motivation also increases gradually as part of a process of maternal sensitisation driven by continuous pup exposure in females not undergoing the hormonal changes associated to pregnancy, delivery and lactation.

Furthermore, we have analysed changes in OXT expression and PRL-derived signalling in the AC/ADP during lactation and under continuous pup exposure. According to our

findings, both OXT neuron number and PRL input over these OXT neurons remain unchanged in postpartum females as compared to virgins, but PRL action onto the AC/ADP increases in postpartum females, specifically in OXT-negative neurons. These findings do not fit the hypothesis of a direct maternal modulation of the activity of this OXT cell population by PRL, but do not rule out the possibility of an indirect regulation through other AC/ADP neuron populations.

Finally, we performed a correlational analysis to link our behavioural and histological measures. This analysis revealed a significant positive correlation between the number of AC/ADP OXT neurons with maternal aggression and a significant negative correlation with motivated pup retrieval. In spite of seeming contradictory at first, these findings fit the explanation that AC/ADP OXT neurons are involved in anxiolysis, leading thus to increased aggression and preventing the expression of a protective mothering style. Moreover, we found a non-significant trend for a negative correlation between pSTAT5-ir density in the AC/ADP and maternal aggression, suggesting a possible inhibitory control of aggressive behaviour through modulation of AC/ADP function. Overall, these data reflect complex and multifactorial roles of OXT in the expression of maternal behaviour, as well as a complex regulatory interaction of PRL with the OXT neuron population of the AC/ADP.

4

GENERAL DISCUSSION

Prolactin is a pleiotropic hormone that has in the brain one of its most remarkable signalling targets. Despite being a polypeptide, this hormone gains access to the CNS bypassing the blood-brain barrier to exert multiple central functions. The present work provides a comprehensive analysis of the neuroanatomical distribution of the signalling exerted by PRL in the mouse brain, both in males and females. In addition, given the close link between PRL, reproductive function and its neuroendocrine control, we have evaluated how gonadal steroids (estradiol and progesterone in females, testosterone in males) contribute to shape the aforementioned patterns of PRL signalling in the male and female brain. The most well studied roles of PRL are related to the period of motherhood, where this hormone drives central changes to adapt the female's physiology and behaviour to the particular demands of motherhood. For this reason, we have also characterised the distribution and extent of the changes in central PRL-derived signalling patterns in the pregnant and lactating female brain. The expression of maternal behaviour is one of the most remarkable maternal adaptations, and includes maternal care driven by a high motivation for pups, and the territorial defence of the pups and nest site, maternal aggression. In this work, we have also analysed the involvement of PRL in the onset of these behaviours, specifically maternal motivation and maternal aggression, with a particular focus on the action of PRL over a particular OXTergic neuron population, located in the AC/ADP.

The following section will be devoted to discuss the functional implications of the findings reported in this work. We will first comment on how PRL might gain access to the brain to produce the signalling patterns we have reported. Then, we will analyse the possible involvement of PRL in the central regulation of central homeostatical and reproductive functions in the brains of males and females (maternal or not). Finally, we will focus on revisiting the roles of PRL and other lactogenic signals mediating the expression of maternal behaviours.

A. PROLACTIN CROSSES THE BLOOD BRAIN BARRIER TO MODULATE BRAIN FUNCTION

The first outcome of our findings throughout the present work is the confirmation of the sensitivity of the brain to PRL. In fact, the brain is responsive to the hormone in males and females in different reproductive stages. It is generally assumed that the central patterns of PRL responsiveness examined in this and other works are elicited by systemic PRL, mainly, if not entirely, of hypophyseal origin. For PRL signalling to occur in the brain, PRL must first gain access to the CNS from the bloodstream. Circulating PRL is classically thought to bypass the Blood-Brain Barrier (BBB) into the cerebrospinal fluid (CSF) at the level of the choroid plexus, by means of a transportation mechanism dependent on the PRLR (Walsh et al. 1987). In addition, circumventricular organs might provide an exceptional access point of PRL to the CSF, too (Ganong 2000).

However, this view has been recently challenged by a study where radiolabelled PRL access to the brain was examined in PRLR-knockout female mice (Brown et al. 2015). This study concluded that the access of PRL into the brain occurs mostly unabated in the absence of the PRLR and, importantly, that the entrance rate through the choroid plexus into the CSF is too slow to explain the relatively fast response of the brain that was observed (45 minutes after exogenous PRL supplementation, see Section 1.2.4). Furthermore, it is important to recall that PRL is a protein of high molecular weight and size, resulting in a relatively low passive diffusion capacity through the neural parenchyma. Considering the relatively short half-life of the PRL molecule (approximately 40 minutes, Yoshida et al. 1991), if the choroid plexus and ventricular system were the major access point of the hormone into the brain, PRL would only reach and produce its signalling in sites close to the ventricular system, before being degraded. This situation could suffice to explain the patterns of PRL-derived signalling found among our sample of male mice, where PRL-responsive brain nuclei were mainly located in the hypothalamus and distributed around the ventral part of the third ventricle. Conversely, it is not coherent with the more extensive patterns reported for virgin female mice, much less for pregnant and lactating specimens. In these animals, lactogenic signalling was found in locations far away from the choroid plexus and the ventricular system, all of which suggests the existence of alternative mechanisms to grant a more uniform access of PRL to the brain. Perhaps, the evident dimorphic differences in central patterns of PRL responsiveness we report in this work are partially

due (in addition to a more restricted PRLR distribution and other conditions) to different systems assisting the access of PRL into the brain, with females depending on alternative or additional mechanisms that would better fit their more extensive response patterns.

A possible alternative access of PRL into the brain, albeit not yet properly studied, could be through the cerebral microvasculature. As in case of many other molecules, PRL could be moved by specific transporters across endothelial cells into the extracellular space of the brain (Dutt et al. 1994). An additional mechanism to explain this process could involve the production of endogenous PRL in the brain. Evidence in the literature points to the existence of PRL circuits in the brain, with PRL-synthetizing neurons located mainly in the hypothalamus and immunoreactive fibres widespread throughout the brain (Paut-Pagano et al. 1993; Mejía et al. 2003). This system could possibly contribute to some degree to elicit the pSTAT5-ir patterns analysed in the present work. In this sense, Torner et al. (2004) showed that PRL is released in a paracrine fashion from the Pa and MPA of female and male rats under certain physiological circumstances (stress, lactation), and that this release can be coupled or not to hypophyseal PRL release and subsequent rise in systemic PRL levels. Although this evidence does not demonstrate the intracerebral release of PRL in response to the elevation of circulating PRL (elicited in our study by exogenous PRL supplementation) it does indeed show that both PRL releasing systems (intracerebral and systemic) are not independent from each other, but work in an interconnected fashion to provide brain and/or body with PRL when required.

B. PROLACTIN REGULATES HOMEOSTATICAL FUNCTIONS UNDER DEMANDING SITUATIONS: STRESS AND MOTHERHOOD

In the present work, we have examined and compared the patterns of PRL responsiveness of the mouse brain in males and females of different reproductive stages. Despite finding an evident sexual dimorphism and substantial differences in responsiveness regarding females on different physiological conditions, we have indeed identified a number of brain nuclei responsive to PRL common to all the described states (males, virgin females, pregnant females and lactating dams). This likely reflects the

existence of ubiquitous roles of PRL in the mouse brain, independent of sex or reproductive conditions.

The most apparent and well-documented example of such role of PRL is the feedback regulation of its own hypophyseal release (Fig I.3). As introduced before, this negative feedback regulation takes place in the TIDA neurons of the Arc and in the PHDA neurons of the Pe, and is mediated by STAT5b (Ma et al. 2005b). Therefore, when circulating PRL levels are high (due to enhanced hypophyseal PRL release or to exogenous PRL supplementation) these dopaminergic neuron populations are increasingly activated by PRL through the Jak/STAT pathway and STAT5b phosphorylation, to inhibit the release of PRL by hypophyseal lactotroph neurons. It is worth recalling, however, that these brain regions also contain other PRL-responsive cell populations not related to PRL feedback regulation (e.g. kisspeptin neurons in the Arc, see below), so that measured levels of pSTAT5-ir do not necessarily predict directly levels of circulating PRL. For this reason, for example, PRL-supplemented male and female mice do show significantly different pSTAT5-ir densities in the Arc (Fig 1.3) despite both likely having equivalent, supraphysiological circulating PRL levels.

Studies in lactating rodents ascribe an important role to PRL in the regulation of energy balance and food intake (Grattan et al. 2001). This physiological trait is controlled by a complex network involving several hypothalamic nuclei, as well as different neurochemical signals (Elmquist et al. 1999). According to our findings, some of the hypothalamic centres belonging to this network (namely the Arc, the VMH, the DM and the Pa) display responsiveness to PRL in the pregnant and lactating brain (Fig 2.1 e-h), but also in the brains of virgin female and the male mice (Fig 1.1 e,f and 1.3). Remarkably, part of these nuclei (the Arc, the VMH and the DM) are also known to express the leptin receptor. Leptin is an endocrine signal secreted by the adipose tissue that is presumed a pivotal role in the neuroendocrine control of food intake as an adiposity signal (Flier 1998). Taken together, our findings and the evidence mentioned above indicate that a similar role to that of PRL in the control of energy balance and food intake during motherhood might also take place in the brain of non-lactating females and males, under certain physiological circumstances concurrent with high levels of circulating PRL.

Prolactin is also proposed to modulate anxiety and stress. First, PRL is a neuroendocrine agent involved in the stress response, as stress leads both to the acute rise in systemic

PRL levels (Fujikawa et al. 2004; Torner 2016), as well as to the paracrine release of PRL from the Pa (Torner et al. 2004). The presence of pSTAT5-ir in the Pa of males and virgin females (Fig 1.1e and 1.3) suggests a feedback effect of PRL modulating the reactivity of the HPA axis, both at a systemic and at a central level (paracrine release form the Pa), an effect recently demonstrated in male mice (Kirk et al. 2017). Although the involvement of PRL in the stress response has been mainly studied in maternal individuals (Grattan 2002), the patterns of PRL responsiveness we found show that this is extensive to non-maternal females and males. This role of PRL would probably be mediated through the action on corticotropin-releasing factor (CRF) or vasopressin (AVP) cells (Mejía et al. 2003; Aguilera et al. 2008). In fact, PRL is a well-documented regulator of the function of magno- and parvocellular vasopressinergic neurosecretory cells at the level of the hypothalamic Pa (Donner and Neumann 2009; Blume et al. 2009; Vega et al. 2010). Although the effect of PRL on these parvocellular AVP cells has been proven to be mediated by ERK/MAPK pathway (Blume et al. 2009), our data in males and females suggest that the Jak/STAT5 signalling cascade can also be involved. This would imply a non-dimorphic regulatory role of PRL on the stress response. Further experiments are needed to evaluate where PRL acts directly onto AVP expressing parvocellular cells in the Pa.

In addition to its effect on the HPA axis and stress response, studies in female rats indicate that PRL has anxiolytic properties when acutely administered i.c.v. or i.v., whereas chronic i.c.v. antisense targeting of the PRLR results in increased anxiety and of the associated HPA axis response (Torner et al. 2001). In contrast to females, PRL has a weak anxiolytic effect in males (Torner et al. 2001). This intersexual difference might be related to our findings of a sexually dimorphic responsiveness to PRL in the central structures directly involved in the regulation of anxiety and emotionality, the Ce and the anterodorsal BST (Davis and Shi 1999). In these nuclei, females show apparent pSTAT5 immunolabelling, especially during pregnancy and lactation (Fig 2.6), whereas males are devoid of pSTAT5-ir in these structures (Fig 1.1c and d).

In both males and females, pSTAT5-ir appears in locations of the Pa where oxytocin (OXT) is present, which suggests a possible action of PRL modulating OXT cells in the Pa. In fact, there is solid evidence demonstrating such an effect in the brain of female rats, both virgin (Blume et al. 2009; Sirzen-Zelenskaya et al. 2011) and lactating (Parker et al. 1991; Ghosh and Sladek 1995). Our data indicate that this role of PRL might be

translatable to males, too. In addition to its conspicuous roles in maternal behaviour regulation, OXT subserves multiple roles in the regulation of social cognition and emotionality, not only in rodents, but also in other mammals, including humans (Kirsch 2015). Hence, the notion that PRL might regulate central OXTergic pathways not only during the postpartum period, but also in non-maternal females and males, might be useful in the neuropsychiatric field.

The essential requisite for PRL to exert the central functions discussed above is the presence of sufficient levels of circulating hormone to allow it to reach the respective brain sites. As our findings reflect, this condition is easily met in the pregnant and lactating female. During pregnancy (Fig I.4), systemic levels of either hypophyseal PRL (early pregnancy) or placental lactogens (late pregnancy) are high, whereas in the postpartum period, circulating PRL levels are permanently increased due to lactation (Anderson et al. 2006). The functional significance of the action of PRL during this periods is to prepare the organism to the highly demanding period of motherhood.

Conversely, in the virgin female and male, circulating PRL levels are lower than in pregnant and lactating females (Guillou et al. 2015), especially in males, where the lack of exogenous PRL supplementation suppresses virtually all PRL-derived signal transduction in the brain (Fig 1.2). Consequently, our findings indicate that proper central responses to PRL in these animals would be facilitated only after a given PRL surge, when circulating PRL levels increase abruptly. Although plasma levels of PRL are subject to circadian rhythmicity in females and males (Guillou et al. 2015), the most prominent elevations of this parameter occur mostly in stressful situations, as the stress response and HPA axis activation lead to the release of both systemic (Fujikawa et al. 2004; Torner 2016) and intracerebral (Torner et al. 2004) PRL (see above). In addition, females experiment an upregulation of the central PRL system during the estrous cycle, promoted by the rise in gonadal steroid levels, granting increased hypophyseal PRL release (Neill 1972) and increased PRLR expression within the brain (Sugiyama et al. 1994). This leads ultimately to the increase of PRL signalling under higher gonadal steroid levels (Fig 1.6). Consequently, it is possible that some of the previously discussed roles of PRL in the brain are also facilitated during the proestrous and estrous phases of the estrous cycle, as gonadal steroid levels are increased.

Summarizing, although PRL-derived signalling in the brain is clearly sexually dimorphic and female biased, our findings indicate that this hormone is able to access and signal in

the brain of both males and virgin females. This signalling would mediate homeostatical responses, mostly geared to adapt the brain to situations of high-energy demand and stress.

C. THE MATERNAL HORMONE IN THE MALE BRAIN: ROLE OF PRL ON MALE SEXUAL BEHAVIOUR

A prominent function of PRL in males is the regulation of sexual behaviour (Freeman et al. 2000). The specific role of PRL in this context is complex and not completely understood. The classical view is that acute systemic PRL rises during mating mediate the postcopulatory refractory period of male sexual behaviour, through both peripheral and central actions (Krüger et al. 2002). This effect is in accordance with evidence on male models of chronic hyperprolactinaemia in certain species like the human and the rat, where sexual behaviour (as well as gonadotrophin secretion) is impaired due to this condition (Drago and Lissandrello 2000; Hernandez et al. 2006; Saito et al. 2013). However, the role of PRL seems to be opposite in other species such as the mouse, where males subject to chronic hyperprolactinaemia display increased rather than suppressed copulatory behaviour and circulating gonadotrophin (FSH, LH) levels (Bartke et al. 1987).

The action of PRL on male copulatory behaviour, either stimulatory or inhibitory, most likely requires the hormone to directly modulate the neural pathways involved in the expression of male sexual behaviour. Importantly, PRL has been proposed to influence attentional, appetitive and consummatory motor processes related to sexual behaviour, integrated by three major central dopaminergic circuits: the incertohypothalamic, mesocorticolimbic and nigrostriatal dopaminergic pathways (Krüger et al. 2002). This hypothesis is based on evidence that peripheral PRL administration alters dopaminergic activity in the main target sites of these pathways (Lookingland and Moore 1984; Chen and Ramirez 1988; Hernández et al. 1994). However, it is important to remark that none of the brain nuclei integrating these dopaminergic pathways (nucleus incertus, ventral tegmental area and substantia nigra) are responsive to PRL in males (according to our evidence, Fig 1.1) and they do not express either form of the PRLR (Bakowska and Morrell 1997; Bakowska and Morrell 2003). Hence, this lactogenic modulation of

dopaminergic tone should be necessarily mediated by an indirect input of PRL on these dopaminergic populations.

An additional candidate nucleus in which PRL might exert its actions related to sexual behaviour is the VMH. This nucleus is sexually dimorphic (Dugger et al. 2007), is known to promote the expression of copulatory behaviour in females (Kow and Pfaff 1998) and to inhibit mounting in male rats (Christensen et al. 1977). According to our results, the VMH is substantially responsive to PRL in males, showing in fact similar levels of pSTAT5-ir as in females (Fig 1.3). In addition, our data on castrated male mice suggest that this responsiveness is also dependent on testosterone (Fig 1.4). Hence, PRL could be able to control male reproductive behaviour also through the VMH in an androgen-dependent fashion. The nature of this control would be species-specific, thus explaining the interspecies differences in the effects of PRL on male sexual behaviour.

In addition to the VMH, evidence has been found during the last decades of the involvement of OXT in the regulation of reproductive behaviour. Research conducted fundamentally in male rats has established that the magno- and parvocellular OXTergic systems in the Pa are responsible for the facilitation of genital reflexes, erection and copulatory behaviour in response to sexual cues. For instance, significant increases in Fos expression (Nishitani et al. 2004; Caquineau et al. 2006) and local OXT release (Waldherr and Neumann 2007) have been found in the OXTergic neurons in the Pa in response to sexual cues or sexual interaction. In addition, paraventricular OXT neurons project (through the spinal cord) to several penile tissues and associated glands, thus facilitating genital reflexes (Veening et al. 2015). As described before, our findings reveal considerable levels of pSTAT5-ir in the Pa of male mice, comprising the magno- and specially the parvocellular division of the nucleus. This responsiveness to PRL is also dependent on testosterone, since castrated males display significantly lower levels of pSTAT5-ir in this nucleus (Fig 1.6). Therefore, PRL is likely able to control copulatory behaviour through the influence of OXTergic cells in the regulation of genital reflexes. Aside from the control of genital reflexes, OXTergic cells can also exert a high-level control of sexual behaviour through their action onto key centres of the hypothalamic circuits for sexual behaviour, such as MPO and VMH. In females, it has been shown that OXT can control the activity of estrogen receptor-expressing VMHvl neurons to regulate lordosis (Numan 2014). The existence of Pa projections to the MPO (Simerly and Swanson 1986; Wang and Swann 2014) and the demonstrated effects of OXT in the

MPO on male copulatory behaviour (Gil et al. 2011), suggest a similar regulatory action of paraventricular OXTergic cells on MPO populations regulating male sexual behaviour, which could in turn be modulated by PRL. Therefore, the presence of abundant pSTAT5-ir in the Pa might constitute the anatomical substrate of an additional mechanism for the influence of PRL on male copulatory behaviour.

D. TUNING THE BRAIN FOR MOTHERHOOD: REVISITING THE ROLE OF LACTOGENIC AGENTS IN THE EXPRESSION OF MATERNAL BEHAVIOURS

The results of the present work show that several brain nuclei belonging to the so-called maternal brain (Lonstein and Gammie 2002; Gammie 2005) display a substantial increase in lactogenic signalling during pregnancy and/or lactation, as compared to the basal condition in virgin females. This indicates that PRL is involved in the endocrine modulation of the neural centres of maternal behaviours, suggesting a possible role of the hormone regulating the onset and/or maintenance of maternal behaviour. Nevertheless, maternal behaviour is a complex behavioural phenomenon with multiple, differentially-regulated components and with remarkable interspecies differences. In this section, we will review the current evidence, provided by our work and that of others, on the involvement of PRL, as a major endocrine signal of pregnancy, in the onset and regulation of different components of maternal behaviour.

Proactive maternal responses

Evidence on the role of endocrine signals of pregnancy and lactation in the control of pup-directed maternal care in mice might seem conflicting, since, opposite to rats, virgin females quickly engage in allomaternal care (Martín-Sánchez et al. 2015b). Herein we will argue, however, that these signals are instrumental for the immediate onset of essential aspects of maternal care and that they might play a key role in the expression of these behaviours in non-maternal individuals.

In this respect, the major finding reported in the present work is the demonstration that high maternal motivation towards pups is dependent on the endocrine input of pregnancy. Even if virgin females engage almost spontaneously in allomaternal care in standard, home-cage and undisturbed conditions, full motivation for pups is not spontaneously expressed even after pup exposure. As shown in Study 3, when faced to a

pup retrieval task in an effort-related decision making paradigm (Yohn et al. 2016; Salamone et al. 2016), where retrieving pups is challenging, only dams will complete the task from the first moment.

This strongly suggests that endocrine signals (including PRL) are responsible for the increase in motivation for pups, by acting pre- and peripartum onto the neural pathways of maternal care. In this sense, the mesocorticolimbic dopaminergic pathway has been proposed as a key element to assign incentive value to pups and their stimuli and to mediate proactive maternal responses (Figs I.7 and I.8). Remarkably, our analysis of lactogenic signalling patterns in pregnant and lactating female mice (chapter 2) found no hint at all of PRL or other lactogens acting directly on this circuit (neither in the VTA originating the projection, nor on the targets of the projection in the striatum, Fig 2.2). Furthermore, neither form of the PRLR is expressed in none of these nuclei in the female rat (Bakowska and Morrell 1997; Bakowska and Morrell 2003), suggesting the same to be true in mice. Nevertheless, the BST-MPA continuum, regarded in this model as the master control directing the activation of the pathway for maternal responses (Fig I.7) does express the PRLR and is increasingly responsive to lactogenic signals during pregnancy and lactation, as indicated by our findings (Fig 2.2 C-E and 2.6). Consequently, as in rats (Bridges et al. 1990b; Bridges and Freemark 1995), the onset of fully-developed maternal care in the mouse might be dependent on the action of PRL in the medial preoptic area during late-pregnancy and the peripartum period, although partial increases can be achieved through pup sensitisation (Fig 3.4 and 3.5, Seip and Morrell 2008).

In line with this, a further point of discussion is the involvement of PRL in the expression of allomaternal behaviour. Even though we have advanced above that virgin female mice easily express allomaternal care (in the absence of the endocrine inputs of pregnancy), evidence stemming from gene deletion studies suggest that PRL might, at some point, play a greater role than expected in mediating this effect. The behavioural characterisation of mouse models with a targeted deletion on the PRLR gene reveals that both pup retrieval (proactive response) and the performance of nursing postures (consumatory response) are severely impaired in both heterozygous knockout dams and homozygous knockout virgins (Bole-feysot et al. 1998; Lucas et al. 1998b). This indicates that the expression of allomaternal care in mice is also dependent on PRL action.

There are at least three hypothesis to fit a role of PRL in alomaternal behaviour facilitation. In the first place, PRL could exert a rapid endocrine action on the brain in response to interaction with pups. In this regard, this work provides an analysis of the effect of continuous pup exposure (the godmother model) in the responsiveness to PRL. This analysis is for now limited to a single nucleus, the hypothalamic AC/ADP. In this region, no significant effect of prolonged pup exposure was found in the levels of PRL-derived signalling (Figs 3.8 B and 3.9 B). Nevertheless, as discussed before, we cannot dismiss the possibility that pup exposure conveys a significant effect on PRL-derived signalling in other brain regions, thereby supporting the hypothesis here discussed. The analysis of the effect of prolonged pup exposure on PRL-derived signalling is going to be extended in the near future to the major sites of the brain involved in maternal behaviour expression.

A second putative mechanism of PRL to mediate allomaternal responsiveness would involve a continuous facilitating input of the hormone. Even if we have found quite extensive patterns of PRL-derived signalling among virgin female mice, with substantial PRL-derived signalling in critical areas like the MPA (Fig 1.1), a continuous input of PRL is difficult to compromise with the general variability we have observed among virgins in our analysis. This adds to the fact that brain PRL responsiveness is subject to gonadal steroid regulation (Fig 1.6) and thus to the variance with the estrous cycle. Hence, our findings do not fit this hypothesis.

Finally, PRL could also exert a sort of “preparatory effect”, hard-wiring the responsiveness to pups permanently in the female’s brain through an acute action at some point during the postnatal development of the female mouse. In support of this hypothesis, it has been shown that prepubertal female mice react with neophobia towards pups, even performing infanticide (Gandelman 1973). However, as puberty advances, the proportion of aversive reactions diminishes as the proportion of allomaternal behaviour-expressing females increases. In addition, Gandelman reports that prepubertal ovariectomy disrupted this increasing allomaternal predisposition. This lies in stark contrast with the situation in rats, which become neophobic to pups with puberty. In sum, the aforementioned evidence on pup-elicited reactions and on PRLR knockout female mice, suggests an interaction of PRL and gonadal steroids during the puberty of the female mouse to permanently facilitate maternal care.

Altogether, female mice display maternal care easily in the absence of endocrine signals of pregnancy, but these signals are crucial for the complete onset of maternal behaviour (including maternal motivation). Consequently, fully-developed maternal behaviour is only elicited during motherhood. In a comparative point of view, mice and rats do not differ as much as it appears at first glance in the relative contribution of endocrine and sensory factors to the regulation of maternal care (Fig I.9). In Numan and Woodside's model for proactive maternal responses (Fig I.7), both species require hormones of pregnancy for the full development and immediate onset of proactive (motivated) maternal care. This endocrine input, in turn, would most likely lead to the priming of the MPA to facilitate the activation of the mesolimbic dopaminergic pathway. Conversely, continuous pup exposure elicits only a slow, progressive and partial increase in pup caregiving, which might also be mediated by endocrine mechanisms involving PRL. Hence, the major difference between rats and mice resides in the fact that adult female laboratory mice lack an active neural pathway mediating avoidance responses to pups. This neural pathway for avoidance responses might be active at some point in the female's life, but appears to be inhibited during puberty (Gandelman 1973), possibly by PRL in interaction with gonadal steroids. Interestingly, the suppression of this aversive pathway seems to be a consequence of artificial selection in mouse housing colonies, since only laboratory mice engage so easily in allomaternal behaviour, whereas feral female mice tend to commit infanticide on unfamiliar pups (D'Amato 1993; Numan and Insel 2003). In sum, PRL might play a greater role than previously thought in the regulation of maternal- and allomaternal responses in the female mouse.

Maternal aggression

In contrast to maternal care, maternal aggression in mice is observed exclusively in dams. Gonadally-intact virgin females, even after prolonged intimate contact with pups, do not attack intruders approaching the nest (Martín-Sánchez et al. 2015a). Therefore, the induction of maternal aggression in mice is most likely dependent on endocrine factors involved in pregnancy and/or lactation, which, like in rats or hamsters (Wise and Pryor 1977; Mayer et al. 1990), would include PRL and placental lactogens. Consistent with this possibility, we found pSTAT5-ir in the nodes of the circuit for maternal aggression in our pregnant-lactating females (Lonstein and Gammie 2002; Gammie 2005), illustrated on Figure I.8 . This network comprises the lateral septum (Brady and Nauta 1953), the BST (Klampfl et al. 2014), the medial and central amygdala (Bosch and Neumann 2010; Unger et al. 2015), the MPA and MPO in the preoptic hypothalamus,

the Pa in the anterior hypothalamus and the VMH in the tuberomammilar hypothalamus (Toth et al. 2010; Lin et al. 2011; Yang et al. 2013), the paraventricular thalamus (PV) and the caudal PAG (Lonstein and Stern 1997), the peripeduncular region (Factor et al. 1993) and the locus coeruleus (LC) in the midbrain. Indeed, virtually every site in this network included in our quantitative evaluation experiments a significant increase in lactogenic signalling during pregnancy and/or lactation (Fig. I.6).

Despite this evidence, the role of PRL in the expression of maternal aggression has been dismissed according to observations that hypophysectomy (Erskine et al. 1980) or bromocriptine treatment (Mann et al. 1980) conducted postpartum, do not disrupt maternal aggression. In addition, Broida et al. (1981) observed no correlation between serum PRL levels and maternal aggression in lactating dams. Instead, pup-derived sensory stimulation of the dams has been proposed as the pivotal factor promoting maternal aggression (Garland and Svare 1988). In this work we show, however, that the lactogenic modulation of brain function leading to a maternal state occurs mainly during late pregnancy (see above). Therefore, prolonged action of lactogenic agents during pregnancy, and not its acute action after parturition, could promote the onset of maternal aggression. Consistent with this, PRL-induced maternal sensitisation in virgin rats is also dependent on prolonged PRL priming, e.g. continuous administration of exogenous PRL (Loundes and Bridges 1986) or prolonged exposure to estradiol eliciting hypophyseal PRL release (Bridges and Ronsheim 1990), rather than acute PRL action. If this were also the case for maternal aggression in mice, the aforementioned lactogenic suppression strategies (hypophysectomy or bromocriptine treatment) applied postpartum would have a minor effect on maternal defence, since these are approaches aimed at testing not the onset but rather the maintenance of this behaviour.

In further support of this hypothesis, Mann et al. (1984) reported aggression in pregnant female mice against conspecific intruders already during gestation day 14, when neither pup stimuli nor hypophyseal PRL can play a role, but lactogens are already acting on the brain, according to our results. Nonetheless, contact with pups and the correct processing of pup-derived stimuli, such as suckling stimulation conveyed by the PIL (Cservenák et al. 2010; Cservenák et al. 2013) or chemosignal stimuli processed by the MePD (Pardo-Bellver et al. 2012), may be major factors in the maintenance (rather than the onset) of maternal aggression. In fact, information derived from pup chemosignals and from suckling stimulation may converge in the medial amygdala, since it receives

projections from the main and accessory olfactory bulbs and the PIL (Cadiz-Moretti et al. 2016). To sum up, evidence in the present work lends further support to the hypothesis that in the mouse, lactogens operating during late pregnancy fulfil an instrumental role in the onset of maternal aggression, through the extensive modulation of the neural pathways integrating this behaviour.

5

CONCLUSIONS

1. Both male and female brains are responsive to PRL, provided sufficient circulating levels of the hormone (e.g. after exogenous administration). However, brain PRL-derived signalling patterns are sexually dimorphic and female biased, in both qualitative and quantitative terms. Signalling in the female brain is most abundant in the hypothalamus, but extensive to the amygdala, BST, septum and to discrete thalamic and midbrain areas. In the male brain, PRL-derived signalling is restricted to circumventricular hypothalamic sites, with the exception of the septofimbrial/triangular septal area and the subfornical organ.

2. Lactogenic signalling in the female brain is regulated by estradiol and progesterone. In the analysed nuclei, estradiol has a general promoting effect of lactogenic signalling, whereas progesterone acting on top of estradiol exerts variable, region-dependent effects, either synergistic or antagonizing.

3. Lactogenic signalling in the male brain is dependent on testosterone. Orchidectomy decreases the density of PRL-derived signalling in the paraventricular and ventromedial hypothalamic nuclei.

4. Lactogenic signalling in the female brain is markedly increased during late pregnancy and the postpartum period, in terms of extension and intensity, as compared to patterns of virgin females. This increase is likely related to the role of lactogenic hormones in physiological and behavioural adaptations to motherhood.

5. This increase in lactogenic signalling during late pregnancy and lactation is observed in all the nodes of the socio-sexual brain, likely driving this network to a maternal state, thus promoting the onset of maternal behaviours.

6. In the maternal period, lactogenic signalling peaks during late pregnancy in most of the analysed nuclei. This indicates that late pregnancy instead of postpartum, is the critical timing for lactogenic hormones to exert maternal adaptations in the brain.

7. Pharmacological suppression of hypophyseal PRL release by means of bromocriptine has no significant effects on the levels of lactogenic signalling in the analysed nuclei of the pregnant brain. This indicates that other lactogenic sources,

likely placental lactogens but also possibly brain PRL, are responsible for the observed signalling patterns.

8. The behavioural assessment of maternal motivation requires the evaluation of proactive maternal responses under challenging conditions. We have validated a novel pup retrieval test, based on reward-based decision making paradigms, for the analysis of maternal motivation in rodents.

9. At first, only dams show pup retrieval in challenging conditions, thus indicating that motivational aspects of maternal behaviours require the action of the endocrine signals of pregnancy/lactation (which include lactogenic hormones).

10. Alternatively, virgin females undergoing prolonged interaction with pups (the so-called *godmothers*) display a moderate increase in motivation for pups, as part of a maternal sensitisation process.

11. The AC/ADP, a cell group located in a critical region for maternal behaviour, undergoes a significant increase in lactogenic signalling during motherhood (late pregnancy and postpartum period). This increase is not centred on its population of oxytocin-expressing cells, but on adjacent, non-oxytocinergic neurons.

12. *Godmothers* show no significant increase in lactogenic signalling in the AC/ADP region as compared to pup-naïve virgins. This suggests that prolonged interaction with pups has no direct effects on central prolactin action, at least in this nucleus.

13. The AC/ADP undergoes no changes in number of oxytocin-expressing neurons, either during the postpartum period or because of prolonged pup exposure. This suggests that, in mice, endocrine- or pup-induced brain changes associated with motherhood are not directly focused on the expression of oxytocin in the preoptic region.

14. Nevertheless, the number of oxytocin neurons in the AC/ADP correlates negatively with measures of motivated pup retrieval in dams and godmothers, and positively with measures of maternal aggression in dams. This suggests a role of the AC/ADP oxytocin population in the reduction of stress and anxiety during motherhood, which would indirectly affect maternal care and aggression.

6

RESUMEN EN CASTELLANO

INTRODUCCIÓN GENERAL

El correcto funcionamiento del organismo depende en gran medida de la interacción recíproca de los sistemas nervioso y endocrino. En este diálogo, el sistema nervioso regula la secreción hormonal sistémica a través del hipotálamo y la hipófisis, pero las hormonas también son capaces de entrar en el encéfalo y regular la función nerviosa. La regulación endocrina del sistema nervioso excluye generalmente la acción directa de hormonas peptídicas, que dada su naturaleza polar no atraviesan libremente la barrera hematoencefálica. La prolactina (PRL) constituye una excepción destacada a esta regla. Esta hormona peptídica parece ser transportada a través de los plexos coroideos (Walsh et al. 1987; Mangurian et al. 1992) al líquido cefalorraquídeo del interior del SNC para regular una multitud de procesos fisiológicos y comportamentales (Freeman et al. 2000). En este sentido, la función más prominente de la PRL es quizás la regulación de la maternidad, no limitada únicamente al desarrollo de la glándula mamaria y la producción de leche (Riddle et al. 1933b), sino consistente en la minuciosa coordinación de la homeostasis y conducta maternal.

Este trabajo se centrará en la caracterización de las acciones de la PRL en el encéfalo del ratón, con particular énfasis en su papel en la neuroendocrinología de la reproducción y la maternidad. En primer lugar, introduciremos la biología molecular de esta hormona, incluyendo su receptor, vías de señalización y regulación, y su interacción con esteroides sexuales. Después, introduciremos los aspectos funcionales de esta hormona, especialmente en aquellos vinculados a la maternidad. Finalmente, nos centraremos en el papel de la PRL en la regulación del comportamiento maternal, que constituye una de las líneas principales de nuestro laboratorio.

A. PROLACTINA – CARACTERÍSTICAS GENERALES

La prolactina es una hormona polipeptídica con una masa molecular de 23 kDa (Shome and Parlow 1977), perteneciente a la familia de citoquinas de tipo I (Rand-Weaver et al. 1991). Su proteína está codificada por un solo gen en el humano y otros primates y por varios genes parálogos en roedores y otros mamíferos (Soares 2004), y a su vez consta de diversas variantes estructurales generadas por ensamblaje alternativo y otras modificaciones posttraduccionales (Freeman et al. 2000). La PRL tuvo su origen evolutivo aparentemente en peces teleósteos o condrictios (Breves et al. 2014; Yamaguchi et al. 2015) asumiendo originalmente funciones relativas a la osmorregulación y/o a reproducción. Eventualmente, intervino en el desarrollo de

glándulas integumentarias (mamarias) y la regulación de sus secreciones (leche) (Horseman and Gregerson 2013).

En mamíferos, la secreción sistémica de PRL se da principalmente en las células lactotróficas de la adenohipófisis ventrolateral (Nakane 1970), aunque otros muchos órganos producen también esta hormona o alguna forma homóloga (Freeman et al. 2000), entre los que destaca el encéfalo. Las principales poblaciones neuronales productoras de PRL se encuentran en el hipotálamo (núcleos paraventricular y supraóptico, Mejía et al. 1997) y accesoriamente en otros centros hipotalámicos y extrahipotalámicos (Roselli et al. 2008), mientras que las proyecciones nerviosas de éstas se distribuyen ampliamente en el encéfalo (Freeman et al. 2000). A su vez, la PRL tiene una gran variedad de tejidos diana, incluyendo los sistemas reproductivos femenino y masculino, la hipófisis, el corazón, los pulmones o los riñones (Freeman et al. 2000). El encéfalo es también un importante órgano diana de la PRL.

B. EL RECEPTOR DE LA PROLACTINA Y SUS VÍAS DE SEÑALIZACIÓN

La PRL ejerce sus acciones biológicas a través del receptor de la PRL (PRLR), un receptor de membrana perteneciente a la superfamilia de receptores de citoquinas de tipo I (Bazan 1990). Éste cuenta con múltiples variantes estructurales (variables entre especies) que difieren únicamente en la longitud de sus dominios intracelulares y se clasifican generalmente como receptores largos o cortos (Bole-feysot et al. 1998). Las formas largas del PRLR son plenamente funcionales, no así las isoformas cortas, cuyo dominio intracelular es incapaz de iniciar las respectivas vías de señalización del receptor (Berlanga et al. 1997).

La figura I.2 esquematiza la variabilidad estructural y las vías de señalización del PRLR. La principal vía de señalización del receptor recibe el nombre de Jak/STAT y está integrada por la kinasa Janus (Jak2) y por las moléculas STAT (Signal Transducers and Activators of Transcription) (Ihle et al. 1994). Esta vía culmina con la fosforilación de STAT5 (a pSTAT5). Una vez fosforilada, pSTAT5 dimeriza y se transloca al núcleo celular, donde mediará las acciones biológicas de la PRL (Bole-feysot et al. 1998). Además, existen otras vías documentadas del PRLR, dependientes por ejemplo de la kinasa MAP u otra kinasas, o de cambios en concentraciones iónicas de potasio (K^+) o calcio (Ca^{2+}) (Buckley et al. 1994; Prevarskaya et al. 1995; Berlanga et al. 1995; al-Sakkaf et al. 1997; Ratovondrahona et al. 1998).

C. REGULACIÓN DE LA SEÑALIZACIÓN POR PROLACTINA EN EL ENCÉFALO

Las acciones pleiotrópicas de la PRL sobre el encéfalo están sujetas a una regulación compleja a diferentes niveles. El primero es la propia secreción sistémica de PRL, que determina los niveles circulantes de la hormona y la cantidad accesible al SNC. Los lactótrofos hipofisarios son células con una elevada actividad espontánea y bajo un control tónico inhibitorio mediado por dopamina (Ben-Jonathan and Hnasko 2001a). Esta dopamina es liberada por neuronas hipotalámicas específicas (neuronas TIDA y PHDA) e inhibe a los lactótrofos hipofisarios vía receptores dopaminérgicos D2 (Caron et al. 1978). A su vez, la PRL activa a las poblaciones neuronales TIDA y PHDA para inhibir su propia secreción (DeMaria et al. 1999), cerrando el bucle de retroinhibición. Este sistema de regulación queda ilustrado en la Figura I.3.

El segundo nivel en la regulación de la acción central de la PRL corresponde al acceso de la hormona al encéfalo. Como se ha detallado antes, este acceso se produce a través de un mecanismo de transporte activo a nivel de los plexos coroideos que parece depender del PRLR (Walsh et al. 1987; Mangurian et al. 1992), y que, por tanto, puede ser regulado a través de la expresión o afinidad de los receptores coroideos de PRL (Mangurian et al. 1992; Tabata et al. 2012) Cabe destacar que, además de este mecanismo, ciertas evidencias sugieren la existencia de mecanismos adicionales independientes del receptor (Brown et al. 2015).

Las acciones centrales de PRL dependen también de la expresión de los PRLR en el encéfalo. La distribución del PRLR ha sido cartografiada en el cerebro de la rata hembra (Bakowska and Morrell 1997), pero la evidencia indica que esta distribución es relativamente variable en función de diferentes situaciones fisiológicas o condicionantes externos, por ejemplo el parto y la presencia de crías (Ma et al. 2005a), la lactancia y el amamantamiento (Pi and Grattan 1999; Pi and Voogt 2001) o el estrés crónico (Faron-Górecka et al. 2014). Como se ha descrito antes con respecto a los plexos coroideos, la actividad del PRLR puede ser regulada a través de modificaciones postraduccionales del receptor, por ejemplo mediante la variación del balance entre isoformas cortas y largas del PRLR. De hecho, la forma corta del PRLR no sólo no es funcional, sino que parece ser capaz de secuestrar isoformas largas formando heterodímeros inactivos (Lesueur et al. 1991; Berlanga et al. 1997). Por último, la acción central de la PRL puede ser regulada a través de las vías de señalización del PRLR. La vía Jak/STAT, por ejemplo, está sujeta a

inhibición por las proteínas SOCS (Suppressors of Cytokine Signalling) (Pezet et al. 1999) o CIS (Cytokine-Inducible SH2-containing proteins) (Masuhara et al. 1997).

Los esteroides sexuales son hormonas estrechamente ligadas a la regulación de la señalización central de PRL. El estradiol y, en menor medida, la progesterona, son promotores de la secreción de PRL o de la expresión de PRLR en el encéfalo y plexos coroideos durante, por ejemplo, el ciclo estral de la rata, la gestación o la lactancia (Sugiyama et al. 1994). Por otro lado, los andrógenos parecen ejercer un efecto inhibitorio sobre la secreción hipofisaria de PRL (O'Hara et al. 2015). Uno de los objetivos principales de este trabajo consistirá en la caracterización del dimorfismo sexual en los patrones encefálicos de señalización por PRL en el ratón, así como su regulación por los respectivos esteroides sexuales.

D. ACCIONES CENTRALES DE LA PROLACTINA Y EL DESARROLLO DEL CEREBRO MATERNAL

Además de su bien conocido papel en la función mamaria (Riddle et al. 1933b), la PRL cuenta actualmente con más de 300 funciones documentadas (Bennett and Morris 1989), muchas de ellas en el sistema nervioso central. Por ejemplo, está implicada en la regulación de diversos circuitos neuropeptidérgicos centrales (oxitocina, vasopresina u hormona liberadora de corticotropinas, entre otros, Aguilera et al. 2008; Blume et al. 2009), del eje hipotálamo-hipofisario-adrenal (HPA, Fujikawa et al. 1995; Torner et al. 2002),del sueño y vigilia (Roky et al. 1995), o en la regulación sexualmente dimórfica del comportamiento sexual (Krüger et al. 2002; Egli et al. 2010), entre otros.

En los mamíferos, la reproducción resulta un desafío importante para el organismo, que requiere una serie de adaptaciones homeostáticas y conductuales para sobrellevar la condición maternal. Gran parte de estas adaptaciones maternales se inician y mantienen por señales endocrinas específicas de la gestación y postparto, entre las que destaca la PRL. Durante el periodo postparto, los niveles elevados de PRL están asegurados por la lactancia, que favorece un estado sostenido de hiperprolactinemia (Andrews et al. 2001b; Cservenák et al. 2010). Durante la gestación, sin embargo, los niveles de PRL circulante disminuyen drásticamente por la inhibición de la secreción hipofisaria de PRL (Fig I.4). Pese a esto, esta supresión queda compensada por los lactógenos placentarios, homólogos estructurales y funcionales de la PRL (Soares et al. 1998a; Linzer and Fisher 1999) secretados por la placenta (Yamaguchi et al. 1992). Uno de los objetivos de este

trabajo consistirá en la caracterización de los patrones encefálicos de señalización por la PRL en la hembra gestante y de su dependencia de PRL hipofisaria o fuentes alternativas como los lactógenos placentarios.

La PRL y sus homólogos lactogénicos son las hormonas maternales por antonomasia, responsables del desarrollo de las principales adaptaciones del cerebro maternal. Entre éstas destacan, por ejemplo, la activación de la ingesta y el anabolismo para asegurar el desarrollo embrionario (Augustine et al. 2008), la estimulación de la neurogénesis en centros olfativos para asegurar el correcto reconocimiento de la descendencia en roedores (Shingo et al. 2003), la regulación del anestro de la lactancia (Araujo-Lopes et al. 2014), la facilitación de la secreción de oxitocina y vasopresina (Van Tol et al. 1988) o la atenuación del eje HPA y la respuesta al estrés (Torner et al. 2001). Una de las adaptaciones maternales más prominentes y que centrará una parte importante del presente trabajo, es la regulación del comportamiento maternal.

E. PROLACTINA Y COMPORTAMIENTO MATERNAL

Introducción al comportamiento maternal

El comportamiento parental se define como una colección de conductas dirigidas a incrementar la supervivencia de la descendencia (Numan & Insel 2003). En mamíferos, es la madre, como responsable última de la lactancia (Kleiman & Malcolm), quien asume la práctica totalidad de éstas conductas, pasando a denominarse comportamientos maternales. Los comportamientos maternales pueden dividirse en aquéllos dirigidos a crías (su búsqueda, su recolección, aseo, termorregulación y amamantamiento) y aquéllos no dirigidos (la construcción del nido y la agresión maternal) (Gammie 2005) (Fig I.5). La importancia del comportamiento maternal va más allá de la supervivencia de la prole, también tiene un impacto positivo sobre su desarrollo cognitivo y emocional, normalmente transmitido a través de mecanismos epigenéticos (Zhang et al. 2013).

Sustrato neural del comportamiento maternal

Como variante especial de conducta social, el comportamiento maternal está controlado por una red altamente interconectada de núcleos telencefálicos, hipotalámicos y mesencefálicos denominada el “Cerebro sociosexual” (Newman 1999) (Fig I.6). Esta red supone el núcleo para la integración de la totalidad de conductas sociales y reproductivas expresadas por cada especie. El comportamiento maternal surgirá

exclusivamente durante la gestación, parto y postparto como resultado de la modificación de los patrones de actividad del Cerebro sociosexual, modificado hacia un estado maternal (el "Cerebro Maternal").

El comportamiento maternal dirigido a crías tiene componentes reflexivos y proactivos. Los componentes proactivos, como la búsqueda de las crías y su recolección en el nido, están motivados por las crías y sus estímulos, que adquieren un carácter reforzante para la madre (Lee et al. 2000; Pereira & Morrell 2011). Esto implica que los circuitos neurales que regulan la recompensa y la motivación (vía dopaminérgica mesocorticolímbica) han de estar integrados, junto al cerebro sociosexual, en la gestión de comportamientos maternales proactivos. Numan y Woodside (2010) desarrollaron un modelo funcional, basado en evidencia en la rata, que integra la acción conjunta del cerebro maternal y los circuitos de recompensa. La rata tiene reacciones peculiares a las crías. Ratas hembra vírgenes sentirán aversión hacia éstas y, sólo tras una exposición continuada de varios días, iniciarán un acercamiento y eventualmente expresarán comportamientos alomaternales. En cambio, las ratas madres primíparas expresarán atracción hacia las crías y el repertorio completo de conductas maternales desde la primera interacción. Este modelo (Fig I.7) detalla cómo ambas respuestas antagónicas (aversión en hembras vírgenes y atracción en madres) están controladas por dos circuitos independientes (ver detalles en Figs I.7 e I.8) que responden a estímulos de crías. Éstos, a su vez, están supeditados a un centro maestro, el área medial preóptica del hipotálamo (MPA) (parte del cerero sociosexual), que dirige la conversión de una a otra conducta inhibiendo una u otra vía. El mediador de la inhibición de respuestas aversivas de vírgenes y la activación de la vía maternal son las hormonas de la gestación, particularmente la acción de la PRL (junto con esteroides sexuales) sobre el MPA. La figura I.8 A resume los centros y conexiones implicados en la gestión del comportamiento maternal proactivo.

Respecto a la agresión maternal, el número de centros nerviosos implicados en su expresión es elevado y sus interconexiones, complejas (Fig I.8 B). Entre los centros implicados, destacan la amígdala medial (MeA, como centro integrador de estímulos quimiosensoriales), el septum lateral (LS) o los núcleos hipotalámicos paraventricular (Pa) o ventromedial (VMH), todos ellos nodos principales del Cerebro sociosexual (Fig I.6). Quizás el centro más relevante implicado en este sistema es la región conformada por el núcleo BST y MPA, entendido como región maternal clave (Numan 1996). Dentro de la fenomenología de la agresión maternal, destaca la atenuación del estrés y

ansiedad que experimentan las madres y que facilita sus reacciones agresivas hacia amenazas peligrosas. Los centros implicados en este fenómeno incluyen el Pa, el BST o la amígdala central (Ce), ente otros.

El nonapéptido oxitocina (OXT) es moduladores bien documentados de la expresión de conductas sociales y reproductivas a lo largo de la evolución de vertebrados (Insel & Young 2000), incluyendo las conductas maternales. Además de su funciones sistémicas como neurohormona (estimulación de la contracción uterina durante el parto y de la eyección de leche en la lactancia, Nishimori et al. 1996; Blanks & Thornton 2003), la OXT también es producida y liberada como neuromodulador en circuitos encefálicos internos (Otero-García et al. 2015). Las principales poblaciones de neuronas oxitocinérgicas cerebrales se ubican en: (1) el BST medial posterior (BSTMP) ventral, (2) el área anterior comisural/anterodorsal preóptica (AC/ADP) del hipotálamo, (3) el Pa, (4) el núcleo supraóptico (SO) del hipotálamo, y (5) la amígdala medial (Otero-García et al. 2015). La OXT tiene un papel demostradp como modulador del comportamiento maternal, estimulando, por ejemplo, el cuidado maternal a crías en hembras vírgenes de ratón (Pedersen & Prange 1979; Pedersen et al. 1982), o la agresión maternal a través de su liberación en ciertos núcleos cerebrales como la Ce (Bosch et al. 2004; Bosch et al. 2005). La OXT juega también un papel fundamental en la modulación del MPA (Champagne et al. 2004) y la vía para conductas maternales proactivas (Pedersen et al. 1994) durante la gestación en la rata (Fig I.7). Sin embargo, el origen concreto de estas proyecciones OXTérgicas moduladoras no es del todo conocido aún. Si bien una parte procede del Pa (Knobloch et al. 2012), no se ha examinado todavía la contribución de otras poblaciones OXTérgicas. Uno de los objetivos del presente trabajo consistirá en analizar la posible relación entre las neuronas OXTérgicas del AC/ADP (localizadas en una posición privilegiada junto al contínuo BST-MPA) y la modulación del comportamiento maternal.

La prolactina y la regulación del comportamiento maternal: análisis comparativo

La maternización del Cerebro Sociosexual que media la expresión de comportamientos maternales depende principalmente de dos tipos de señales: estímulos sensoriales de crías y estímulos endocrinos de la gestación y lactancia (incluyendo la PRL). La acción específica y la contribución relativa de cada componente sobre el Cerebro sociosexual permiten la estabilización de diferencias interespecíficas en la expresión de comportamientos maternales.

Como antes se ha descrito, en ratas, las hembras vírgenes inicialmente neofóbicas hacia crías pasan a ser completamente maternales al experimentar la gestación. Esta conversión la produce la modulación del MPA por las señaes hormonales de la gestación, específicamente por los lactógenos (PRL y/o lactógenos placentarios), actuando de forma sinérgica con el estradiol (E) y la progesterona (P) (Bridges et al. 1985; Bridges & Ronsheim 1990; Bridges & Freemark 1995; Bridges et al. 1996). Los estímulos sensoriales derivados de crías, en cambio, tendrían un mayor protagonismo en la rata en el mantenimiento del comportamiento maternal durante el periodo postparto (Numan & Insel 2003).

En contraste con la rata, los ratones hembra vírgenes exhiben conductas alomaternales de cuidado de crías muy fácilmente (Stolzenberg & Rissman 2011; Martín-Sánchez et al. 2015b). Esto llevaría a suponer que la PRL y otras señales endocrinas de la gestación tienen una importancia secundaria en la inducción del comportamiento maternal (las vírgenes no las han experimentado). Sin embargo, este fenómeno se da exclusivamente respecto al cuidado maternal, no frente a la agresión maternal (expresada exclusivamente por hembras maternales). Además, mutantes KO para el PRLR exhiben déficits significativos en la expresión de conductas maternales. Todo esto apunta a una mayor relevancia de la PRL en el control de estos comportamientos en el ratón. Este trabajo pretende, entre otros, contextualizar mejor la acción de PRL sobre las vías de control del comportamiento maternal (proactivo y agresión) y sobre sistemas moduladores accesorios como el oxitocinérgico.

F. OBJETIVOS DE ESTE TRABAJO

I. Describir los patrones básicos de señalización por PRL en el encéfalo de ratones hembra y macho y su posible dimorfismo sexual.

II. Analizar la regulación específica de estos patrones por testosterona (machos) y estradiol y progesterona (hembras).

III. Examinar los cambios en los patrones basales de señalización cerebral por PRL de hembras producidos durante la gestación y lactancia.

IV. Evaluar la contribución de PRL hipofisaria frente a otras fuentes (lactógenos placentarios) a la conformación de los mencionados patrones de señalización por PRL durante la gestación.

V. Investigar la acción lactogénica sobre la población oxitocinérgica específica del AC/ADP en vírgenes y madres lactantes.

VI. Desarrollar un nuevo modelo comportamental para el estudio de la motivación maternal en hembras de ratón lactantes, hembras vírgenes expuestas a crías y vírgenes no expuestas a crías.

VII. Analizar la influencia de PRL y la acción de OXT en la expresión de dos conductas maternales: la recogida de crías y la agresión maternal.

1

DIMORFISMO SEXUAL E INFLUENCIA DE HORMONAS ESTEROIDEAS EN LA REACTIVIDAD CEREBRAL A PROLACTINA

1.1. OBJETIVOS

El primer objetivo de este estudio es la descripción exhaustiva de los patrones de distribución de células sensibles a PRL en el encéfalo de ratones hembra y macho y la identificación de diferencias sexualmente dimórficas. Además, pretendimos explorar la contribución de hormonas esteroideas sexuales femeninos (estradiol y progesterona) y masculinos (testosterona) a la modulación de dichos patrones en hembras y machos, respectivamente. Como estrategia para la obtención de los citados patrones de sensibilidad central a PRL, empleamos la detección inmunohistoquímica de la formas fosforilada de STAT5 (pSTAT5, ver sección II de la Introducción).

1.2. METODOLOGÍA

1.2.1. Animales y diseño experimental

Para este estudio se empleó un total de 30 ratones adultos de la cepa CD1, 18 hembras y 12 machos, con edades comprendidas entre 10 y 24 semanas.

Las hembras experimentales fueron ovarectomizadas (sección 1.2.2) y posteriormente sometidas a un tratamiento de sustitución hormonal, consistente en la administración de estradiol (mediante implantes de liberación lenta), progesterona (mediante inyección i.p.) o vehículo (aceite) (sección 1.2.3). Estas hembras fueron divididas aleatoriamente en 3 grupos experimentales según el tratamiento recibido: (1) grupo EP (estradiol y progesterona), (2) grupo EO (estradiol y vehículo), y (3) grupo OO (vehículo, control). Tras 7 días de tratamiento hormonal, las hembras recibieron una suplementación de PRL ovina (sección 1.2.3) y fueron perfundidas 45 minutos después (Brown et al. 2010) para la detección inmunohistoquímica de pSTAT5.

Los machos experimentales fueron asignados aleatoriamente a dos grupos experimentales: (1) Intactos y (2) Castrados (que fueron esterilizados, ver sección 1.2.2). Imitando el procedimiento al que las hembras fueron sometidas, los machos de ambos grupos recibieron implantes e inyecciones de vehículo. Siete días después, recibieron una suplementación equivalente de PRL y fueron perfundidos 45 minutos la suplementación.

1.2.2. Ovarectomía y orquidectomía

Las hembras y machos experimentales fueron sometidos a esterilización a las 10 semanas (hembras) y 12 semanas (machos) de edad. Para ambos procedimientos quirúrgicos se empleó ketamina i.p. (Imalgene 500, 75mg/kg) y medetomidina (Domtor, 1 mg/kg) para la anestesia, tartrate de butorfanol 1% (Torbugesic, 20 µl s.c.) como analgésico sedante y hidrocloruro de atipamezol (Antisedan, 1 mg/kg) para revertir los efectos de la anestesia tras la cirugía. Los animals tuvieron como mínimo 7 días de recuperación después de la cirugía.

1.2.3. Tratamiento hormonal

En función de su grupo experimental, las hembras ovarectomizadas recibieron estradiol, progesterona y/o vehículo. El estradiol fue administrado mediante implantes subcutáneos de liberación lenta, rellenos con una solución de 20 µg/ml de β-estradiol (Sigma) en aceite de girasol. En cambio, la progesterone fue administrada de forma aguda mediante una inyección subcutánea de 500 µg, disuelta también en aceite de girasol. Este protocol de sustitución hormonal está diseñado para emular la dinámica hormonal del estro (Rissman 1997).

Tres horas después de la administración de progesterone (o vehículo), machos y hembras recibieron una dosis i.p. de 5 mg/kg de PRL ovina (25 UI/mg) para asegurar niveles elevados y equivalents de PRL circulante en ambos sexos y, 45 minutos después, fueron perfundidos.

1.2.4. Perfusión y procesado histológico

Los animales experimentales fueron perfundidos usando paraformaldehido 4% en tampón fosfato 0,1M a pH 7,4 (PB) como fijador. El tejido cerebral fue extraído y postfijado por inmersión en el mismo fijador durante la noche y después crioprotegido en una solución de sacarosa 30% en PB. Finalmente, el tejido fue cortado al micrótomo de congelación (Microm HM-450) en 5 series paralelas de secciones coronales de 40 µm y las series almacenadas a -20 ºC.

1.2.5. Detección inmunohistoquímica de pSTAT5

La inmunohistoquímica se realizó siguiendo un protocolo adaptado de Brown y colaboradores (2010). Éste comprende: (1) un proceso inicial de exposición antigénica

(incubaciones secuenciales en tampón TRIS pH 10 a 85ºC); (2) inhibición de peroxidasas endógenas; (3) bloqueo de uniones inespecíficas; (4) incubación en anticuerpo primario anti pSTAT5 (Rabbit-anti-pSTAT5 Tyr694; Cell Signalling Technology) diluido 1:500 durante 72 horas; (5) incubación en anticuerpo secundario biotinilado (goat anti-rabbit IgG biotinilado, Vector) diluido 1:200 durante 90 minutos y (6) amplificación de la señal mediante incubación en complejo ABC (ABC Elite kit; Vector). Por último, se sometió al tejido a un revelado permanente con 3-3'-, diaminobenzidina 0,005% (Sigma) y H_2O_2 0,01%. Finalmente, el tejido revelado fue montado y cubierto.

1.2.6. Análisis de inmunorreactividad pSTAT5

Para el análisis microscópico de las preparaciones histológicas obtenidas se empleó un microscopio óptico Leitz DMRB (Leica). Para el mapeo de los patrones de inmunorreactividad pSTAT5 (pSTAT5-ir) se registró la presencia o ausencia de pSTAT5-ir en todo núcleo cerebral en todos los especímenes analizados. Además, se llevó a cabo un muestreo cuantitativo de la densidad de células pSTAT5-positivas en una selección de núcleos cerebrales con pSTAT5-ir tanto en machos como en hembras. Para este muestreo, se tomó fotomicrografías de los núcleos de interés de ambos hemisferios cerebrales usando una cámara Leica DFC495 asociada al microscopio y siguiendo "frames" determinados para cada núcleo (Fig 1.5). Después, se analizó las imágenes obtenidas usando el software ImageJ y se cuantificó automáticamente la densidad media interhemisférica de células pSTAT5 positivas para cada núcleo muestreado.

1.2.7. Análisis estadístico

Los datos resultantes fueron sometidos a análisis estadístico con el software SPSS. En primer lugar, se comparó la densidad de pSTAT5-ir en los núcleos analizados entre machos (intactos) y hembras (grupo EP), aplicando un test-t para muestras independientes o, como alternativa no paramétrica cuando los datos no se distribuían normalmente, un ANOVA de Kruska-Wallis. Para la comparación entre los tres grupos de hembras (OO; EO; EP) se empleó un ANOVA de Kruskal Wallis (datos no distribuidos normalmente) con análisis post-hoc de Dunnett. Finalmente, para la comparación de machos intactos y castrados, se empleó también un test-t o un ANOVA de Kruskal Wallis (en ausencia de normalidad).

1.3. DISCUSIÓN

En este estudio se ha examinado los patrones de señalización central mediada por PRL en hembras y macho de ratón de la cepa CD1. Para este propósito, hemos empleado machos y hembras suplementados con una dosis de PRL exógena que aseguró niveles circulantes suprafisiológicos de la hormona, evitando así el sesgo debido a la diferencia sustancial en niveles de PRL circulante en machos y hembras. En este análisis, hemos hallado un dimorfismo sexual evidente en favor de hembras, que presentan patrones de señalización mediada por PRL más extensos y abundantes que machos. Los patrones descritos en machos quedan restringidos al hipotálamo, a núcleos cercanos al sistema ventricular. En hembras, además de los anteriores, pSTAT5 se observa también en núcleos amigdalinos, en el BST, septum y, en menor mediad, en núcleos talámicos y mesencefálicos.

Aun así, los machos presentan una respuesta encefálica clara a PRL tras la suplementación hormonal, lo que indica que esta hormona ejerce funciones centrales también en machos. Éstas se darían siempre y cuando los niveles circulantes y/o intracerebrales de PRL sean lo suficientemente altos, lo que en machos puede ocurrir durante la cópula (Kamel et al. 1977) o durante la respuesta al estrés (Torner et al. 2004), entre otros.

Por otro lado, hemos evaluado también cómo los esteroides sexuales femeninos (estradiol y progesterona) contribuyen a modular la sensibilidad central a PRL. Para ello, hemos analizado la expresión de los patrones centrales de señalización mediada por PRL en hembras ovarectomizadas y tratadas con vehículo, estradiol, o estradiol más progesterona. El tratamiento de estradiol produjo una tendencia generalizada al incremento de la inmunorreactividad de pSTAT5, que llevó a aumenos significativos respecto a los controles (tratados con vehículo) en MePD, LSV, AC/ADP, PIL y VLPAG. Con respecto a la acción del estradiol, la progesterona produjo efectos variables según región: o bien sinérgicos (MePD, PIL), neutros (LSV, VLPAG) o incluso antagónicos (AC/ADP) respecto al estradiol. En suma, la señalizaión lactogénica central se halla bajo la influencia de los niveles circulantes de estradiol y progesterona, de forma compleja y neuroanatómicamente heterogénea.

Del mismo modo, hemos evaluado también el papel de la testosterona en la modulación de la sensibilidad central a PRL en ratones macho. En este caso, la supresión de la

testosterona sistémica mediante castración produjo una disminución significativa de la densidad de señalización mediada por PRL específicamente en el SFi del septum y el Pa y VMH en el hipotálamo. Esto indica que la testosterona contribuye a mantener la sensibilidad a PRL en estas regiones cerebrales.

2

CARACTERIZACIÓN DE LA SENSIBILIDAD ENCEFÁLICA A PROLACTINA A LO LARGO DEL CICLO REPRODUCTIVO DE LA HEMBRA DEL RATÓN

2.1. OBJETIVOS

Durante la gestación y la lactancia, el encéfalo experimenta una serie de adaptaciones maternales, cambios en la regulación fisiológica y conductual encaminados a afrontar la maternidad (Grattan 2001). Éstas están mediadas entre otros por la PRL. El objetivo principal de este estudio es caracterizar la variación de la sensibilidad central a PRL en el cerebro de la hembra del ratón durante los periodos críticos de la gestación y la lactancia, para lo que se caracterizará los patrones cerebrales de pSTAT5-ir en hembras gestantes tardías, lactantes y vírgenes (controles). Existe evidencia de que durante la segunda mitad de la gestación, la secreción hipofisaria de PRL queda inhibida en favor de otras hormonas lactogénicas como los lactógenos placentarios (PLs) (Soares 1998; Soares 2004). Este estudio pretende también explorar la contribución de agentes lactogénicos hipofisarios y no hipofisarios a la modulación lactogénica del encéfalo durante la gestación. Para este cometido, se incluirá un grupo adicional de hembra gestantes donde la secreción hipofisaria de PRL fue inhibida farmacológicamente mediante bromocriptina (sección 2.2.2).

2.2. METODOLOGÍA

2.2.1. Animales y diseño experimental

En este estudio se empleó 32 hembras de ratón adultas de la cepa CD1, de entre 8 y 12 semanas de edad. De éstas, 27 fueron asignadas aleatoriamente a 4 grupos experimentales diferentes: (1) Vírgenes (n=7); (2) Gestantes (n=6); Gestantes + bromocriptina (n=8); y (4) Lactantes (n=6). Las hembras del grupo Vírgenes fueron perfundidas directamente. Las hembras del resto de grupos fueron cruzadas con machos reproductores durante 4 días y posteriormente estabuladas en parejas hasta el día de su perfusión. Las hembras de los Grupos Gestante y Gestante + bromocriptina fueron perfundidas 18 días después de comenzar su cruzamiento, evaluando el día exacto de gestación a posteriori a través del grado de desarrollo de sus fetos. Las hembras del grupo Lactante fueron perfundidas en el día 6 postparto.

2.2.2. Inhibición farmacológica de la secreción hipofisaria de prolactina

Para este procedimiento se empleó la bromocriptina, agonista D2 dopaminérgico que inhibe a los lactótrofos hipofisarios emulado la acción de las neuronas TIDA y PHDA (Fig I.3). Se administró dos inyecciones s.c. consecutivas de una solución de 100 µg de

bromocriptina (2-Bromo-α-ergocriptina, Sigma-Aldrich) en etanol 10%, 19 y 2 horas antes de su perfusión, respectivamente.

2.2.3. Perfusión, procesado histológico e inmunohistoquímica para pSTAT5

Ver secciones 1.2.4 y 1.2.5.

2.2.4. Análisis de inmunorreactividad pSTAT5

Tanto el análisis cualitativo como el cuantitativo se realizaron siguiendo la metodología descrita en la sección XX. Los "frames" de muestreo empleados están recogidos en la figura 2.5.

2.2.5. Análisis estadístico

Se aplicó un contraste ANOVA de 1 vía en cada núcleo muestreado para analizar las diferencias en densidad de pSTAT5-ir en los grupos de Vírgenes, Gestantes y Lactantes. Se empleó el mismo contraste estadístico para evaluar el efecto de la inhibición hipofisaria de PRL, comparando las densidades de pSTAT5-ir en cada núcleo muestreado entre los grupos de Gestantes y Gestantes + bromocriptina.

2.3. DISCUSIÓN

En este capítulo, se ha caracterizado cómo varían los patrones de señalización mediada por PRL en el encéfalo del ratón a lo largo del ciclo reproductivo femenino (en hembras vírgenes, gestantes y lactantes). Hemos hallado patrones aumentados (en términos de extensión y de densidad de marcaje) en hembras gestantes y lactantes respecto a vírgenes. Además, nuestros resultados sugieren que estos patrones son generados por lactógenos alternativos a la PRL hipofisaria.

Con respecto a los patrones de señalización de PRL obtenidos en hembras vírgenes, cabe destacar que éstos son relativamente similares pero también más variables que los patrones analizados en vírgenes ovarectomizadas y tratadas con estradiol y progesterona (capítulo 1). Esta comparación apunta a que la PRL puede ejercer un rango muy variable de actividad central, oscilando entre prácticamente nula y moderadamente elevada. El factor causal más probable para esta variabilidad son los esteroides sexuales, como apunta nuestra evidencia (capítulo 1) y la de otros (Sugiyama et al. 1994).

El hallazgo más significativo de este estudio es el marcado incremento en señalización lactogénica producido en el encéfalo gestante y lactante. Este incremento es patente en términos de extensión (implicando que muchas regiones cerebrales se vuelven sensibles a la PRL durante estos periodos) y de densidad (en la mayoría de centros basalmente sensibles a PRL se produce un aumento significativo de la misma). Es importante destacar que, al menos en términos cuantitativos, este aumento no se produce en todas las regiones analizadas de forma homogénea a lo largo de estos periodos. Así pues, los picos de señalización lactogénica aparecen generalmente durante la gestación (en CeM, MePD, LSV, BSTMPM, Pa, SO, VMHvl y VLPAG). De estos, en la mayoría de casos los niveles elevados persisten durante postparto (mantenidos probablemente por los elevados niveles de PRL circulante derivados de la lactancia), pero en algunos centros disminuyen de nuevo hasta niveles basales. Esta evidencia tiene la importante implicación de que la actividad lactogénica que media las principales adaptaciones maternales centrales se da preparto, de forma que la madre primípara estaría ya completamente adaptada a las necesidades del periodo maternal desde el parto y el primer contacto con sus crías. Excepcionalmente, dos de las regiones estudiadas (AC/ADP y MPO) presentaron un aumento moderado de señalización lactogénica durante la gestación, que sin embargo alcanza su máximo durante la lactancia, lo que sugiere un papel de la PRL en estos núcleos en el mantenimiento de ciertas adaptaciones maternales.

Los núcleos encefálicos integrados en los patrones obtenidos en hembras gestantes y lactantes en este trabajo reflejan la participación de PRL en la inducción de las adaptaciones maternales más destacadas (Grattan 2001), que incluyen: la modulación de circuitos oxitocinérgicos y vasopresinérgicos (Ghosh & Sladek 1995; Sirzen-Zelenskaya et al. 2011), la atenuación de la respuesta al estrés (Torner et al. 2002), la activación de ingesta y anabolismo (Sauvé & Woodside 2000), o, notablemente, la inducción del comportamiento maternal (Slotnick et al. 1973; Lonstein & Gammie 2002; Gammie 2005; Brunton & Russell 2008).

Finalmente, en relación al origen de la actividad lactogénica central descrita en este estudio, la inhibición de la secreción hipofisaria de PRL en hembras gestantes mediante la administración de bromocriptina tuvo un efecto virtualmente nulo sobre la extensión y densidad de sus respectivos patrones de señalización lactogénica. Esto demuestra que el agente causal de estos patrones es un lactógeno alternativo. Esta actividad tendrá,

pues, su origen probable en los lactógenos placentarios, sustitutos funcionales de la PRL (Bridges and Freemark 1995) producidos por la placenta durante la segunda mitad de la gestación. Sin embargo, un posible origen alternativo y no excluyente es la PRL intracerebral (Paut-Pagano et al. 1993; Grattan and Kokay 2008).

3

COMPORTAMIENTO MATERNAL, PROLACTINA Y OXITOCINA – ANÁLISIS DE COMPORTAMIENTOS MATERNALES ESPECÍFICOS Y EXPRESIÓN DE pSTAT5 Y OXITOCINA EN EL AC/ADP

3.1. OBJETIVOS

Durante la maternidad, las hembras adquieren una motivación incrementada por las crías y sus estímulos, que facilitan la ejecución de comportamientos maternales proactivos. En el ratón, este tipo de conductas maternales parecen expresarse sin la necesidad de cambios endocrinos asociados a la gestación y lactancia, ya que las hembras vírgenes emprenden el cuidado de crías ajenas con facilidad. Sin embargo, se desconoce todavía si la expresión de estas conductas maternales va acompañada de un incremento de motivación maternal inducido por la interacción con crías, o si, por el contrario, la motivación maternal sólo puede incrementarse con la acción apropiada de agentes endocrinos maternales. Este estudio pretende, en primer lugar, caracterizar la contribución relativa de los agentes endocrinos maternales (que incluyen a la PRL) y el contacto con crías y sus estímulos al incremento de motivación maternal. Para este cometido, se ha desarrollado un nuevo test comportamental que permite analizar la motivación maternal de forma más específica (ver sección 3.2.1).

El nonapéptido oxitocina (OXT) cumple una función clave como neuromodulador del comportamiento maternal proactivo (Numan & Woodside 2010; Bosch & Neumann 2012). El segundo objetivo de este estudio consiste en analizar la interacción de la exposición prolongada a crías, por un lado, y la PRL (como hormona principal en la gestación y lactancia), por otro, sobre los circuitos OXTérgicos centrales y sobre la expresión de conductas maternales proactivas. Este análisis se centrará concretamente en la población OXTérgica específica del área anterior comisural/anterodorsal preóptica del hipotálamo (AC/ADP), que ocupa un lugar privilegiado en la región preóptica medial, crítica para la regulación del comportamiento maternal (Numan et al. 1988; Tsuneoka 2013). El análisis consistirá en la cuantificación de la expresión y coexpresión de OXT y pSTAT5 (como marcador de señalización mediada por PRL) en este núcleo y en la búsqueda de correlaciones significativas entre estos parámetros histológicos y las medidas conductuales de comportamiento maternal motivado obtenidas previamente.

Por último, emprenderemos un análisis histológico y correlacional equivalente con una muestra adicional de hembras de ratón que ha sido testada en agresión maternal, otro componente clave del comportamiento maternal con una dependencia clara de señales endocrinas.

3.2. METODOLOGÍA

3.2.1. Animales, diseño experimental y análisis conductual

Motivación maternal

En este experimentó se empleó un total de 66 hembras adultas de ratón de la cepa CD1, de 11 semanas de edad. De éstas, 30 fueron asignadas aleatoriamente a 3 grupos experimentales: (1) Vírgenes (n=10); (2) Comadres (vírgenes expuestas de forma prolongada a crías, n=10) y Madres (en día postparto 6, n=9). Las hembras restantes fueron empleadas como acompañantes o como donantes de crías. Estas hembras fueron sometidas a una versión modificada del test de recogida de crías, en la que 4 crías se encuentran separadas del nido por una barrera de 10 cm de altura que la hembra debe superar para efectuar la recogida (Fig 3.1). Se sometió a las hembras experimentales a una sesión diaria del test, de 10 minutos de duración, comenzando en el día postparto 2 y durante 3 días. En la mañana del día postparto 5, las hembras experimentales fueron perfundidas. Las sesiones fueron grabadas en vídeo y las grabaciones analizadas por un observador ciego a las condiciones experimentales, que registró los siguientes parámetros: latencia al primer contacto con crías, latencia a la recogida de cada cría (4 en total), tiempo total de contacto con crías y número de cruces de barrera.

Agresión maternal

La muestra en la que se evaluó la agresión maternal consta de 27 hembras de ratón CD1 adultas, divididas en los mismos grupos experimentales: Vírgenes (n=9); Comadres (n=9) y Madres (n=9). La metodología del análisis conductual queda recogida en un trabajo previo de nuestro grupo de investigación (Martín-Sánchez et al. 2015a).

3.2.2. Perfusión y procesado histológico

Ver sección 1.2.4.

3.2.3. Inmunofluorescencia doble para pSTAT5 y oxitocina

Este protocolo comprendió las siguientes fases: (1) un proceso doble de exposición antigénica (incubación en solución de H_2O_2 y NaOH a pH > 13; incubación en solución de glicina y dodecilsulfato de sodio); (2) un bloqueo de autofluorescencia (incubación en borhidruro sódico); (3) un bloqueo de uniones inespecíficas; (4) incubación conjunta de

72 horas en anticuerpos primarios, rabbit-anti-pSTAT5 Tyr694 1:500 (ver 1.2.3) y mouse-anti-oxitocina monoclonal 1:200 (Dr. Harold Gainer, NIH Cat#PS38); (5) incubación conjunta en anticuerpos secundarios fluorescentes, goat anti rabbit IgG conjugado con Alexa Fluor 488 (1:200) y goat-anti-mouse IgG conjugado con Rhodamine Red (1:250); y (6) tinción nnuclear con DAPI (600 nM). Finalmente, se montó y cubrió los cortes histológicos con medio de montaje fluorescente (Fluorsave).

3.2.4. Análisis histológico

El marcaje fluorescente se analizó con un microscopio confocal Leica TCS SP8. Se realizó escaneos secuenciales triples de un "frame" determinado del AC/ADP (Fig 3.2) en los canales para DAPI, Alexa Fluor 488 (AVP) y Rodamina (OT). Las longitudes de onda de excitación fueron 405 nm para DAPI, 488 nm para Alexa Fluor 488 y 559 nm para Rodamina Red-X. Las longitudes de onda de emisión fueron 461, 520 y 591 respectivamente. Las secciones del plano Z se tomaron con 4 µm de separación y a un aumento de 20x.

Tras el muestreo, un observador ciego a las condiciones experimentales del mismo analizó los stacks (pilas de imágenes) obtenidos para cuantificar: (1) el número medio (interhemisférico) de neuronas OXTérgicas; (2) la densidad media (interhemisférica) de pSTAT5-ir; (3) el porcentaje de inmunorreactividad pSTAT5 sobre neuronas OXT-positivas; y (4) el porcentaje de inmunorreactividad pSTAT5 sobre neuronas OXT-negativas. Este análisis se realizó con el software Image J, de forma manual usando la herramienta contador de células en todos los casos salvo en la cuantificación de pSTAT5-ir, donde se plicó un protocolo automatizado similar al empleado en los estudios anteriores.

3.2.5. Análisis estadístico

Datos conductuales

El análisis de motivación maternal mediante recogida de crías comprendió, en primer lugar, la comparación entre grupos experimentales de la latencia a contacto con crías, tiempo total de contacto y número de cruces de barreras. Ante la falta de normalidad de estos datos, se empleó un test ANOVA de Kruskal Wallis para cada variable y cada sesión de conducta. Después, para evaluar la recogida de crías en cada grupo experimental, se aplicó un análisis de supervivencia log Rank (Kaplan Meier) a las latencias de recogida de

la primera y cuarta cría en cada sesión. Finalmente, para obtener una medida del aprendizaje o mejora de cada grupo en la tarea en cuestión, se comparó las latencias a la recogida de la primera cría dentro de cada grupo entre cada sesión, mediante un test Wilcoxon de muestras emparejadas.

Datos histológicos

Para analizar la expresión de pSTAT5 y OXT en el AC/ADP en ambas muestras (recogida de crías y agresión maternal), se aplicó ANOVAs de una vía discretos, con comparaciones post-hoc según Bonferroni. Para comparar los pocentajes relativos de expresión pSTAT5 sobre neuronas OXT-positivas y –negativas, se aplicó en primer lugar un ANOVA de dos vías mixto (con población neuronal y grupo experimental como factores independiente y de medidas repetidas, respectivamente), seguidos de ANOVAs de una vía discretos para cada muestra y cada población neuronal, en busca de diferencias entre grupos experimentales.

Análisis correlacional

Finalmente, se comprobó la asociación de las variables histológicas obtenidas con las variables conductuales de recogida de crías y de agresión maternal (en sus respectivas muestras experimentales). Cuando se cumplía el requisito de normalidad en los datos, se empleó el método paramétrico de Pearson. Cuando no se cumplía la normalidad, se transformó logarítmicamente los datos. En caso de que aún tras esto no se cumpliera normalidad, se aplicó el método no paramétrico de correlación de Spearman.

3.3. DISCUSIÓN

En este capítulo, hemos desarrollado y validado un nuevo test conductual para la evaluación de la motivación maternal. También hemos analizado los cambios en la expresión de OXT y en el input de PRL en la región AC/ADP del hipotálamo, tanto durante el periodo maternal como resultando de la exposición prolongada a crías. Finalmente, hemos explorado correlaciones existentes entre los datos conductuales e histológicos recogidos.

La motivación maternal se define como un estado sostenido en el que las crías adquieren propiedades reforzantes para la madre, desencadenando conductas maternales proactivas (Olazabal et al. 2013). La recogida de crías supone la conducta

maternal proactiva por excelencia, por lo que habitualmente se emplea como aproximación para la evaluación de la motivación maternal. Sin embargo, en ratones hembra vírgenes, dada su facilidad para expresar conductas alomaternales, el test clásico de recogida de crías (en condiciones estándar) puede llevar a conclusiones erróneas o poco precisas. Por esta razón, hemos desarrollado un nuevo test que pretende evaluar la recogida de crías en un contexto adverso (acceso a crías limitado por barreras), que enfrente al animal a la decisión de ejercer un esfuerzo significativo para realizar la conducta y así obtener la recompensa.

Nuestros resultados confirman la validez de este test. Hemos hallado diferencias significativas y previamente obviadas en la motivación por crías en el grupo de madres respecto a vírgenes (Figs 3.4 y 3.5). Esto confirma que los eventos endocrinos de la gestación y postparto son esenciales para el completo desarrollo de la motivación maternal. Por otro lado, hemos determinado que las hembras vírgenes pueden también adquirir una cierta motivación incrementada por crías, si bien de forma gradual, si éstas se encuentran expuestas de forma continua a crías (grupo comadres, Figs 3.4 y 3.5).

El sistema OXTérgico cerebral es un candidato clave para experimentar adaptaciones maternales. Dentro de este sistema, hemos estudiado en profundidad los cambios en el input de PRL (como mediadora de adaptaciones maternales) sobre la población OXTérgica del AC/ADP asociados tanto a la maternidad como a la exposición a crías. Respecto a OXT, no hemos encontrado cambios en el número de neuronas OXTérgicas en ningún caso (Figs 3.8 y 3.10). Respecto a PRL, hemos hallado un aumento significativo en la densidad de células pSTAT5-positivas asociado al periodo postparto, pero no a la exposición a crías (Figs 3.8 y 3.10). Sin embargo, el incremento asociado al periodo maternal parece centrarse en neuronas no OXTérgicas del AC/ADP, mientras que la expresión de pSTAT5 sobre la población OXTérgica permance constante. Esto sugiere que, pese a que esta población se hace sensible a estímulos de crías durante el periodo maternal (Tsuneoka et al. 2013), este cambio probablemente no se da en virtud de una acción directa de la PRL (si bien no queda excluída la posibilidad de una acción de PRL sobre otros centros dentro de las vías que procesan los estímulos de crías). Además, la ausencia de cambios en la expresión de pSTAT5 sobre el AC/ADP en comadres indica que, en el AC/ADP, no se produce ninguna acción o cambio mediado por PRL en respuesta a la exposición continuada a crías.

Por último, hemos explorado posibles correlaciones entre nuestras medidas conductuales (motivación maternal y agresión maternal) y nuestras medidas histológicas en el AC/ADP. Respecto a motivación maternal, hemos hallado correlaciones negativas en madres y comadres entre el número de neuronas OXTérgicas en el AC/ADP y el número total de crías recogidas (Fig 3.9). Con relación a la agresión maternal, hemos hallado una correlación positiva en madres entre el número de neuronas OXtérgicas del AC/ADP y la latencia a la primera agresión (Fig 3.11). Aunque aparentemente contradictorias, las correlaciones halladas podrían indicar un papel de la población OXTérgica del AC/ADP en la liberación de OXT asociada a la respuesta al estrés. Por un lado, la respuesta al estrés conlleva la liberación de OXT en ciertas regiones, con un efecto anxiolítico (Blume et al. 2008). Por otro, mayores niveles de estrés se vinculan a menor expresión de agresión maternal y a mayor atención y cuidado a las crías (Bosch 2011). De estar la población OXTérgica del AC/ADP implicada en la respuesta al estrés, pues, la liberación de OXT desde ésta tendría un efecto anxiolítico, promoviendo la agresión maternal y una atención a crías menos acusada.

4

CONCLUSIONES

1. En el ratón, tanto el encéfalo masculino como el femenino muestran reactividad evidente a la prolactina, siempre y cuando los niveles circulantes de la hormona sean suficientemente altos (p.e. tras administración exógena). Sin embargo, estos patrones son sexualmente dimórficos en favor de hembras. La señalización mediada por prolactina en el encéfalo femenino está presente en el hipotálamo, amígdala, BST, septum y en áreas talámicas y mesencefálicas discretas, mientras que en el macho está restringida a regiones hipotalámicas circunventriculares, con la excepción del núcleo septofimbrial y el órgano subfornical.

2. La señalización lactogénica en el encéfalo femenino está regulada por estradiol y progesterona. En los núcleos analizados, el estradiol tiene un efecto general promotor, mientras que la interacción de la progesterona con el estradiol tiene efectos regionales variables, o bien sinérgicos, o antagónicos.

3. La señalización lactogénica en el encéfalo masculino es dependiente de testosterona. La castración provoca una disminución de la densidad de señalización lactogénica en los núcleos hipotalámicos paraventricular y ventromedial.

4. La señalización lactogénica en el cerebro femenino aumenta considerablemente durante la gestación tardía y postparto, tanto en extensión como en intensidad. Este aumento está probablemente relacionado con el papel de las hormonas lactogénicas en la inducción de adaptaciones fisiológicas y conductuales a la maternidad.

5. Este aumento en la señalización lactogénica durante la gestación tardía y postparto se observa en todos los nodos del cerebro sociosexual. Esto sugiere que las hormonas lactogénicas están probablemente modulando esta red neural hacia un estado maternal, promoviendo así la expresión de comportamientos maternales.

6. Durante el periodo maternal, en la mayoría de los núcleos analizados, la señalización lactogénica alcanza su máximo durante la gestación tardía. Esto indica que es la gestación tardía y no el postparto el periodo crítico para la inducción de las adaptaciones cerebrales a la maternidad.

7. La supresión farmacológica de la secreción hipofisaria de prolactina mediante bromocriptina no atenúa significativamente los niveles de señalización lactogénica medidos en el encéfalo gestante. Esto indica que las hormonas responsables de la señalización lactogénica observada son de origen no hipofisario, probablemente lactógenos placentarios y/o prolactina intracerebral.

8. La correcta evaluación de la motivación por las crías requiere la medición de respuestas maternales proactivas bajo condiciones adversas para la madre. Con este fin, hemos diseñado y validado un nuevo test de recogida de crías para la que se requiere un elevado esfuerzo.

9. En estas condiciones sólo las madres realizan la recogida de crías desde el primer test, lo que indica que el desarrollo de los aspectos motivacionales de la conducta maternal requiere de la acción de señales endocrinas de la gestación y lactancia (incluyendo hormonas lactogénicas).

10. Alternativamente, las hembras vírgenes sujetas a una interacción prolongada con crías (*comadres*) experimentan un incremento moderado de motivación por las crías, como parte del proceso de sensibilización maternal.

11. El AC/ADP, núcleo localizado en una región crítica para el comportamiento maternal, experimenta un incremento significativo de señalización lactogénica durante el periodo maternal (gestación tardía y postparto). Este incremento no ocurre en su población de neuronas oxitocinérgicas, sino en neuronas no oxitocinérgicas adyacentes.

12. Las *comadres* no muestran ningún incremento en señalización lactogénica en la región del AC/ADP en comparación con vírgenes sin contacto con crías. Esto sugiere que la interacción prolongada con crías no tiene efectos directos sobre la acción central de prolactina, al menos en este núcleo.

13. El AC/ADP no sufre ninguna variación en el número de neuronas oxitocinérgicas asociada al periodo postparto ni a la exposición prolongada a crías en *comadres*. Esto sugiere que, en el ratón, los cambios neurales inducidos por agentes endocrinos o estímulos de crías durante el periodo maternal no afectan directamente a la expresión de oxitocina.

14. Aún así, el número de neuronas oxitocinérgicas del AC/ADP correlaciona negativamente con las medidas de recogida (motivada) de crías en madres y *comadres*, y positivamente con las medidas de agresión maternal en madres. Esto sugiere un papel de estas neuronas oxitocinérgicas en la reducción de estrés y ansiedad durante la maternidad, que a su vez afectaría indirectamente al cuidado y agresión maternales.

7

REFERENCES

Aguilera G, Subburaju S, Young S, Chen J (2008) The parvocellular vasopressinergic system and responsiveness of the hypothalamic pituitary adrenal axis during chronic stress. Prog Brain Res 170:29–39. doi: 10.1016/S0079-6123(08)00403-2

Akinci MK, Johnston G a (1993) Sex differences in the effects of acute swim stress on binding to GABAA receptors in mouse brain. J Neurochem 60:2212–2216.

al-Sakkaf KA, Dobson PR, Brown BL (1997) Prolactin induced tyrosine phosphorylation of p59fyn may mediate phosphatidylinositol 3-kinase activation in Nb2 cells. J Mol Endocrinol 19:347–50.

Andersen SL, Tomada A, Vincow ES, et al (2008) Preliminary Evidence for Sensitive Periods in the Effect of Childhood Sexual Abuse on Regional Brain Development. J Neuropsychiatr 20:292–301. doi: 10.1176/appi.neuropsych.20.3.292

Anderson GM, Beijer P, Bang AS, et al (2006) Suppression of prolactin-induced signal transducer and activator of transcription 5b signaling and induction of suppressors of cytokine signaling messenger ribonucleic acid in the hypothalamic arcuate nucleus of the rat during late pregnancy and lactation. Endocrinology 147:4996–5005. doi: 10.1210/en.2005-0755

Anderson GM, Kieser DC, Steyn FJ, Grattan DR (2008) Hypothalamic prolactin receptor messenger ribonucleic acid levels, prolactin signaling, and hyperprolactinemic inhibition of pulsatile luteinizing hormone secretion are dependent on estradiol. Endocrinology 149:1562–70. doi: 10.1210/en.2007-0867

Andrews ZB, Kokay IC, Grattan DR (2001a) Dissociation of prolactin secretion from tuberoinfundibular dopamine activity in late pregnant rats. Endocrinology 142:2719–24. doi: 10.1210/endo.142.6.8196

Andrews ZB, Kokay IC, Grattan DR (2001b) Dissociation of prolactin secretion from tuberoinfundibular dopamine activity in late pregnant rats. Endocrinology 142:2719–2724. doi: 10.1210/en.142.6.2719

Araujo-Lopes R, Crampton JR, Aquino NSS, et al (2014) Prolactin regulates kisspeptin neurons in the arcuate nucleus to suppress LH secretion in female rats. Endocrinology 155:1010–20. doi: 10.1210/en.2013-1889

Augustine R a, Ladyman SR, Grattan DR (2008) From feeding one to feeding many: hormone-induced changes in bodyweight homeostasis during pregnancy. J Physiol 586:387–397. doi: 10.1113/jphysiol.2007.146316

Bakowska JC, Morrell JI (1997) Atlas of the neurons that express mRNA for the long form of the prolactin receptor in the forebrain of the female rat. J Comp Neurol 386:161–177. doi: 10.1002/(SICI)1096-9861(19970922)386:2<161::AID-CNE1>3.0.CO;2-#

Bakowska JC, Morrell JI (2003) The distribution of mRNA for the short form of the prolactin receptor in the forebrain of the female rat. Mol Brain Res 116:50–58. doi: 10.1016/S0169-328X(03)00213-4

Bartke A, Morgan WW, Clayton RN, et al (1987) Neuroendocrine studies in hyperprolactinaemic male mice. J Endocrinol 112:215–20.

Baum MJ, Bakker J (2013) Roles of sex and gonadal steroids in mammalian pheromonal communication. Front Neuroendocrinol 34:268–84. doi: 10.1016/j.yfrne.2013.07.004

Bayerl DS, Klampfl SM, Bosch OJ (2014) Central V1b Receptor Antagonism in Lactating Rats: Impairment of Maternal Care But Not of Maternal Aggression. J Neuroendocrinol 26:918–926. doi: 10.1111/jne.12226

Bayless DW, Shah NM (2016) Genetic dissection of neural circuits underlying sexually dimorphic social behaviours. Philos Trans R Soc Lond B Biol Sci 371:20150109. doi: 10.1098/rstb.2015.0109

Bazan JF (1990) Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci U S A 87:6934–8.

Ben-Jonathan N, Hnasko R (2001a) Dopamine as a prolactin (PRL) inhibitor. Endocr Rev 22:724–63. doi: 10.1210/edrv.22.6.0451

Ben-Jonathan N, Hnasko R (2001b) Dopamine as a prolactin (PRL) inhibitor. Endocr Rev 22:724–763. doi: 10.1210/er.22.6.724

Bennett BA, Morris M (1989) An immunochemical and biochemical study of catecholaminergic activity in dissociated hypothalamic cultures. Brain Res

491:109–15.

Bennett E, McGuinness L, Gevers EF, et al (2005) Hypothalamic STAT proteins: regulation of somatostatin neurones by growth hormone via STAT5b. J Neuroendocrinol 17:186–94. doi: 10.1111/j.1365-2826.2005.01296.x

Bergan JF, Ben-Shaul Y, Dulac C (2014) Sex-specific processing of social cues in the medial amygdala. Elife. doi: 10.7554/eLife.02743

Berlanga JJ, Fresno Vara JA, Martín-Pérez J, García-Ruiz JP (1995) Prolactin receptor is associated with c-src kinase in rat liver. Mol Endocrinol 9:1461–7. doi: 10.1210/mend.9.11.8584023

Berlanga JJ, Garcia-Ruiz JP, Perrot-Applanat M, et al (1997) The short form of the prolactin (PRL) receptor silences PRL induction of the beta-casein gene promoter. Mol Endocrinol 11:1449–57. doi: 10.1210/mend.11.10.9994

Berridge KC, Robinson TE (1998) What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? Brain Res Brain Res Rev 28:309–69.

Besnard I, Auclair V, Callery G, et al (2014) Antipsychotic-drug-induced hyperprolactinemia: physiopathology, clinical features and guidance. Encephale Feb;40:86–94. doi: 10.1016/j.encep.2012.03.002.

Binart N, Bachelot A, Bouilly J (2010) Impact of prolactin receptor isoforms on reproduction. Trends Endocrinol Metab 21:362–368. doi: 10.1016/j.tem.2010.01.008

Bintarningsih, Lyons WR, Johnson RE, Li CH (1958) Hormonally-induced lactation in hypophysectomized rats. Endocrinology 63:540–8. doi: 10.1210/endo-63-5-540

Blanks AM, Thornton S (2003) The role of oxytocin in parturition. BJOG An Int J Obstet Gynaecol 110:46–51. doi: 10.1016/S1470-0328(03)00024-7

Blume A, Bosch OJ, Miklos S, et al (2008) Oxytocin reduces anxiety via ERK1/2 activation: Local effect within the rat hypothalamic paraventricular nucleus. Eur J Neurosci 27:1947–1956. doi: 10.1111/j.1460-9568.2008.06184.x

Blume A, Torner L, Liu Y, et al (2009) Prolactin activates mitogen-activated protein

kinase signaling and corticotropin releasing hormone transcription in rat hypothalamic neurons. Endocrinology 150:1841–9. doi: 10.1210/en.2008-1023

Bole-feysot C, Goffin V, Edery M, et al (1998) Prolactin (PRL) and Its Receptor : Actions , Signal PRL Receptor Knockout Mice. Endocr Rev 19:225–268. doi: 10.1210/er.19.3.225

Bosch OJ (2011) Maternal nurturing is dependent on her innate anxiety: The behavioral roles of brain oxytocin and vasopressin. Horm Behav 59:202–212. doi: 10.1016/j.yhbeh.2010.11.012

Bosch OJ, Krömer SA, Brunton PJ, Neumann ID (2004) Release of oxytocin in the hypothalamic paraventricular nucleus, but not central amygdala or lateral septum in lactating residents and virgin intruders during maternal defence. Neuroscience 124:439–48. doi: 10.1016/j.neuroscience.2003.11.028

Bosch OJ, Meddle SL, Beiderbeck DI, et al (2005) Brain oxytocin correlates with maternal aggression: link to anxiety. J Neurosci 25:6807–6815. doi: 10.1523/JNEUROSCI.1342-05.2005

Bosch OJ, Neumann ID (2010) Vasopressin released within the central amygdala promotes maternal aggression. Eur J Neurosci 31:883–891. doi: 10.1111/j.1460-9568.2010.07115.x

Bosch OJ, Neumann ID (2012) Both oxytocin and vasopressin are mediators of maternal care and aggression in rodents: From central release to sites of action. Horm Behav 61:293–303. doi: 10.1016/j.yhbeh.2011.11.002

Brady J V, Nauta WJ (1953) Subcortical mechanisms in emotional behavior: affective changes following septal forebrain lesions in the albino rat. J Comp Physiol Psychol 46:339–46.

Breves JP, McCormick SD, Karlstrom RO (2014) Prolactin and teleost ionocytes: new insights into cellular and molecular targets of prolactin in vertebrate epithelia. Gen Comp Endocrinol 203:21–8. doi: 10.1016/j.ygcen.2013.12.014

Bridges R, Zarrow MX, Gandelman R, Denenberg VH (1972) Differences in maternal responsiveness between lactating and sensitized rats. Dev Psychobiol 5:123–127.

doi: 10.1002/dev.420050205

Bridges RS (2015) Neuroendocrine regulation of maternal behavior. Front Neuroendocrinol 36:178–96. doi: 10.1016/j.yfrne.2014.11.007

Bridges RS, DiBiase R, Loundes DD, Doherty PC (1985) Prolactin stimulation of maternal behavior in female rats. Science 227:782–4.

Bridges RS, Freemark MS (1995) Human placental lactogen infusions into the medial preoptic area stimulate maternal behavior in steroid-primed, nulliparous female rats. Horm Behav 29:216–26. doi: 10.1006/hbeh.1995.1016

Bridges RS, Hays LE (2005) Steroid-induced alterations in mRNA expression of the long form of the prolactin receptor in the medial preoptic area of female rats: Effects of exposure to a pregnancy-like regimen of progesterone and estradiol. Mol Brain Res 140:10–16. doi: 10.1016/j.molbrainres.2005.06.011

Bridges RS, Lupini CE (1991) Measurement of rat placental lactogen-II in cerebrospinal fluid during late pregnancy in rats using the Nb2 node lymphoma cell bioassay.

Bridges RS, Mann PE, Coppeta JS (1999) Hypothalamic involvement in the regulation of maternal behaviour in the rat: inhibitory roles for the ventromedial hypothalamus and the dorsal/anterior hypothalamic areas. J Neuroendocrinol 11:259–66.

Bridges RS, Numan M, Ronsheim PM, et al (1990a) Central prolactin infusions stimulate maternal behavior in steroid-treated, nulliparous female rats. Proc Natl Acad Sci U S A 87:8003–8007. doi: 10.1073/pnas.87.20.8003

Bridges RS, Numan M, Ronsheim PM, et al (1990b) Central prolactin infusions stimulate maternal behavior in steroid-treated, nulliparous female rats. Proc Natl Acad Sci U S A 87:8003–7.

Bridges RS, Robertson MC, Shiu RP, et al (1996) Endocrine communication between conceptus and mother: placental lactogen stimulation of maternal behavior. Neuroendocrinology 64:57–64.

Bridges RS, Ronsheim PM (1990) Prolactin (PRL) regulation of maternal behavior in rats: bromocriptine treatment delays and PRL promotes the rapid onset of behavior. Endocrinology 126:837–48. doi: 10.1210/endo-126-2-837

Broida J, Michael SD, Svare B (1981) Plasmin prolactin levels are not related to the initiation, maintenance, and decline of postpartum aggression in mice. Behav Neural Biol 32:121–5.

Brown RE, Murdoch T, Murphy PR, Moger WH (1995) Hormonal Responses of Male Gerbils to Stimuli from Their Mate and Pups. Horm Behav 29:474–491. doi: 10.1006/hbeh.1995.1275

Brown RSE, Herbison a E, Grattan DR (2011) Differential changes in responses of hypothalamic and brainstem neuronal populations to prolactin during lactation in the mouse. Biol Reprod 84:826–836. doi: 10.1095/biolreprod.110.089185

Brown RSE, Kokay IC, Herbison AE, Grattan DR (2010) Distribution of prolactin-responsive neurons in the mouse forebrain. J Comp Neurol 518:92–102. doi: 10.1002/cne.22208

Brown RSE, Wyatt AK, Herbison RE, et al (2015) Prolactin transport into mouse brain is independent of prolactin receptor. FASEB J. doi: 10.1096/fj.15-276519

Brunton PJ, Russell JA (2008) The expectant brain: adapting for motherhood. Nat Rev Neurosci 9:11–25. doi: 10.1038/nrn2280

Buckley AR, Rao YP, Buckley DJ, Gout PW (1994) Prolactin-induced phosphorylation and nuclear translocation of MAP kinase in Nb2 lymphoma cells. Biochem Biophys Res Commun 204:1158–64. doi: 10.1006/bbrc.1994.2584

Buonfiglio DC, Ramos-Lobo AM, Silveira MA, et al (2015) Neuronal STAT5 signaling is required for maintaining lactation but not for postpartum maternal behaviors in mice. Horm Behav 71:60–8. doi: 10.1016/j.yhbeh.2015.04.004

Burton KA, Kabigting EB, Clifton DK, Steiner RA (1992) Growth hormone receptor messenger ribonucleic acid distribution in the adult male rat brain and its colocalization in hypothalamic somatostatin neurons. Endocrinology 131:958–63. doi: 10.1210/endo.131.2.1353444

Cádiz-Moretti B, Otero-García M, Martínez-García F, Lanuza E (2016) Afferent projections to the different medial amygdala subdivisions: a retrograde tracing study in the mouse. Brain Struct Funct 221:1033–65. doi: 10.1007/s00429-014-

0954-y

Calcagnoli F, Meyer N, de Boer SF, et al (2014) Chronic enhancement of brain oxytocin levels causes enduring anti-aggressive and pro-social explorative behavioral effects in male rats. Horm Behav 65:427–433. doi: 10.1016/j.yhbeh.2014.03.008

Canetti L, Bachar E, Galili-Weisstub E, et al (1997) Parental bonding and mental health in adolescence. Adolescence 32:381–94.

Capozzi A, Scambia G, Pontecorvi A, Lello S (2015) Hyperprolactinemia: pathophysiology and therapeutic approach. Gynecol Endocrinol 31:506–10. doi: 10.3109/09513590.2015.1017810

Caquineau C, Leng G, Guan XMM, et al (2006) Effects of alpha-melanocyte-stimulating hormone on magnocellular oxytocin neurones and their activation at intromission in male rats. J Neuroendocrinol 18:685–91. doi: 10.1111/j.1365-2826.2006.01465.x

Caron MG, Beaulieu M, Raymond V, et al (1978) Dopaminergic receptors in the anterior pituitary gland. Correlation of [3H]dihydroergocryptine binding with the dopaminergic control of prolactin release. J Biol Chem 253:2244–53.

Champagne FA, Chretien P, Stevenson CW, et al (2004) Variations in nucleus accumbens dopamine associated with individual differences in maternal behavior in the rat. J Neurosci 24:4113–23. doi: 10.1523/JNEUROSCI.5322-03.2004

Champagne F, Diorio J, Sharma S, Meaney MJ (2001) Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. Proc Natl Acad Sci 98:12736–12741. doi: 10.1073/pnas.221224598

Chandrashekar V, Bartke A (1988) Influence of endogenous prolactin on the luteinizing hormone stimulation of testicular steroidogenesis and the role of prolactin in adult male rats. Steroids 51:559–76.

Chen JC, Ramirez VD (1988) In vivo dopaminergic activity from nucleus accumbens, substantia nigra and ventral tegmental area in the freely moving rat: basal neurochemical output and prolactin effect. Neuroendocrinology 48:329–35.

Christensen LW, Nance DM, Gorski RA (1977) Effects of hypothalamic and preoptic

lesions on reproductive behavior in male rats. Brain Res Bull 2:137–41.

Consiglio AR, Bridges RS (2009) Circulating prolactin, MPOA prolactin receptor expression and maternal aggression in lactating rats. Behav Brain Res 197:97–102. doi: 10.1016/j.bbr.2008.08.006

Consiglio AR, Lucion AB Lesion of hypothalamic paraventricular nucleus and maternal aggressive behavior in female rats. Physiol Behav 59:591–6.

Cservenák M, Bodnár I, Usdin TB, et al (2010) Tuberoinfundibular peptide of 39 residues is activated during lactation and participates in the suckling-induced prolactin release in rat. Endocrinology 151:5830–5840. doi: 10.1210/en.2010-0767

Cservenák M, Szabó ÉR, Bodnár I, et al (2013) Thalamic neuropeptide mediating the effects of nursing on lactation and maternal motivation. Psychoneuroendocrinology 38:3070–84. doi: 10.1016/j.psyneuen.2013.09.004

D'Amato F (1993) Effect of Familiarity with the Mother and Kinship on Infanticidal and Alloparental Behaviour in Virgin House Mice. Behaviour 124:313–326.

D'Amato FR, Scalera E, Sarli C, Moles A (2005) Pups call, mothers rush: does maternal responsiveness affect the amount of ultrasonic vocalizations in mouse pups? Behav Genet 35:103–12. doi: 10.1007/s10519-004-0860-9

Davis M, Shi C (1999) The extended amygdala: are the central nucleus of the amygdala and the bed nucleus of the stria terminalis differentially involved in fear versus anxiety? Ann N Y Acad Sci 877:281–91.

DeMaria JE, Lerant AA, Freeman ME (1999) Prolactin activates all three populations of hypothalamic neuroendocrine dopaminergic neurons in ovariectomized rats. Brain Res 837:236–41.

DeVito WJ, Connors JM, Hedge GA (1987) Immunoreactive prolactin in the rat hypothalamus: in vitro release and subcellular localization. Neuroendocrinology 46:155–61.

Donadio MVF, Gomes CM, Sagae SC, et al (2006) Estradiol and progesterone modulation of angiotensin II receptors in the arcuate nucleus of ovariectomized and lactating rats. Brain Res 1083:103–9. doi: 10.1016/j.brainres.2006.02.018

Donner N, Neumann ID (2009) Effects of chronic intracerebral prolactin on the oxytocinergic and vasopressinergic system of virgin ovariectomized rats. Neuroendocrinology 90:315–22. doi: 10.1159/000225986

Douglas AJ, Meddle SL, Toschi N, et al (2005) Reduced activity of the noradrenergic system in the paraventricular nucleus at the end of pregnancy: Implications for stress hyporesponsiveness. J Neuroendocrinol 17:40–48. doi: 10.1111/j.1365-2826.2005.01272.x

Drago F, Bohus B, Gispen WH, et al (1983) Prolactin-enhanced grooming behavior: interaction with ACTH. Brain Res 263:277–82.

Drago F, Lissandrello CO (2000) The “low-dose” concept and the paradoxical effects of prolactin on grooming and sexual behavior. Eur J Pharmacol 405:131–137. doi: 10.1016/S0014-2999(00)00678-6

Drago F, Pellegrini-Quarantotti B, Scapagnini U, Gessa GL (1981) Short-term endogenous hyperprolactinaemia and sexual behavior of male rats. Physiol Behav 26:277–9.

Dugger BN, Morris JA, Jordan CL, Breedlove SM (2007) Androgen receptors are required for full masculinization of the ventromedial hypothalamus (VMH) in rats. Horm Behav 51:195–201. doi: 10.1016/j.yhbeh.2006.10.001

Dungan HM, Clifton DK, Steiner RA (2006) Minireview: kisspeptin neurons as central processors in the regulation of gonadotropin-releasing hormone secretion. Endocrinology 147:1154–8. doi: 10.1210/en.2005-1282

Dutt A, Kaplitt MG, Kow LM, Pfaff DW (1994) Prolactin, central nervous system and behavior: a critical review. Neuroendocrinology 59:413–9.

Egli M, Leeners B, Kruger THC (2010) Prolactin secretion patterns: basic mechanisms and clinical implications for reproduction. Reproduction 140:643–654. doi: 10.1530/REP-10-0033

Erskine MS, Barfield RJ, Goldman BD (1980) Postpartum aggression in rats: I. Effects of hypophysectomy. J Comp Physiol Psychol 94:484–494. doi: 10.1037/h0077686

Factor EM, Mayer AD, Rosenblatt JS (1993) Peripeduncular nucleus lesions in the rat: I. Effects on maternal aggression, lactation, and maternal behavior during pre- and

postpartum periods. Behav Neurosci 107:166–85.

Fahrbach SE, Morrell JI, Pfaff DW (1985) Possible role for endogenous oxytocin in estrogen-facilitated maternal behavior in rats. Neuroendocrinology 40:526–32.

Faron-Górecka A, Kuśmider M, Kolasa M, et al (2014) Prolactin and its receptors in the chronic mild stress rat model of depression. Brain Res 1555:48–59. doi: 10.1016/j.brainres.2014.01.031

Flannelly KJ, Kemble ED, Blanchard DC, Blanchard RJ (1986) Effects of septal-forebrain lesions on maternal aggression and maternal care. Behav Neural Biol 45:17–30.

Fleming AS, Cheung U, Myhal N, Kessler Z (1989) Effects of maternal hormones on "timidity" and attraction to pup-related odors in female rats. Physiol Behav 46:449–53.

Fleming AS, Kraemer GW, Gonzalez A, et al (2002) Mothering begets mothering: the transmission of behavior and its neurobiology across generations. Pharmacol Biochem Behav 73:61–75.

Fleming AS, Luebke C (1981) Timidity prevents the virgin female rat from being a good mother: emotionality differences between nulliparous and parturient females. Physiol Behav 27:863–8.

Francis D, Diorio J, Liu D, Meaney MJ (1999) Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science 286:1155–8.

Freeman ME, Kanyicska B, Lerant A, Nagy G (2000) Prolactin: structure, function, and regulation of secretion. Physiol Rev 80:1523–631.

Fujikawa T, Soya H, Tamashiro KLK, et al (2004) Prolactin prevents acute stress-induced hypocalcemia and ulcerogenesis by acting in the brain of rat. Endocrinology 145:2006–13. doi: 10.1210/en.2003-1446

Fujikawa T, Soya H, Yoshizato H, et al (1995) Restraint stress enhances the gene expression of prolactin receptor long form at the choroid plexus. Endocrinology 136:5608–13. doi: 10.1210/endo.136.12.7588315

Furigo IC, Kim KW, Nagaishi VS, et al (2014a) Prolactin-sensitive neurons express estrogen receptor-α and depend on sex hormones for normal responsiveness to prolactin. Brain Res 1566:47–59. doi: 10.1016/j.brainres.2014.04.018

Furigo IC, Kim KW, Nagaishi VS, et al (2014b) Prolactin-sensitive neurons express estrogen receptor-α and depend on sex hormones for normal responsiveness to prolactin. Brain Res 1566:47–59. doi: 10.1016/j.brainres.2014.04.018

Furigo IC, Metzger M, Teixeira PDS, et al (2016) Distribution of growth hormone-responsive cells in the mouse brain. Brain Struct Funct. doi: 10.1007/s00429-016-1221-1

Gala RR (1990) The physiology and mechanisms of the stress-induced changes in prolactin secretion in the rat. Life Sci 46:1407–20.

Gala RR, Shevach EM (1994) Evidence for the release of a prolactin-like substance by mouse lymphocytes and macrophages. Proc Soc Exp Biol Med 205:12–9.

Gammie SC (2005) Current models and future directions for understanding the neural circuitries of maternal behaviors in rodents. Behav Cogn Neurosci Rev 4:119–135. doi: 10.1177/1534582305281086

Gandelman R (1973) The ontogeny of maternal responsiveness in female Rockland-Swiss albino mice. Horm Behav 4:257–68.

Ganong WF (2000) Circumventricular organs: Definition and role in the regulation of endocrine and autonomic function. Clin Exp Pharmacol Physiol 27:422–427. doi: 10.1046/j.1440-1681.2000.03259.x

Garland M, Svare B (1988) Suckling stimulation modulates the maintenance of postpartum aggression in mice. Physiol Behav 44:301–5.

Ghosh R, Sladek CD (1995) Role of prolactin and gonadal steroids in regulation of oxytocin mRNA during lactation. Am J Physiol 269:E76-84.

Gil M, Bhatt R, Picotte KB, Hull EM (2011) Oxytocin in the medial preoptic area facilitates male sexual behavior in the rat. Horm Behav 59:435–43. doi: 10.1016/j.yhbeh.2010.12.012

Gill-Sharma MK (2009) Prolactin and male fertility: the long and short feedback regulation. Int J Endocrinol 2009:687259. doi: 10.1155/2009/687259

Gimpl G, Fahrenholz F (2001) The oxytocin receptor system: structure, function, and regulation. Physiol Rev 81:629–83.

Goffin V, Kinet S, Ferrag F, et al (1996) Antagonistic properties of human prolactin analogs that show paradoxical agonistic activity in the Nb2 bioassay. J Biol Chem 271:16573–9.

Gong Y, Ishida-Takahashi R, Villanueva EC, et al (2007) The long form of the leptin receptor regulates STAT5 and ribosomal protein S6 via alternate mechanisms. J Biol Chem 282:31019–31027. doi: 10.1074/jbc.M702838200

Goodson JL (2005) The vertebrate social behavior network: Evolutionary themes and variations. Horm Behav 48:11–22. doi: 10.1016/j.yhbeh.2005.02.003

Goudreau JL, Lindley SE, Lookingland KJ, Moore KE (1992) Evidence that hypothalamic periventricular dopamine neurons innervate the intermediate lobe of the rat pituitary. Neuroendocrinology 56:100–5.

Goupille O, Daniel N, Bignon C, et al (1997) Prolactin signal transduction to milk protein genes: carboxy-terminal part of the prolactin receptor and its tyrosine phosphorylation are not obligatory for JAK2 and STAT5 activation. Mol Cell Endocrinol 127:155–69.

Grattan DR (2002) Behavioural significance of prolactin signalling in the central nervous system during pregnancy and lactation. Reproduction 123:497–506. doi: 10.1530/reprod/123.4.497

Grattan DR, Kokay IC (2008) Prolactin: A pleiotropic neuroendocrine hormone. J Neuroendocrinol 20:752–763. doi: 10.1111/j.1365-2826.2008.01736.x

Grattan DR, Pi XJ, Andrews ZB, et al (2001) Prolactin receptors in the brain during pregnancy and lactation: implications for behavior. Horm Behav 40:115–124. doi: 10.1006/hbeh.2001.1698

Grosvenor CE, Picciano MF, Baumrucker CR (1993) Hormones and growth factors in milk. Endocr Rev 14:710–728. doi: 10.1210/edrv-14-6-710

Guillou A, Romanò N, Steyn F, et al (2015) Assessment of lactotroph axis functionality in mice: longitudinal monitoring of PRL secretion by ultrasensitive-ELISA. Endocrinology en.2014-1571. doi: 10.1210/en.2014-1571

Gur R, Tendler A, Wagner S (2014) Long-Term Social Recognition Memory Is Mediated by Oxytocin-Dependent Synaptic Plasticity in the Medial Amygdala. Biol Psychiatry 76:377–386. doi: 10.1016/j.biopsych.2014.03.022

Hansen S (1989) Medial hypothalamic involvement in maternal aggression of rats. Behav Neurosci 103:1035–46.

Hansen S, Ferreira A (1986) Effects of bicuculline infusions in the ventromedial hypothalamus and amygdaloid complex on food intake and affective behavior in mother rats. Behav Neurosci 100:410–5.

Hansen S, Harthon C, Wallin E, et al (1991) Mesotelencephalic dopamine system and reproductive behavior in the female rat: effects of ventral tegmental 6-hydroxydopamine lesions on maternal and sexual responsiveness. Behav Neurosci 105:588–98.

Hauser H, Gandelman R (1985) Lever pressing for pups: evidence for hormonal influence upon maternal behavior of mice. Horm Behav 19:454–68.

He Z, Ferguson SA, Cui L, et al (2013) Development of the sexually dimorphic nucleus of the preoptic area and the influence of estrogen-like compounds. Neural Regen Res 8:2763–74. doi: 10.3969/j.issn.1673-5374.2013.29.008

Hernandez ME, Soto-Cid A, Rojas F, et al (2006) Prostate response to prolactin in sexually active male rats. Reprod Biol Endocrinol 4:28. doi: 10.1186/1477-7827-4-28

Hernández ML, Fernández-Ruiz JJ, Navarro M, et al (1994) Modifications of mesolimbic and nigrostriatal dopaminergic activities after intracerebroventricular administration of prolactin. J Neural Transm Gen Sect 96:63–79.

Horseman ND, Gregerson KA (2013) Prolactin actions. J Mol Endocrinol 52:R95–R106. doi: 10.1530/JME-13-0220

Horseman ND, Yu-Lee LY (1994) Transcriptional regulation by the helix bundle peptide

hormones: Growth hormone, prolactin, and hematopoietic cytokines. Endocr Rev 15:627–649. doi: 10.1210/er.15.5.627

Hu ZZ, Dufau ML (1991) Multiple and differential regulation of ovarian prolactin receptor messenger RNAs and their expression. Biochem Biophys Res Commun 181:219–25.

Ihle JN, Witthuhn BA, Quelle FW, et al (1994) Signaling by the cytokine receptor superfamily: JAKs and STATs. Trends Biochem Sci 19:222–7.

Insel TR, Harbaugh CR (1989) Lesions of the hypothalamic paraventricular nucleus disrupt the initiation of maternal behavior. Physiol Behav 45:1033–41.

Insel TR, Young LJ (2000) Neuropeptides and the evolution of social behavior. Curr Opin Neurobiol 10:784–9.

Kamel F, Wright WW, Mock EJ, Frankel AI (1977) The influence of mating and related stimuli on plasma levels of luteinizing hormone, follicle stimulating hormone, prolactin, and testosterone in the male rat. Endocrinology 101:421–9. doi: 10.1210/endo-101-2-421

Kawauchi H, Sower SA (2006) The dawn and evolution of hormones in the adenohypophysis. Gen Comp Endocrinol 148:3–14. doi: 10.1016/j.ygcen.2005.10.011

Kelly PA, Tsushima T, Shiu RP, Friesen HG (1976) Lactogenic and growth hormone-like activities in pregnancy determined by radioreceptor assays. Endocrinology 99:765–74. doi: 10.1210/endo-99-3-765

Kim S, Strathearn L (2016) Oxytocin and Maternal Brain Plasticity. New Dir Child Adolesc Dev 2016:59–72. doi: 10.1002/cad.20170

Kirk SE, Xie TY, Steyn FJ, et al (2017) Restraint stress increases prolactin-mediated phosphorylation of signal transducer and activator of transcription 5 in the hypothalamus and adrenal cortex in the male mouse. J Neuroendocrinol. doi: 10.1111/jne.12477

Kirsch P (2015) Oxytocin in the socioemotional brain: implications for psychiatric disorders. Dialogues Clin Neurosci 17:463–76.

Kishi K, Hirashiba M, Hasegawa Y (1991) Gestational profiles of rat placental lactogen-II (rPL-II) and growth hormone (GH) in maternal and fetal serum, amniotic fluid, and placental tissue. Endocrinol Jpn 38:589–595.

Klampfl SM, Brunton PJ, Bayerl DS, Bosch OJ (2014) Hypoactivation of CRF Receptors, Predominantly Type 2, in the Medial-Posterior BNST Is Vital for Adequate Maternal Behavior in Lactating Rats. J Neurosci 34:9665–76. doi: 10.1523/JNEUROSCI.4220-13.2014

Kleiman DG, Malcolm J (1981) The Evolution of Male Parental Investment in Mammals.

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

Kohmoto K, Tsunasawa S, Sakiyama F (1984) Complete amino acid sequence of mouse prolactin. Eur J Biochem 138:227–37.

Kokay IC, Petersen SL, Grattan DR (2011) Identification of prolactin-sensitive GABA and kisspeptin neurons in regions of the rat hypothalamus involved in the control of fertility. Endocrinology 152:526–35. doi: 10.1210/en.2010-0668

Kow LM, Pfaff DW (1998) Mapping of neural and signal transduction pathways for lordosis in the search for estrogen actions on the central nervous system. Behav Brain Res 92:169–80.

Krebs DL, Hilton DJ (2001) SOCS proteins: negative regulators of cytokine signaling. Stem Cells 19:378–87. doi: 10.1634/stemcells.19-5-378

Krüger THC, Haake P, Hartmann U, et al (2002) Orgasm-induced prolactin secretion: feedback control of sexual drive? Neurosci Biobehav Rev 26:31–44.

Ladyman SR, Fieldwick DM, Grattan DR (2012) Suppression of leptin-induced hypothalamic JAK/STAT signalling and feeding response during pregnancy in the mouse. Reproduction 144:83–90. doi: 10.1530/REP-12-0112

Larsen CM, Grattan DR (2010a) Prolactin-induced mitogenesis in the subventricular zone of the maternal brain during early pregnancy is essential for normal postpartum behavioral responses in the mother. Endocrinology 151:3805–3814. doi:

10.1210/en.2009-1385

Larsen CM, Grattan DR (2010b) Prolactin-induced mitogenesis in the subventricular zone of the maternal brain during early pregnancy is essential for normal postpartum behavioral responses in the mother. Endocrinology 151:3805–14. doi: 10.1210/en.2009-1385

Lebrun JJ, Ali S, Ullrich A, Kelly PA (1995) Proline-rich sequence-mediated Jak2 association to the prolactin receptor is required but not sufficient for signal transduction. J Biol Chem 270:10664–70.

Lee A, Clancy S, Fleming AS (2000) Mother rats bar-press for pups: effects of lesions of the mpoa and limbic sites on maternal behavior and operant responding for pup-reinforcement. Behav Brain Res 108:215–31.

Lesueur L, Edery M, Ali S, et al (1991) Comparison of long and short forms of the prolactin receptor on prolactin-induced milk protein gene transcription. Proc Natl Acad Sci U S A 88:824–8.

Li C, Chen P, Smith MS (1999) Neuropeptide Y and tuberoinfundibular dopamine activities are altered during lactation: Role of prolactin. Endocrinology 140:118–123. doi: 10.1210/en.140.1.118

Lin D, Boyle MP, Dollar P, et al (2011) Functional identification of an aggression locus in the mouse hypothalamus. Nature 470:221–226. doi: 10.1038/nature09736

Linzer DI, Fisher SJ (1999) The placenta and the prolactin family of hormones: regulation of the physiology of pregnancy. Mol Endocrinol 13:837–40. doi: 10.1210/mend.13.6.0286

Lobie PE, García-Aragón J, Lincoln DT, et al (1993) Localization and ontogeny of growth hormone receptor gene expression in the central nervous system. Brain Res Dev Brain Res 74:225–33.

Lonstein JS, De Vries GJ (2000) Maternal behaviour in lactating rats stimulates c-fos in glutamate decarboxylase-synthesizing neurons of the medial preoptic area, ventral bed nucleus of the stria terminalis, and ventrocaudal periaqueductal gray. Neuroscience 100:557–68.

Lonstein JS, Gammie SC (2002) Sensory, hormonal, and neural control of maternal aggression in laboratory rodents. Neurosci Biobehav Rev 26:869–888. doi: 10.1016/S0149-7634(02)00087-8

Lonstein JS, Stern JM (1997) Role of the midbrain periaqueductal gray in maternal nurturance and aggression: c-fos and electrolytic lesion studies in lactating rats. J Neurosci 17:3364–78.

Lookingland KJ, Moore KE (1984) Effects of estradiol and prolactin on incertohypothalamic dopaminergic neurons in the male rat. Brain Res 323:83–91.

Loundes DD, Bridges RS (1986) Length of prolactin priming differentially affects maternal behavior in female rats. Biol Reprod 34:495–501.

Lucas BK, Ormandy CJ, Binart N, et al (1998a) Null mutation of the prolactin receptor gene produces a defect in maternal behavior. Endocrinology 139:4102–4107. doi: 10.1210/endo.139.10.6243

Lucas BK, Ormandy CJ, Binart N, et al (1998b) Null mutation of the prolactin receptor gene produces a defect in maternal behavior. Endocrinology 139:4102–7. doi: 10.1210/endo.139.10.6243

Lyons DJ, Hellysaz a., Broberger C (2012) Prolactin Regulates Tuberoinfundibular Dopamine Neuron Discharge Pattern: Novel Feedback Control Mechanisms in the Lactotrophic Axis. J Neurosci 32:8074–8083. doi: 10.1523/JNEUROSCI.0129-12.2012

Ma E, Lau J, Grattan DR, et al (2005a) Male and female prolactin receptor mRNA expression in the brain of a biparental and a uniparental hamster, phodopus, before and after the birth of a litter. J Neuroendocrinol 17:81–90. doi: 10.1111/j.1365-2826.2005.01278.x

Ma FY, Anderson GM, Gunn TD, et al (2005b) Prolactin specifically activates signal transducer and activator of transcription 5b in neuroendocrine dopaminergic neurons. Endocrinology 146:5112–5119. doi: 10.1210/en.2005-0770

Machado RB, Rocha MR, Suchecki D (2017) Brain prolactin is involved in stress-induced REM sleep rebound. Horm Behav 89:38–47. doi: 10.1016/j.yhbeh.2016.12.004

Mackinnon DA, Stern JM (1977) Pregnancy duration and fetal number: effects on maternal behavior in rats. Physiol Behav 18:793–7.

Maeda T, Ikegami H, Sakata M, et al (1996) Intraventricular administration of estradiol modulates rat prolactin secretion and synthesis. J Endocrinol Invest 19:586–92. doi: 10.1007/BF03349022

Maestripieri D, Alleva E (1991) Litter defence and parental investment allocation in house mice. Behav Processes 23:223–230. doi: 10.1016/0376-6357(91)90052-2

Maestripieri D, Badiani A, Puglisi-Allegra S (1991) Prepartal chronic stress increases anxiety and decreases aggression in lactating female mice. Behav Neurosci 105:663–8.

Maestripieri D, D'Amato FR (1991) Anxiety and maternal aggression in house mice (Mus musculus): a look at interindividual variability. J Comp Psychol 105:295–301.

Mangurian LP, Walsh RJ, Posner BI (1992) Prolactin enhancement of its own uptake at the choroid plexus. Endocrinology 131:698–702. doi: 10.1210/endo.131.2.1639017

Mann MA, Konen C, Svare B (1984) The role of progesterone in pregnancy-induced aggression in mice. Horm Behav 18:140–60.

Mann M, Michael SD, Svare B (1980) Ergot drugs suppress plasma prolactin and lactation but not aggression in parturient mice. Horm Behav 14:319–328.

Marano RJ, Ben-Jonathan N (2014) Minireview: Extrapituitary prolactin: an update on the distribution, regulation, and functions. Mol Endocrinol 28:622–33. doi: 10.1210/me.2013-1349

Martinez-Ferre A, Martinez S (2012) Molecular regionalization of the diencephalon. Front Neurosci 6:73. doi: 10.3389/fnins.2012.00073

Martinez-Garcia F, Novejarque A, Gutiérrez-Castellanos N, Lanuza E (2012) Piriform Cortex and Amygdala. In: The Mouse Nervous System. pp 140–172

Martín-Sánchez A, McLean L, Beynon RJ, et al (2015a) From sexual attraction to maternal aggression: When pheromones change their behavioural significance. Horm Behav 68C:65–76. doi: 10.1016/j.yhbeh.2014.08.007

Martín-Sánchez A, Valera-Marín G, Hernández-Martínez A, et al (2015b) Wired for motherhood: induction of maternal care but not maternal aggression in virgin female CD1 mice. Front Behav Neurosci 9:197. doi: 10.3389/fnbeh.2015.00197

Masuhara M, Sakamoto H, Matsumoto A, et al (1997) Cloning and characterization of novel CIS family genes. Biochem Biophys Res Commun 239:439–46. doi: 10.1006/bbrc.1997.7484

Matthews J, Almlöf T, Kietz S, et al (2005) Estrogen receptor-alpha regulates SOCS-3 expression in human breast cancer cells. Biochem Biophys Res Commun 335:168–74. doi: 10.1016/j.bbrc.2005.07.057

Mattson BJ, Williams S, Rosenblatt JS, Morrell JI (2001) Comparison of two positive reinforcing stimuli: pups and cocaine throughout the postpartum period. Behav Neurosci 115:683–94.

Mayer AD, Freeman NCG, Rosenblatt JS (1979) Ontogeny of maternal behavior in the laboratory rat: Factors underlying changes in responsiveness from 30 to 90 days. Dev Psychobiol 12:425–439. doi: 10.1002/dev.420120503

Mayer AD, Monroy MA, Rosenblatt JS (1990) Prolonged estrogen-progesterone treatment of nonpregnant ovariectomized rats: factors stimulating home-cage and maternal aggression and short-latency maternal behavior. Horm Behav 24:342–64.

Meaney MJ (2001) Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu Rev Neurosci 24:1161–92. doi: 10.1146/annurev.neuro.24.1.1161

Mehta MA, Golembo NI, Nosarti C, et al (2009) Amygdala, hippocampal and corpus callosum size following severe early institutional deprivation: the English and Romanian Adoptees study pilot. J Child Psychol Psychiatry 50:943–51. doi: 10.1111/j.1469-7610.2009.02084.x

Mejía S, Morales MA, Zetina ME, et al (1997) Immunoreactive prolactin forms colocalize with vasopressin in neurons of the hypothalamic paraventricular and supraoptic nuclei. Neuroendocrinology 66:151–9.

Mejía S, Torner LM, Jeziorski MC, et al (2003) Prolactin and 16K Prolactin Stimulate

Release of Vasopressin by a Direct Effect on Hypothalamo-Neurohypophyseal System. Endocrine 20:155–162. doi: 10.1385/ENDO:20:1-2:155

Melcangi RC, Giatti S, Garcia-Segura LM (2015) Levels and actions of neuroactive steroids in the nervous system under physiological and pathological conditions: Sex-specific features. Neurosci Biobehav Rev. doi: 10.1016/j.neubiorev.2015.09.023

Mitra SW, Hoskin E, Yudkovitz J, et al (2003) Immunolocalization of estrogen receptor α in the mouse brain: Comparison with estrogen receptor β. Endocrinology 144:2055–2067. doi: 10.1210/en.2002-221069

Morgan HD, Watchus JA, Milgram NW, Fleming AS (1999) The long lasting effects of electrical simulation of the medial preoptic area and medial amygdala on maternal behavior in female rats. Behav Brain Res 99:61–73.

Morris JA, Jordan CL, Breedlove SM (2008) Sexual dimorphism in neuronal number of the posterodorsal medial amygdala is independent of circulating androgens and regional volume in adult rats. J Comp Neurol 506:851–9. doi: 10.1002/cne.21536

Muccioli G, Di Carlo R (1994) Modulation of prolactin receptors in the rat hypothalamus in response to changes in serum concentration of endogenous prolactin or to ovine prolactin administration. Brain Res 663:244–50.

Mütze J, Roth J, Gerstberger R, Hübschle T (2007) Nuclear translocation of the transcription factor STAT5 in the rat brain after systemic leptin administration. Neurosci Lett 417:286–291. doi: 10.1016/j.neulet.2007.02.074

Naftolin F, Ryan KJ, Davies IJ, et al (1975) The formation of estrogens by central neuroendocrine tissues. Recent Prog Horm Res 31:295–319.

Nakane PK (1970) Classifications of anterior pituitary cell types with immunoenzyme histochemistry. J Histochem Cytochem 18:9–20.

Neill J (1972) Sexual Differences in the Hypothalamic Regulation of Prolactin Secretion12. Endocrinology 90:1154–1159. doi: 10.1210/endo-90-5-1154

Neill JD (1970) Effect of "stress" on serum prolactin and luteinizing hormone levels during the estrous cycle of the rat. Endocrinology 87:1192–7. doi:

10.1210/endo-87-6-1192

Neumann ID, Torner L, Wigger A (2000) Brain oxytocin: differential inhibition of neuroendocrine stress responses and anxiety-related behaviour in virgin, pregnant and lactating rats. Neuroscience 95:567–75.

Neumann ID, Toschi N, Ohl F, et al (2001) Maternal defence as an emotional stressor in female rats: correlation of neuroendocrine and behavioural parameters and involvement of brain oxytocin. Eur J Neurosci 13:1016–24.

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. doi: 10.1111/j.1749-6632.1999.tb09271.x

Nicoletti F, Drago F, Speciale C, et al (1983) Role of prolactin-opiate interactions in the central regulation of pain threshold. Cephalalgia 3 Suppl 1:31–4.

Nishimori K, Young LJ, Guo Q, et al (1996) Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. Proc Natl Acad Sci U S A 93:11699–704.

Nishitani S, Moriya T, Kondo Y, et al (2004) Induction of Fos immunoreactivity in oxytocin neurons in the paraventricular nucleus after female odor exposure in male rats: effects of sexual experience. Cell Mol Neurobiol 24:283–91.

Nogami H, Hoshino R, Ogasawara K, et al (2007) Region-specific expression and hormonal regulation of the first exon variants of rat prolactin receptor mRNA in rat brain and anterior pituitary gland. J Neuroendocrinol 19:583–93. doi: 10.1111/j.1365-2826.2007.01565.x

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 29:23–51. doi: 10.1002/(SICI)1098-2302(199601)29:1<23::AID-DEV2>3.0.CO;2-O

Numan M (2014) Neurobiology of social behavior : toward an understanding of the prosocial and antisocial brain. Elsevier Academic Press, Amsterdam

Numan M, Corodimas KP, Numan MJ, et al (1988) Axon-sparing lesions of the preoptic

region and substantia innominata disrupt maternal behavior in rats. Behav Neurosci 102:381–96.

Numan M, Insel TR (2003) The Neurobiology of Parental Behavior. Springer, New York

Numan M, McSparren J, Numan MJ (1990) Dorsolateral connections of the medial preoptic area and maternal behavior in rats. Behav Neurosci 104:964–79.

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 29:23–51. doi: 10.1002/(SICI)1098-2302(199601)29:1<23::AID-DEV2>3.0.CO;2-O

Numan M, Numan MJ, Marzella SR, Palumbo A (1998) Expression of c-fos, fos B, and egr-1 in the medial preoptic area and bed nucleus of the stria terminalis during maternal behavior in rats. Brain Res 792:348–52.

Numan M, Numan MJ, Schwarz JM, et al (2005) Medial preoptic area interactions with the nucleus accumbens-ventral pallidum circuit and maternal behavior in rats. Behav Brain Res 158:53–68. doi: 10.1016/j.bbr.2004.08.008

Numan M, Stolzenberg DS (2009) Medial preoptic area interactions with dopamine neural systems in the control of the onset and maintenance of maternal behavior in rats. Front Neuroendocrinol 30:46–64. doi: 10.1016/j.yfrne.2008.10.002

Numan M, Stolzenberg DS (2008) Hypothalamic interaction with the mesolimbic dopamine system and the regulation of maternal responsiveness. In: Neurobiology of the Parental Brain. pp 3–22

Numan M, Woodside B (2010) Maternity: neural mechanisms, motivational processes, and physiological adaptations. Behav Neurosci 124:715–741. doi: 10.1037/a0021548

Numan M, Young LJ (2016) Neural mechanisms of mother–infant bonding and pair bonding: Similarities, differences, and broader implications. Horm Behav 77:98–112. doi: 10.1016/j.yhbeh.2015.05.015

O'Hara L, Curley M, Tedim Ferreira M, et al (2015) Pituitary androgen receptor signalling regulates prolactin but not gonadotrophins in the male mouse. PLoS One

10:e0121657. doi: 10.1371/journal.pone.0121657

Olazábal DE, Pereira M, Agrati D, et al (2013) New theoretical and experimental approaches on maternal motivation in mammals. Neurosci Biobehav Rev 37:1860–1874. doi: 10.1016/j.neubiorev.2013.04.003

Otero-García M, Agustín-Pavón C, Lanuza E, Martínez-García F (2015) Distribution of oxytocin and co-localization with arginine vasopressin in the brain of mice. Brain Struct Funct. doi: 10.1007/s00429-015-1111-y

Otero-Garcia M, Martin-Sanchez A, Fortes-Marco L, et al (2014) Extending the socio-sexual brain: Arginine-vasopressin immunoreactive circuits in the telencephalon of mice. Brain Struct Funct 219:1055–1081. doi: 10.1007/s00429-013-0553-3

Pardo-Bellver C, Cádiz-Moretti B, Novejarque A, et al (2012) Differential efferent projections of the anterior, posteroventral, and posterodorsal subdivisions of the medial amygdala in mice. Front Neuroanat 6:1–26. doi: 10.3389/fnana.2012.00033

Parkening TA, Collins TJ, Smith ER (1982) Plasma and pituitary concentrations of luteinizing hormone, follicle-stimulating hormone and prolactin in aged, ovariectomized CD-1 and C57BL/6 mice. Exp Gerontol 17:437–43.

Parker SL, Armstrong WE, Sladek CD, et al (1991) Prolactin stimulates the release of oxytocin in lactating rats: evidence for a physiological role via an action at the neural lobe. Neuroendocrinology 53:503–10.

Parmigiani S, Palanza P, Rodgers J, Ferrari PF (1999) Selection, evolution of behavior and animal models in behavioral neuroscience. Neurosci Biobehav Rev 23:957–970. doi: 10.1016/S0149-7634(99)00029-9

Paut-Pagano L, Roky R, Valatx JL, et al (1993) Anatomical distribution of prolactin-like immunoreactivity in the rat brain. Neuroendocrinology 58:682–95.

Paxinos G, Franklin KBJ (2004) The Mouse Brain in Stereotaxic Coordinates. Academic Press

Pedersen CA, Ascher JA, Monroe YL, Prange AJ (1982) Oxytocin induces maternal behavior in virgin female rats. Science 216:648–50.

Pedersen CA, Caldwell JD, Johnson MF, et al (1985) Oxytocin antiserum delays onset of ovarian steroid-induced maternal behavior. Neuropeptides 6:175–82.

Pedersen CA, Caldwell JD, Walker C, et al (1994) Oxytocin activates the postpartum onset of rat maternal behavior in the ventral tegmental and medial preoptic areas. Behav Neurosci 108:1163–71.

Pedersen CA, Prange AJ (1979) Induction of maternal behavior in virgin rats after intracerebroventricular administration of oxytocin. Proc Natl Acad Sci U S A 76:6661–5.

Pedersen CA, Vadlamudi S V., Boccia ML, Amico JA (2006) Maternal behavior deficits in nulliparous oxytocin knockout mice. Genes, Brain Behav 5:274–281. doi: 10.1111/j.1601-183X.2005.00162.x

Pereira M, Morrell JI (2011) Functional Mapping of the Neural Circuitry of Rat Maternal Motivation: Effects of Site-Specific Transient Neural Inactivation. J Neuroendocrinol 23:1020–1035. doi: 10.1111/j.1365-2826.2011.02200.x

Pereira M, Morrell JI (2009) The changing role of the medial preoptic area in the regulation of maternal behavior across the postpartum period: facilitation followed by inhibition. Behav Brain Res 205:238–48. doi: 10.1016/j.bbr.2009.06.026

Pezet A, Favre H, Kelly PA, Edery M (1999) Inhibition and restoration of prolactin signal transduction by suppressors of cytokine signaling. J Biol Chem 274:24497–502.

Pfaff DW (1982) Motivational Concepts: Definitions and Distinctions. In: The Physiological Mechanisms of Motivation. Springer New York, New York, NY, pp 3–24

Pi X, Voogt JL (2001) Mechanisms for suckling-induced changes in expression of prolactin receptor in the hypothalamus of the lactating rat. Brain Res 891:197–205. doi: 10.1016/S0006-8993(00)03212-1

Pi XJ, Grattan DR (1999) Increased expression of both short and long forms of prolactin receptor mRNA in hypothalamic nuclei of lactating rats. J Mol Endocrinol 23:13–22. doi: 10.1677/jme.0.0230013

Prevarskaya NB, Skryma RN, Vacher P, et al (1995) Role of tyrosine phosphorylation in

potassium channel activation. Functional association with prolactin receptor and JAK2 tyrosine kinase. J Biol Chem 270:24292–9.

Rand-Weaver M, Noso T, Muramoto K, Kawauchi H (1991) Isolation and characterization of somatolactin, a new protein related to growth hormone and prolactin from Atlantic cod (Gadus morhua) pituitary glands. Biochemistry 30:1509–15.

Rastrelli G, Corona G, Maggi M (2015) The role of prolactin in andrology: what is new? Rev Endocr Metab Disord 16:233–48. doi: 10.1007/s11154-015-9322-3

Ratovondrahona D, Fahmi M, Fournier B, et al (1998) Prolactin induces an inward current through voltage-independent Ca2+ channels in Chinese hamster ovary cells stably expressing prolactin receptor. J Mol Endocrinol 21:85–95.

Repetti RL, Taylor SE, Seeman TE (2002) Risky families: family social environments and the mental and physical health of offspring. Psychol Bull 128:330–66.

Rheingold H (1963) Maternal behavior in mammals. London ;J. Wiley, New York

Riddle O, Bates R, Dykshorn S (1933a) The preparation, identification and assay of prolactin—a hormone of anterior pituitary. Am J Physiol 191–216.

Riddle O, Bates RW, Dykshorn SW (1933b) The preparation, identification and assay of prolactin—a hormone of the anterior pituitary. Am J Physiol Content 105:191–216.

Riecher-Rössler A, Schmid C, Bleuer S, Birkhäuser M (2009) Antipsychotics and hyperpolactinaemia: pathophysiology, clinical relevance, diagnosis and therapy. Neuropsychiatr Klin Diagnostik, Ther und Rehabil Organ der Gesellschaft Österreichischer Nervenärzte und Psychiater 23:71–83.

Rissman EF, Early AH, Taylor JA, et al (1997) Estrogen receptors are essential for female sexual receptivity. Endocrinology 138:507–10. doi: 10.1210/endo.138.1.4985

Robertson MC, Friesen HG (1981) Two forms of rat placental lactogen revealed by radioimmunoassay. Endocrinology 108:2388–90. doi: 10.1210/endo-108-6-2388

Roky R, Obál F, Valatx JL, et al (1995) Prolactin and rapid eye movement sleep regulation. Sleep 18:536–42.

Rood BD, De Vries GJ (2011) Vasopressin innervation of the mouse (Mus musculus) brain and spinal cord. J Comp Neurol 519:2434–74. doi: 10.1002/cne.22635

Rood BD, Stott RT, You S, et al (2013) Site of origin of and sex differences in the vasopressin innervation of the mouse (Mus musculus) brain. J Comp Neurol 521:2321–2358. doi: 10.1002/cne.23288

Roselli CE, Bocklandt S, Stadelman HL, et al (2008) Prolactin Expression in the Sheep Brain. Neuroendocrinology 87:206–215. doi: 10.1159/000114643

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

Rui H, Djeu JY, Evans GA, et al (1992) Prolactin receptor triggering. Evidence for rapid tyrosine kinase activation. J Biol Chem 267:24076–81.

Rutter M, Kumsta R, Schlotz W, Sonuga-Barke E (2012) Longitudinal studies using a "natural experiment" design: the case of adoptees from Romanian institutions. J Am Acad Child Adolesc Psychiatry 51:762–70. doi: 10.1016/j.jaac.2012.05.011

Saito TR, Terada M, Oláh M, Nagy GM (2013) The Role of Prolactin in the Regulation of Male Copulatory Behavior. In: Nagy, György M., Toth BE (ed) Prolactin. InTech,

Salamone JD, Yohn SE, López-Cruz L, et al (2016) Activational and effort-related aspects of motivation: neural mechanisms and implications for psychopathology. Brain 139:1325–1347. doi: 10.1093/brain/aww050

Sapsford TJ, Kokay IC, Östberg L, et al (2012) Differential sensitivity of specific neuronal populations of the rat hypothalamus to prolactin action. J Comp Neurol 520:1062–1077. doi: 10.1002/cne.22775

Sauvé D, Woodside B (2000) Neuroanatomical specificity of prolactin-induced hyperphagia in virgin female rats. Brain Res 868:306–14.

Scott N, Prigge M, Yizhar O, Kimchi T (2015) A sexually dimorphic hypothalamic circuit controls maternal care and oxytocin secretion. Nature 525:519–22. doi: 10.1038/nature15378

Scully KM, Gleiberman AS, Lindzey J, et al (1997) Role of estrogen receptor-alpha in the anterior pituitary gland. Mol Endocrinol 11:674–81. doi: 10.1210/mend.11.6.0019

Seip KM, Morrell JI (2008) Exposure to pups influences the strength of maternal motivation in virgin female rats. Physiol Behav 95:599–608. doi: 10.1016/j.physbeh.2008.09.003

Semaan SJ, Tolson KP, Kauffman AS (2013) The development of kisspeptin circuits in the Mammalian brain. Adv Exp Med Biol 784:221–52. doi: 10.1007/978-1-4614-6199-9_11

Shamgochian MD, Avakian C, Truong NH, et al (1995) Regulation of prolactin receptor expression by estradiol in the female rat brain. Neuroreport 6:2537–41.

Shingo T, Gregg C, Enwere E, et al (2003) Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. Science 299:117–120. doi: 10.1126/science.1076647

Shome B, Parlow AF (1977) Human pituitary prolactin (hPRL): the entire linear amino acid sequence. J Clin Endocrinol Metab 45:1112–1115. doi: 10.1210/jcem-45-5-1112

Shrenker P, Bartke A (1987) Effects of hyperprolactinaemia on male sexual behaviour in the golden hamster and mouse. J Endocrinol 112:221–8.

Simerly RB, Swanson LW (1986) The organization of neural inputs to the medial preoptic nucleus of the rat. J Comp Neurol 246:312–42. doi: 10.1002/cne.902460304

Sinha YN, Salocks CB, Wickes MA, Vanderlaan WP (1977) Serum and pituitary concentrations of prolactin and growth hormone in mice during a twenty-four hour period. Endocrinology 100:786–91. doi: 10.1210/endo-100-3-786

Sirzen-Zelenskaya A, Gonzalez-Iglesias AE, Boutet de Monvel J, et al (2011) Prolactin Induces a Hyperpolarising Current in Rat Paraventricular Oxytocinergic Neurones. J Neuroendocrinol 23:883–893. doi: 10.1111/j.1365-2826.2011.02207.x

Sjoeholm A, Bridges RS, Grattan DR, Anderson GM (2011) Region-, neuron-, and signaling pathway-specific increases in prolactin responsiveness in reproductively experienced female rats. Endocrinology 152:1979–1988. doi: 10.1210/en.2010-

1220

Slotnick BM, Carpenter M Lou, Fusco R (1973) Initiation of maternal behavior in pregnant nulliparous rats. Horm Behav 4:53–59. doi: 10.1016/0018-506X(73)90016-0

Soares MJ (2004) The prolactin and growth hormone families: pregnancy-specific hormones/cytokines at the maternal-fetal interface. Reprod Biol Endocrinol 2:51. doi: 10.1186/1477-7827-2-51

Soares MJ, Müller H, Orwig KE, et al (1998a) The uteroplacental prolactin family and pregnancy. Biol Reprod 58:273–84.

Soares MJ, Müller H, Orwig KE, et al (1998b) The uteroplacental prolactin family and pregnancy. Biol Reprod 58:273–284. doi: 10.1095/biolreprod58.2.273

Solomon NG, French JA (1997) Cooperative breeding in mammals. Cambridge University Press

Stanić D, Dubois S, Chua HK, et al (2014) Characterization of aromatase expression in the adult male and female mouse brain. I. Coexistence with oestrogen receptors α and β, and androgen receptors. PLoS One 9:e90451. doi: 10.1371/journal.pone.0090451

Stern JM, Mackinnon DA (1976) Postpartum, hormonal, and nonhormonal induction of maternal behavior in rats: effects on T-maze retrieval of pups. Horm Behav 7:305–16.

Stolzenberg DS, Rissman EF (2011) Oestrogen-Independent, Experience-Induced Maternal Behaviour in Female Mice. J Neuroendocrinol 23:345–354. doi: 10.1111/j.1365-2826.2011.02112.x

Stoop R (2012) Neuromodulation by oxytocin and vasopressin. Neuron 76:142–59. doi: 10.1016/j.neuron.2012.09.025

Sugiyama T, Minoura H, Kawabe N, et al (1994) Preferential expression of long form prolactin receptor mRNA in the rat brain during the oestrous cycle, pregnancy and lactation: hormones involved in its gene expression. J Endocrinol 141:325–33.

Svare B, Mann M a, Broida J, Michael SD (1982) Maternal aggression exhibited by hypophysectomized parturient mice. Horm Behav 16:455–461.

Tabata H, Kobayashi M, Ikeda JH, et al (2012) Characterization of multiple first exons in murine prolactin receptor gene and the effect of prolactin on their expression in the choroid plexus. J Mol Endocrinol 48:169–176. doi: 10.1530/JME-11-0122

Terkel J, Bridges RS, Sawyer CH (1979) Effects of transecting lateral neural connections of the medial preoptic area on maternal behavior in the rat: nest building, pup retrieval and prolactin secretion. Brain Res 169:369–80.

Torner L (2016) Actions of Prolactin in the Brain: From Physiological Adaptations to Stress and Neurogenesis to Psychopathology. Front Endocrinol (Lausanne) 7:25. doi: 10.3389/fendo.2016.00025

Torner L, Maloumby R, Nava G, et al (2004) In vivo release and gene upregulation of brain prolactin in response to physiological stimuli. Eur J Neurosci 19:1601–1608. doi: 10.1111/j.1460-9568.2004.03264.x

Torner L, Nava G, Dueñas Z, et al (1999) Changes in the expression of neurohypophyseal prolactins during the estrous cycle and after estrogen treatment. J Endocrinol 161:423–32.

Torner L, Toschi N, Nava G, et al (2002) Increased hypothalamic expression of prolactin in lactation: Involvement in behavioural and neuroendocrine stress responses. Eur J Neurosci 15:1381–1389. doi: 10.1046/j.1460-9568.2002.01965.x

Torner L, Toschi N, Pohlinger A, et al (2001) Anxiolytic and anti-stress effects of brain prolactin: improved efficacy of antisense targeting of the prolactin receptor by molecular modeling. J Neurosci 21:3207–14.

Toth M, Fuzesi T, Halasz J, et al (2010) Neural inputs of the hypothalamic “aggression area” in the rat. Behav Brain Res 215:7–20. doi: 10.1016/j.bbr.2010.05.050

Tsuneoka Y, Maruyama T, Yoshida S, et al (2013) Functional, anatomical, and neurochemical differentiation of medial preoptic area subregions in relation to maternal behavior in the mouse. J Comp Neurol 521:1633–63. doi: 10.1002/cne.23251

Tsuneoka Y, Tsukahara S, Yoshida S, et al (2017) Moxd1 Is a Marker for Sexual Dimorphism in the Medial Preoptic Area, Bed Nucleus of the Stria Terminalis and Medial Amygdala. Front Neuroanat 11:26. doi: 10.3389/fnana.2017.00026

Unger EK, Burke KJ, Yang CF, et al (2015) Medial Amygdalar Aromatase Neurons Regulate Aggression in Both Sexes. Cell Rep 10:453–462. doi: 10.1016/j.celrep.2014.12.040

Vaiserman AM (2015) Epigenetic programming by early-life stress: Evidence from human populations. Dev Dyn 244:254–65. doi: 10.1002/dvdy.24211

Vaisse C, Halaas JL, Horvath CM, et al (1996) Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. Nat Genet 14:95–7. doi: 10.1038/ng0996-95

van Leengoed E, Kerker E, Swanson HH (1987) Inhibition of post-partum maternal behaviour in the rat by injecting an oxytocin antagonist into the cerebral ventricles. J Endocrinol 112:275–82.

Van Tol HH, Bolwerk EL, Liu B, Burbach JP (1988) Oxytocin and vasopressin gene expression in the hypothalamo-neurohypophyseal system of the rat during the estrous cycle, pregnancy, and lactation. Endocrinology 122:945–51. doi: 10.1210/endo-122-3-945

Veening JG, de Jong TR, Waldinger MD, et al (2015) The role of oxytocin in male and female reproductive behavior. Eur J Pharmacol 753:209–28. doi: 10.1016/j.ejphar.2014.07.045

Vega C, Moreno-Carranza B, Zamorano M, et al (2010) Prolactin promotes oxytocin and vasopressin release by activating neuronal nitric oxide synthase in the supraoptic and paraventricular nuclei. Am J Physiol Regul Integr Comp Physiol 299:R1701-8. doi: 10.1152/ajpregu.00575.2010

Waldherr M, Neumann ID (2007) Centrally released oxytocin mediates mating-induced anxiolysis in male rats. Proc Natl Acad Sci U S A 104:16681–4. doi: 10.1073/pnas.0705860104

Walsh RJ, Slaby FJ, Posner BI (1987) A Receptor-Mediated Mechanism for the Transport

of Prolactin from Blood to Cerebrospinal Fluid*. Endocrinology 120:1846–1850. doi: 10.1210/endo-120-5-1846

Walters CA, Daly DC, Chapitis J, et al (1983) Human myometrium: a new potential source of prolactin. Am J Obstet Gynecol 147:639–44.

Wang J, Swann JM (2014) Connections of the magnocellular medial preoptic nucleus (MPN mag) in male Syrian hamsters. II. The efferents. Neuroscience 274:102–18. doi: 10.1016/j.neuroscience.2014.05.014

Waters MJ (2015) The growth hormone receptor. Growth Horm IGF Res. doi: 10.1016/j.ghir.2015.06.001

Wise DA, Pryor TL (1977) Effects of ergocornine and prolactin on aggression in the postpartum golden hamster. Horm Behav 8:30–9.

Yamaguchi M, Ogren L, Endo H, et al (1992) Production of mouse placental lactogen-I and placental lactogen-II by the same giant cell. Endocrinology 131:1595–1602. doi: 10.1210/endo.131.4.1396305

Yamaguchi Y, Takagi W, Kuraku S, et al (2015) Discovery of conventional prolactin from the holocephalan elephant fish, Callorhinchus milii. Gen Comp Endocrinol 224:216–227. doi: 10.1016/j.ygcen.2015.08.020

Yang CF, Chiang MC, Gray DC, et al (2013) Sexually Dimorphic Neurons in the Ventromedial Hypothalamus Govern Mating in Both Sexes and Aggression in Males. Cell 153:896–909. doi: 10.1016/j.cell.2013.04.017

Yip SH, Eguchi R, Grattan DR, Bunn SJ (2012) Prolactin Signalling in the Mouse Hypothalamus is Primarily Mediated by Signal Transducer and Activator of Transcription Factor 5b but not 5a. J Neuroendocrinol 24:1484–1491. doi: 10.1111/j.1365-2826.2012.02357.x

Yohn SE, Collins SL, Contreras-Mora HM, et al (2016) Not All Antidepressants Are Created Equal: Differential Effects of Monoamine Uptake Inhibitors on Effort-Related Choice Behavior. Neuropsychopharmacology 41:686–694. doi: 10.1038/npp.2015.188

Yoshida Y, Kawasaki Y, Morikawa N, et al (1991) [A kinetic study on serum prolactin

concentration in the thyrotropin-releasing hormone test]. Kaku Igaku 28:585–90.

Zhang TY, Labonté B, Wen XL, et al (2013) Epigenetic mechanisms for the early environmental regulation of hippocampal glucocorticoid receptor gene expression in rodents and humans. Neuropsychopharmacology 38:111–23. doi: 10.1038/npp.2012.149

Ziegler TE, Snowdown CT (2000) Preparental hormone levels and parenting experience in male cotton-top tamarins, Saguinus oedipu. Horm Behav 38:159–67. doi: 10.1006/hbeh.2000.1617