Characterization of maternal behaviours in mice.

Pheromonal control and nonapeptidergic substrate

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C e r t i f i q u e

Que **Na Ana Martín Sánchez**, Màster en Neurociències per la *Universitat de València*, ha realitzat sota la nostra direcció el treball titulat ***"Characterization of maternal behaviours in mice. Pheromonal control and nonapeptidergic substrate"*** per a l'obtención del grau de Doctor en Neurociències Bàsiques i Aplicades.

La qual cosa signem a Burjassot, el dia 16 de juny de 2015

Fernando Martínez-García, PhD Carmen Agustín-Pavón, PhD

VNIVERSITAT DE VALÈNCIA

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H e r e b y c e r t i f y

*That **Ms. Ana Martín Sánchez**, MSc in Neuroscience at the University of València, has performed, under our supervision the work entitled: "Characterization of maternal behaviours in mice. Pheromonal control and nonapeptidergic substrate" to obtain the PhD in Basic and Applied Neurosciences.*

What we sign as required in Burjassot, on June the 16th 2015

Fernando Martínez-García, PhD Carmen Agustín-Pavón, PhD

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"El jardín de la neurología brinda al investigador espectáculos cautivadores y emociones artísticas incomparables"

Santiago Ramón y Cajal. Nobel en Fisiología o Medicina (1906)

"Nobody said it was easy"

The Scientist, Coldplay (2002)

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AC3 adenylyl cyclase type 3

AC anterior commisure

ADH antidiuretic hormone

ADP anterodorsal preoptic area

AOB accessory olfactory bulb

AHM anterior hypothalamic nucleus

AVP argine-vasopressin

AVPergic vasopressinergic

AVPR arginine-vasopressin receptor

AVP1aR arginine-vasopressin receptor type 1a

AVP1bR arginine-vasopressin receptor type 1b

BLA basolateral amygdala;

BMA basomedial amygdala;

BST bed nucleus of stria terminalis

vBST ventral part of bed nucleus of stria terminalis

Ce central amygdala

DA dopamine

D1 dopamine receptor type 1

D2 dopamine receptor type 2

ERA English and Romanian Adoptees

ERα estrogen receptor α

ERβ estrogen receptor β

HAB low anxiety-related behaviours

HPA hypothalamic-pituitary-adrenal axis

i.p intraperitoneal

LAB low anxiety-related behaviours

LS lateral septum

Me medial amygdala

MeEA medial extended amygdala

MePV posteroventral medial amygdala

MePD posterodorsal medial amygdala

MOB main olfactory bulb

MPOA medial preoptic area

MUPs major urinary proteins

rMup 20 recombinant major urinary protein type 20

rMup 3 recombinant major urinary protein type 3

NAcc nucleus accumbens

NMDA N-metil-D-aspartate

OT oxytocin

OTergic oxytocinergic

OT-ir oxytocin immunoreactive

OTR oxytocin receptor

Pa paraventricular nucleus

PAG periaqueductal grey

PFC prefrontal cortex

PRL prolactin

PPD postpartum day

Pmv ventral premammillary nucleus

PRG progesterone;

r-darcin darcin recombinant

SBN socio-sexual brain network

SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel

STAT signal transducer and activator of transcription

s.c subcutaneous

TIDA tuberoinfundibular dopaminergic neurones

TPH2 tryptophan hydroxylase-2

TRPC2 transient receptor potential channel 2

VMH ventromedial hypothalamic nucleus

VNO vomeronasal organ

VP ventral pallidum

VR vomeronasal receptors

V2R vomeronasal type 2 receptor

V1R vomeronasal type 1 receptor

VTA ventral tegmental area.

1. GENERAL INTRODUCTION

Parental behaviour occurs in a wide variety of vertebrates and invertebrates, but it is specially important in mammals and birds (Numan and Insel, 2003). This type of behaviour was defined by Numan and Insel as '*any behaviour of a member of a species toward a reproductively immature conspecific that increases the probability that the recipient will survive to maturity*' (Numan and Insel, 2003). In mammals, the mother is the main caregiver of the young due to lactation, and in some species, e.g. house mouse (*Mus musculus*), communal breeding is displayed by dams (Manning *et al*., 1995; Weidt *et al*., 2014).

In some species, males express parental behaviour, what is known as paternal behaviour. Alloparental behaviours are performed by some species of mammals, including humans. Alloparental behaviour is a caregiving behaviour directed toward a conspecific infant by an individual who is not related genetically with the young (Riedman, 1982). In these cases, clearly, processes not related to the physiological events occurring during pregnancy and lactation contribute to eliciting the parental response.

Parental care, and in particular maternal behaviour, has a deep impact in the development of newborns. Both the quality and quantity of this behaviour affect the development of the offspring. In this sense, maternal behaviour is important because it influences some phenotypical aspects of infants (Li *et al*., 2015; Meaney, 2001; Pan *et al*., 2014; Pedersen *et al*., 2011). In fact, some studies in humans have revealed that disruption of mother-infant interactions during the early postnatal period, such as maternal separation, alters the neuroendocrine regulations, and produce cognitive and psychomotor retardation (Mehta *et al*., 2009; Rutter *et al*., 2012). One of the major systems directly altered in the disruption of mother-infant bonding is the hypothalamic-pituitary-adrenal axis (HPA), which influences the stress responses in both the dam and the young.

1.1 MATERNAL BEHAVIOUR AND OFFSPRING DEVELOPMENT

Research with children, who have been reared in deprived conditions provides an important opportunity to study the possible relationship between early negative experiences and subsequent

brain structures development. For instance, a study of English and Romanian Adoptees (ERA) reveals that children that had spent their early life living in institutional deprivation conditions showed difficulties in different areas; i.e. quasi-autism, disinhibited attachment, impaired cognition and inattention/overactivity (Mehta *et al*., 2009). When compared with controls, children raised in these conditions showed no differences in hippocampal or corpus callosum size but had greater amygdala volumes. The volume of this nucleus was positively correlated to the time spent in institutions. The authors suggest that a larger amygdala volume might underlay a disrupted affective processing (Mehta *et al*., 2009).

Experimental studies with animals using tightly controlled conditions have revealed that, among other factors, abnormalities in maternal responsiveness induce a substantial variation in upregulation of hippocampal glucocorticoid receptor and hypothalamic corticotropin-releasing factor, along with stress-related hormone levels. These variations affect directly the normal function of HPA axis (Meaney, 2001; Vaiserman, 2015). Ultimately, all these effects together alter what is known as stress reactivity of the offspring during adulthood (Champagne *et al*., 2003; Francis *et al*., 1999; Li *et al*., 2015; Meaney, 2001; Suomi, 2006).

The stress reactivity not only mediates long-term responses to stress (Li *et al*., 2015) but also determines individual differences in the vulnerability to develop some affective disorders (Zhang *et al*., 2013). Different studies correlate affective disorders with low quality of the maternal care received. For instance, low maternal care is associated with significantly increased risk of child neglect/abuse and depression

in later life of infants (Andersen *et al*., 2008; Canetti *et al*., 1997; Numan and Insel, 2003; Repetti *et al.*, 2002). In addition, individual features maternal behaviour are transmitted from one generation of females to the next (Figure 1) (Francis *et al*., 1999; Li *et al*., 2015).

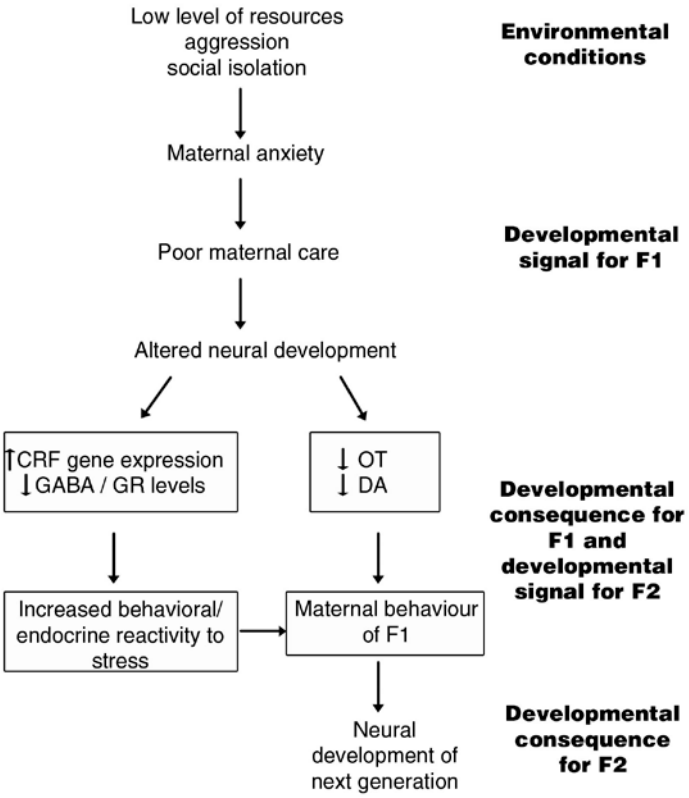

Figure 1. Variations in maternal care alter the development of neural systems that mediate stress reactivity.
Individual differences in stress reactivity could influence maternal behaviour in next generations. CRF, corticotropin-releasing factor; DA, dopamine; GR, glucorticoid receptor; OT, oxytocin. Adapted from Meaney (2001).

There is strong evidence supporting that environmental regulation during postnatal period can alter the development of

responses to stress in later life. Since infant rodents HPA shares many molecular and biological features with the human HPA (Gilles *et al*., 1996; Walker *et al*., 1986), the rodent might be regarded as a good animal model to study this issue.

In rats, maternal separation is a paradigm that has been used to analyse the influence of maternal care on development of the stress response and the responses to stressors experienced during adulthood. The maternal separation paradigm consists of extended periods of mother-infant separation of as much as 24 hours. When reared using this protocol, 7-day old pups show a higher plasma corticosterone levels in comparison with controls in response to novelty *(*Rosenfeld *et al*., 1992). These findings provide evidence for the importance of maternal care as a regulator of a normal psychiatric development of the offspring. Thus, analysing how mother-infant interaction is essential for understanding how optimum maternal care improves pup fitness, and what mechanistic processes might explain that some mothers that do not display correct bonding with their infants. For this reason, animal models are needed to study the neural substrate of maternal behaviour to promote physical and mental health in future generations.

Our knowledge on the neural and endocrine basis of maternal behaviours is mainly based on studies carried out in rats along the last 50 years. However, the last decade has witnessed an increased interest in the mouse, mainly because of the advantageous use of genetically modified individuals in behavioural neurosciences. These studies have revealed important, unexpected differences in maternal behaviour between both species, mice resembling more to primates

(including humans) than rats. Therefore, we first describe the features of maternal behaviours in rats and we review the experiments that have revealed its hormonal and non-hormonal basis. Then, we analyse data from the mouse, which is the species employed in our experiments.

1.2 HORMONAL AND NON-HORMONAL BASIS OF MATERNAL BEHAVIOUR IN RATS

In rodents, researchers have differentiated between maternal behaviours that are directed to pups from those that are not (Numan and Insel, 2003). Thus, maternal behaviours include pup-directed responses i.e. retrieval and grouping pups in nest, crouching over pups, pup-licking/grooming, and nursing. Non-pup directed behaviours include nest building and its maintenance, and maternal aggression (Numan and Insel, 2003), aimed at to defending the nest (Vom Saal *et al*., 1995). Non-pup-directed behaviours also include increased food consumption and diminished anxiety with associated increases in exploratory activity (Bridges, 2015). In preparation for birth, pregnant females show high levels of aggression towards intruders and increased nest building (Caughey *et al*., 2011). At birth, most parturient females ingest amniotic fluid and placenta. This last process might have a dual function: to provide a source of nutrition and hormones (e.g. placental lactogens) to the mother and to remove olfactory cues to surrounding putative predators (Bridges, 2015).

Recently, the accurate 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 behaviour.

Most researches have used the rat as the species of choice to study the hormonal and neural basis of the mentioned maternal behaviours. At the moment of parturition, the primiparous female rat displays the full repertoire of maternal care on her first exposition to her own pups or to foster ones (Numan and Insel, 2003). By contrast, virgin adult rats do not display alloparental care when they are exposed for the first time to foster pups and may even occasionally show infanticide behaviours (Numan and Insel, 2003). This differential response towards infants suggests that pup stimuli are not enough to elicit spontaneous maternal care in rats. In fact, nulliparous adult rats need a sensitization period before expressing pup retrieval and other kinds of maternal care (Fleming and Rosenblatt, 1974c; Rosenblatt, 1967). Virgin female rats not treated with hormones need between 3-4 days to tolerate the presence of pups and at least 5-7 days exposition period to express maternal-like behaviours (Fleming and Luebke, 1981; Rosenblatt, 1967). Numan and Woodside (2010) interpreted this sensitization process (Numan and Woodside, 2010), as follows: '*novel pup stimuli initially arouse fear-related processes and avoidance and defensive behaviours in virgins, but after several days of pup exposure the virgin habituates to fear-arousing stimuli of the young, which allows proximal contact to occur*' (Numan and Woodside, 2010). This interpretation is key to understand the basis of

the motivational model of maternal behaviour in rats, that we will describe below.

Both treatment with physiological levels of progesterone plus oestradiol in virgin females (Bridges, 1984) and blood transfusions from a parturient female to virgin female rats (Terkel and Rosenblatt, 1972), facilitate the onset of maternal behaviour in these females. The importance of progesterone was demonstrated using pregnancy termination models, developed by Rosenblatt and colleagues. Early pregnancy termination via hysterectomy –removing uterus, placentas and pups- on days 15-17 of pregnancy is enough to shorten to one day the latency of the onset of maternal behaviours in presence of pups 48h later. Moreover, if females are hysterectomized and ovariectomized and treated with oestradiol, and then presented with pups 48h later, they show maternal care from the first moment. This is due to hysterectomy resulting in a reduction of progesterone levels similar to that occuring at parturition (Numan and Insel, 2003). Thus, a treatment mimicking the endocrine events of the peripartum period, namely withdrawal of progesterone and rising of oestradiol, together with the administration of prolactin, facilitate the onset of maternal behaviour in virgin rats (Bridges and Ronsheim, 1990; Bridges *et al*., 1990). This gonadal steroid profile not only stimulates maternal care in virgin female rats but also maternal-like aggression (Mayer *et al*., 1990).

Reproductive experience reduces the dependency of the onset of maternal behaviour on hormonal events, since multiparous females are less dependent upon neuroendocrine events on maternal behaviour than primiparous ones (Numan and Insel, 2003).

All these pieces of evidence suggest that promotion of maternal behaviour in rats is under hormonal control during adulthood. However, juvenile 22-24-day-old rats express high levels of maternal behaviour. Numan and Insel (2003) suggest that during adolescence there is a switch that changes the physiology of the female rat showing hormone-independent maternal behaviour to a strict hormonal regulation of maternal care during adulthood.

In summary, all these studies have shed light about hormonal basis of maternal behaviours in rats. Now we will describe the neural control of these behaviours.

1.2.1 Regulation of responsiveness to pup stimuli

As we saw above, virgin rats are neophobic towards pups when they are exposed to them for the first time. Virgin females need a sensitization period of 5-7 days before displaying retrieval and licking-grooming behaviours. This sensitization period is not observed in lactating female rats since they are fully maternal from the moment of parturition. Numan and Woodside, (2010) proposed the approach-avoidance motivational model of the onset of maternal care in rats (Figure 2). This model proposes that the hormonal events during late pregnancy and lactation tune the preference of dams towards pups and their stimuli that in this way become attractive (Fleming *et al*., 1989; Kinsley and Bridges, 1990; Numan and Stolzenberg, 2008). Finally, pup-attraction is maintained only during early postpartum period and it disappears during the middle and late postpartum period (Mattson *et al*., 2001).

These data indicate that the pregnancy hormones, i.e. oestradiol, progesterone and lactogens, act directly on neurons of some brain centres to facilitate the onset of maternal behaviours. This brain centres would constitute the *core* of the maternal behaviour circuits. On the one hand, the activation of some of its connections may be inhibiting subsequent neural pathways that promote defensive responses toward pups e.g. pup avoidance. On the other hand, neurons of this brain centres would be connected to other brain areas that elicit approach and attraction to the pups.

Lesion experiments using axon-sparing neurotoxic agents indicate a key role of the medial preoptic area (MPOA) in the control of maternal behaviours (Numan *et al*., 1988). In fact, the MPOA is enriched in neurons expressing receptors for sex steroids (Simerly *et al*., 1990) and prolactin (Anderson *et al*., 2006; Consiglio and Bridges, 2009), In this section we will review the evidence supporting a role of the MPOA and its connections on avoidance vs attraction towards pup stimuli in the brain of female rats.

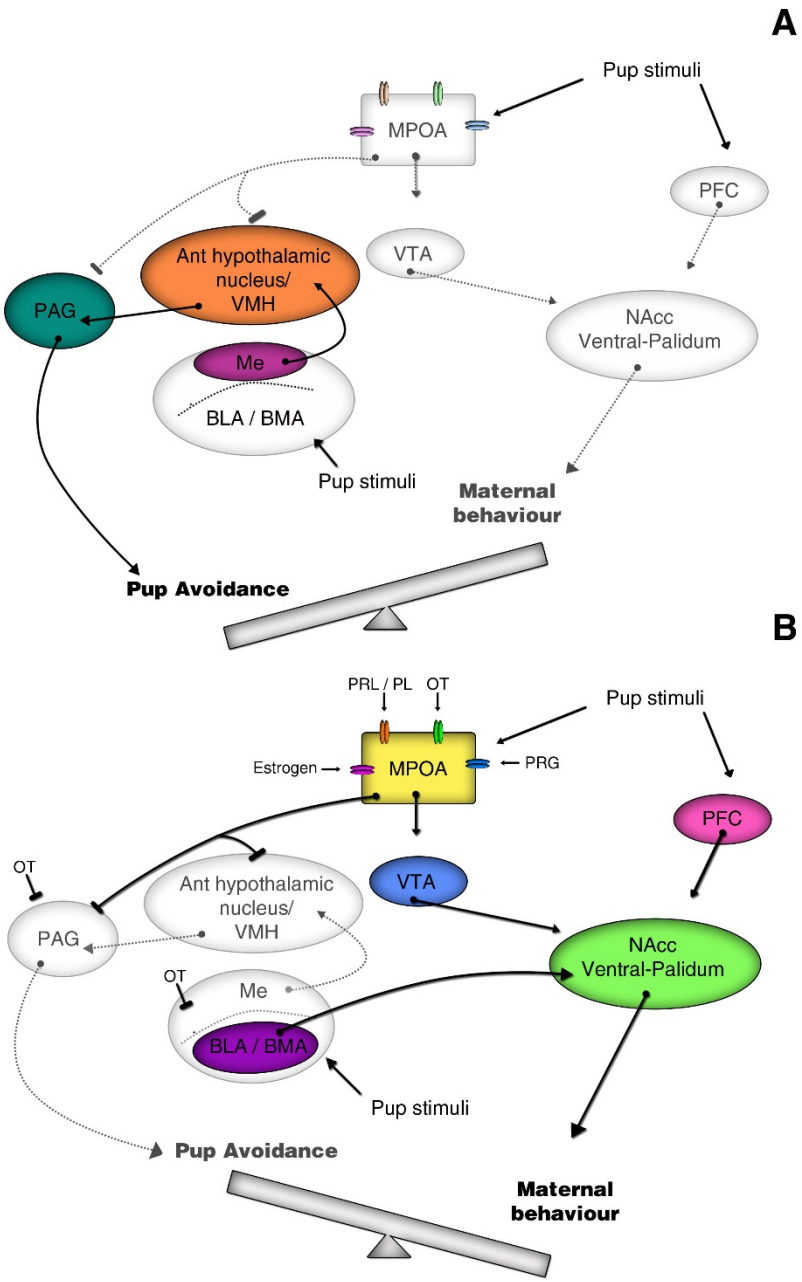

Figure 2. The proposed neural circuitry controlling approach-avoidance and maternal behaviour in rats.

Pup stimuli could influence MPOA, amygdala and PFC. (A) In virgin rats, pup stimuli could activate pup-avoidance responses trough Me-AHN/VMH pathway. (B) In lactating females, hormones prime MPOA to become responsive to pup stimuli in two ways. On the one hand, MPOA activates VTA dopaminergic neurons, resulting in the activation of the mesolimbic system. On the other hand, MPOA inhibits the AHN/VMH, depressing the pup avoidance response. Additionally, OT could modulate several neural centres to elicit maternal behaviour. Abbreviations: Ant hypothalamic nucleus, anterior hypothalamic nucleus; BLA, basolateral amygdala; BMA, basomedial amygdala; Me, medial amygdala; MPOA, medial preoptic area; NAcc, nucleus accumbens; PAG, periaqueductal grey; PFC, prefrontal cortex; PL, placental lactogens/placental lactogens ; PRG, progesterone; OT, oxytocin; VMH, ventromedial hypothalamic nucleus; VTA, ventral tegmental area. Adapted and modified from Numan and Woodside, 2010.

1.2.1.1. Neural pathways of pup-avoidance in rats

Olfactory cues from pups activate pup-avoidance neural pathways in virgin female rats. Numan and Woodside (2010) report in a theoretical review a wide range of anatomical and functional evidence supporting this idea. The neural circuitry underlying pup avoidance is summarized in Figure 2A.

First, anosmia shortened maternal sensitization in virgin naïve rats (Fleming and Rosenblatt, 1974a,1974b) . The olfactory information from main and accessory olfactory bulb relay into the medial amygdala (Me) in rats (Canteras *et al*., 1995). In fact, excitotoxic lesions of this amygdaloid nucleus shorten maternal sensitization in virgin female rats (Fleming *et al*., 1980; Numan and Woodside, 2010). Since Me-lesioned virgin female rats take 2-3 days of cohabitation with pups, in comparison to 7-9 days in non-lesioned controls. Further studies demonstrated that this amygdala-induced facilitation of maternal behaviour in naïve females is dependent on the integrity of the medial preoptic area (MPOA) (Fleming *et al*., 1983).

This evidence and the direct inputs to the Me from the olfactory bulbs (Gutiérrez-Castellanos *et al*., 2010) suggest that pup-derived stimuli could activate Me neurons. The projections of Me to the caudal part of anterior hypothalamic nucleus (AHM), and to the ventromedial hypothalamic nucleus (VMH) (Pardo-Bellver *et al*., 2012; Sheehan *et al*., 2001) could promote pup-avoidance responses in virgin females (Figure 2A), since, as we saw above, excitotoxic amino acid lesions of Me promote maternal responsiveness in hormone-primed virgin female rats (Bridges *et al*., 1999).

The pup-avoidance neural pathway also involves the periaqueductal grey (PAG) as a final central node (Figure 2A). Some regions of PAG that are associated with fear-related behaviours and avoidance responses receive afferents from the AHM and VMH (Risold *et al*., 1994). Once again, evidence of the role of the PAG in maternal behaviours comes from excitotoxic lesions (Sukikara *et al*., 2006).

1.2.1.2. The neural model of proactive maternal responses

By contrast to virgin females, lactating female rats do not avoid pups when they are exposed to newborns for the first time. The differential behavioural response between a primiparous and a virgin female rat is that dams have a different hormonal background that prepares the brain for motherhood.

In lactating dams exposed to pups, neurons in MPOA co-express both Fos protein (commonly used as a marker of neuronal activity) and glutamate decarboxlyase, the synthesizing enzyme for the classical

inhibitory neurotransmitter gamma-amino butyric acid (GABA) (Lonstein and De Vries, 2000). Thus, these inhibitory cells -in conjunction with other possible routes- would suppress the pup-avoidance neural pathway in primiparous female rats, whereas voluntary proactive maternal responses would be activated through MPOA projections to mesolimbic dopaminergic system (Figure 2B) (Numan and Stolzenberg, 2009; Numan *et al*., 2005b). As mentioned previously, proactive responses include only pup retrieval, but not nursing behaviour.

The onset of proactive responses is facilitated by the hormone-priming action on MPOA neurons. As Figure 2B shows, oestrogens, prolactin and placental lactogens, progesterone and oxytocin act on MPOA, activating its projections to ventral tegmental area (VTA), which releases dopamine (DA) into nucleus accumbens (NAcc). The complex formed by NAcc-Ventral pallidum would process and control the motivated response towards pup stimuli. Numan and Woodside (2010) postulate that DA release in the NAcc makes ventral pallidum easily excited by pup stimuli inputs from prefrontal cortex (PFC) and the basolateral/basomedial amygdaloid nuclei (BLA/BMA).

The BLA/BMA receives direct and indirect inputs from the Me, cortical amygdala and piriform cortex (Martínez-García *et al*., 2012). This allows convergence of olfactory and vomeronasal inpunts onto BLA/BMA neurons. In this way, the Me/BLA/BMA projections to the MPOA, NAcc and VP might provide the circuit with pup-derived chemosensory –and somatosensory- stimuli (Cádiz-Moretti *et al*., 2014). Once pup-avoidance is overcome, proximal stimuli would be responsible for most proactive pup-care responses. In this respect,

pheromones are crucial for pup survival. A pheromone is a chemical excreted or secreted by an individual that is able to elicit stereotyped reactions in other individuals (Karlson and Luscher, 1959). Thus, the preputial glands of rat pups secrete dodecyl propionate to promote the specific licking of pup's anogenital areas, facilitating defecation. Detection of this pheromone by dam's vomeronasal organ is crucial because those non-licked pups cannot defecate and die (Brouette-Lahlou *et al*., 1999).

In summary, in virgin females the inhibitory Me-to-AHN/VMH-to-PAG defensive pathway is more active in response to pup stimuli, promoting pup-avoidance (Fleming and Luebke, 1981; Fleming *et al*., 1980), whereas in postpartum hormone-primed females, MPOA interacts with mesolimbic dopaminergic system, the BLA/BMA, Me, NAcc and VP to elicit proactive maternal responses.

1.2.2 The neural pathways underlying maternal aggression

The putative neural circuits that regulate certain components of maternal behaviours, including maternal aggression, in rodents have been widely reviewed (Lonstein and Gammie, 2002; Numan and Woodside, 2010; Numan and Stolzenberg, 2008; Numan and Insel, 2003). Different studies involving brain lesions, pharmacological approaches –as well as genetic manipulation in mice- support the idea that two different pathways regulate pup-care and maternal aggression in the brain of females.

Some putative effector centres of maternal aggression have been identified. For instance, Hansen (1989), using electrolytic lesions of the ventral part of ventromedial hypothalamic nucleus in lactating rats, observed a reduction in maternal aggression but not in maternal care. Other hypothalamic areas, such as ventral premammillary nucleus (PMv) might be important for aggressive behaviour, because lesions in this centre abolish maternal aggression (Motta *et al*., 2013). PMv receives projections from the medial amygdala (Me). The Me is a key node in *the socio-sexual brain*, composed of anterior (MeA), posteroventral (MePV) and posterodorsal (MePD) subregions. These subdivisions respond to pheromones and are related to affiliative and defensive behaviours (Baum and Bakker, 2013; Bergan *et al*., 2014; DiBenedictis *et al*., 2012). Both olfactory and vomeronasal subsystems project directly to these amygdaloid regions (Cádiz-Moretti *et al.*, 2014). Interestingly, recent studies suggest that MePD is a key nucleus for the onset of maternal aggression due to specific aromatase-expressing MePD neurons in females (Unger *et al*., 2015). However, the MePD should be considered more as an integrative centre rather than an effector nucleus. Its inactivation has phenotypes similar to vomeronasal removal, suggesting a general deficit in the processing and integrating of both vomeronasal and olfactory information.

The neural basis of maternal care and aggression also share lateral septum (LS), paraventricular nucleus (Pa) and the continuum of the medial preoptic area, extending to the ventral part of bed nucleus of stria terminalis (MPOA-vBST). While LS is related to social recognition in females (Ferguson *et al.*, 2000) and facilitates some

aspects of pup retrieval and aggression (Gammie, 2005), Pa is linked to the secretion of oxytocin during pup suckling in rats (Neumann *et al*., 1993) and with the onset of maternal care and aggression (Consiglio and Lucion, 1996 ; Insel and Harbaugh, 1989). Aside from that, MPOA-vBST seems to be the neural core of maternal responsiveness (Numan and Numan, 1996). The latter centre could be the convergent point where both maternal care and maternal aggression pathways could be coordinated via the hormone-primed action –including nonapeptides, as we will discuss below.

1.3 HORMONAL AND NON-HORMONAL BASIS OF MATERNAL BEHAVIOUR IN MICE

Most of the data reported so far were obtained in the rat. However, these studies strongly contrast with data obtained from primates (Maestripieri and Wallen, 1995) which suggest that virgin female macaques display spontaneous maternal behaviour. For this reason, the rat does not seem the best species to understand some aspects of neuroendocrinology of maternal behaviour of primates, including humans (Numan and Insel, 2003).

The mouse is nowadays the species of choice for studies of maternal behaviour. In contrast to rats (Numan and Insel, 2003) , virgin juvenile mice ignore pups (Alsina-Llanes *et al*., 2015; Gandelman, 1973). However, this situation reverts by cohabitation with their newborn siblings (Alsina-Llanes *et al*., 2015). Irrespective of this previous experience, virgin female laboratory mice show nearly immediate maternal behaviour during adulthood (Calamandrei and Keverne, 1994; Stolzenberg and Rissman, 2011). Thus, although there might be minor inter-strain differences (Parmigiani *et al.*, 1999) in outbred –e.g. Rockland-Swiss-, inbred –C56BL/6J- our hybrid strains -129/Sv- most of the virgin females retrieve pups to a nest site in a short -15min- pup-retrieval test (Lucas *et al*., 1998, Numan and Insel, 2003). In spite of this minor inter-strain variability, all these studies suggest that laboratory mice show an intrinsic motivation that triggers maternal behaviour almost spontaneously, in contrast to rats

In this sense, the mouse model of maternal behaviour might be closer to primates, including humans, than the rat model (Alsina-

Llanes *et al*., 2015; Gandelman *et al*., 1970; Stolzenberg and Rissman, 2011). Since virgin female mice do not avoid pups when they are exposed for the first time, the approach-avoidance motivational model of the onset of maternal behaviour that we have described does not fit the situation in the mouse. Nevertheless, this model might still be taken as a reference for the characterization of the neurobiological basis of maternal behaviour in mice.

1.3.1 Hormones and maternal behaviour in mice

The data reviewed above suggest that maternal care in mice is independent of endocrine events, i.e. levels of steroid hormones or prolactin. Nevertheless, disruption of prolactin signalling through the deletion of the prolactin receptor gene severely impairs the expression of several forms of maternal care (Lucas *et al*., 1998; but see Buonfiglio *et al*., 2015;). Thus, prolactin could be modulating some aspects of maternal behaviour in mice.

Whereas maternal care seems to be independent of the hormonal state of females in mice, the hormonal influence on maternal aggression is less clear.

Maternal aggression has been described as a defence of the nest against potential infanticide intruders (Vom Saal and Howard, 1982), rather than as a defence of territory. In this paradigm, the lactating female act as resident and a male is often used as an intruder. In comparison with intermale aggression, maternal aggression is qualitatively different, being less ritualized and more violent (Ervin *et al*., 2015; Parmigiani *et al*., 1998). Garland and Svare

(1988) proposed that maternal aggression could depend only on pup contact, specifically on the somatosensory stimulus provided by suckling, but not on endogenous prolactin sources. In fact, studies conducted in parallel by Erskine *et al*., (1980a) in rats and Mann *et al*., (1980) in mice, which removed hypophyseal prolactin by hypophysectomy and pharmacological treatment with ergot drugs, respectively, showed no effect of the lack of prolactin on maternal aggression. In addition, Svare and Gandelman (1976a) observed that a hormonal treatment leading to nipple growth allowed pup suckling, inducing aggression in virgin female mice. In the same vein, McDermott and Gandelman (1979) reported that some virgin female mice exposed to 1-day-old pups for 9 days displayed maternal aggression, and those females showed enlarged and more numerous nipples than their counterparts that, in the same conditions, were not aggressive. This indicates that a continuous exposure to pups for 9 days had caused hormonal changes leading to both nipple growth and aggressiveness, by means of unknown mechanisms. Nevertheless, a key role of endocrine agents in maternal aggression is further supported by the expression of maternal aggression before parturition, in late-pregnant mice (Mann *et al*., 1984) –what is sometimes called pregnancy-induced aggression- and rats (Caughey *et al*., 2011), in which no nipple stimulation by pups has occurred yet.

In summary, adult laboratory female mice show nearly spontaneous pup retrieval and pup care, even if virgin. This suggests that pup-directed components of maternal behaviour are largely independent of endocrine factors but elicited simply by pup-stimuli. By contrast, there are conflicting views on the physiological control of

maternal aggression, with some authors suggesting it to depend on suckling stimulation rather than endocrine agents.

1.3.2 Olfaction and maternal care in mice

Like rats, mice are macrosmatic animals and their behaviours are guided by the detection of scent marks. In rodents, the sense of smell is composed by two major sensory systems. These systems are the olfactory and vomeronasal or accessory olfactory system, with five different sensory organs (for a review see by Munger *et al*., 2009). Briefly, the vomeronasal organ (VNO) detects pheromones that can stimulate male and female social behaviours, as well as trigger endocrine responses (Wysocki and Lepri, 1991). By contrast, the olfactory epithelium detects many of airborne odorants that can influence all kinds of behaviours, including social, predatory, anti-predatory, foraging and so on. The vomeronasal and olfactory pathways run in parallel, partially segregated pathways (Figure 3) (Boehm *et al*., 2005; Halpern and Martínez-Marcos, 2003) that nevertheless converge in some amygdaloid nuclei (Cádiz-Moretti *et al*., 2014).Therefore, chemosignals detected by both the main and accessory olfactory systems mediate socio-sexual interactions, including intersexual attraction, agonistic behaviours e.g. intermale and maternal aggression, and maternal care.

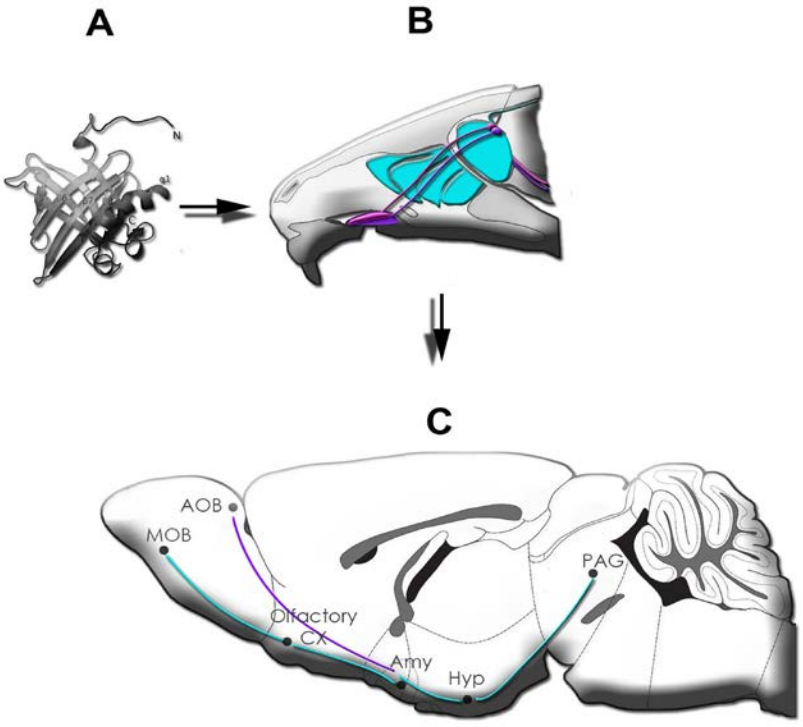

Figure 3. Encoding social information.

Scent marks (A) are detected by peripheral organs such as the vomeronasal organ (purple) and/or main olfactory epithelium (blue), among others (B). These chemosignals are integrated in the brain through their projections to accessory (AOB) and main olfactory bulb (MOB), respectively, and then to limbic e.g. amygdala (Amy) and hypothalamic areas (Hyp), to act onto motor regions, e.g. periacqueductal grey (PAG) (C). In the context of intraspecific communication mediated by chemosignals in mice, male sexual pheromones could be the sign stimuli that promote stereotyped behaviours depending on the state of female.

The olfactory cues derived from pups seem crucial to guide maternal responses. Early studies found that impairment in olfaction could directly affect maternal behaviour. On the one hand, lesions of the olfactory epithelium using $ZnSO_4$ infusion into the nasal cavity and/or lesion of the main olfactory bulbs have mild and severe effects, respectively, on nest building and maternal care (Vandenbergh, 1973).

Additionally, Gandelman and collaborators observed that olfactory bulb removal totally eliminated maternal behaviour in pregnant and non-pregnant females (Gandelman et al., 1971) and could even promote cannibalism –pup-eating- in lactating dams (Gandelman *et al.*, 1972).

A possible explanation for this strange behaviour could be that non-olfactory effects of the lesion, such as stress, might induce pup killing. For instance, olfactory bulb lesion in rodents has been employed as a model for depression in both rats (Song and Leonard, 2005) and mice (Sato *et al*., 2010). The behavioural, physiological and neurotransmission alterations induced by bulbectomy are similar to those observed in clinical patients with depression symptoms. In addition, bulbectomy has a deep impact on maternal care. For instance, bulbectomised lactating females spend less time licking/grooming and crouching over their pups, and increase total time out of nest (Sato *et al*., 2010). The authors noted that these deficits reduced pup survival, but they do not mention any cannibalistic behaviour.

Recent research has also supported the importance of neonatal-derived chemosignals in maternal behaviour. Null mutations for type 3 adenylyl cyclase ($AC3^{-/-}$), one member of the transduction pathway to which some odorant receptors are coupled, were used to evaluate the role of olfaction in maternal behaviour. The mutation in AC3 cause anosmia in mutant individuals (Wong *et al*., 2000). Both virgin and postpartum female mice with this type of mutation failed the pup retrieval assay and did not construct well-defined nests (Wang and Storm, 2011). In the same vein, the loss of function of the sodium

channel $Na_{v1.7}$ using conditional mutants (Weiss *et al*., 2011), which also causes anosmia, showed that mutant mice did not display pup retrieval behaviour. All these data suggest that the main olfactory system is involved in the detection of pup stimuli, which are important for the onset of maternal behaviour in mice. A likely hypothesis is that pup-derived olfactory cues might also protect pups from maternal cannibalistic behaviour, a role that has been demonstrated for auditory cues in birds (Lorenz, 1963).

By contrast, the accessory olfactory system seems to play a minor role in the maternal responsiveness. Thus, VNO lesions have no effect on nest building and maternal care, (Bean and Wysocki, 1989), whereas homozygous deficiency in the transient receptor potential channel type 2 (TRPC2), a cation channel expressed in the VNO (Leypold *et al.*, 2002), have only minor deficits in maternal care and nest building (Hasen and Gammie, 2011, 2009; Kimchi *et al*., 2007).

1.3.3 Olfaction and maternal aggression in mice

The accessory olfactory system has a critical role in detection of socio-sexual cues. Whereas TRPC2 mutation caused minor deficits in maternal care, mutant males and nursing females not only showed an electrophysiological reduced response to pheromones but also failed to initiate aggressive attacks on intruder males (Hasen and Gammie, 2011; Kimchi *et al*., 2007; Leypold *et al*., 2002; Stowers *et al*., 2002). However, data on constitutive mutations should be taken catiously. Null mutations could have several effects in the normal development of mice, having different phenotype results than lesions or genetic silencing or depletion during adulthood. In case of $TRPC2^{-/-}$

mice, they seem to retain some type of vomeronasal function (Kelliher *et al*., 2006; Kim *et al*., 2011). Moreover, TRPC2$^{-/-}$ mutants probably have altered olfactory sensitivity (Omura and Mombaerts, 2014).

During the last decade, the number of mutations targeting the vomeronasal receptors (VR) has increased, providing relevant information about their role in maternal aggression. For instance, homozygous mice lacking non-classical class I major histocompatibility genes (also called *ΔH2-Mv*mice) produced by chromosome engineering, not only show reduced response to several vomeronasal type 2 receptors (V2R) ligands, but also fail to display maternal aggression in the presence of castrated male intruders swabbed with urine from intact males (Leinders-Zufall *et al.*, 2014). These data indicate that this subpopulation of V2R-expressing cells is critical for the expression of aggression by lactating females. Concerning V1R, knockouts for the Gαi2 (Norlin *et al*., 2003) also show defective maternal aggression behaviour. This suggests that simultaneous signalling through both V1R and V2R is required for the expression of aggression. When one or the other vomeronasal subsystems fail, no aggression is displayed (Chamero *et al*., 2011). Some previous studies by Del Punta *et al*., (2002), who used chromosome-engineering technology to generate a mouse line with a deletion of a cluster of 16 genes that code for V1R, also reveal that this cluster is important for promoting maternal aggression. Thus, VNO signalling seems to be required for normal performance of maternal aggression.

The main olfactory system seems also involved in maternal aggression. Mutants for AC3$^{-/-}$ do not display maternal aggression toward male intruders (Wang and Storm, 2011), probably because they cannot detect the non-volatile pheromonal cues from males. As Mandiyan *et al*., (2005) suggest, one of the main effects of odorants is to promote VNO-mediated chemoinvestigation requiring contact with the substrate and vomeronasal pumping. Therefore, anosmic mutants (in this case AC3) do not sniff at conspecifics or their derived-stimuli and, consequently, they may not have access to vomeronasal stimuli, in spite of their having a functional VNO. An alternative explanation would be that since AC3$^{-/-}$ females have no maternal behaviour, not even nest building, there is no nest to defend. In other words, the lack of maternal aggression is a consequence of the dramatic disturbance of other aspects of maternal behaviour.

1.3.4 Male pheromones: from sexual attraction to maternal aggression

In the previous section we have reviewed the experimental evidence that indicates a crucial role of vomeronasal and olfactory stimuli in the control of pup-directed and non-pup-directed aspects of maternal behaviour in mice. In fact, in mice and many other rodents, most social interactions are mediated by chemosignals. Mice are strongly territorial. Males own a territory and they are constantly marking it with urine spots, especially on their boundaries, where territorial disputes with competitor males can occur. Females trespass male territorial boundaries and countermark with urine spots. Urine seems, therefore, the main vehicle for chemical advertising (Wolff and Powell, 1984).

Current views on animal chemical communication fully support this idea (Hurst and Beynon, 2004). Thus, very likely, in mice most pheromones are urinary proteins of the lipocalin family, which have an internal pocket that binds low molecular weight hydrophobic volatiles (Novotny *et al*., 1999). These so called major urinary proteins (MUPs) are encoded by a cluster of >21 genes chromosome 4 (Hurst and Beynon, 2013). There is a clear sexual dimorphism in urinary protein secretion in laboratory mice (Hurst and Beynon, 2013; Hurst, 1987). Adult males produce two to eight-fold more urinary protein than females of the C57 lineage (Cheetham *et al*., 2009).

In this respect, previous work of our laboratory demonstrated that female mice are innately attracted by non-volatile male pheromones contained in male-soiled bedding (Moncho-Bogani *et al*., 2002). Male pheromones were demonstrated to be rewarding for virgin females (Martínez-Ricós *et al*., 2007), which detect it with their vomeronasal organ (Martínez-Ricós *et al*., 2008). Subsequent work of other groups demonstrated that the pheromone responsible for sexual attraction in mice was a male-specific atypical MUP named *darcin* (also known as Mup20, Figure 4) (Roberts *et al*., 2010). Darcin by itself stimulates attraction and associative learning (Lanuza *et al*., 2014; Martínez-Ricós *et al*., 2007; Roberts *et al*., 2012), whereas other MUPs do not show this bioactivity (Roberts *et al*., 2012). MUPs themselves are apparently detected by the VNO (Chamero *et al*., 2007) and also act as reservoirs of the volatiles they bind in their hydrophobic pockets. This MUP associated volatiles are released slowly (Hurst and

Beynon, 2013) and can be detected by VNO and/or main olfactory epithelium.

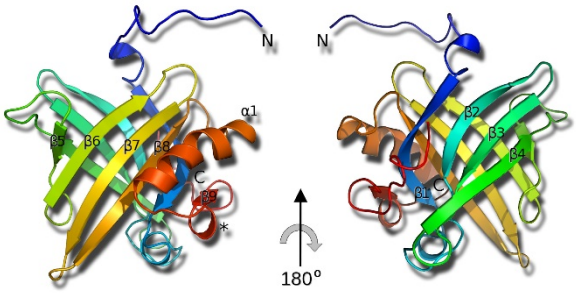

Figure 4.Tridimensional structure of male sexual pheromone darcin (Mup 20). Adapted from Phelan *et al*., 2014.

Females in estrous that contact darcin during associative learning experiment are subsequently attracted to the associated airborne odours of a particular male (Roberts *et al*., 2012). In fact, this odour conditioning can also be induced by pairing darcin with a non-mouse neutral odorant (Lanuza *et al*., 2014). This demonstrates that darcin is likely the primary sexual pheromone of male mice, since it is attractive itself and confers attractiveness to odorants by means of Pavlovian associative learning. The attractive properties of darcin are observed in virgin females but also in pregnant ones up to at least 2-5 days prior parturition (Roberts *et al*., 2014).

The attractive male sexual pheromones could also modulate other kind of behaviours. Thus, Chamero *et al*., (2007) reported that a pool of mice MUPs obtained in *Escherichia coli* using recombinant technology –thus, free of mouse-derived volatiles- was able to induce intermale territorial aggression. A recent study has demonstrated that

recombinant Mup20 and Mup3 -rMup 20 and rMup3, respectively- contain significant aggression-promoting bioactivity in inter-male agonistic encounters (Kaur *et al*., 2014). Thus, the same male chemosignal could trigger either attraction or aggression, depending on the context and the sex of the recipient.

1.3.5 Maternal behaviour, pheromones and social brain network

All the information reviewed so far indicates that the influence of pheromones on socio-sexual interactions is mediated by direct or indirect olfactory and/or vomeronasal inputs to the neural centres of the so-called '*socio-sexual brain network (SBN)'* (Newman, 1999). The SBN is a group of interconnected centres of the forebrain and midbrain that express receptors for steroids –likely reflecting sexually dimorphic steroid-modulated social behaviours- and whose lesions or inactivation alter social interaction. In her original definition of the SBN Newman, (1999) include in the network the lateral septum (LS), –medial- extended amygdala (MeEA), medial preoptic area (MPOA), anterior (AHM) and ventromedial (VMH) nuclei of hypothalamus and periaqueductal grey of the midbrain (PAG) (Figure 5). Thus, the Me is a key role of the SBN that, as discussed above –see section 1.2.2- is clearly involved in the control of maternal behaviour. The same is valid for MPOA. This is not surprising since maternal behaviours are part of the repertoire of social behaviours.

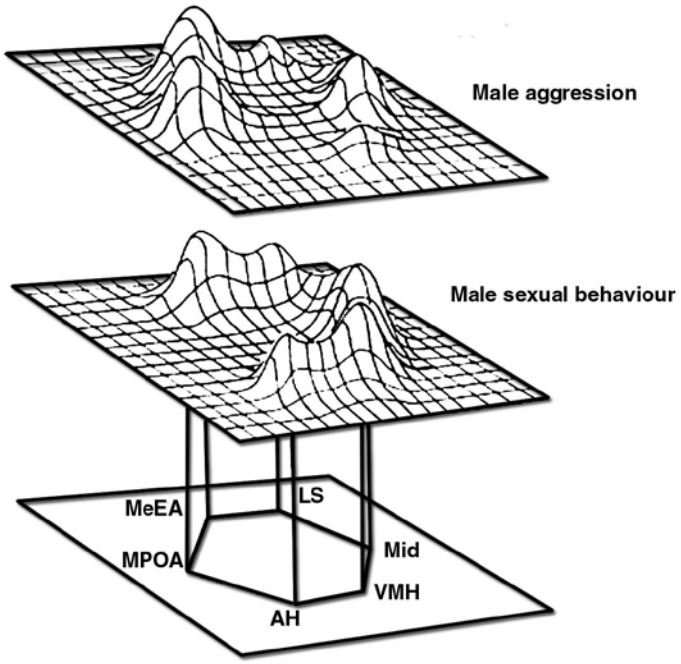

Figure 5. The social behaviour network (SBN).

The network is composed of six main nodes: the medial extended amygdala (MeEA), lateral septum (LS), medial preoptic area (MPOA), anterior hypothalamus (AH), ventromedial hypothalamus (VMH) and midbrain (Mid). The schema represents two different pattern of activation of early gene among the nodes of the network, for two different behavioural outputs, e.g. male aggression and male sexual behaviour. Adapted and modified from Newman (1999)

Anatomical data in mice indicate that the Me (Novejarque *et al*., 2011; Pardo-Bellver *et al*., 2012) projects directly to specific regions of the ventral striato-pallidum, known to be involved in processing of rewarding stimuli. In fact, lesions of the portion of the medio-ventral striato-pallidum abolish the innate attraction toward male sexual pheromones in female mice (Agustín-Pavón *et al*., 2014; DiBenedictis

et al., 2015, 2014). This indicates that this portion of the striatopalidum is the interface of the SBN and the brain system of motivation. As such, probably it is not only involved in intersexual attraction through pheromones (Agustín-Pavón *et al*., 2014) but also in motivational aspects of maternal behaviour –see section 1.2.1.2.

In conclusion, the nuclei of the socio-sexual network might control intraspecific interactions, e.g. deciding whether attacking or, on the contrary, expressing affiliative, parental or sexual behaviour towards conspecifics. Our hypothesis is that during the peripartum period there are changes in the socio-sexual brain that might be responsible for the different responses elicited by the same chemosignal in female mice. In this context, some of the nuclei of the SBN contain neurons that express the nonapeptides arginine-vasopressin and oxytocin, which are known to participate in the control of socio-sexual behaviour. It is thus likely that pregnancy, parturition and lactation provoke changes in the neurons expressing these nonapeptides.

1.4 NONAPEPTIDERGIC NUCLEI AND SOCIAL BEHAVIOUR

As already discussed, social behaviours including affiliative, agonistic and sexual behaviours –including pair bonding- as well as parental behaviours depend on a well-defined evolutionary conserved circuit called the socio sexual network (SBN). The nonapeptides vasopressin (AVP) and oxytocin (OT) participate in the control of the majority of these behaviours (Insel and Young, 2000). These nonapeptides only differ at the 3rd and 8th positions of the amioacids, being the difference in the 8th position their most distinguishing feature (Figure 6).

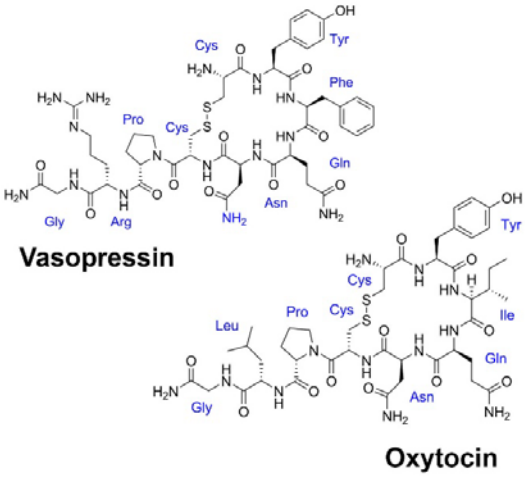

Figure 6. Molecular structure of vasopressin and oxytocin.
Secondary structure of vasopressin (AVP) and oxytocin (OT) showing the disulphide bond between cysteines. Source: Public domain

Oxytocin and vasopressin are well-known as peripheral neurohormones, which are released into the bloodstream by the

neurosecretory hypothalamic neurons of the preoptic hypothalamic area at the posterior pituitary gland (Figure 7).

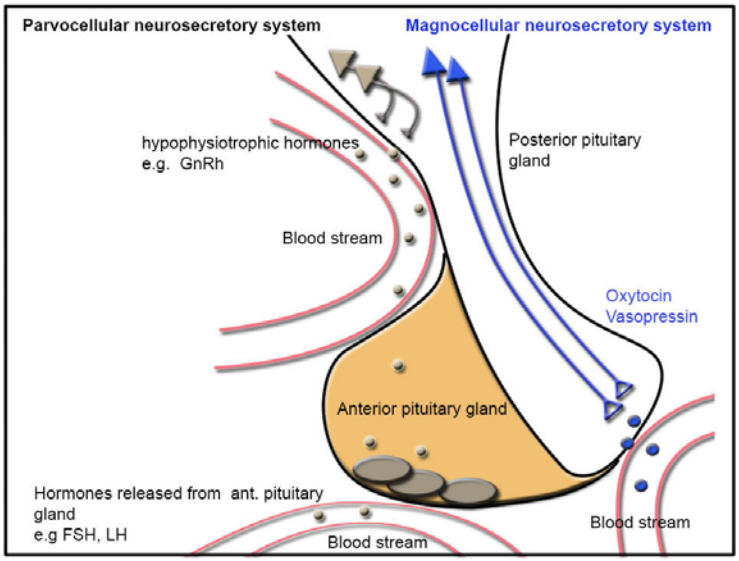

Figure 7. The two main neurosecretory systems in the central nervous system.

In the hypothalamus there are two neurosecretory systems: the magnocellular (blue) and the parvocellular ones (grey). The magnocellular neurosecretory system projects to posterior pituitary gland, also named neurohypophysis. These neurons release directly AVP and OT into the blood stream acting as neurohormones.

OT promotes milk ejection during lactation (Nishimori *et al*., 1996) and uterine contraction during parturition, whereas AVP increases blood pressure via antidiuretic – thus, also known as antidiuretic hormone, ADH- and vasoconstrictor effects. Experimental evidence indicates that nonapeptides cannot cross back the blood-brain barrier after being released as hormones (Neumann and Landgraf, 2012). However, they are also produced and released centrally by several brain nuclei, and therefore they can act as

neurotransmitters or neuromodulators in several brain circuits. As we will see, OT and AVP are important neurotransmitters in the SBN.

In every function, either as neurotransmitters or neurohormones, nonapeptides act through specific receptors, one OT receptor (OTR) and three AVP receptors (AVPR). The OTR is expressed both in the brain and peripheral target organs and tissues, i.e. mammary gland, uterine myometrium and cardiac muscle of heart, among others (Neumann and Landgraf, 2008). Concerning AVP, cells in the central nervous system express two well-characterized receptors for AVP, namely types 1a (AVP1aR) and 1b (AVP1bR) (Neumann and Landgraf, 2008). In spite of their name these receptors are not absolutely specific. Thus, AVP binds with similar affinity to OTR as it does to AVP receptors. On the other hand, OT is more specific for OTR, but it can still bind AVP receptors with a 100-fold lower affinity (Manning *et al*., 2012). This receptor 'promiscuity' has led to some difficulties for the understanding the functions of AVP and OT. For instance, in mice but not in human, both AVP and OT mediate the contractile reaction in myometrial smooth muscle via the OTR (Kawamata *et al*., 2003). A similar situation probably happens in several circuits of the central nervous system (Stoop, 2012).

Recent studies using genetically modified lines of mice lacking receptors for nonapeptides have shed light on the differential contribution of the AVP and OT in socio-sexual behaviours and their functions within the central nervous system, which are reviewed next. In the present doctoral thesis, we are interested in the role of nonapeptidergic system as regulators of maternal care and aggression. In this respect, it is important to keep in mind that, besides

nonapeptides, other neurotransmitter systems are also interacting in the regulation of maternal behaviour, including maternal care and aggression. Thus, prolactin (Mann and Bridges, 2001), dopamine (Numan and Stolzenberg, 2009), female sex steroid hormones (Brunton and Russell, 2010), or corticotropin-releasing factor (D'Anna and Gammie, 2009; Gammie *et al*., 2005), are also contributors to the expression of maternal behaviour.

The central distribution of AVP is largely differentiated in two cell populations. On the one hand, immunodetection of AVP showed that the most intensely AVP-immunostained cells correspond to the magnocellular neurosecretory cell groups of the hypothalamus, in the supraoptic, suprachiasmatic and paraventricular nuclei. Other populations of AVP cells are sparsely distributed in the caudal part of the bed nucleus of the *stria terminalis* (Otero-Garcia *et al*., 2014; Rood *et al*., 2013). The last populations of AVPergic cells shows a clear sexual dimorphism with more cells in males, in which AVP production is testosterone dependent (Otero-Garcia *et al*., 2014; Rood *et al*., 2013). This cell group gives rise to widespread sexually dimorphic AVP projections to every centre of the SBN. This is one of the neurochemical features of this neural network that explains the important role of AVP in the control of social behaviours. Moreover, these properties illustrate the sexual dimorphism of social brain as a substrate of the markedly dimorphic repertoire of social conducts.

Genetic approaches have linked the AVP1aR to spatial memory, olfactory deficits and, in voles, pair bonding (Bielsky *et al*., 2004; Egashira *et al*., 2004; Young and Wang, 2004; Wersinger *et al*., 2007). On the other hand, the AVP1bR is highly expressed in the

anterior pituitary, where it might have an important role in the neuroendocrinology of stress. Additionally, this receptor is also expressed in olfactory bulb, piriform cortical layer II, septum, cortex, hippocampus, paraventricular nucleus, cerebellum and red nucleus (Caldwell *et al*., 2008). AVP1bR knockout mice ($^{-/-}$) provided evidence on the role of this receptor in the normal display of social behaviours. Thus mutant mice showed altered aggression, social motivation and individual recognition, demonstrated through the Bruce effect in female laboratory mice (Caldwell *et al*., 2008; Wersinger *et al*., 2004, 2002). Other behaviours, such as social recognition, seem to be mediated by both AVP1aR and 1bR (Caldwell *et al*., 2008). Nevertheless, knockout mice for AVP1aR (AVP1aR $^{-/-}$) show mild deficits in social behaviours, such as a somewhat a shorter latency to attack in resident-intruder aggression test, accompanied by olfactory deficits that alter discrimination of social odours (Wersinger *et al*., 2007).

Concerning OT, besides its role as neurohormone, also acts as a neurotransmitter in specific brain regions (Castel and Morris, 1988; Otero-García *et al*., 2015) where it might modulate prosocial behaviours. Most of OT-immunoreactive (OT-ir) somata are located in four areas: a cell group is located next to the anterior commissure, extending into the bed nucleus of *stria terminalis* (BST), cell bodies are also present in the paraventricular hypothalamic nucleus (Pa) cohabitating with AVP cells, the supraoptic nucleus, and the medial amygdala (Otero-García *et al*., 2015). Additionally, some cells are scattered in hypothalamic locations, next to the third ventricle (Otero-García *et al*., 2015).

The rostral edge of the Pa expresses frequent OT/ AVP double labelling, with a general dominance of OT over AVP immunoreactivity. As Otero-García and collaborators (2015) described, these nonapeptidergic cells extend from the anterior commisure to the anterodorsal preoptic area (AC/ADP) and are consequently located between the MPOA and the ventral part of the medial BST, a crucial anatomical region for the expression of maternal behaviour in the rat (Numan and Numan, 1996; Numan *et al*., 2005b; Olazábal *et al*., 2002) and mouse (Tsuneoka *et al*., 2013). In fact, Numan *et al*., (2005b) reported that NMDA (N-metil-D-aspartate) lesions in a region that extended MPOA abolished maternal behaviour (see above). Additionally, Numan and Woodside, (2010) postulate that the proactive maternal behaviours could be modulated by the oxitocinergic projections to MPOA region. This suggests that the region involved in maternal care could include the AC/ADP along with MPOA-vBST.

Several experiments looking at memory, social bonding and reproductive and maternal behaviours in oxytocin deficient ($OT^{-/-}$) and OTR deficient ($OTR^{-/-}$) mice have provided some evidence about the role of this nonapeptide. OT defective male mice show deficits in social recognition (Ferguson *et al*., 2000), and display less ultrasound vocalizations in comparison with their littermates as well as increased inter-male aggression (Winslow *et al*., 2000).

1.4.1 Nonapeptides and maternal care

During motherhood, the activity of nonapeptidergic systems is increased due to physiological adaptations, specifically the hormonal

changes taking place during gestation, parturition and lactation (Bosch and Neumann, 2012). Pedersen and collaborators reported the earliest evidence of OT and AVP as promoters of maternal behaviours in rats (Pedersen and Prange, 1979; Pedersen *et al*., 1994, 1982). Intracerebroventricular infusions of OT stimulate spontaneous maternal care in virgin rats without previous sensitization period (Pedersen and Prange, 1979). Later, Pedersen *et al.*, (1982) reported that central infusions with AVP in virgin female rats also enhanced a rapid onset of maternal care. However, AVP provoked a longer onset in maternal care than OT infusions. Pharmacological approaches also support the implication of the AVP in maternal care. Intracerebroventricular infusions of antagonists of AVP1aR diminish both arched-back posture and the time in direct contact with pups. In addition, when the antagonists were directly injected in Pa, they reduced anxiety-related behaviours (Bayerl *et al*., 2016). However the blockade does not affect to licking-grooming behaviour in dam rats (Bosch and Neumann, 2008). Additionally, AVP but not OT central infusions enhance maternal care in dams (Bosch and Neumann, 2008).

Some studies with female mice have shown that, surprisingly, female $OT^{-/-}$ mice do not have any defect in parturition, although their nipples do not eject milk. Thus, newborn pups from these females die shortly after birth (Nishimori *et al*., 1996). Other lines of evidences have shown that $OTR^{-/-}$ female mice have impaired maternal bonding, thus suggesting a key role of central OT on mother-infant interaction (Takayanagi *et al*., 2005).

Data in humans and rodents suggest a functional link between mother's anxiety and the level of maternal care (Numan and Insel,

2003). OT and AVP presumably synthesized within either Pa or other sources might mediate this fine-tuned regulation of the mother's innate anxiety. OT could be acting centrally to promote maternal care, possibly by reducing anxiety in lactating females (Numan and Woodside, 2010). The AVP contribution has recently been studied in rodents using rat (Bosch and Neumann, 2010, 2008) and mouse strains (Kessler *et al*., 2011) selected for either high or low anxiety-related behaviours (HAB and LAB, respectively).These selected lines showed behavioural differences during lactation. Since HAB dams show markedly increased maternal care with respect to LAB ones, anxiety could be underlying maternal care differences from both strains (Bosch and Neumann, 2008; Bosch, 2011). In fact, whereas the blockade on AVP1aR decreases the high level of maternal care in HAB dams, chronic infusions of AVP increases maternal responsiveness in LAB ones (Bosch and Neumann, 2008; Kessler *et al*., 2011). These pieces of evidence suggest that the AVP is a putative candidate involved in hypoanxiety and hyperanxiety in LAB and HAB rodents, respectively (Landgraf *et al*., 2007).

Additional evidence on the relationship between stress and maternal behaviour arises from the finding that in mice chronic stress during pregnancy results in high scores of maternal care after parturition (Hillerer *et al*., 2011). In humans, functional studies indicate that infant stimuli activate the OT central neuroendocrine responses in a way that is dependent on mother-infant attachment (Strathearn *et al*., 2009).

1.4.2 Nonapeptides and maternal aggression

The oxytocinergic (OTergic) and vasopressinergic (AVPergic) systems have also been related to the regulation of maternal aggression, and it is possible that this behaviour might be linked to anxiety. Early studies with non-selected strains of mice for anxiety-related behaviours showed that those lactating females with higher scores of maternal aggression were less anxious (Maestripieri and D'Amato, 1991). In contrast, further studies with HAB lactating Wistar rats revealed that they display elevated levels of maternal aggression (Bosch and Neumann, 2010) and release more OT within both Pa and Ce during maternal defence (Bosch *et al*., 2005, 2004).

However, whereas OT seems directly involved in the expression of maternal aggression, the role of AVP is not clearly understood. On the one hand, AVP promotes nest defence when it is released within central amygdala (Ce) and thee amount of AVP released within Ce correlates with the agonistic behaviour in lactating female rats. (Bosch and Neumann, 2010). This contrast with the fact that there are no changes in Pa AVP release between HAB and LAB dams during maternal aggression test (Bosch and Neumann, 2010). By contrast, Nephew *et al*., (2010) found opposite results using Sprague-Dawley lactating rats. In this case, intracerebroventricular AVP injections decreased maternal aggression in multiparous rats (Nephew *et al*., 2010). The authors attribute these differences to differences in local manipulation and the use of different rat strains.

In summary, both AVPergic and OTergic systems seem to be involved in both components of maternal behaviour i.e. maternal care

and aggression, but further research is necessary to deepen our knowledge about the implications of both nonapeptides for maternal aggression.

The present doctoral thesis aims to study the extrinsic and intrinsic factors that affect maternal behaviours in outbred CD-1 mice. To these aims, we will first characterize the maternal sensitization process in this strain. We will perform tests that would reveal whether maternal aggression is promoted only by pup exposition –an extrinsic factor- or it depends on maternal hormonal background. Next, we will study whether there is a differential responsiveness toward male sexual chemosignals derived from the intruder that could affect maternal-like aggressive behaviours in both lactating and non-lactating females. These cues are considered as putative extrinsic factors that elicit maternal aggression in dams. Finally, we will study the OTergic and AVPergic systems in dams and maternally-sensitized virgin females to check whether there are changes in both circuitries that could underlie behavioural differences. In summary, we seek to deepen our knowledge on the neurobiological basis of maternal care and aggression.

2. AIMS

1. To characterize two different models of maternal sensitization in outbred virgin female mice of the CD1 strain.
2. To investigate whether prolonged and continuous intimate contact with pups is sufficient to induce nest defence in virgin female mice.
3. To investigate whether male chemosignals, including the attractive male sexual pheromone darcin, promote maternal aggression.
4. To investigate the possible changes occurring during lactation in the expression and co-expression of AVP and OT in brain nuclei involved in maternal behaviour.

3. MATERIAL AND METHODS

3.1 STUDY 1: CHARACTERIZATION OF MATERNAL BEHAVIOUR IN CD-1 FEMALE MICE

These experiments three main aims. First we wanted to assess, using an outbred strain CD-1 outbred strain, whether maternal care is really spontaneous in mice or if, in contrast a sensitization process is needed for virgin female to display fully maternal care. Second, we wanted to validate a sensitization procedure that we have designed, which we call *the godmother*. And third, we aim at testing whether

prolonged, intimate contact with pups leading otherwise full maternal behaviour in virgin females –the godmothers- elicits maternal aggression in different experimental conditions.

3.1.1 Mice

Except when otherwise started in our work we use chemically naïve females Moncho-Bogani *et al*., (2002), these are females bred in the absence of adult males and male-derived compounds. To do so, we housed pregnant females isolated from males, following Moncho-Bogani *et al*., (2002). Nineteen days after parturition, all pups were separated by sex, before puberty. Female siblings were brought to a clean room in a complete absence of adult male chemical signals.

After weaning females were kept in groups of 6-8 animals per cage, in propylene plastic cages (145 mm wide, 465 mm length, and 215 mm high; Panlab) until they were used for behavioural experiments when they were adults -9-12 weeks of age. Then, females were housed in black propylene plastic cages (145 mm wide, 465 mm length, and 215 mm high) in a room maintained at 24°C, 60–80% relative humidity and a 12:12 h light:dark cycle, with lights on at 08:00 hours, and ad libitum access to water and food (Teklad Global 14% Protein Rodent Maintenance Diet, Harlan). Cages were cleaned weekly, except during postpartum days, when dams were left undisturbed until the end of the experiment. For sample size in each experiment, see Table 1.

Males were purchased from Janvier Labs (France) at the age of 6 weeks. They were housed in a separate room and left to habituate

for at least one week before any experimental procedure. Males were kept in similar conditions as the females except that housed individually in 215 x 465 x 145 h mm cages (Panlab). This housing condition is thought to enhance territoriality of the males (Miczek and O'Donnell, 1978), and avoids that they become subordinates. Intact males were randomly assigned to two conditions: stud males or intruder males. Stud males were not used in behavioural experiments. For sample size in each experiment, see Table 1.

Experiment	Group	Sex	Number
Pup-directed maternal behaviour test	Dams	Female	7
	Godmothers	Female	7
	Pup-sensitized	Female	7
	Donor dams	Female	8
	Accompanying females	Female	21
Maternal aggression test	Dams	Female	9
	Godmothers	Female	9
	Pup-sensitized	Female	9
	Pup-naïve	Female	9
	Intruder, intact	Male	15
Maternal-like aggression in godmothers in the presence and the absence of pups	Dams	Female	6
	Godmothers	Female	6
	Intruder, intact	Male	7
Total			**110**

Table 1. Summary of maternal care and aggression experiments, including number and type of experimental females, non-experimental females and male intruders

3.1.2 Models of maternal sensitization

As we have mentioned in the introduction, maternal behaviours can be classified as pup-directed (maternal care) and non-pup-directed (maternal aggression) behaviour. To shed light into the contribution of experience and contact with pups in the different components of maternal behaviour, we first characterized pup-directed behaviours in both lactating and non-lactating females. Previous studies have suggested that virgin females can express maternal care in response just to by contact with pups, in the absence of hormonal changes taking place during gestation, parturition and lactation (Alsina-Llanes *et al.*, 2015; Stolzenberg and Rissman, 2011). This process is called maternal sensitization, and the aim of the first experiments was to characterize two different protocols of maternal sensitization in virgin CD1 females, namely pup-sensitized virgins and godmothers.

A. Pup-sensitized virgin female mice. This protocol was adapted from the one classically used in rats (Fleming and Luebke, 1981). We have adapted used this procedure to mice by placing foster pups -irrespective their sex- from a donor dam into the home cage of a virgin female for 2 hours per day. Females have never had previous experienced with males or male-derived signals (chemically-naïve females) prior to the experiment and therefore, these pup-sensitized females become sensitized during the three of four days of the experiment. This allow us analysing the sensitization process. This sensitization procedure was repeated during three (in Section 3.1.3.) or four consecutive days (in Section 3.1.4).

b. Godmothers. In this case we used a more naturalistic sensitization process. We placed together pregnant females with virgin, chemically-naïve females under communal conditions, following Gandelman *et al.*, (1970). The virgin and pregnant females were siblings, and they were housed together when pregnancy was evident. These virgin females, which name *godmothers*, were exposed to pups from the moment of parturition and share pup-care with the dams. We hypothesise that this procedure will result in a complete sensitization already at postpartum day 2.

3.1.3 Test of pup-directed maternal behaviour

For this experiment, adult virgin females were randomly assigned to three groups: experimental dams, experimental godmothers and pup-sensitized females. Females assigned to be dams were paired with a stud male (3 dams/male) for 4 days. After mating, males were removed and pregnant females were housed in pairs with an accompanying female homogenize the housing conditions in all the groups.

The experimental groups are shown in Figure 8. In groups 1 and 2, pregnant dams were housed with a sister (the godmother). They remained together during the rest of gestation, parturition, at postpartum day 0 (PPD 0) and lactation. Whereas in group 1 the dam was the experimental animal whose maternal behaviour was observed, in group 2 the behaviour of the godmothers was studied (Figure 8).

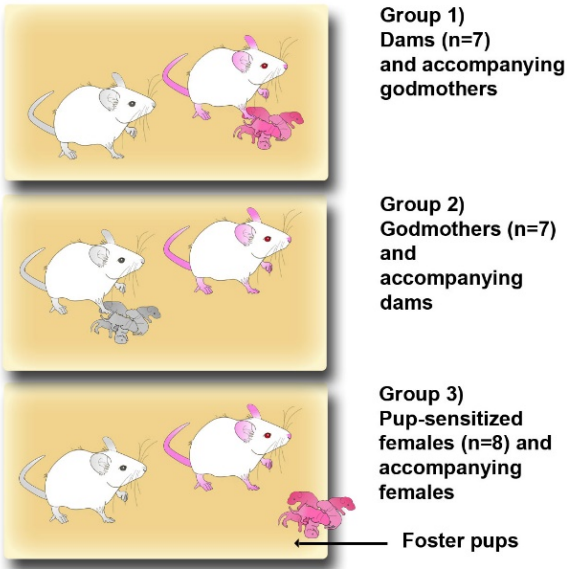

Figure 8. Protocols for maternal sensitization.

Sketch of the three experimental groups: Group 1) dams and accompanying godmothers; Group 2) Godmothers and accompanying dams; and Group 3) Pup-sensitized females and accompanying virgin females. Pup-sensitized females were isolated and exposed to pups 2h-daily. Pink-coloured drawings represent the experimental females and black and white drawings are the accompanying females.

Finally, for the group 3, pup-sensitized females, two virgin females were housed together. One of them was sensitized for 2 hours

per day exposition to foster pups throughout the experimental phase (see below).

Our earlier observations indicated that litter size from female CD1 mice oscillated from 7 to 16 pups. Previous results obtained by Maestripieri and Alleva (1990). This study revealed that maternal aggression is dependent on the size of litter, so that dams with litters of 8 or 12 displayed similar levels of aggression, but were more aggressive than dams with only 4 pups. Therefore, litters were culled to 8 pups on PPD1 in all of our experiments to unify the conditions between experiments. Pups were not selected by sex.

Pup-directed maternal behaviour tests were performed daily in the females' home cages from PPD 2 to 4 between 09:00–14:00h. Females were brought to the testing room in their home cage immediately before the test. Since females were housed in pairs (dam/godmother in groups 1 and 2, two virgin females in group 3), the accompanying female was removed prior to the test and put in an adjacent clean cage.

Logically, mothers are separated from the pups during a test of maternal care for just a few seconds. However, the accompanying females are separated from the pups for about 45 minutes, while the experimental female is performing the test. This long separation might dramatically change the maternal behaviour of the female. This effect of maternal separation has been reported in the rat. Thus, a short separation (less than 1h) of rat dams from pups increase dramatically maternal care (Pryce *et al*., 2001). Our observations in mice support these data (see BOX 1).

BOX 1: In a pilot study, we tested some accompanying dams after being briefly isolated from the pups during the test of their companion godmothers. This brief separation altered all the measures of maternal behaviour (Figure 9, see below for the definition of maternal behaviour measures).

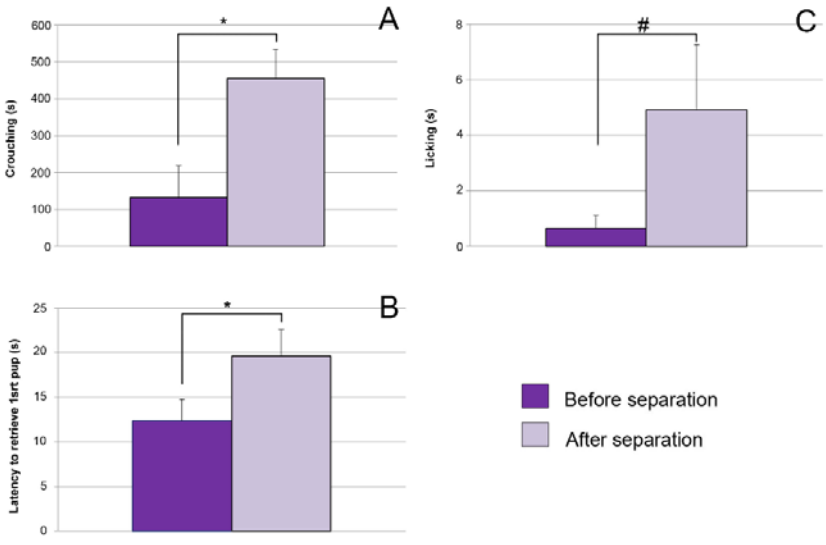

Figure 9. Separation of dams from pups during 45 minutes increases maternal responsiveness.

Dams (n=7) spend more time crouching over pups (A) and tend to lick more time in a maternal behaviour test (B) after 45-minutes of separation. By contrast, dams were slower in pup retrieval test (C) after this isolation period, indicating that isolation is a stressful situation and they failed to retrieve pups quickly. (Wilcoxon test; *p>0.5; #p=0.08).

In the groups of dams and godmothers, the eight pups were briefly removed with a clean spatula and scattered through the home cage. Pup-sensitized females received 8 stimulus pups from a donor dam. The experimenter scattered the pups with a clean spatula along the whole perimeter of the cage, approximately, as Figure 10 shows. Testing lasted 40 minutes and behaviours were video-recorded for their offline analysis.

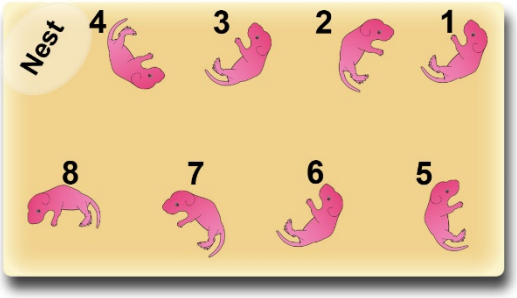

Figure 10. Sketch of the pup location for pup retrieval test.

After the test, the accompanying females were immediately returned to the home cage. By contrast, pup-sensitized females were left undisturbed with the pups for 2 hours. After this exposure period, pups were returned to their home cage with the donor dams, and pup-sensitized females reunited with their cage mates. The full procedure was repeated for 3 consecutive days.

3.1.4 Maternal aggression test

Maternal aggression is modulated by both intrinsic, e.g. endocrine factors, and extrinsic factors, e.g. stimuli arising from the pups or the intruders, or mixed factors, e.g. stressors and/or stress hormones (Bosch, 2013). This experiment was designed to check the

role of endocrine factors *vs* pup stimuli, as well as, the importance of the condition of the intruder, such as the necessity of testosterone-dependent factors in male intruders for being attacked. We specifically checked whether testosterone dependent pheromones in the intruder elicit maternal aggression.

In this experiment we checked whether the different protocols that induce pup-directed maternal behaviour could also elicit maternal aggression. Thus, we performed maternal aggression tests using the three experimental groups and conditions defined in 3.1.3, namely dams, godmothers and pup-sensitized females. In this experiment, we added a fourth experimental group of pup-naïve females as a negative control. These virgin females had no previous experience with pups, so we expected that they would display a very low or null level of aggressiveness towards intruders in the cage.

Maternal aggression tests were performed in the females' home cages (Figure 11) between 09:00–14:00h on PPD 5. We selected this time point because maternal aggression shows the highest expression level on PPD 3, 4 and 5, and then it declines during the second postpartum week (Gandelman, 1972; Ghiraldi *et al*., 1993; Lonstein and Gammie, 2002). Females were brought to the testing room in their home cage. Then, pups were removed prior to the tests (Lonstein and Gammie, 2002; Svare *et al*., 1981), to avoid infanticide behaviour by the male intruder (Vom Saal and Howard, 1982). Since maternal aggression declines after 5 hours of separation of the pups (Gandelman, 1972), this brief separation did not alter the level of aggressiveness. During the tests, pups were left in a clean cage close to their home cage where the maternal aggression test are performed.

This ensures exposure to the female being tested to odours and vocalizations of the pups during the test.

Figure 11. Screenshot of a video of black propylene plastic home cage used for maternal aggression tests.

Pups were removed before each test, and the intruders introduced in the female home cage. Tests were video-recorded to register aggressive behaviours. On the right: lactating female; on the left: male intruder.

Since females were housed in pairs (dam/godmother; two virgin females), the female that was not going to be tested was removed and were left in separate adjacent cages. In the case of virgin females, also caged in pairs, the procedure was identical except that there were no pups.

For the aggression test, an unrelated, adult male was put into the female's cage on the opposite corner of nest emplacement and behaviour was recorded for 5 minutes. Intruders were different from the stud males. To avoid damage to the males and a possible effect of experience, a male was used only once as an intruder for a dam. In addition, the same male was not used more than three aggression tests.

3.1.5 Effect of the presence of pups during the maternal aggression tests with godmothers

In order to evaluate whether godmothers would display aggressive behaviours in the presence of pups to defend, we performed a resident-intruder test with and without pups present in the test. In this test, we also analysed the interaction of godmothers with males, i.e general sniffing and anogenital approaches. These tests were performed following the same protocol as in Section 3.1.4. On PPD4, dams and pups were removed from the home cage and they were placed in an adjacent cage. Then, an unrelated male was put into the female's cage for 5 minutes. On PPD5, the resident-intruder test was repeated but this time pups were not removed. In the event of any infanticide behaviour, the test was finished immediately by removing the intruder. The accompanying dams were tested immediately after the godmothers in the absence of pups, as a positive control for maternal aggression.

3.1.6 Behavioural recording

All behavioural tests were video-recorded and an observer blind to the experimental conditions scored the behavioural responses using SMART 2.5 (Panlab S.L., Barcelona, Spain). Behavioural responses were manually scored using the plugging *event recorder*, and we assessed the frequency and total duration of each type of behaviour. The software provides an ethogram of discrete behaviours displayed by the experimental animal (Figure 12).

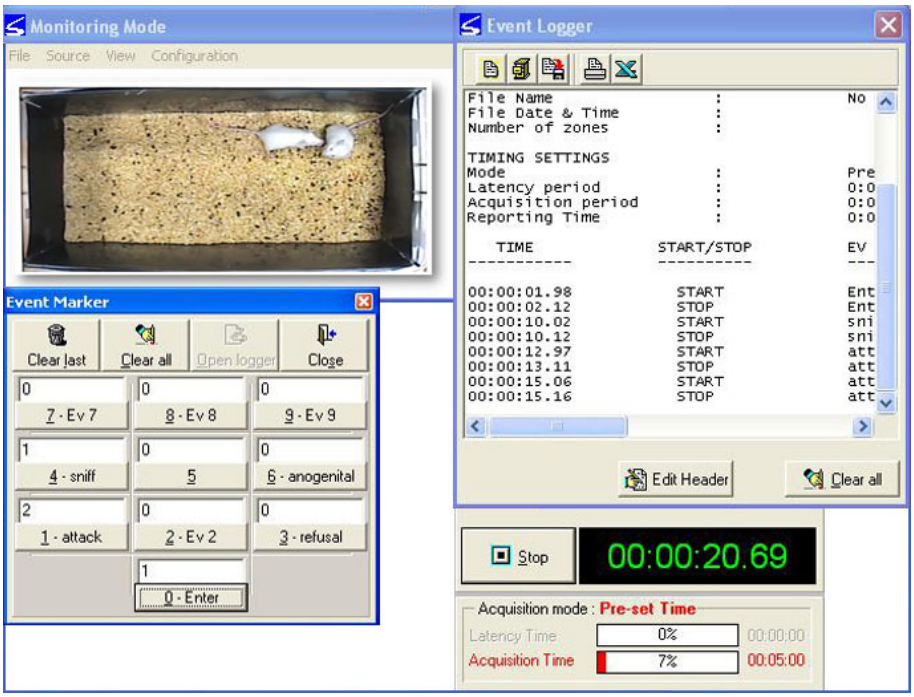

Figure 12. Screenshot showing a maternal aggression test played in 2.5 SMART. The SMART software allows manual scoring of all behavioural measures using the plugging event recorder. Before starting the recording, the test duration (lower right) is set. Each behavioural response is assigned to a key of the keyboard, as the screen

of event marker shows (lower left). The software gives information about the latency, frequency and also describes the total duration of each type of behaviour.

For pup-directed maternal behaviour tests, we scored the latency to sniff the first pup and to retrieve the first three pups during the first 300s of the 40 minutes tests. If a female did not retrieve the three pups in this time, it was assigned a latency value of 300s. In addition, the observer scored the number of events that a female tried to carry a pup with the mouth to nest site unsuccessfully as failure in pup retrieval. Fifteen minutes after starting the test, the observer scored other maternal behaviours for 10 minutes (between minute 15 and 25) following Pryce *et al*., (2001). Specifically, the observer scored *crouching* defined as an immobile posture with all four limbs supported, acquiring a slightly arched position over the pups, so that pups had access to the female's ventral surface. Moreover, we scored *pup licking/grooming*, defined as an active behaviour in which the female caught a pup with both forelimbs and then approached it to the mouth/nose. To score the frequency of crouching and pup licking/grooming we adapted the protocol by Stolzenberg and Rissman, (2011). We observed the mice every 15 seconds during the 10-minute period of observation (40 total time slots), and counted a positive event if the females were expressing crouching and/or pup licking/grooming behaviours at the time of each observation (Figure 13).

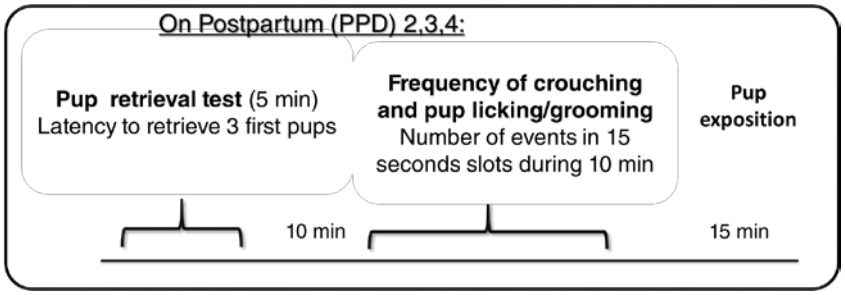

Figure 13. Protocol and behavioural measurements.

In order to evaluate whether virgin mice show neophobic responses towards pups – similar to virgin rats during the sensitization period (Fleming and Luebke, 1981) - we scored the frequency of risk-assessment behaviour, an innate response when they are confronted toward fear-evoking stimuli. For this measure, we followed the definition of risk-assessment behaviour by Papes *et al*., (2010), as a stereotypical cautious investigative approach characterized by a low-lying extended body posture.

In maternal aggression tests, we scored typical parameters of aggressiveness during the five-minute test i.e. number of attacks, total attack duration and latency to the first attack. It should be noted that we scored separately the attacks and refusal behaviours towards the intruder. Since a refusal is a sudden and aggressive response of the female, it could be confused with an attack. For this reason we defined a rejection or refusal response as a sudden attack behaviour of the female in response to a male approach, consisting of kicks using any of four limbs. In this case, the male is chasing the female to approach her. By contrast, an attack was defined as a female spontaneously

and actively biting or kicking the intruder, without previous approach of the intruder to the female.

Finally, we also measured non-aggressive social interactions. To evaluate the social interaction we scored the total time that the experimental females spent sniffing at any part of the body of the intruder. Sexual-like approaches of females to the intruder males were scored as the number of times that a female investigated the anogenital zone of the intruder.

3.1.7 Statistical analysis

Data were analysed using the software IBM SPSS Statistics 19.0. First of all, we checked whether the data fulfilled the conditions of an ANOVA, i.e. normality (Kolmogorov-Smirnov‘s test), homoscedasticity (Levene’s test) and sphericity (Mauchly’s test). When normality was violated, data was either log-transformed (log[X+1]) and then the ANOVA performed, or non-parametric analyses were used using Wilcoxon test for related samples and Kruskal-Wallis test, followed by a Dunn’s post-hoc, for non-related samples. Significance was set at $p < 0.05$.

3.2 STUDY 2: MATERNAL AGGRESSION-PROMOTING STIMULI

The aim of this study was to try to identify the key stimuli of the intruders eliciting maternal aggression in lactating dams. To do so, we first checked whether castrated males elicited similar levels of aggression than intact ones, to check if testosterone-dependent factors are involved. Previous studies indicate that maternal aggression is strongly influenced by the gonadal status and reproductive condition of intruder mice (Rosenson and Asheroff, 1975). Lactating females attack more fiercely intact mice than gonadectomised intruders. These data suggest that at least some of the male and female chemosignals that promote maternal aggression are dependent on sexual steroids. Then, we used castrated male intruders sprayed with urine or recombinant darcin, the attractive male sexual pheromone, to check if chemosignals were effective in eliciting maternal aggression. Finally, we analysed the possible territorial character of maternal aggression by performing encounters between dams and males in a neutral arena.

3.2.1 Mice

All females were bred in house following the same procedure than in Study 1. The groups of females and the sample size of each group are indicated in Table 2 .

Experiment	Group	Sex	Number
Maternal aggression test	Dams	Female	9
	Godmothers	Female	9
	Virgin	Female	9
	Intruder, intact	Male	15
	Intruder, castrated	Male	15
Aggression-promoting properties of r-darcin in lactating females	Dams	Female	10
	Godmothers	Female	10
	Castrated + PB (0.05M, pH 7.6)	Male	12
	Castrated + r-darcin (1 mg/ml in PB 0.05M pH 7.6)	Male	12
	Castrated + complete male urine	Male	12
Effect of the presence of pups during the maternal aggression tests with godmothers	Dams	Female	11
	Godmothers	Female	11
	Castrated + PB (0.05M, pH 7.6)	Male	12
	Castrated + r-darcin (1 mg/ml in PB 0.05M pH 7.6)	Male	12
	Castrated + complete male urine	Male	12

Maternal aggression in neutral arena	Dams	Female	6
	Godmothers	Female	6
	Castrated + complete male urine	Male	7
Total			**190**

Table 2. Summary of maternal aggression experiments, including number and type of experimental females and male intruders.

Males were purchased from Janvier Labs (France) at the age of 6 weeks. They were housed in a separate room and they were treated throughout as Study 1. The groups employed and the sample size per group are indicated in Table 2 .

Intact males were randomly assigned to two conditions: intruders or stud males. Like in Study 1, stud males were never used in behavioural experiments.

In the experiments of this study we used castrated males that had their testes surgically removed via scrotal access surgery (see below, Surgery and Table 2). After orchidecthomy, these animals were housed in groups of 7 –castrated males are not aggressive. Castrated males were used in behavioural tests at least three weeks after surgery, to ensure the loss of circulating testosterone.

3.2.2 Stimuli

In some of the experiments castrated intruders were impregnated with stimuli in order to test their ability to trigger aggressive behaviour in females. Specifically urine of intact males and recombinant darcin (r-darcin) were used.

Male urine was provided by Janvier Labs (France) from healthy, adult stud male mice of the Swiss-albino CD1 strain (outbred).

The male sexual pheromone darcin (Mup 20) was kindly provided by Dr Jane L. Hurst and Dr Robert J. Beynon from the Institute of Integrative Biology at the University of Liverpool (Phelan *et al*., 2014; Roberts *et al*., 2010; Roberts *et al*., 2012). In order to test possible aggression-triggering properties of this male sexual pheromone, we used the recombinant darcin (r-darcin) obtained by heterologous expression of the recombinant protein in *Escherichia coli* according to Roberts *et al.*, (2010). We received r-darcin diluted in PBS 0'05M, pH=7.4 (5.4 mg/ml). We diluted this solution to a final concentration of 1 µg/ µl, which is the concentration found in the urine of male wild mice. Aliquotes of 25 µl were stored at -20ºC for subsequent use.

3.2.3 Surgery

Adult males were anaesthetized with an i.p. injection of ketamine (75 mg/kg) and medetomidine (1 mg/kg). For analgesia, mice received a s.c. injection of butorfanol (5 mg/kg, Torbugesic, Pfizer, New York, USA). Eye drops were applied to prevent eye ulceration during surgery. Males were gonadectomized via a single midline

incision on the scrotal sac. After surgery, skin was sutured and atipamezole hydrochloride was administered (1 mg/kg i. p.; Antisedan, Pfizer) to reverse anaesthesia. Castrated males were used for the maternal aggression tests at least three weeks after gonadectomy to ensure low levels of circulating testosterone.

3.2.4 Maternal aggression test

The test of maternal aggression were performed as in Study 1 (see section 3.1.4). In these experiments, we aimed at testing the ability of different conditions e.g. intact, castrated, castrated bearing specific stimuli, as promoters of maternal aggression.

To do so, adult, virgin females were randomly assigned to three experimental groups: dams, godmothers and pup-naïve virgin females. Each female was confronted with an intact and a castrated male on two consecutive days, (between PPD 3 and 5). The order of tests was counterbalanced for each group of females. In order to minimize possible damage to the males and to avoid possible effects of their experience on subsequent tests, each male was confronted with only one lactating dam and was used in no more than two maternal aggression tests in total.

3.2.5 Analysing of mice urinary proteins

Since most of the studies on aggression are based on inter-male aggression, where the intruder is by definition a male, attempts to identify aggression-promoting chemosignals have focused on testosterone-dependent urine-borne compounds. Specifically, Chamero *et al*., (2007) demonstrated a key role of major urinary

proteins in inducing intermale aggression in resident-intruder tests. Therefore, we analysed the protein content of our intact and castrated males. Additionally, we compared them with that of female urine using a single electrophoresis.

3.2.5.1 Electrophoresis of mice urinary proteins

To do so, we collected urine from all three kinds of animals by means of a modification of a protocol for urine collection with mild intervention described by Kurien *et al*.,(2004). We obtained fresh, clean samples for the analysis of the entire urine by pressing the bladder manually, holding the animal over a Petri dish. After micturition, we transferred the urine from the dish to sterile centrifuge tubes using 20 µl-calibrated micropipettes.

This procedure was repeated for each animal, changing the Petri dish between each experimental group to avoid contamination of samples. All the urine collected from the same experimental group -female, intact or castrated male- was collected in the same microcentrifuge tube, mixed and stored at -20ºC for subsequent analysis in 20 µl aliquotes.

Generally, spontaneous urine collection is best performed immediately upon entering the animal room. Kurien and collaborators (2004) report that one micturition from a mouse produce approximately 30- 100 µl of urine. However, we generally collected less volume, depending on the animal. For this reason and to avoid handling habituation by mice, we alternated the animals and they were not used more than twice in the same week.

In order to visualize urinary proteins of the three experimental groups, we performed a protein separation according to their mass using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Lanuza *et al.*, 2014). The first step was the dilution of urine samples 1:1 with denaturalization buffer (which composition is shown in Table 3) in a capped microcentrifuge tube, vortexed to mix, boiled for 5 min and then centrifuged for 5 min at 10 000 rpm. After these steps, the samples were then allowed to cool before sample loading. Samples of male urine were further diluted using dilution buffer (composition shown in Table 3), samples of male urine were brought to a final 1:6 dilution, whereas female urine samples were not further diluted (final dilution 1:2. See Table 4). This allowed direct comparison of male and female urine protein species, in spite of the difference in total urinary protein content between the sexes –which in male urine is 3-fold as compared to female urine, Table 4.

Using a PhastGel system (General Electrics), electrophoresis was run under reducing conditions at a constant 200 V on a 20% polyacrylamide gel (PhastGel Homogeneous e 20, GE). We charged 2 µl of low range molecular weight markers (Sigmamarker low range, M3913, St Louis, MO, U.S.A.) and they were used for comparison with our samples. Following electrophoresis, protein bands were visualized using PhastGel Blue (0.1%) solution and differentiated in a solution of methanol:acetic acid:distilled H_2O (30:10:60 v/v/v).

Content	Denaturalization buffer	Dilution buffer
Buffer TRIS–HCl	20 mM pH=8.0	10 mM pH=8.0
Sodium dodecyl sulphate, SDS	5 % w/v	2,5 % w/v
Mercaptoethanol	10 %	5 %
Ethylenediaminetetraacetic acid, EDTA	2 mM	1 mM
Bromophenol blue	0,05%	0,05%

Table 3. Protocol of preparation of denaturalization and dilution buffers used in SDS-PAGE.

Adapted by Lanuza *et al.*, (2014)

Content of proteins	Male	Female or castrated male
Urine	12-13 mg/ml	3 mg /ml
Denaturalization solution (1:2)	6-6,5 mg/ml	1,5 mg/ml
Final dilution (1:6)	2 mg/ml	NO

Table 4. Relative content of urinary proteins in males, castrated males and females in CD-1 mice previously and after dilution steps.

Adapted by Lanuza *et al.*, (2014)

3.2.6 Aggression-promoting properties of recombinant darcin in lactating females

One of the proteins isolated from intact male urine is darcin (Roberts *et al*., 2010; 2012). In an experiment of this study, we tested the aggression-promoting properties of the attractive male pheromone darcin (Roberts *et al*., 2010; 2012) in lactating dams, and godmothers. To do so, we compared the attacks of both types of females towards three groups of castrated males swabbed,

respectively, with saline solution (vehicle), recombinant darcin (r-darcin; Roberts *et al*., 2010) at natural concentration in saline, and full urine of gonadally intact males.

Adult females were randomly assigned to two groups, dams and godmothers, as in Section 3.1.5. These females were tested for maternal aggression using castrated males as intruders.

Castrated male intruders were randomly assigned to one of three groups, and were swabbed in the neck and anogenital region with one of the three different stimuli: animals in the control group were sprayed with 10µl of phosphate buffer in each zone (PB 0.05M, pH 7.6); in the r-darcin group, males were rubbed with 10 µl of r-darcin in each zone (1 mg/ml in PB 0.05M pH 7.6). In the urine group, castrated males were swabbed with 5 µl of complete urine from intact male from Swiss albino mice in each zone (Janvier Labs, France). Before applying each stimulus, we supplemented the stimulus solution with 0.5-1 µl of TBS-Tx100 (1%) to ensure that that the stimulus soaked the hair of the animal and did not slip off.

Aggression tests were performed and measured as described in Section 3.1.4. Each female was confronted with a castrated male intruder from the control, r-darcin and urine groups, in three aggression tests performed over three consecutive days, corresponding to PPD 3-5 for the dams. The order of male presentation was counterbalanced for each group of females. As we have described previously, each male was used in no more than two tests and never used twice with a lactating dam, to avoid damage and the possible influence of male experience on the outcome of the test.

3.2.7 Maternal aggression in neutral arena

Maternal aggression has been classically described as maternal protection ensuring the wellness of the offspring in the presence of potential infanticide intruders. To our knowledge, there are no previous studies that evaluate maternal aggression like a territorial defence behaviour. In this experiment, we evaluated the level of aggression of lactating females and godmothers in a neutral arena, where females were placed before a male intruder. Our hypothesis was that lactating females are not unselectively aggressive in response to an unfamiliar intruder due to their lactation hormonal status; rather, maternal aggression would be triggered only in presence of a nest to defence. Thus, we expect that dams would be aggressive toward intruders only when they are in their own territory.

In order to test this hypothesis, we evaluated maternal aggression in a neutral arena. To do so, each female was individually placed in a 220 x 220 x 145 h mm cage. After a 2-minute habituation period, we placed a castrated male (n=7) swabbed with complete male urine, as described in Section 3.2.6. on PPD3. Aggression tests were performed and measured as described for Section 3.1.4. As we have described, each male was used in no more than two tests and never used twice with a lactating dam, to avoid damage and the possible influence of male experience on the outcome of the test.

3.2.8 Behavioural measures

All tests were video-recorded and an observer blind to the experimental conditions scored behavioural measurements using the

plugging *event recorder* of SMART 2.5 (Panlab S.L., Barcelona, Spain), as described in Study 1 (see section 3.1.6.)

3.2.9 Statistical analysis

All data from the experiments of this study were analysed using the software IBM SPSS Statistics 19.0, with the exception of data derived from Experiment 2.

As in Study 1, we checked whether the data fulfilled the conditions of an ANOVA, i.e. normality (Kolmogorov-Smirnov's test), homoscedasticity (Levene's test) and sphericity (Mauchly's test). When normality was violated, data was either log-transformed (log[X+1]) or we performed non-parametric analyses. Since data from Experiment 2 were not normal even after log-transformation and there was a cut-off to the right (>300s), we used Log-rank tests (latency to first attack) and randomization tests (duration of attacks). Both kind of tests are more robust and adequate for this type of data (Adams and Anthony, 1996; Good, 2005). This analysis was carried out with R free-software (Development Core Team; 2008. Viena, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org).

Randomization test consist of generating 999 additional data sets by permutating the correspondence between each female-male confrontation and the group of females (dams, godmothers and virgin females) with the original observations on attack duration. The F statistic (used in ANOVA) is obtained for the original data (F1) and for each randomly generated data set F2-F1000. Under the null hypothesis of no difference among groups F1 is distributed as the F2

to F1000 and any order of F1- F1000 values has the same probability, i.e. any rank for F1 in the series is equiprobable. If r1 is the rank of F1 value in the series of F values F1-F1000, the p-value for a two-tailed test is calculated as the fraction of 2*(r1/1000) (if r1<500) or 2*(1000-r1/1000) (if r1>500).

In the rest of tests data were analysed using an ANOVA of repeated measurements or non-parametric statistics as Wilcoxon test for related samples and Kruskal-Wallis test, followed by a Dunn's post-hoc, for non-related samples. Significance was set at $p < 0.05$.

3.3 STUDY 3: DISTRIBUTION OF NONAPEPTIES IN MATERNAL BRAIN NUCLEI

The third goal of this work has been to explore possible changes in the nonapeptidergic central systems related to motherhood and maternal behaviours. To do so we have compared the distribution of arginine-vasopressin (AVP) and oxytocin (OT) in the brain of virgin females (pup-naïve or godmothers) and dams, using immunohistochemical techniques.

3.3.1 Mice

Females from Study 2 (3.2.4 ; n=9 dams, n= 9 godmothers, n=9 virgin pup-inexperienced females) were used to map the distribution of OTergic cell bodies in selected nuclei of the maternal brain using immunoperoxidase techniques. Females from Study 1 (section 3.1.5; n=6 dams, and n=6 godmothers) were used to map the distribution of both OT and AVP in double immunofluorescence preparations. We focused in the regions of the brain where most cell bodies giving rise to central nonapeptidergic pathways are located, namely the region of the anterior commissure and anterodorsal preoptic area (AC/ADP) and the paraventricular nucleus Pa (Otero-García *et al*., 2014; 2015).

3.3.2 Perfusion, fixation and sectioning

On PPD 6, after behavioural tests, animals were deeply anaesthetized using an overdose of pentobarbital (i.p. injection of 120 mg/kg of body weight of the pentobarbital-based solution reported by Shipley and Adamek, 1984). Then, animals were killed by transcardial

perfusion of phosphate saline solution 0.1M (PBS) using a peristaltic pump (5.5 ml/min for 5 minutes) followed by 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4 (same flux for 12 minutes). Brains were carefully removed from the skull and immediately postfixed in the same fixative solution for 4 h. Then, the brains were placed into 30% sucrose solution (in 0.01M PBS, pH 7.6, 4^0C) until they sank. Finally, the brains were frozen and we obtained 40-µm-thick coronal sections with a freezing microtome (Microm HM-450, Walldorf, Germany). Free-floating sections were collected in four parallel sets.

3.3.3 Immunohistochemistry for the detection of oxytocin

A series of coronal sections of each animal of Study 2 was processed for the permanent inmunostaining of OT using the indirect avidin–biotin complex procedure adapted from Otero-García *et al*., (2015). Briefly, sections: (i) incubated in 1 % hydrogen peroxide (H_2O_2) in 0.05 M TRIS-buffered saline, pH 7.6 (TBS), for 30 min at room temperature (RT, approximately 25ºC) for endogenous peroxidase inactivation; (ii) preincubated in 0.05M TRIS buffered saline pH 7.6 (TBS) with 0.2% Triton X-100, 1% bovine serum albumin (BSA) and 4% normal goat serum at RT for 1 hour as a blocking solution; (iii) incubated in the primary antibody solution (1:25000, rabbit anti-oxytocin IgG, Millipore Cat#AB911) in TBS, with 0.3 % Triton X-100, 1 % BSA and 4 % normal goat serum (NGS) during overnight period at 4 ºC; (iv) incubated in diluted biotinylated secondary antibody (1:200, goat anti-rabbit IgG, Vector Labs, BA-1000) in TBS for 90 min at RT; (v) incubated in avidin–biotin-peroxidase complex (ABC Elite kit; Vector Labs, PK-6200) in TBS for 90 min at RT.

After each step, sections were washed in TBS (5, 10 and 10 min) except after step (ii). After ABC incubation, the sections were rinsed in TBS (5, 10 and 10 min) and TRIS buffer (TB) 0.05 M, pH 8 (5, 10 and 10 min). The histochemical detection of the resulting peroxidase activity was performed by incubation in 0.003 % H_2O_2 and 0.025 % 3,30-diaminobenzidine (Sigma) in TB for about 20 min. The sections were rinsed thoroughly in TB and mounted onto gelatinized slides, dehydrated in ethanol, cleared with xylene and coverslipped with Entellan.

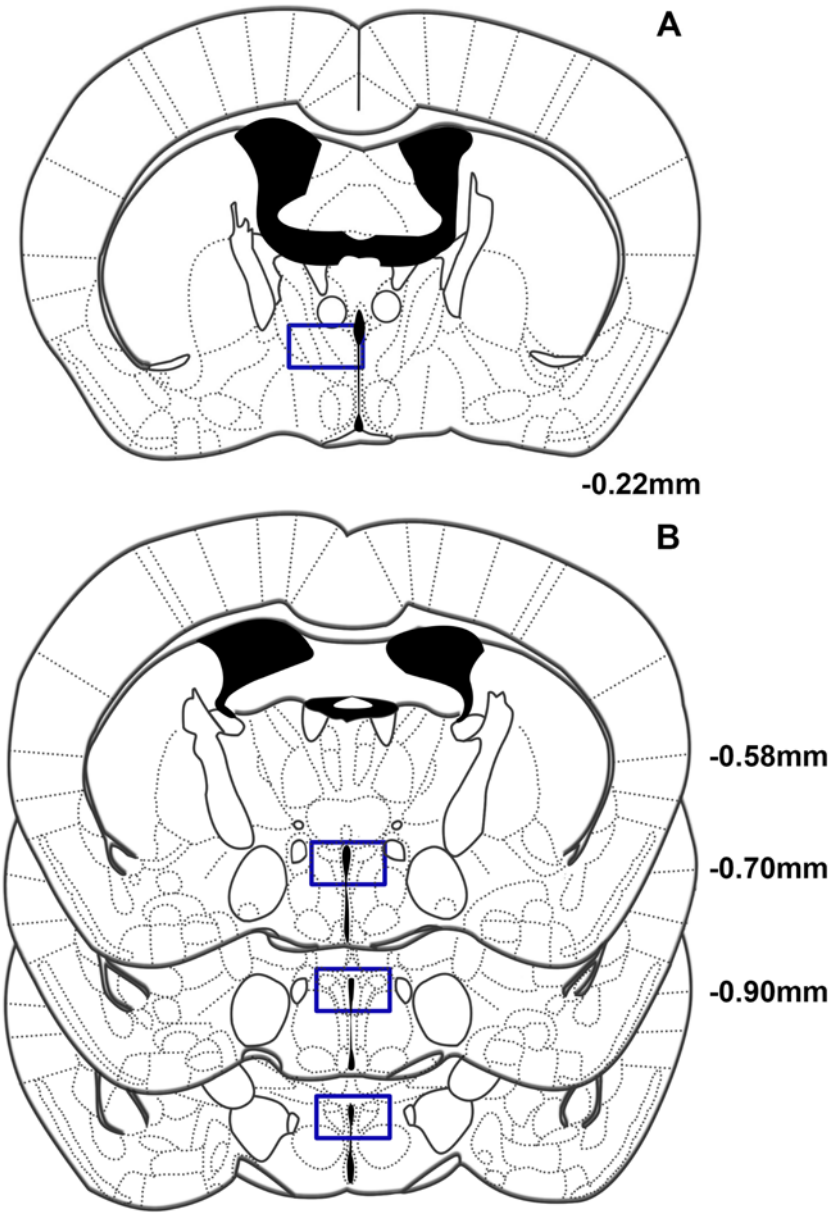

Figure 14. Location of ROI for the anatomical quantification of neuropeptides.
The ROI was located in each hemisphere for the quantification of single OT-ir cells in AC/ADP (A; distance to Bregma -0.22mm) and three different sections of Pa (B; distance

to Bregma -0.58mm, -0.70mm and -0.94mm, respectively). Adapted from Paxinos and Franklin (2004).

3.3.4 Quantification of oxytocin cells

In order to compare the number of OT-immunoreactive cells in each nucleus, we focused in selected regions of interest (ROI) of the AC/ADP region (Figure 14A) or the Pa (Figure 14B). In AC/ADP, we used the level -0.22mm relative to Bregma (Figure 14A) according to the mouse stereotaxic atlas (Paxinos and Franklin, 2004). Since Pa is a very heterogeneous nucleus along the rostro-caudal axis, we evaluated the number of OT-positive cells in three separate levels, -0.58, -0.70 and -0.94mm relative to Bregma (Figure 14B). Figures were elaborated with Adobe Photoshop CS6 software.

Photographs of the ROIs were taken in both hemispheres using a microscope Leitz DMRB (Leica AG, Germany) with a digital camera (Leica DFC300 FX) using 10x magnification. Background light was subtracted using Image J software.

An observer blind to the experimental conditions performed the quantification manually. This observer used the *cell counter* plugin of Image J software to count the number of OT-ir cells of each ROI. We used the sum of cells in each hemisphere to evaluate whether there were differences in the number of OTergic cells in this nuclei in the females with different conditions.

3.3.5 Statistical analysis

Data were analysed using the software IBM SPSS Statistics 19.0. First of all, we checked whether the data fulfilled the conditions for applying an ANOVA, e.g. normality (Kolmogorov-Smirnov‘s test), and homoscedasticity (Levene’s test). If so, they were evaluated using one-way ANOVA with GROUP as inter-subject variable, followed by Bonferroni’s posthoc pairwise comparisons. In addition, we performed a linear regression analysis between the different measurements (number of OT-ir cell bodies in AC/ADP, number of OT-ir cell bodies in the three subregions of Pa, attack duration and number of attacks) on the whole set of data of all three groups of mice (dams, godmothers and virgin females) using Pearson’s coefficient. Significance was set at $p < 0.05$.

3.3.6 Double immunohistochemistry for arginine-vasopressin and oxytocin

Two out of four parallel series obtained from six dams and six godmothers of Study 2 were employed for immunofluorescence for simultaneous immunolabelling of OT and AVP following Otero-García *et al.*, (2015). In brief, sections were: (i) incubated in 1% sodium borohydride in TBS at room temperature (RT, approximately 25ºC) for 30 minutes to supress autofluorescence; (ii) preincubated in 4% normal goat serum in 0.05M TRIS buffered saline pH 7.6 (TBS) with 0.3% Triton X-100, at RT for 1 hour, for blocking unspecific labelling; (iii) incubated in the mixture of primary antibodies, rabbit anti-vasopressin IgG (1:2500; Millipore, AB1565) and mouse anti-oxytocin, monoclonal IgG (1:200; Dr. Harold Gainer, NIH, PS38), diluted in TBS

with 0.2% Triton X-100 with 4% normal goat serum (48h at 4ºC); (iv) incubated with fluorescent-labelled secondary antibodies (90 min at RT) diluted in TBS with 0.2% Triton X-100, Alexa Fluor 488-conjugated Goat anti-rabbit IgG (1:250; Jackson ImmunoResearch, 111-545-003) and Rhodamine Red X-conjugated goat anti-mouse IgG (1:250; Invitrogen R6393). After each step, sections were washed in TBS (except between steps ii and iii). To reveal the cytoarchitecture, sections were counterstained prior to mounting by bathing them for 45 seconds in 600nM DAPI (4',6-diamino-2-fenilindol) at room temperature. Sections were finally rinsed thoroughly in TB and mounted onto gelatinized slides and cover-slipped with fluorescence mounting medium (Dako, Glosrup, Denmark). Immunofluorescence was analysed with an Olympus FV1000 Confocal Microscope System, mounted on an inverted microscope. Triple scans were made to identify DAPI, Alexa Fluor 488 (AVP) and Rhodamine Red X (OT).

Excitation wavelengths were 405 nm for DAPI, 488 nm for Alexa Fluor 488 and 559 nm for Rhodamine Red X. Emission wavelengths were 461, 520 and 591 respectively. Z-sections with a distance of 4 microns of separation were taken at 10x magnifications through the regions of interest. To minimize channel spillover, the images were sequentially acquired and saved as OIF files. The stacks obtained were further processed with Image J to optimize brightness and contrast. No manipulation of individual image elements was performed. Figures were elaborated with Adobe Photoshop CS6.

3.3.7 Quantification of arginine-vasopressin and oxytocin doubly and singly labelled cells

We used ImageJ to obtain stacks from the pictures taken with the confocal microscope. Then, an observer blind to the experimental conditions manually counted the number of AVPergic and OTergic single cells and the level of co-localization of both neuropeptides.

To do so, we opened the OIF files directly on ImageJ and we split the image into separated windows for each channel and assigned the corresponding –artificial- colour to each channel (blue for DAPI, green for Alexa Fluor 488 and red for Red Rhodamine-X) to observe the immunoreactive cells. Next, we obtained the merge colour in a different window to observe the double immunostained cells, comparing this image to the source and distinguishing single immunoreactive cells for either AVPergic or OTergic immunoreactivity, or double-labelled cells that co-express both AVP and OT.

Using the *multi-point* tool of ImageJ software, we counted the total number of cells in two adjacent optical sections of the 4-5 optical sections of each stack, moving the frame in the Z-axis. We counted the single-stained cells (Figure 15A and B) and double-stained cells (Figure 15C) confirming individually the presence of OT and AVP, using the view of specific colour channels.

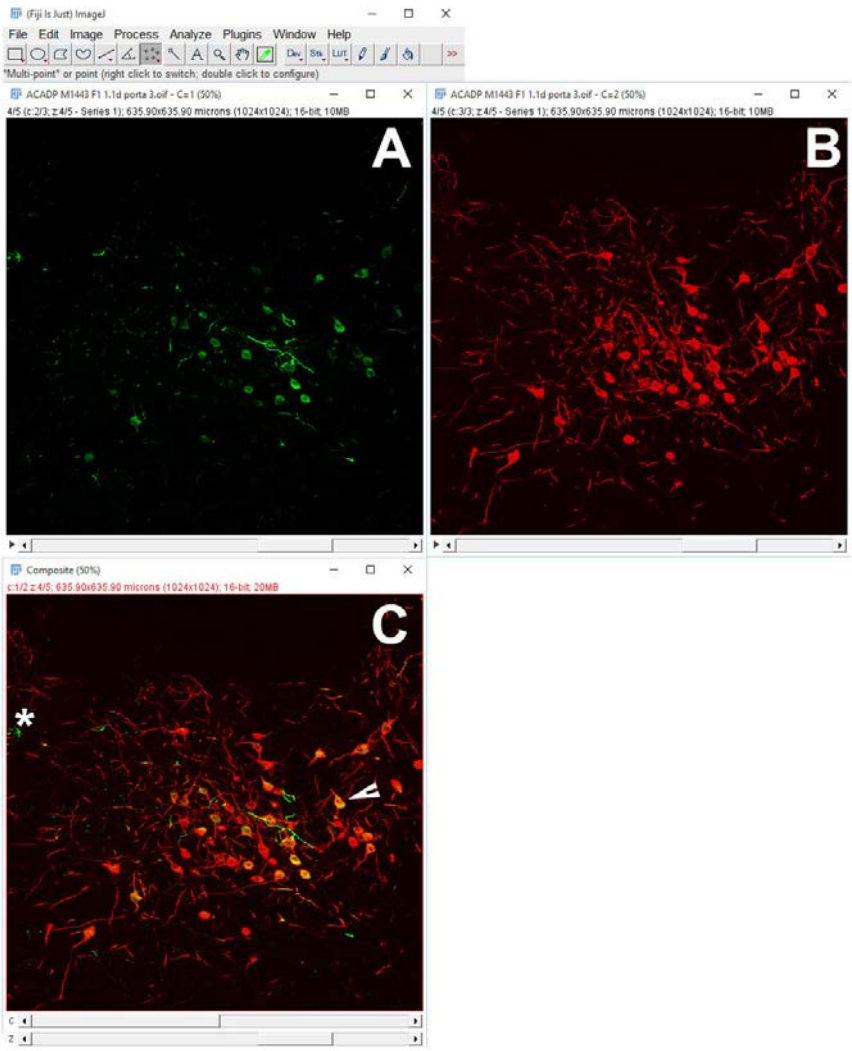

Figure 15. Quantification of AVPergic and OTergic cells

Screenshot of ImageJ with frames of AVPergic (A; in green) and OTergic (B; in red) cells. The merged image (C) shows the co-localization of both peptides (yellow) in some of the cells (white arrow shows a specific example). As we can observe, there are also AVP-ir fibers (green) that do not co-localized with OT-ir elements (white asterisk).

3.3.8 Variables

We scored the number of OT-ir or AVP-ir single-labelled somata, and the number of somata that expressed both neuropeptides in the ROIs of AC/ADP and the three subregions of Pa. Additionally, in the two groups of females (dams and godmothers), we analysed the percentage of nonapeptidergic cells that expressed a) only OT; b) only AVP or c) that co-expressed both nonapeptides.

3.3.9 Statistical analysis

Data were analysed using the software IBM SPSS Statistics 19.0. First of all, we checked whether the data fulfilled the conditions of normality (Kolmogorov-Smirnov‘s test) and homoscedasticity (Levene’s test). Next, data were evaluated using t-Student test. In addition, we performed a linear regression among the different parameters (the number of both single and double immunoreactive cells, the number of cells that only express either OT or AVP, attack duration and number of attacks) of both groups of mice (dams and godmothers) using Pearson’s coefficient. Significance was set at $p < 0.05$.

3.4 ETHICAL ASPECTS

For the present doctoral thesis, we used experimental adult mice of Swiss albino CD-1 strain (outbred, 107 males and 125 females; Charles River Laboratories, France; Janvier Labs, France). Animals were treated throughout according to the European Communities Council Directive of 24th November 1986 86/609/ECC, and, accordingly, experimental procedures were approved by the Committee of Ethics on Animal Experimentation of the University of Valencia.

4. RESULTS

4.1 STUDY 1: CHARACTERIZATION OF MATERNAL BEHAVIOUR IN CD-1 FEMALE MICE

4.1.1 Godmothers and pup-sensitized females express maternal care with different time course

In this experiment we compared the maternal behaviours expressed by virgin female mice subjected to two different protocols of maternal sensitization (see Section 3.1.2.).

First, we checked whether virgins displayed any avoidance or neophobic response towards pups, as has been described for virgin rats (Fleming and Luebke, 1981). To do so, we measured the latency

to approach and sniff pups in the first pup-retrieval test. Data violated the assumptions of normality, so we analysed the log(X+1) transformed data by means of an ANOVA with repeated measurements with TEST (Day 1, Day 2 and Day 3) as intra-subject variable and GROUP (Dams, Godmothers and Pup-sensitized females) as inter-subject variable. The test showed neither significant effects of TEST ($F_{2,38}$ =0.59, p>0.1) and GROUP ($F_{2,19}$ = 2.959; p>0.05) nor interaction between the factors TESTxGROUP ($F_{4,38}$ =0.588, p>0.1). Thus, latency to sniff the first pup was similar in all the females, suggesting that they were not neophobic towards pups irrespective of their status (data not illustrated).

The lack of neophobic responses was further confirmed by the low levels of, risk-assessment behaviour displayed by all the groups. A Kruskal-Wallis test revealed that there were no significant differences between groups across test days (Day 1: χ^2=4.5, Day 2: χ^2=3.852 and Day 3: χ^2=0.095; p>0.1 in all cases; data not shown). Thus, data suggest that virgin females with no previous experience are not neophobic toward pups.

Next, we analysed the difference between the latency to sniff the first pup and the retrieval as an approximate measure of the motivation for pups. We analysed log(X+1) transformed data by means of ANOVA with TEST (Day 1, Day 2 and Day 3) as within-subject variable and GROUP (Dams, Godmothers and Pup-sensitized females) as between-subject variable. This analysis revealed significant effects of the factors TEST ($F_{2,38}$=4.746, p=0.014;), and GROUP ($F_{2,19}$=15.167, p<0.001), as well as a significant interaction TESTxGROUP ($F_{4,38}$=2.707, p=0.044). This interaction was further

explored by analysing the simple effect of GROUP within each TEST. The results indicate that there are differences between females in all three testing days (Day 1, $F_{1,19}$=57.917, p<0.001; Day 2, $F_{1,19}$=4.915, p=0.019; Day 3, $F_{1,19}$=5.539, p=0.013). Pairwise comparisons between GROUPS showed statistically significant differences between dams and pup-sensitized females (p<0.001) and godmothers and pup-sensitized females (p=0.02) (Figure 16) but no global differences between dams and godmothers.(p=0.09). Post-hoc analysis with the Bonferroni correction revealed that both godmothers (p<0.001) and pup-sensitized females (p<0.001) were slower to retrieve the first pup after the first sniff as compared to dams on Day 1. Thus, dams are highly motivated and retrieve pups as soon as they locate them. The analysis also showed significant differences between godmothers and pup-sensitized females in this measure (p<0.001), suggesting a higher motivation for pups in godmothers than in pup-naïve virgins. On the next days, only pup-sensitized females behaved different from the lactating females (Day 2, p=0.03 and Day 3, p=0.011), whereas godmothers were as quick as dams to retrieve the first pup after sniffing it (Figure 16).

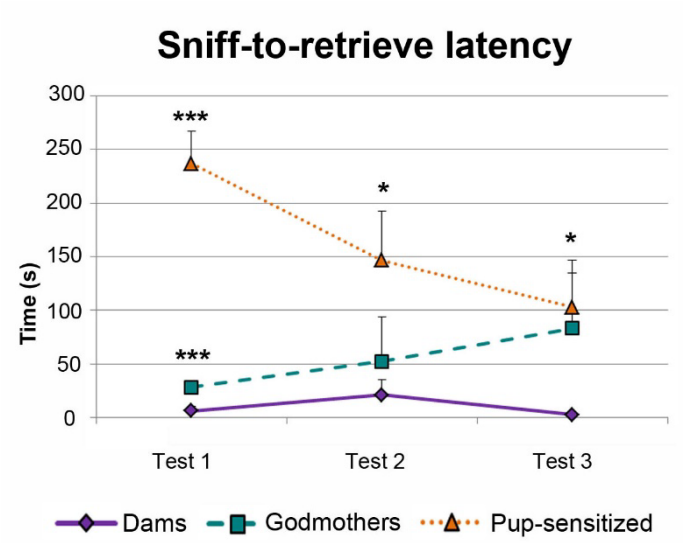

Figure 16. Time difference between first sniff and retrieval the first pup.

Time between first sniff and pup-retrieval shown by dams, godmothers, and pup-sensitized females in each test day. ANOVA of repeated measurements indicated that dams initiated faster pup retrieval after sniffing the first pup than pup-sensitized females across the three days of testing. Godmothers were slower than dams only on the first day. Data are represented as mean+SEM. *p<0.05, ***p<0.001

We analysed the latency to retrieve the first three pups as the log(X+1) transformed data by means of a two-way ANOVA for repeated measurements, with TEST (Day 1, Day 2 and Day 3) as within-subject variables and GROUP (Dams, Godmothers and Pup-sensitized females) as between-subject variable. For all the pups, the results revealed a significant effect of both TEST (Pup 1: $F_{2,38}$ =4.273, p=0.021; Pup 2: $F_{2,38}$ =6.095; and Pup 3: $F_{2,38}$ =7,358; p values < 0.005) and GROUP ($F_{2,19}$ = 11.445; $F_{2,19}$ = 9.747, $F_{2,19}$ = 7.828; p <0.003 in all cases). Concerning differences between groups, dams showed a significantly shorter latency to retrieve pups than pup-sensitized

virgins ($p<0.001$ for the three pups). Godmothers showed the same retrieval latency than dams ($p>0.12$, $p>0.32$, $p>0.54$ for pups 1, 2 and 3 respectively), while differences between godmothers and pup-sensitized females bordered on significance ($p=0.06$, $p=0.05$, $p=0.07$ for pups 1, 2 and 3 respectively).

Only data from the second pup showed significant TESTxGROUP interaction ($F_{4, 38} = 2.841$; $p<0.05$; Figure 17). Further analysis of this interaction by means of a multivariate test of the effect of TEST within each GROUP indicates that whereas dams and godmothers show a steady performance in pup retrieval through the days ($p>0.18$ in both cases), virgin pup-sensitized females significantly ($p=0.004$) improve in their performance. Post-hoc pairwise comparisons indicate that retrieval latency significantly decreases between days 1 and 2 ($p=0.018$) and days 1 and 3 ($p=0.003$) in pup-sensitized females, but did not differ between days 2 and 3. The comparison of the performance of the different females within each day (Figure 17) revealed that dams and godmothers did not differ in any of the days ($p>0.07$), whereas pup-sensitized females showed significantly longer pup retrieval latency than dams on days 1 ($p<0.001$) and 2 ($p=0.038$), and nearly reached a performance similar to dams on day 3 ($p=0.06$). Differences between godmothers and pup-sensitized virgins were restricted to day 1 ($p<0.001$).

In conclusion, godmothers were not significantly different from dams in any measure of the pup-retrieval test, and so we could consider that they are fully maternal from the first testing day, albeit they might be less motivated in the first testing day (see above). By

contrast, pup-sensitized females needed two sensitization sessions to display fully maternal pup-retrieval behaviour.

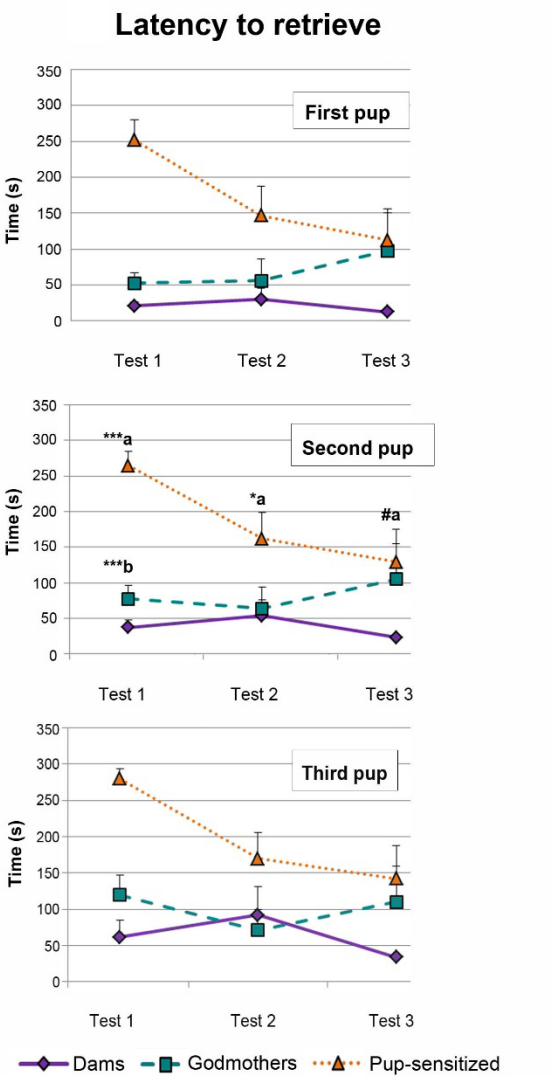

Figure 17. Latency to retrieve the first three pups.

Time that dams, godmothers, and pup-sensitized females took to retrieve the first, second and third pup. ANOVA of repeated measurements indicates that pup-

sensitized females were slower to retrieve the second pup than dams on first and second days of test. Pup-sensitized females retrieved the second pup slower than godmothers only on the first day. a) Comparison pup-sensitized vs dams; b) comparison godmothers vs pup-sensitized. Data are represented as mean+SEM. For inter-female comparisons within the same test day: #p=0.06, *p<0.05, ***p<0.001. Groups with similar means are indicated with the same letter (a, b) while different letters (a vs b) are indicative of significant differences between measurements (p<0.05).

In order to check whether differences in pup retrieval between females could be attributed to differential motor performance, we analysed the frequency of failures in pup retrieval behaviour between groups using a Kruskal-Wallis test. A failure was a pup retrieval in which the pup falls down from the mouth of the female before she deposited it in the nest. All experimental groups displayed a low rate of failures in pup retrieval and no statistical differences in this measure were observed across tests (Day 1, χ^2=2.637; Day 2, χ^2= 4.359; Day 3, χ^2= 1.799; p>0.2 in all cases, data not shown). Therefore, differences between females do not seem due to different motor performance but to motivational aspects of behaviour.

Fifteen minutes after the onset of the pup-retrieval test we evaluated the frequency of other maternal behaviours, namely crouching over the pups and licking/grooming them. We analysed differences in frequency of crouching by means of an ANOVA with TEST (Day 1, Day 2 and Day 3) as within-subject variable and GROUP (Dams, Godmothers and Pup-sensitized females) as between-subject variable. Results showed that there were significant differences in crouching between female groups ($F_{2, 19}$= 4.364, p=0.028, Figure 18), significant effect of TEST ($F_{2,18}$= 4.683, p=0.023), and a marginally

significant TESTxGROUP interaction ($F_{4,38}$= 2.239, p=0.083). Further comparisons between groups showed statistically significant differences between godmothers and dams, indicating that godmothers displayed crouching more frequently than dams across test days (p=0.025), but there were no differences between godmothers and pup-sensitized virgins (p>0.5) or between dams and pup-sensitized females (p>0.3). Further, whereas frequency of crouching did not vary across tests in dams and godmothers, post-hoc analysis revealed that frequency of crouching increased in pup-sensitized females, so that crouching at day 3 was significantly higher than at day 1 (p=0.026) and day 2 (p=0.009) in this group (Figure 18).

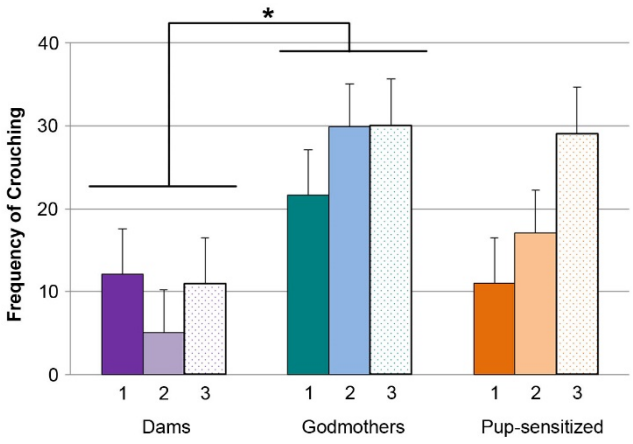

Figure 18. Godmothers showed crouching more frequently than dams and pup-sensitized females.

Number of events (out of 40 total) of crouching during the 10 min observation period. Godmothers displayed crouching more often than dams, but not than pup-sensitized females. In addition, frequency of crouching did not vary in dams and godmothers across tests, whereas it increased in the third day in pup-sensitized females. Data are represented as mean+SEM. *p<0.05

We also analysed log(X+1) transformed data of licking/grooming by means of an ANOVA with TEST (Day 1, Day 2 and Day 3) as within-subject variable and GROUP (Dams, Godmothers and Pup-sensitized females) as between-subject variable. The analysis showed a significant effect of the variable GROUP ($F_{2, 19}$ = 13.553, $p<0.001$, Figure 19), but no effect of TEST or TESTxGROUP interaction. Post-hoc comparisons between groups confirmed that pup-sensitized females overexpressed licking behaviour as compared with both dams ($p>0.001$) and godmothers ($p= 0.021$), which did not differ between them ($p=.0.157$).

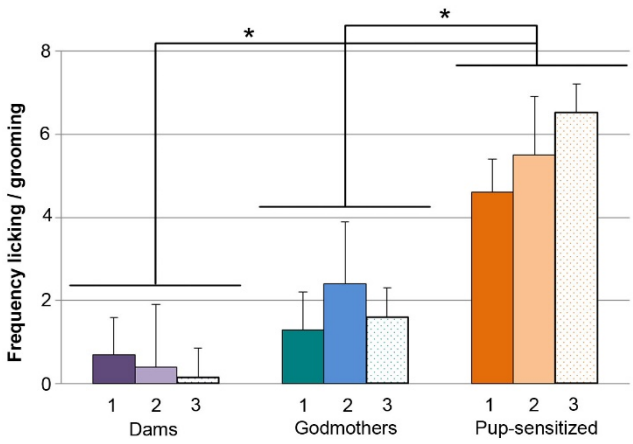

Figure 19. Pup-sensitized females showed more frequent pup licking/grooming than dams and godmothers.

Number of events (out of 40 total) of pup licking/grooming during the 10 min observation period. Overall, pup-sensitized females displayed more often pup licking/grooming behavior than dams and godmothers Analysis was performed in log(X+1) transformed data. Data are represented as mean+SEM (non-transformed, raw data). *$p<0.05$

4.1.2 Contact with pups fails to elicit maternal aggression

Since both procedures of maternal sensitisation employed (pup-sensitisation and godmothers) were able to induce virtually full maternal care, we wondered whether these sensitisation procedures would be able to induce the expression of nest defence in virgin females. Therefore, we applied a Kruskal-Wallis test to evaluate differences in latency to attack, total attack duration and frequency of attacks in dams, godmothers, pup-sensitized and pup-naïve virgin females when confronted to an adult male intruder. Data revealed global, statistically significant differences in the latency to attack ($H= 23.2$; $p>0.001$ Figure 20A), total attack duration ($H= 26.3$ $p>0.001$; Figure 20B) and frequency of attacks ($H= 26.4$; $p>0.001$ Figure 20C). The analysis of attack latency showed that dams displayed faster attacks than godmothers ($p=0.001$), pup-sensitized ($p=0.023$) and pup-naïve females ($p<0.001$). However, there were no significant differences between the different groups of virgin females (godmothers, pup-sensitized and pup-naïve females; p values>0.1). Concerning attack duration, dams spent more time attacking intruders and a higher frequency of aggressive behaviours as compared to all the non-lactating female groups (all of $p< 0.005$). Pairwise comparisons between godmothers, pup-sensitized and pup-naïve virgins showed that all of them behaved in a similar way, with low level of aggressiveness ($p>0.1$ in all cases).

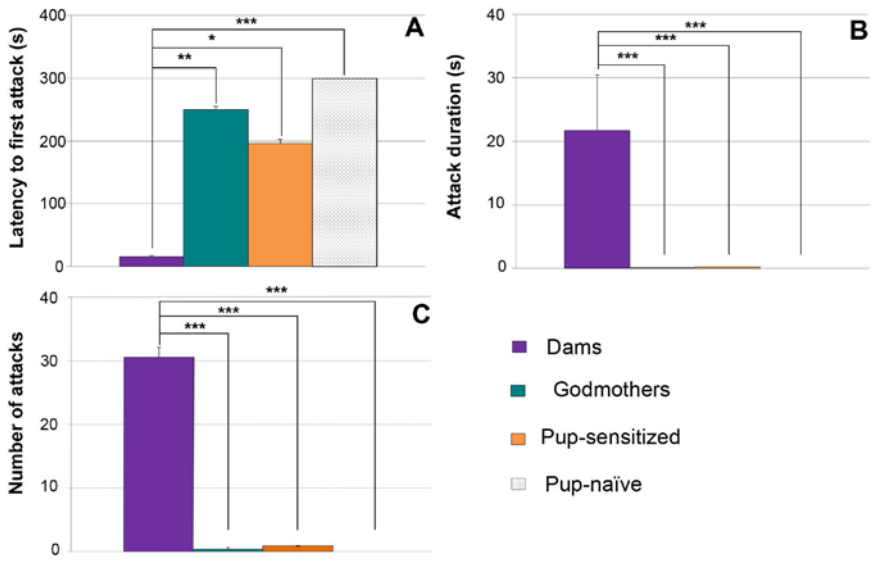

Figure 20. Both maternal sensitization models failed to promote maternal aggression

(A) Time that dams, godmothers, pup-sensitized and pup-naïve females took to initiate the first attack. Kruskal-Wallis test indicated that dams were faster than the rest of groups to initiate maternal aggression toward a male intruder. Dams also displayed longer attack duration (B) and (C) higher number of attacks toward male intruders in comparison with godmothers, pup-sensitized females and non-sensitized virgin females. Data are represented as mean + SEM. *p<0.05; **p<0.01; ***p<0.001.

4.1.3 Godmothers do not display nest defence irrespective of the presence of pups

Contrary to pup-sensitized or pup-naïve virgin females, godmothers have a nest to defend in their home cage. Even so, our previous experiments indicate that in absence of pups, they do not attack male intruders. The next experiment was aimed at checking whether they might show maternal aggression in the presence of

pups. We applied a Wilcoxon test to analyse the aggressive behaviours in the same group of godmothers in two experimental conditions, namely in absence and presence of pups. In the latter situation, we observed one infanticide event by a male intruder, and that test was finished at that point.

Results revealed that there were no statistically significant differences between both tests. Thus, godmothers behaved in a similar way in absence and presence of pups, showing low levels of aggression in all the measures (latency to first attack Figure 21A), total time duration of attacks (Figure 21B) and frequency of attacks (Figure 21C, $p>0.4$ in all of cases).

Since godmothers did not attack males, we checked whether they expressed social (i.e, sniffing) or sexual (i.e. anogenital approaches, lordosis) interactions with the intruders. A Wilcoxon test revealed that the time that godmothers spent sniffing the males was not different in the two experimental conditions (with and without pups, $p=0.463$). Further, a Mann-Whitney test between godmothers and a group of dams in the without pups condition revealed that godmothers sniffed at intruder males significantly more than dams ($p=0.026$; Figure 21D).

The number of times that godmothers investigated the anogenital region of the males was not significantly different in the presence and the absence of pups (Wilcoxon test, $p=0.273$; Figure 21E). In addition, godmothers displayed a higher number of anogenital approaches to intruder males as compared to dams (Mann-Whitney test, $p=0.004$; Figure 21E). Finally, we did not observe any lordosis.

Thus, godmothers did not attack males irrespective of the presence of pups, but displayed more socio-sexual interactions with males than dams.

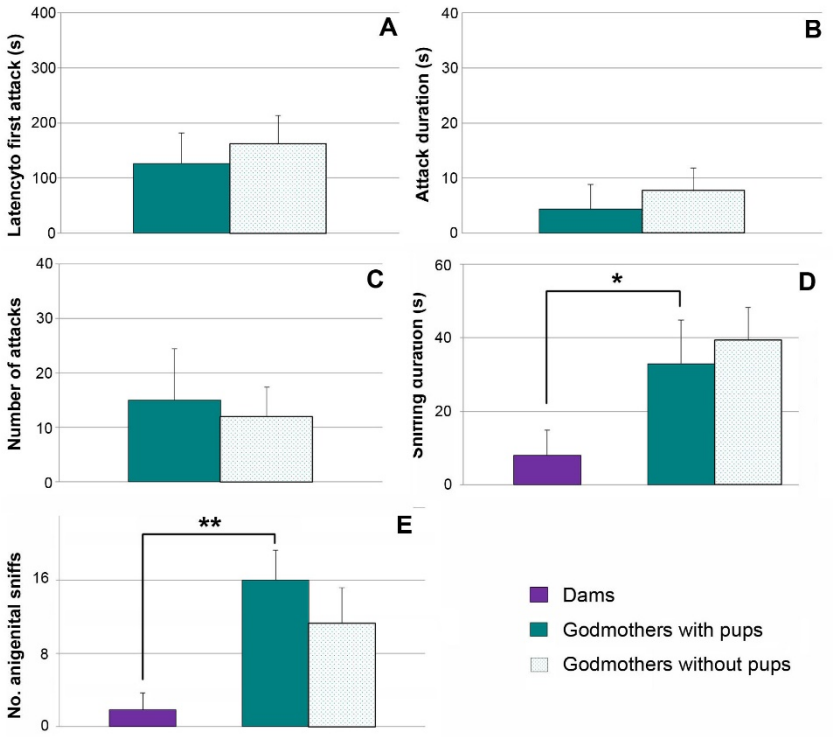

Figure 21. Godmothers are not aggressive irrespective of the presence of pups in the nest, and display more socio-sexual investigation of intruders.

(A) Time (mean + SEM) that godmothers took to initiate the first attack, (B) attack duration and (C) number of attacks toward male intruders was low in godmothers, and was not altered in the presence of the pups. The socio-sexual interactions with the male (D, time spent sniffing the intruder; E, frequency of anogenital approaches) were higher in godmothers than in dams in a similar context. *p<0.05; **p<0.01.

4.2 STUDY 2: MATERNAL AGGRESSION-PROMOTING STIMULI

4.2.1 Intruder testosterone-dependent chemosignals promote maternal aggression

This experiment was aimed at identifying the male chemosignals that induce attacks to intruders (maternal aggression) in lactating females. To do so, we compared the aggressiveness towards two different intruders, gonadally-intact and castrated males. The main difference between these types of intruders is that intact males produce male sexual pheromones that are secreted in urine. However, gonadectomy abolishes testosterone production and, consequently, the production of testosterone-dependent pheromones (e.g. including sexual pheromones).

For total duration of attacks, the data did not fulfil the conditions for an ANOVA even after logarithmic transformation. Therefore, comparison of the total duration of attack to intact and castrated males by the different types of females was performed by means of a randomization test. The results of this analysis indicated that all groups of females behaved similarly when the intruder was a castrated male ($p= 0.256$; Figure 22A), but attack duration to intact intruders significantly differed among females ($p=0.002$). Pairwise comparisons, revealed that dams displayed longer attacks than godmothers ($p= 0.001$) or pup-naïve virgin females ($p<0.001$), but no differences were found in the time of attack between pup-naïve virgin and godmother females ($p=0.215$). Thus, lactating females attacked

intact males more than virgin females, which showed similar attack duration irrespective of their experience with pups.

We analysed the attack latency using a log-rank test. This analysis showed differential responses of the females as a function of the gonadal status of the male intruder. Thus, the groups of females did not differ on the latency when the intruder is a castrated male (X^2= 1.7 on 2 df, p =0.432), but the effect of female is highly significant when the intruder is an intact male (X^2= 9.8 on 2 df, p= 0.00762, Figure 22B). Post-hoc analysis of this effect confirms differences between dams and godmothers (X^2= 4 on 1 df, p=0.0452), and between dams and pup-naïve virgin females (X^2= 8.1 on 1 df, p=0.00452). On the other hand, there are no differences between godmothers and pup-naïve females (X^2= 0.5 on 1 df, p=0.47).

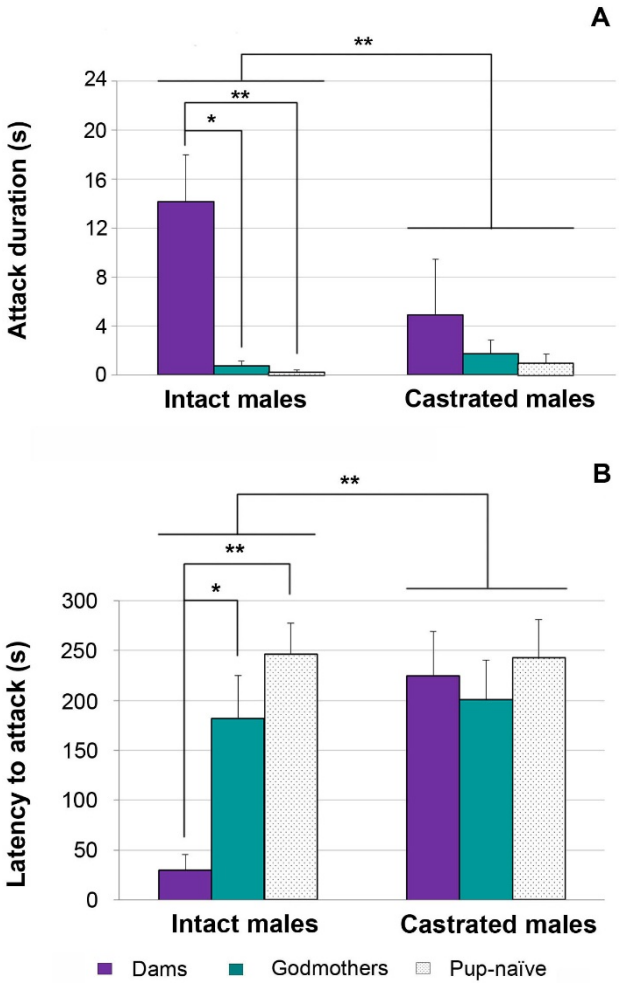

Figure 22. Total attack duration and latency to attack to intact or castrated male intruders by females

(A) Total time (mean+SEM) that dams (n=9), godmothers (n=9) and virgin pup-naïve females (n=9) spent attacking intact (left) and castrated (right) male intruders. Randomization statistics indicate that dams (but not virgin females, irrespective of their contact with pups) attacked intact male intruders for a longer time than castrated male intruders (randomization tests). (B) Bar histogram illustrating the time (mean+SEM) that dams (n=9), godmothers (n=9) and pup-naïve virgin females (n=9)

took to initiate an attack on intact (left) and castrated (right) male intruders. Log-rank tests indicated that dams attacked intact male intruders (but not castrated intruders) with a shorter latency than did either pup-naïve virgin females or godmothers (*p< 0.05; **p < 0.03). A female outlier in the godmother group has been excluded from the figure, but not from the statistical analysis.

These results lead to two main conclusions. First, only dams attack intruders and, second, godmothers and pup-inexperienced virgin females show identical behavioural response towards intruders, i.e. virtually no attacks. This clearly indicates that even continuous, close contact with the pups for 4 days and the presence of a nest to defend in the home cage (see Study 1) are not sufficient conditions to induce attacks to intruders (maternal aggression).

Therefore our data indicate a causal relationship between physiological processes related to pregnancy, parturition and lactation and the induction of maternal aggression. The second conclusion derived from our results is that lactating dams attack intact much more than castrated male intruders, thus replicating previous observations in CD1 mice (Rosenson and Asheroff, 1975). In the context of chemical communication this supports the idea that chemosignals reflecting maleness are potent cues that elicit attacks from lactating dams. Thus, the next experiment sought to compare the composition of the urine of intact and castrated males to look for maternal aggression-promoting chemosignals.

4.2.2 Protein content of the mouse urine

Mice commonly excrete lipocalins of 18-19kDa named major urinary proteins (MUPs). Most of these proteins are synthesised in the liver, secreted into serum and then rapidly excreted to urine (Hurst and Beynon, 2013). Although there are other sources of pheromones including submaxillary, lacrimal and preputial glands (Kimoto *et al*., 2007; Zhang *et al*., 2008), MUPs are viewed as important chemosignals with a clear pheromonal role on their own (Roberts *et al*., 2010).

An electrophoresis of urine of males (intact and castrated) and females reveals two main bands present in all the samples, plus an additional band exclusive of intact males. (Figure 23). This band corresponds, therefore, to a testosterone-dependent urinary protein that was described by Roberts *et al*., (2010) as a MUPs of 18893Da that shows an unusually high mobility for its size on reducing SDS-PAGE and appears as a band equivalent to 16 kDa. Roberts *et al*., (2010) christened this protein darcin (after Mr Darcy, the main character of the Jane Austen novel *Pride and Prejudice*). Similar results to ours have been reported by Hoffman *et al*., (2015) for the urine of CD-1 mice.

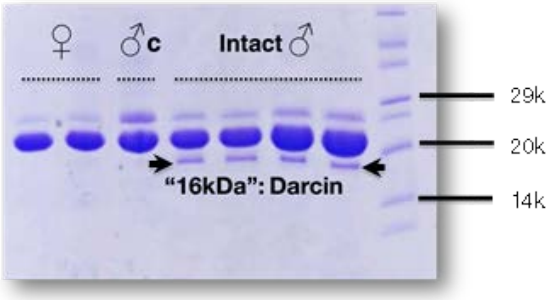

Figure 23. Electrophoresis of female, castrated male and intact male urine showing a testosterone-dependent MUP (darcin).

4.2.3 The male sexual pheromone darcin induces maternal aggression in dams

To investigate the inherent property of darcin as a trigger of aggression in lactating dams, we compared the attacks of dams and godmothers (negative control) to castrated male intruders swabbed with saline (control), gonadally-intact male urine and recombinant darcin (r-darcin). Since godmothers show full maternal behaviour but no maternal aggression from the first day of testing in the previous study, these females could be proper negative controls to evaluate different responses to male pheromones in the context of maternal aggression.

We analysed the results using log(X+1) transformed data by means of an ANOVA with repeated measurements comparing the total duration of attack between FEMALES (godmothers and dams) and STIMULI (control, r-darcin and urine). The ANOVA revealed a significant main effect of FEMALE ($F_{1,18} = 134.41$, $p < 0.001$) and STIMULUS ($F_{2,36} = 8.59$, $p = 0.001$) but no significant interaction between these factors ($p > 0.4$). These results indicate that both types

of females discriminate the chemosignals with which castrated male intruders are swabbed in the same manner (Figure 24A). However, our data clearly show that attacks displayed by lactating females are longer than the ones from godmothers (x12 times more). Pairwise comparisons of the different STIMULI, using Bonferroni correction, indicate that females spent more time attacking intruders swabbed with urine ($p = 0.001$) or with r-darcin ($p = 0.014$) than with phosphate buffer. In addition, the total duration of attacks was very similar towards castrated males swabbed with urine or r-darcin ($p=1$). In conclusion, our data demonstrate that darcin is an aggression-promoting chemosignal for lactating females. In fact, when applied to castrated male intruders, r-darcin induces the same level of maternal aggression that full urine of an intact male does, even if darcin is odourless to the human nose or, in the conditions of our test, might only bind odorants from the castrated male.

The results of the ANOVA analysing latency to attack revealed highly significant effects of both FEMALE ($F_{1,18} = 17.303$, $p = 0.001$) and STIMULUS ($F_{2,36} = 3.762$, $p = 0.033$), but no interaction between these factors ($p>0.4$; (Figure 24B). A detailed analysis of these results indicates that dams attacked with much shorter latency (mean ± SEM; 23.21 ± 19.64 s) than godmothers (140.82 ± 19.35 s). On the other hand, post-hoc analysis of the main effect of STIMULUS indicates that females showed a significantly longer latency to attack saline-sprayed castrated males than those swabbed with urine ($p = 0.018$), whilst r-darcin rendered attack latencies that were more similar to male urine ($p=1$) than to saline ($p= 0.072$, see Figure 24B). These results indicate that females, whether lactating females or godmothers, discriminated

between the three kinds of stimuli, with r-darcin eliciting a response similar to urine. Moreover, as in experiment 3.2.4, dams readily attacked male intruders, whereas godmothers displayed a very long latency to attack.

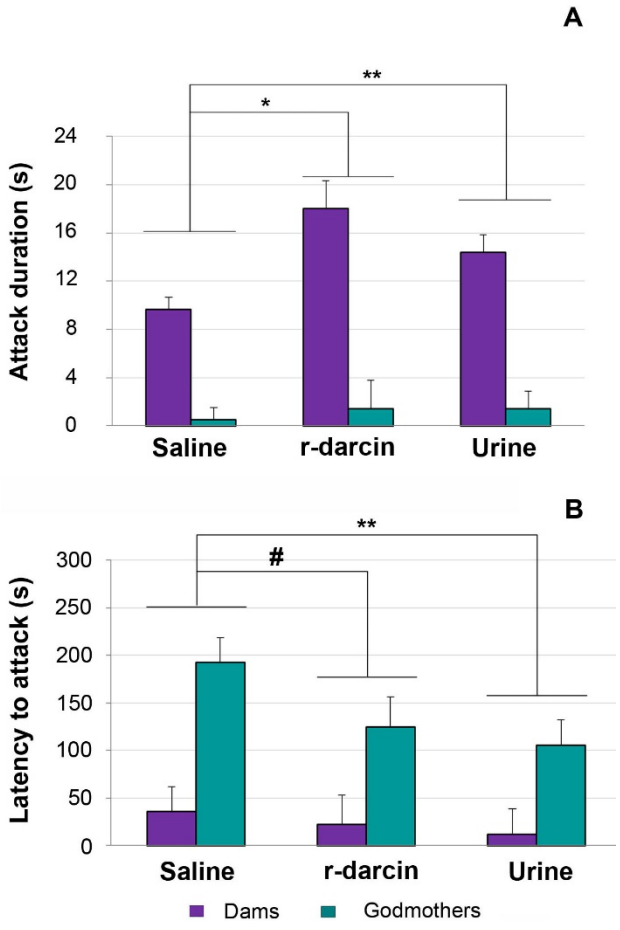

Figure 24. Male chemosignals increase total attack duration and reduce latency to attack in dams.

(A) Total time (mean + SEM) that dams (n = 10) and godmothers (n = 10) attacked castrated intruders that were swabbed with phosphate buffered saline, r-darcin and urine of gonadally intact males. Dams attacked for much longer (≈×12) than

godmothers, whilst urine- and darcin-swabbed castrated male intruders were attacked similarly, and much more than saline-treated controls (B)Time (mean±SEM) that dams (n=10) and godmothers (n=10) took to initiate an attack on castrated males that were swabbed with phosphate buffered saline, r-darcin and urine of gonadally intact males. There was a highly significant effect of the FEMALE, with dams attacking much earlier than godmothers (repeated measures ANOVA of log-transformed data, see text). In addition, male urine and r-darcin strongly reduce latency as compared to phosphate buffered saline (p-values derived from ANOVA and post hoc analysis of log-transformed data; $p<0.05$; #$p = 0.072$; **$p <0.01$).

4.2.4 Dams do not display maternal aggression in a neutral arena

To assess the aggressiveness of both lactating females and godmothers in a neutral arena, we scored the number of attacks toward castrated males bearing saline solution, r-darcin or male urine.

Dams and godmothers were not aggressive in general (Table 5). Those females that displayed aggressiveness showed a very low number of attacks toward castrated males, whatever the stimulus that they were sprayed with. We analysed possible differences between both groups of females using a Mann-Whitney *U* test for two non-related samples. The analysis revealed that there were no significant differences between females across intruders (castrated males swabbed with saline, $p=0.97$; with r-darcin, $p= 0.74$; or with male urine, $p=0.74$).

Proportion of non-aggressive females	Castrated males		
	Saline	r-darcin	Urine
Dams	*9/10	10/10	*9/10
Godmothers	9/10	9/10	10/10

Table 5. Proportion of non-aggressive females that were confronted toward castrated males sprayed with saline, r-darcin and male urine in a neutral arena. At least, 90% of Dams (n=10) and godmothers (n=10) were not aggressive when they had not a nest to defend. (*the aggressive females spent less than 1 s attacking intruder).

Additionally to number of attacks, we measured the total duration of the attacks of lactating females and godmothers towards the three types of castrated males. We analysed differences between females using a Mann-Whitney *U* test for two non-related samples. Dams and godmothers did not attack intruders, independently on the stimuli that were swabbed in neck and anogenital zone. The analysis revealed that there were no significant differences in total attack duration between females across intruders (castrated males sprayed with saline, p=0.97; with r-darcin, p= 0.74; or with male urine, p=0.74).

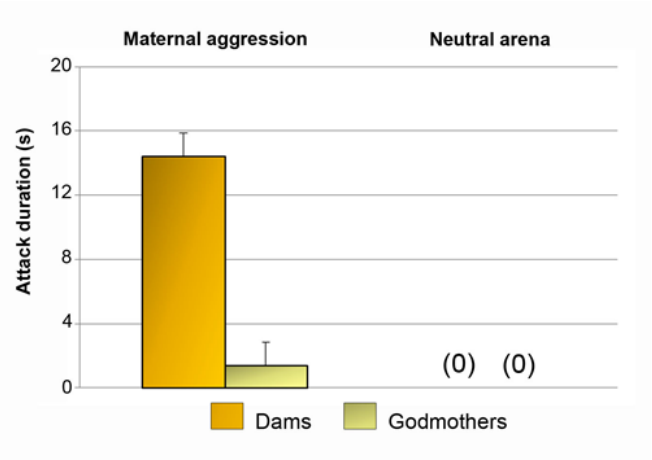

Figure 25. Maternal aggression is a territorial nest defense.
Comparison of time (mean ± SEM) that dams and godmothers spent attacking male intruder in home cage (left side) and in neutral arena (right side). Dams and godmothers did not attack castrated males swabbed with male urine when the test is performed in a neutral arena. Data from attack duration in maternal aggression context are extracted from previous experiments made in our group (Figure 24).

Finally, since our previous results showed that females not attacking the males (godmothers, virgin females) were instead interacting socio-sexually with them (see Figure Figure 21D and E), we investigated the socio-sexual behaviour of the females with the intruder male in their encounters in a neutral arena. To do so, we analysed differences in sniffing episodes directed to the body (head-snout, back) or the ano-genital region of the male intruder between both groups of females (dams and godmothers) using a Mann-Whitney *U* test for two non-related samples. Dams and godmothers did not differ in the total time sniffing the males (p=0.684; Figure 26A) or anogenital approaches (p=0.280; Figure 26B). These results contrast with the previous shown in Figure 21, showing that dams

virtually display no socio-sexual behaviour when they are attacking male intruders, but they do socially investigate males when in a neutral arena. In conclusion, maternal aggression is a territorial behaviour, expressed only in the proximity of the nest to defend.

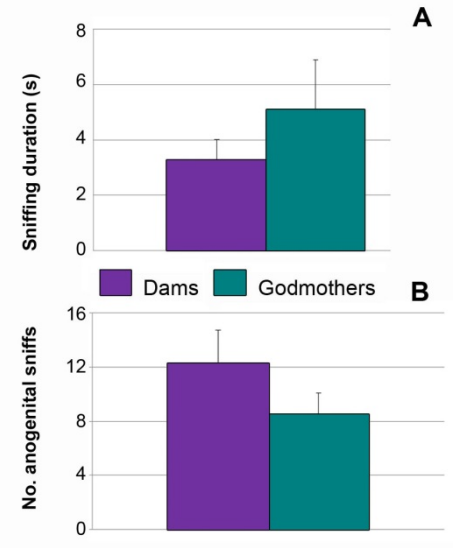

Figure 26. Dams and godmothers did not differ in socio-sexual investigation of a stimulus conspecific in a neutral arena.

(A) Time (mean+SEM) that dams and godmothers spent sniffing the intruder. (B) Number of anogenital approaches (mean + SEM) of dams and godmothers to intruders.

4.3 STUDY 3: ANALYSIS OF OXYTOCIN AND ARGININE-VASOPRESSIN IN SELECTED NUCLEI OF THE MATERNAL BRAIN

After behavioural analysis, animals from studies 1 and 2 were perfused and their brains processed for immnunohistochemistry to reveal the nonapeptidergic cell bodies of their brains. This allowed comparing the amount of cells expressing OT and/or AVP in selected brain nuclei of the brain of dams, virgin females having had no contact with pups and sensitised virgin females (godmothers), to explore changes induced by pregnancy and/or lactation or by contact with pups.

4.3.1 Lactation does not affect the number of OT-ir somata in AC/ADP or Pa

We first analysed whether either lactation or prolonged exposure to pups could modulate the total number of OTergic somata in the continuum AC/ADP and Pa in single stained DAB preparations (Figure 27). To do so, we measured the total number of OT-ir cells in dams, godmothers and virgin females that had never been in contact with pups. To analyse these data, we used one-way ANOVA.

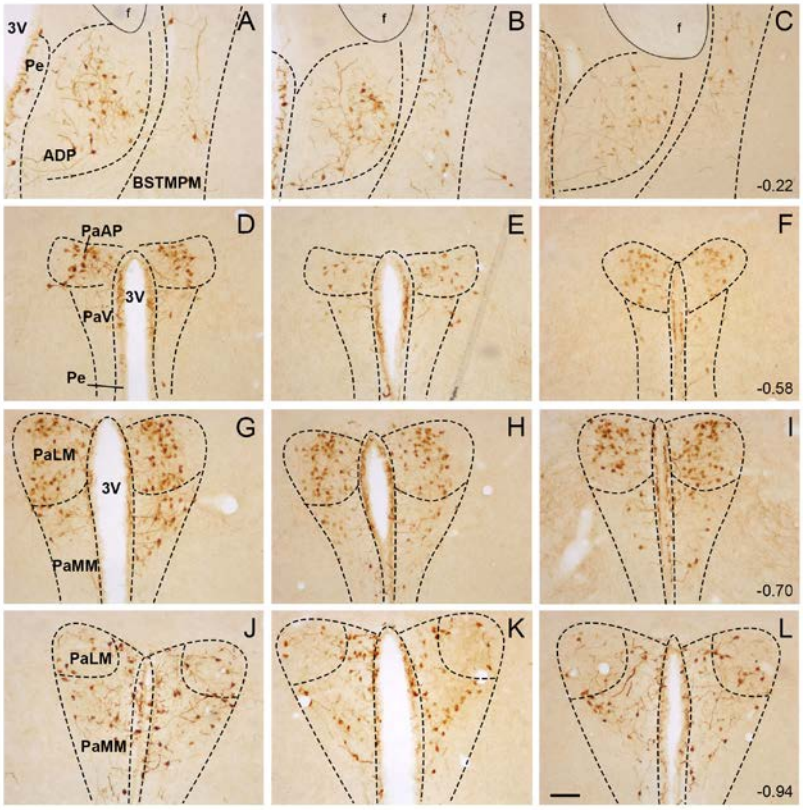

Figure 27. Oxytocin immunostaining in dams, godmothers and virgin females.

The images show OT-immunoreactivity in dams (A,D,G,J), godmothers (B,E,H,K) and virgin females (C,F,I,L). We analysed total number of somata in the anterior commissure anterodorsal preoptic nucleus area (AC/ADP) and paraventricular nucleus of the hypothalamus (Pa). Numbers in the right column refer to Bregma (-0.22mm: A,B,C; -0.58mm: D,E,F; -0.70mm: G,H,I; and -0.94mm:J,K,L). All images were taken at 10X. Scale bar: 100µm.

Quantitative analysis of the OT-ir in both regions revealed that there were not statistical differences in the number of OT-ir cells between dams, godmothers and virgin females in both nuclei (Figure 28, $p>0.05$). These data suggest that there is no variation in the

number of OT cells in these maternal regions caused either by the hormonal changes of pregnancy and lactation or by exposure to pup stimuli, as dams and godmothers had similar number of OT-ir cells than virgin females that had never contacted pups.

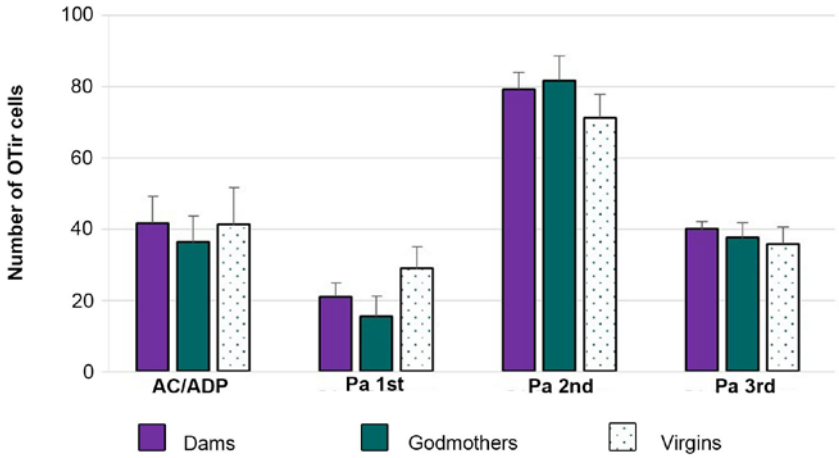

Figure 28. Neither maternity nor exposure to pups change the number of OT-ir cell bodies in AC/ADP and Pa.

Bar histogram showing the mean (+SEM) number of OT-ir cells in AC/ADP (-0.22mm) and the three anteroposterior levels of Pa. (Pa 1st: -0.58mm; Pa 2nd: -0.70mm; and Pa 3rd: -0.94mm to Bregma) in dams, godmothers and virgin pup-inexperienced females. A one-way ANOVA revealed no differences in the number of OT-ir somata between groups ($p>0.05$).

4.3.2 The number of OT-ir somata in the AC/ADP correlates with maternal aggression

Next, we checked whether there was a correlation between the total number of somata in AC/ADP and Pa and the aggressive behaviour displayed by the three groups of females when they were

confronted with intact males. In dams (Fig. 29), but not godmothers or pup-naïve virgin females ($p>0.1$; data not shown), a significant Pearson's correlation was found between the number of somata in AC/ADP (-0.22mm to Bregma) and both variables of aggressive behaviour, the number of attacks ($p<0.01$, $R=0.817$; Figure 29A) and total attack duration ($p<0.05$, $R=0.7$; Figure 29B) to the intruder. Thus, the more aggressive dams were the higher number of OT-ir somata displayed in their AC/ADP. This correlation was not significant for the number of OT-ir cells in any of the levels of Pa ($p>0.05$). These data suggest that OTergic cells in the AC/ADP might be involved in the expression of aggressiveness in dams specifically during lactation.

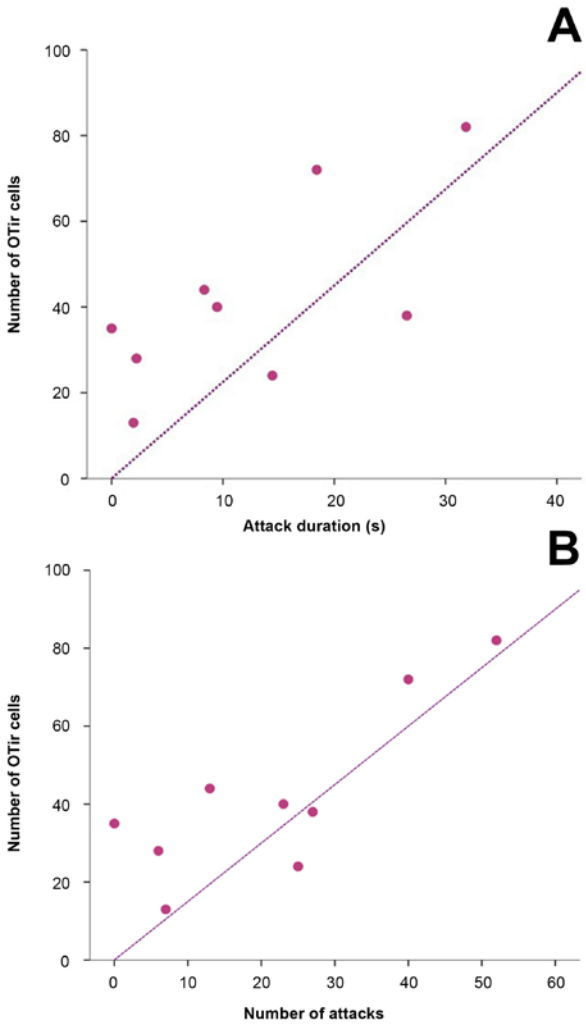

Figure 29. The number of OTergic somata in AC/ADP (-0.22mm to Bregma) correlates with aggression in dams.

Pearson's correlation showed that level of aggressiveness correlates both variables, the number of attacks ($p<0.01$, $R=0.817$; A) and total attack duration ($p<0.05$, $R=0.7$; B).

4.3.3 Lactation does not affect the number or proportion of single- and double- stained nonapeptidergic cells

Next, we sought to assess whether lactation could increase the co-localization of AVP and OT in Pa and AC/ADP, as it was described in the supraoptic nucleus in rats (Mezey and Kiss, 1991). These authors suggest that the co-expression of both nonapeptides vary during postpartum period, with a maximum peak during first week of lactation. Thus, we analysed the number of OTergic, AVPergic and co-expressing AVP-OT somata in the continuum AC/ADP and in the Pa in double immunofluorescent preparations. Since we did not find any difference between godmothers and inexperienced virgin females, we did not include this latter group in this experiment and used godmothers as a control.

As in the case of OT immunoperoxidase preparations, we found no significant differences between the number of cells expressing OT between dams and godmothers. The same was true for the number of AVP-ir cells or doubly labelled (OT-ir plus AVP-ir) neurons (Figure 30). Further, there were no significant differences in the percentage of only OT-ir and only AVP-ir somata relative to the population of nonapeptidergic cells in the AC/ADP (Table 6).

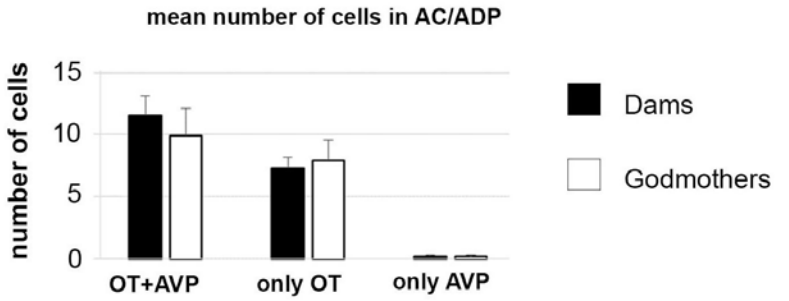

Figure 30. Lactation does not increase the number of OT-ir, AVP-ir or co-labelled cells in the AC/ADP region.

Bar histogram showing the mean+SEM number of cells of each frame that co-expresses both OT and AVP cells (OT+AVP) and the number of cells that only express OT or AVP (only OT or only AVP, respectively) in the AC/ADP of dams and godmothers. Student's t test revealed that there were no significant differences in the number of the OTergic, AVPergic or co-labeled somata between both groups (all cases $p>0.1$).

Also in agreement with previous results, Student's t tests revealed no significant differences in the number of OTergic and AVPergic cells or in co-labelled cells throughout any portion of Pa (Figure 31A, B and C, respectively and Figure 32). Again, the percentage of nonapeptidergic cells that were positive for OT or AVP in each ROI was not significantly different between godmothers and lactating dams (Table 6; all cases $p>0.05$).

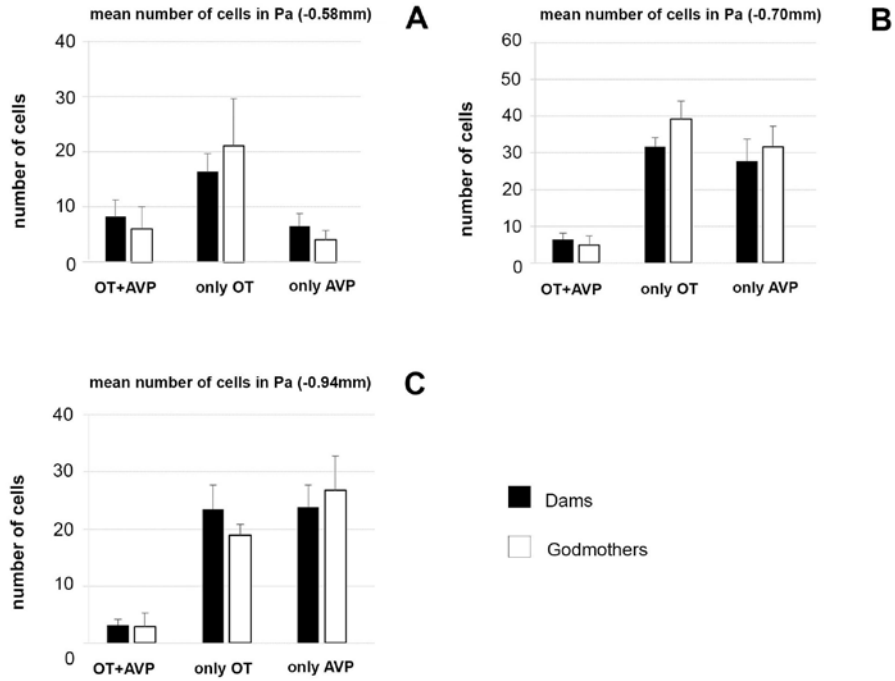

Figure 31. Lactation does not affect the number of nonapeptidergic cells in the Pa.

Bars represent the mean+SEM of the number of cells in each level of the Pa of dams and godmothers that co-express both OT and AVP (OT+AVP) and the number of cells that only express OT or AVP (only OT or only AVP, respectively) (A, -0.58mm; B, -0.70mm; C, -0.94mm; relative to Bregma). Student's test revealed no significant differences in the number of the OT-ir, AVP-ir or co-labelled somata between both groups ($p>0.1$ in all cases).

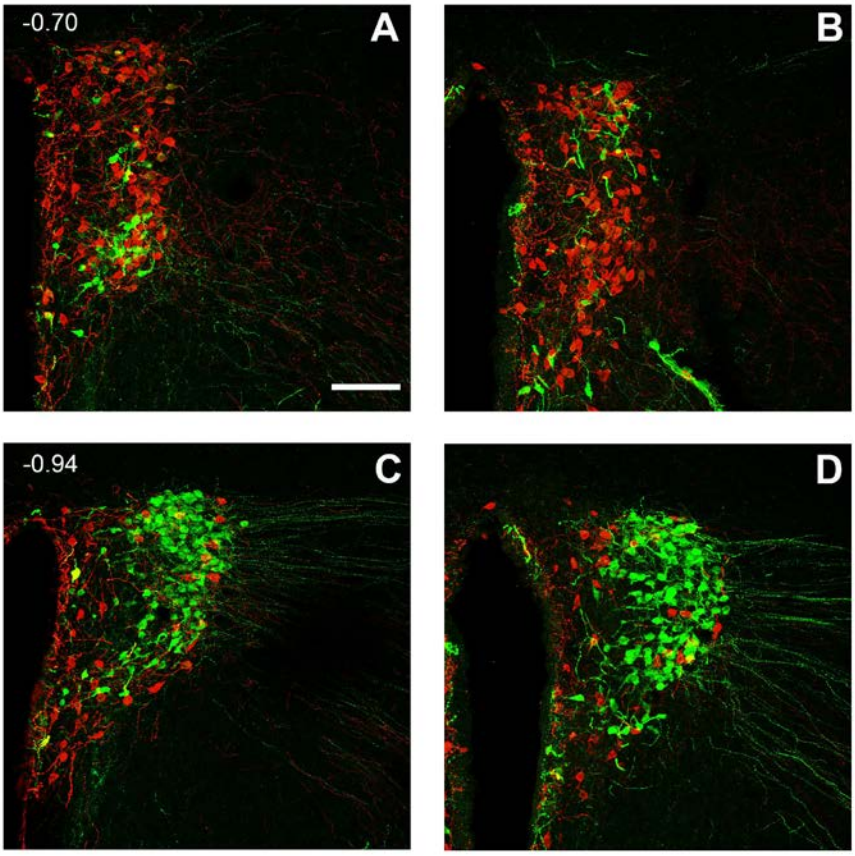

Figure 32. Simultaneous immunofluoresce for oxytocin (red) and vasopressin (green) in two coronal sections of the Pa of a dam and a godmother.

Representative pictures of Pa of one dam (A, C) and a godmothers (B, D), illustrating that there is no variation in the expression of single- or dobly-stained cells. (Scale bar: 100 µm, valid also for all illustrations; distance from Bregma is indicated in mm).

In summary, our data suggest that the total or relative numbers of nonapeptidergic subpopulation do not change during the postpartum period indicating that lactation does not substantially modify the number of cells that express OT or AVP in these nuclei related to maternal behaviour.

Dams			
	% Nonapeptide-ir cells expressing only OT	% Nonapeptide-ir cells expressing only AVP	% Nonapeptide-ir cells expressing OT + AVP
AC/ADP	39.17	0.63	59.44
Pa (-0.58 mm)	51.70	20.91	27.39
Pa (-0.70 mm)	49.15	40.79	10.06
Pa (-0.94 mm)	47.08	47.06	5.86
Pa total	49.14	36.66	14.20

Godmothers			
	% Nonapeptide-ir cells expressing only OT	% Nonapeptide-ir cells expressing only AVP	% Nonapeptide-ir cells expressing OT + AVP
AC/ADP	44.74	0.92	54.34
Pa (-0.58mm)	71.35	11.86	16.79
Pa (-0.70mm)	52.92	41.82	5.26
Pa (-0.94mm)	39.73	52.43	7.83
Pa total	50.54	37.97	11.49

Table 6. Distribution of nonapeptidergic cell bodies immunoreactive for OT, AVP or both in the different nuclei of the brain of dams and godmothers.

Percentage of nonapeptidergic cells expressing only OT, only AVP or both nonapeptides (OT+AVP) in the AC/ACP, the three levels of the Pa separately and the whole Pa (Pa total) in dams and godmothers. A one-way ANOVA revealed no statistical differences (all cases $p>0.2$).

4.3.4 Correlation between nonapeptidergic neurons and aggression in dams

We analysed whether we could replicate the positive correlation that we found in DAB stained cells for OT in the AC/ADP with behavioural aggression parameters in dams. Additionally, we explored whether aggressiveness correlated with the number of AVP-ir or double stained cells in AC/ ADP and Pa.

We could not replicate the statistically significant correlation between the aggression parameters and the number of OTergic

somata, but we did find the same trend towards a higher number of OT-ir cells in the AC/ADP of dams displaying higher total attack duration (p=0.130, R=0.689; Figure 33A) and higher number of attacks (p=0.150, R=0.664; Figure 33B). It should be noted that for this experiment we used a lower number of animals, n=6 per group, than for the previous experiment, in which we had n=9 per group. Since the trend was maintained in spite of the smaller sample and different technique, a further investigation of the relationship between OT-ir cells in the AC/ADP and maternal aggression is warranted.

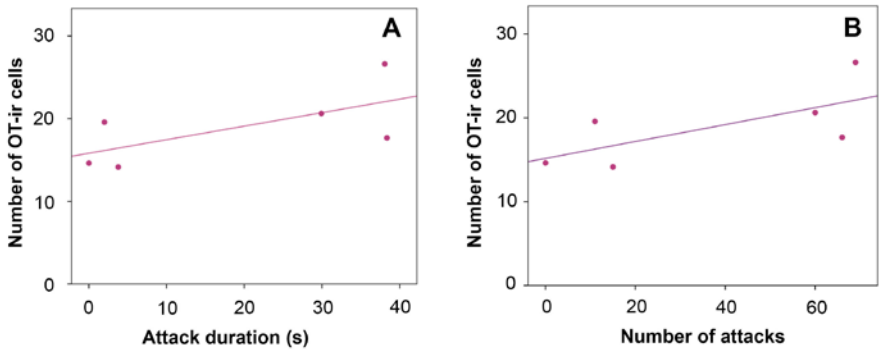

Figure 33.The number of OT-ir somata in all AC/ADP trends to positively correlate with aggression in dams.

Pearson's correlation showed that level of aggressiveness is near to correlate with both variables,) total attack duration (p=0.130, R=0.689, A) and the number of attacks (p=0.150, R=0.664; B).

Finally, we did not find any significant correlation between AVP-ir somata in AC/ADP and Pa and maternal aggression parameters in dams or godmothers (all cases p>0.2), or of the number of somata co-expressing OT and AVP in both nuclei with aggressiveness (p>0.1; data not shown).

In summary, the number of cells expressing OT, AVP or both nonapeptides in the AC/ADP and Pa is not affected by the hormonal events taking place during lactation. However, we found a positive and significant correlation between the number of OT-ir somata and aggressive responses in dams in one of the experiments that should be further explored in the future.

5. DISCUSSION

The present doctoral thesis constitutes the first detailed description of maternal behaviour, both in dams and virgin females, in the outbred CD1 strain of mice. In addition, we carried out a exploratory analysis of the nonapeptidergic cells in two brain nuclei related to maternal behaviour (AC/ADP and Pa), to investigate the possible effects on these nuclei of the hormonal events taking place during pregnancy and lactation or of prolonged exposure to pup-derived stimuli.

We will first discuss our findings on the characterization of maternal care of CD1 mice. As in other species, primiparous female mice show maternal care from the moment of delivery. Previous data indicated that virgin female mice show immediate onset of maternal

care when they are exposed for the first time to pups. However, our data suggest that full maternal care is not completely spontaneous, but rather nearly spontaneous, since virgin female mice that have never had contact with pups needed at least two sensitization sessions to display levels of maternal care comparable to those of dams. In any case, our results show that virgin mice are not neophobic towards pups, and this lack of neophobia could account for the rapid sensitization shown by virgin females.

To our knowledge, our study is the first including the investigation of both components of maternal behaviour, namely, maternal care and aggression, in the same set of experiments and individuals. Previous data usually evaluated aggression and maternal care separately. Our data strongly suggest that maternal care and maternal aggression have different neuroendocrine basis and time course, since the maternal sensitization protocols we used were able to induce the former but not the later.

We devote the second part of the discussion to our results on the aggression-promoting properties of male derived-stimuli for dams. Maternal aggression tests showed that dams attacked intact males more times and for longer than they attacked castrated males. We analysed the aggression-eliciting properties of male sexual pheromones contained in urine, the production of which is dependent on testosterone. In particular, we investigated the aggression-promoting properties of the pheromone darcin. We demonstrate that both urine and darcin alone elicit the same levels of maternal aggression in dams. Thus, the same pheromone elicits two opposite emotional behaviours depending on the recipient: attraction in virgin

females (Roberts *et al*., 2010) and aggression in lactating dams (our results).

Finally, we will discuss the role of nonapeptides in the maternal brain and their possible role in modulating maternal aggression. Our data suggest that OT, but not AVP, in the AC/ADP, a nucleus contained in a key region for maternal behaviour, could be related to the aggressive responses during peripartum period, but more data is needed to confirm this relationship.

5.1 EXPOSURE TO PUPS ELICITS PUP-DIRECTED MATERNAL BEHAVIOUR IN VIRGIN FEMALE MICE

We have characterized and validated two protocols of maternal sensitization in virgin female laboratory mice of the outbred strain CD-1. Thus, we adapted the classical protocol, in which virgin females were exposed daily for 2 h to pups. In addition, we designed a protocol in which virgin females, which we named godmothers, share pup care with dams from parturition. This latter protocol presents some advantages. First, it is an easy protocol that minimizes handling of the animals –and therefore, stress. Second, continuous exposure to pups in the home cage of the godmothers induces the quick and full expression of pup-directed behaviours already evident at postpartum day 2.

In both models, we have studied the expression of the two main components of maternal behaviour, namely pup-directed (Numan and Insel, 2003; Rosenblatt, 1967) and non-pup-directed behaviours i.e., maternal aggression (Numan and Insel, 2003). We

extend previous findings in other mice strains, showing that contact with foster pups is able to almost spontaneously induce the former but not the latter kind of maternal behaviour in virgin female mice of the CD1 strain.

We compared pup-oriented behaviours induced by the two different protocols of exposure to pups in non-lactating females with the full maternal behaviour expressed by lactating dams with their own pups. Both protocols of maternal sensitization successfully induced pup-directed behaviours with a different time course, so that godmothers were indistinguishable from dams in most behavioural aspects from the first testing day (on PPD 2), whereas pup-sensitized females took at least two sensitization sessions to express similar levels of maternal-like behaviour. This result is consistent with a previous study in another strain of mice (Stolzenberg and Rissman, 2011). This study indicates that, whereas pup-inexperienced females need only 2 days of exposure to pups for expressing pup retrieval behaviour in the home cage, this period is not enough to be responsive to pups in a novel place, such as a T-maze. For this reason, the choice of the test environment is crucial to observe maternal responsiveness to pups in nulliparous female mice.

Godmothers, exposed to pups from the moment of parturition, were as quick as dams in the pup-retrieval test. By contrast, pup-sensitized females were slower than dams during both the first day of testing—when they had contact with pups for the first time—and the second. Across tests, pup-sensitized females got experience with pups and they learned to retrieve them faster. Thus, during the third

test, after 2 days of 2 h exposure to pups, pup-sensitized females showed the same speed than dams in pup retrieval.

This quick induction of maternal care observed in adult virgin mice contrasts with the situation in virgin adult rats, which need a full week of sensitization to pups to express maternal-like behaviour (Fleming and Luebke, 1981). In fact, virgin adult female rats express neophobic avoidance responses when they are presented for the first time with pups. This avoidance lasts for at least 2–3 days, and after 7 days of exposure, virgin female rats express maternal care. Thus, it is possible that the interspecies difference is due to a reduced neophobic response in mice to pups as compared to rats. Actually, we did not find differences in the latency to approach and sniff the pups between groups. This indicates that virgin females with no previous experience with pups show no aversion for them, as they approach pups with a similar latency than dams. In addition, stimuli inducing fear or anxiety usually elicit risk assessment behaviour in rodents. Our findings reveal an extremely low level of risk assessment episodes toward pups, which are similar in dams and both groups of virgin females. This indicates that, in contrast to rats, adult CD1 female mice do not display avoidance responses or fear/anxiety toward pups.

Regarding pup licking/grooming, pup-sensitized females overexpress this behaviour as compared to dams and godmothers. We hypothesize that these high levels of licking/grooming might correlate with an increased investigative behaviour induced by novelty (Rinaldi *et al*., 2010). Thus, dams and godmothers recognize their young or familiar pups, respectively and, after retrieving them in the nest, express low levels of pup licking/grooming. By contrast, virgin

females in the process of sensitization show a higher level of licking/grooming, likely because pups are novel stimuli for them. This hypothesis is in agreement with a previous study by Stolzenberg and Rissman (2011), in which they observed that experienced females were faster in pup retrieval but expressed lower frequency of licking/grooming than females that had never had previous contact with pups. In addition, Alsina-Llanes *et al*., (2015) also showed that more experienced female mice spend less time both licking pups and in the nest during the pup-retrieval tests. This further supports that pups are neither aversive, nor fear eliciting but, on the contrary, highly attractive stimuli for previously pup-inexperienced virgin females.

The results on pup licking/grooming contrast with those on crouching. Godmothers showed higher frequency of crouching than dams. The relatively short time that dams spent crouching over their pups is somewhat surprising. We speculate that dams might actually spend more time in the nest while nursing undisturbed, but under the experimental conditions in a room different from the homeroom, and in the absence of any threat, they might be more willing to leave the nest and explore the surroundings—this is the most common behaviour shown by the dams. Indeed, in agreement with this hypothesis, it has been proposed that anxiety levels are decreased and exploratory activity increased in lactating females (Bridges, 2015). Godmothers, on the contrary, might be more anxious than dams when left alone with pups, so they are highly motivated to retrieve pups and stay with them in the nest.

In summary, we have shown that godmothers are as fast as dams in the pup retrieval test from the first test day and that they

overexpress crouching, suggesting that these virgin accompanying females care for foster pups as much as lactating females do. These results are in agreement with a previous study about communal nesting in laboratory mice by Gandelman *et al*., (1970), who observed that virgin females accompanying the dams cared for the young even more than lactating females did in about half of the observations, noting that accompanying females could even act as "midwives"—helping dams during delivery and eating the placenta.

Although we have observed that both lactating and non-lactating females care for the youngsters, previous results suggest that the motivation towards them might be higher in dams. Thus, Hauser and Gandelman, (1985) performed an operant task in which they presented a pup as a reinforcer for lever-pressing. They observed that lactating female mice pressed a lever at much higher rate than virgin females did, suggesting that pups are more effective reinforcers for lactating females than for virgins. The appetitive process underlying maternal behaviour has been also evaluated throughout postpartum period (Mattson *et al*., 2001). Dams were tested for place preference to evaluate the rewarding properties of pups. Pup exposure was provided in one chamber and injection of cocaine in the other one. Lactating female rats only showed preference for pup stimuli during early lactation, whereas cocaine showed stronger reinforcing value than pups for dams in the middle-late postpartum period (Mattson *et al*., 2001).

In order to investigate whether dams in our study were more motivated to retrieve pups, we measured the time difference between sniffing and retrieving the first pup. Dams readily retrieved pups as

soon as they sniffed them, so that the difference between both behaviours tended to 0 in all the tests. However, in both godmothers and pup-sensitized females there was a variable time lapse between the moment of first sniff and retrieval in the first test day. This result supports that the motivation towards pups is higher in lactating than in non-lactating female mice. Thus, hormonal events during pregnancy and lactation might promote changes in motivational responses in lactating females. If so, lactating female mice would differ from virgin females in their brain dopaminergic circuitry and/or in motivation modulatory systems, as we discuss below.

5.2 PUP-SENSITISATION FAILED TO INDUCE MATERNAL AGGRESSION

Contact with pups that induced full pup-directed maternal behaviour was not enough to promote maternal aggression, a separate component of the maternal behavioural repertoire. Indeed, in our experiments, neither constant contact with pups for 5 full days (godmothers) nor 2h daily exposure to pups, were able to trigger aggressive behaviour in virgin female mice. The lack of aggression in godmothers is especially relevant.

Maternal aggression only occurs near the nest, as dams do not attack unknown adult males if encounters occur in an environment other than the dam's home cage (Experiment 3.2.7). This is an interesting finding of the present study, demonstrating that maternal aggression is a territorial behaviour, as also occurs in inter-male aggression (Miczek and O'Donnell, 1978; Miczek *et al*., 2001). In this doctoral thesis, all dams included in maternal aggression tests in their

home cages attacked intact male intruders. By contrast, in a neutral arena only 10% of dams (1/10) attacked the male intruder. Since the aggressive female spent less than 1 second attacking the intruder, we conclude that, in general, maternal aggression is displayed specifically in the dam's territory, next to her nest.

The lack of aggression of pup-sensitized females in Experiment 3.1.4 could be attributed to the lack of a stable nest to defend in their home cages. By contrast, the godmothers had a nest to defend but, even so, they did not express maternal-like fighting. This demonstrates that prolonged (5 days), intimate contact with pups is not able, *per se*, to elicit maternal aggression in virgin female mice, and is consistent with other study performed in rats (Erskine *et al*., 1980b). Therefore, in contrast to maternal care, maternal aggression seems to require physiological changes occurring only in the dams, likely related to endocrine agents acting during pregnancy, parturition and/or lactation.

This finding contrasts, however, with the results by McDermott and Gandelman, (1979) who showed that some virgin female mice displayed aggression after 9 days of continuous exposure to 1 day-old pups that were renewed daily. However, the protocol reported by these authors (1-day old pup exposition during 9 days) does not reflect a naturalistic scenario. In other words, it does not seem likely that a female wild mouse will find every day new one day-old pups during 9 consecutive days to become aggressive.

Anyhow, there is conflicting evidence as to what are the factors promoting the onset of maternal aggression. Early studies suggested

that sensory cues from the pups were the key factor triggering maternal aggression in mice. In particular suckling-induced nipple stimulation—but not lactation—would trigger and maintain maternal aggression in mouse dams (Svare *et al*., 1980). This was supported by two main lines of evidence. On the one hand, parturient females that were thelectomyzed prepartum or immediately postpartum were not aggressive (Svare and Gandelman, 1976b). On the other hand, Svare and Gandelman, (1976a) ovariectomized virgin females and treated them with oestradiol benzoate and progesterone for 19 days to induce nipple growth. Then they fostered them with pups that were observed to attach themselves to the nipples. These so treated virgin females exhibited aggression, but not milk production. This finding suggests a causal role of nipple stimulation, instead of endocrine factors, in maternal aggression onset and maintenance. The lack of aggression in our godmothers suggests, on the contrary, that endocrine agents rather than pup-derived stimulation are necessary to induce nest defence.

A key role of endocrine agents in maternal aggression is further supported by the expression of maternal aggression before parturition, in late-pregnant mice (Mann *et al*., 1984, who called it pregnancy-induced aggression) and rats (Caughey *et al*., 2011), in which no nipple stimulation by pups has occurred yet. Maybe it is the hormonal stimuli leading to nipple growth, together with the presence of a nest to defend, rather than nipple stimulation *per se*, what causes maternal attacks. This would fit both our results and those from other studies. Thus, in the experiments by Svare and Gandelman, (1976a), the hormonal treatment leading to nipple growth rather than

subsequent pup suckling might have induced aggression. Alternatively, it is possible that both factors act in a synergic way to promote and maintain aggression. In the same vein, McDermott and Gandelman (1979) reported that those virgin females exposed to 1-day-old pups for 9 days that displayed maternal aggression showed enlarged and more numerous nipples than their counterparts that, in the same conditions, were not aggressive. This indicates that, by means of unknown mechanisms, continuous exposure to pups for 9 days might have caused hormonal changes in some of the females leading to both nipple growth and aggressiveness. Apparently these hormonal changes did not occur in our godmothers even if they had spent 5 days caring for pups, likely because of the presence in the same cage of the dam nursing the pups for most of the time.

Finally, sensory stimulation might be important for maintaining rather than for triggering maternal aggression. In rats, dams need contact with pups to express high levels of aggression. When lactating rats are separated from their litter more than 4h, they show a decrease in maternal attacks (Stern and Kolunie, 1993). These authors suggested that maternal aggression in rats does not depend on suckling but on somatosensory stimulation by pups, since they showed that maternal aggression is lost upon anaesthesia of the ventral surface of the dams. Since the display of aggressive behaviour becomes independent of circulating hormones after about day 5 of lactation (Erskine *et al*., 1980a), it is likely that endocrine factors promote an enduring modification of neural pathways controlling aggression during late pregnancy and first postpartum days. Thus,

endocrine factors might be responsible for the onset of the aggressive state, whereas contact with pups is necessary to maintain it.

Regarding mice, maternal aggression has been proposed to be dependent primarily on pup contact, specifically by the suckling of pups (Garland and Svare, 1988). Hypophysectomy (Erskine *et al*., 1980a) or treatment with ergot drugs (Mann *et al*., 1980), suppressing hypophyseal prolactin (PRL) release, do not impair maternal aggression when applied during postpartum period. Moreover, plasmatic PRL levels during lactation do not correlate with the intensity of the aggressive outputs (Broida *et al*., 1981).

Garland and Svare, (1988) proposed that, relative to the presence of maternal aggression, there are three phases in the postpartum period: 1) the first one would be characterised by suckling stimulation as a necessary event to promote postpartum aggression, (although it is in disagreement with prepartum aggression observed by Mann *et al*., 1984); 2) a midlactational phase in which suckling stimulation is not necessary to maintain the aggressiveness, and other kind of pup-derived stimuli might play an important role in the expression of dam's behaviour; and 3) late postpartum period during second week of lactation in which aggression declines in parallel with the absence of suckling. During lactation, maternal aggression could be maintained by exteroceptive pup-derived stimuli. In fact, dams separated from pups by a wire mesh screen still show high maternal attacks (Bean and Wysocki, 1989; Svare and Gandelman, 1973), and these conditions are able to reinstate aggressive behaviour after a 5-h separation period.

In conclusion, at least in mice, maternal aggression and pup-directed behaviours seem to have different underlying neural and endocrine mechanisms (Figure 34). Our results point to the existence of some physiological events during pregnancy, delivery and lactation that elicit increased motivation for pups and stimulate aggressive behaviour. Likely, these events might induce central changes in neural pathways controlling these behavioural components. As we will discuss now, data on the nature of maternal aggression-promoting pheromones give support to this view.

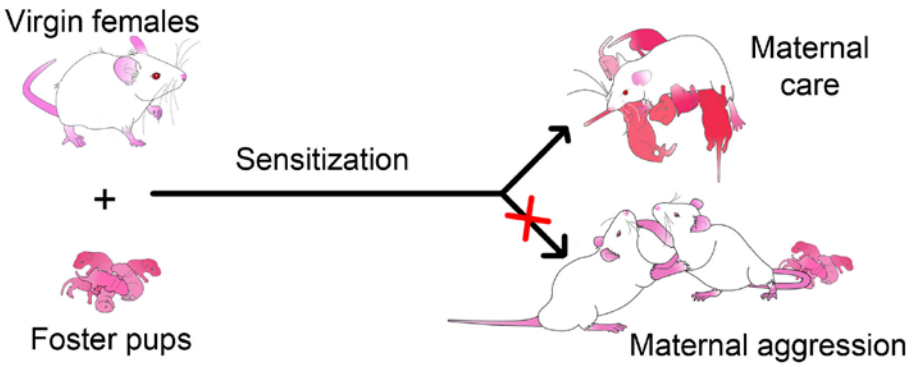

Figure 34. Sketch of maternal behaviour sensitization by pup-stimuli in virgin female mice.

Virgin female mice exposed to foster pups show maternal care after a short period of maternal sensitization but not maternal aggression. This fact shows the hormonal and non-hormonal factors that promote maternal behavioural repertoire in CD-1 virgin females.

5.3 FROM ATTRACTION TO AGGRESSION: LACTATION CHANGES THE EMOTIONAL VALUE OF SEXUAL PHEROMONES

5.3.1 Darcin is the attractive male sexual pheromone in mice

Urine is one of the most important sources of chemosignals in mice, and urine marking and countermarking is a conspicuous behaviour exhibited of by male mice (see Kaur *et al*., (2014)). To date, an attractive non-volatile pheromone has been identified in the urine of male mice, which has been named darcin (Roberts *et al*., 2010). As we have mentioned before, darcin is a member of the major urinary protein family, MUP20 (MGI:3651981) which binds with high affinity small lipophilic molecules also present in male (but not female) urine in a testosterone dependent way (e.g. thiazoline and brevicomin, Robertson *et al*., 1993) . Although some pheromonal activities have been proposed for thiazoline, including attraction (together with brevicomin, but not alone), oestrous synchronisation and puberty acceleration *(*Jemiolo *et al*., 1989, 1985; Novotny *et al*., 1999), it is not needed for intersexual attraction since r-darcin obtained in *E. coli*, free of ligands, is attractive as compared to buffer or to full female urine. In fact, another MUP isoform that also binds thiazoline (MUP 18694Da) shows no attractive properties (Roberts *et al*., 2010), thus demonstrating that darcin has actual pheromonal properties, rather than being a simple odorant or pheromone reservoir.

The attraction that female mice display for male sexual pheromones reflects the reinforcing properties of these pheromones

for females. This was first shown by Martínez-Ricós *et al*., (2007), who tested the ability of bedding soiled by gonadally intact males, females or castrated males to induce place preference. To do so, adult, chemically naïve females were run in successive preference tests (one test per day for four consecutive days) of soiled *vs* clean bedding, with the soiled bedding presented in the same location in the cage in every test. On the next day, a clean *vs* clean bedding preference test revealed that only females which were exposed to bedding soiled by intact males, but not those which were exposed to female or castrated male cues, had developed a conditioned place preference for the location of the cage where soiled bedding had been presented. In addition, if a platform separated the females from the bedding, leaving access only to the volatile odorants emanating from the bedding, neither a preference during the four test days nor a place preference was observed. Therefore, some non-volatile chemosignals contained in the bedding soiled by males, but not bedding soiled by castrated males or females, are both attractive and rewarding/reinforcing -able to generate place preference- for females.

More recently, Roberts *et al*., (2012) replicated and extended these findings by using the urinary pheromone darcin. They demonstrated that darcin is the key component underlying the rewarding properties of male urine for females. In fact, r-darcin alone induces place preference. Interestingly, darcin not only induces attraction and spatial learning in females but also in males that, nevertheless, are less attracted to their own than to a competitor's urine. In addition, darcin promotes intermale aggression (Kaur *et al*., 2014).

On the other hand, urinary volatiles that had become secondary attractors by means of their association with darcin - volatiles were found not to be not primarily attractive- were not able to induce place preference. All these findings strongly suggest that darcin is the main attractive sexual pheromone of male mice, due to its rewarding properties for females, which mediate an interesting type of spatial learning especially adaptive for mate search (see discussion in Roberts *et al*., (2012)).

5.3.2 Pheromones and behaviour: darcin as the maternal aggression-promoting pheromone

Female laboratory mice constitute an interesting model to analyse how chemosignals are processed for the control of behaviour. In general, laboratory female mice do not attack intruders to defend their territory or to get access to possible mates, but they are usually engaged in affiliative interactions with conspecifics (although inter-female aggression is present in some wild-stock mice, Stockley *et al.*, 2013). However, as we have described and fully discussed, during perpartum period, dams not only show pup-directed maternal behaviour, but also attack conspecifics approaching the nest, a behaviour that is only expressed near the nest (pup defence or maternal aggression).

Analysis of the changes that occur in female social behaviour after parturition and their physiological substrates constitutes an interesting issue that could prove very helpful for understanding the neuroendocrinology of social interactions. A first question in this respect is whether pheromone sensing is equally involved in female-

to-male reactions throughout the life of the female. In this respect, two alternative (but not necessarily exclusive) possibilities exist.

On the one hand, changes in female physiology (e.g. endocrine state) and/or behavioural stimulation (e.g. interaction with pups) occurring through pregnancy, parturition and lactation might alter the pattern of receptor expression in the vomeronasal and/or olfactory epithelia (e.g. Alekseyenko *et al.*, 2006), or the responsiveness of olfactory and/or vomeronasal sensory neurons to their ligands (Dey *et al.*, 2015). A similar phenomenon has been suggested for the vomeronasal system of males in relation to their paternal *vs.* infanticide behaviour (Tachikawa *et al*., 2013). As a consequence, lactating dams might be able to detect new chemosignals derived from conspecifics or, alternatively, they might fail to detect chemosignals that they detected before. This would result in a change in social behaviour. On the other hand, females might detect the same male pheromones throughout their lives, but changes in the central nervous system during pregnancy, parturition and lactation would alter the response to these chemosignals thus modifying the social behaviour of females.

The results of Study 2 lead to several main conclusions with respect to this question. First of all, only dams attack intruders and, more importantly, godmothers and pup-naïve virgin females show identical, non-aggressive behavioural response towards intact male intruders (0.76±0.36s, 0.26±0.14 s attack time, godmothers and pup-naïve, respectively). This fact suggests that only lactating females respond in an aggressive manner toward male conspecifics when they were placed in the female's home cage.

Additionally, not only dams attacked more vigorously but also they were much quicker than non-lactating females to initiate the attacks towards intact males (see Results). However, when dams were confronted to castrated males, they did not differ from non-lactating females in terms of total attack duration and attack latency. These results indicate that testosterone-dependent chemosignals emitted by males that change the dams' behaviour, as occurs in intermale aggression (Kaur *et al*., 2014; Stowers *et al*., 2002). Thus, since in this type of tests no training period is necessary, the aggressive behaviour observed might rely exclusively on innate and stereotyped behaviours initiated by pheromones.

Our results have two major additional implications. On the one hand, we have demonstrated that darcin is a robust and extrinsic aggression-promoter chemosignal for lactating females. In fact, when we swabbed castrated male intruders with r-darcin, this induces the same level of maternal aggression and with the same latency that full urine of an intact male does in dams (see Figure 24). Importantly, this maternal aggression promotion occurs even if darcin is odourless or, in the conditions of our test, it could be binding some castrated male's odorants. In other words, lactating females might recognize the same maleness in the both full urine and r-darcin diluted in saline solution. For this reason, darcin contained in both male urine and saline buffer promotes aggressiveness in lactating females.

Therefore, even if there are additional secreted or excreted substances that have been proposed to be sexual pheromones, both volatiles (α- and β-farnesenes, dehydro-exo-brevicomin (brevicomin) and 2-sec-butyl-4,5-dihydrothiazole, e.g. see Jemiolo *et al*., 1985) and

non-volatile compounds (e.g. Exocrine Secreted Peptide 1 or ESP-1; Kimoto *et al*., 2005; Haga *et al*., 2010), darcin is a urinary protein acting as promoter of both attraction to males (Roberts *et al*., 2010) intermale aggression (Kaur *et al*., 2014) and maternal aggression (our results). Being a large protein, of nearly 19 kDa molecular weight, darcin is probably detected by V2R, located in the basal part of VNO (Chamero *et al*., 2012). It is feasible that the volatile components of the urine might act promoting the approach to conspecifics, so the odourless darcin becomes available after close contact.

If the very same molecule (darcin) induces different behavioural responses in virgin (attraction) and lactating females (aggression), the change in behavioural response towards males that takes place around parturition does not involve a modification of the vomeronasal organ response to chemosignals (e.g. changes in expression of V2R receptors). On the contrary, the most parsimonious explanation for this change is that the same receptor detects darcin throughout female's life, but the behavioural response to this stimulus changes. In this case, we hypothesize that postpartum circulating hormones might be regulating the response to darcin, by changing the emotional value of this pheromone from attraction to aggression-promoting stimulus. This probably occurs through modifications of the socio-sexual brain, which could be induced by a mixture of endocrine agents and sensory inputs -chemosignals and, possibly, nipple stimulation- acting around the time of parturition.

We started exploring this idea by means of an analysis of the possible changes occurring in nonapeptidergic cells in two brain nuclei involved in maternal behaviour. Since OT and AVP have been

implicated in both social behaviour and chemosignal processing, these nonapeptides are likely candidates to be modulated by the factors operating during pregnancy and the peripartum period (as discussed in Section 5.4).

5.3.3 Variability in attraction and aggression: possible roles of stress and illness

Our experiments confirm previous findings in CD1 mice (Rosenson and Asheroff, 1975) indicating that lactating dams attacked intact males much more than castrated ones. Since castrated male intruders do not display aggressive behaviour and evoke minimal aggression by the resident both in intermale (Chamero *et al*., 2007; Stowers *et al*., 2002) and maternal aggression tests (our findings), any attack to them by the resident when they are sprayed with chemosignals (urine, darcin or other specific putative pheromones or pheromone mixtures) can be unambiguously attributed to the tested chemosignals.

Although the results of our experiments are largely consistent in that point, dams' aggression towards castrated males apparently differs between experiments 3.1.4 and 3.2.6. Attacks occurred earlier and had longer durations in Experiment 3.1.4 than in Experiment 3.2.6. We have not analysed this difference, because experiments were not run in parallel and, even if the conditions were similar, many variables may affect aggressiveness. For instance, running a pup-retrieval test just before the maternal aggression test results in a high rate of attacks to both intact and castrated male intruders (unpublished results). Probably, stress due to maternal separation from pups could have

enhanced aggressiveness in dams. This is an interesting issue that requires further investigations.

On the other hand, other responses to pheromones are also influenced by external factors. Thus, darcin is exclusively present in the intact male urine but not in castrated male or female urine (Figure 23). Although attractiveness of male urine for virgin females is attributed to darcin (Roberts *et al*., 2010; 2012), this pheromone is apparently present in urine from males parasitized by the intestinal nematode *Aspiculuris tetraptera,* even if their urine has lost its attractiveness for females (Lanuza *et al*., (2014). These findings suggest that the attractiveness of darcin can be overcome by aversion to illness chemosignals secreted into the urine of infected animals. How both chemosignals interact in the brain circuitry involved in sociosexual behaviours is the subject of incipient research in different rodents (Arakawa *et al*., 2010; Boillat *et al*., 2015; Gil-Solsona *et al*., 2016).

5.4 THE NUMBER OF NONAPEPTIDERGIC CELLS IS NOT AFFECTED BY MATERNITY OR PUP-SENSITIZATION

After the characterization of maternal care and aggression in CD-1 female mice, we sought to investigate possible adaptations in the maternal brain that could explain the increased motivation for pup stimuli and the expression of nest defence in dams. Specifically, we hypothesised that modifications of the nonapeptidergic systems in the brain of dams could account as intrinsic factors regulating maternal aggression.

We first sought to quantify the number of cells expressing OT, AVP or both nonapeptides in the AC/ADP and Pa, two nuclei that might be involved in maternal behaviour and display variable proportions of both nonapeptides (Otero-García *et al*, 2015). For example, pharmacological blockade of the receptors for AVP and OT in the MPOA –a region that includes the AC/ADP- impair the onset of maternal care in the rat (Pedersen *et al*., 1994).

Our results using both immunoperoxidase and immunofluorescence were coincident in revealing no significant differences in the number of OTergic or AVPergic cells (immunofluorescence only) between lactating and non-lactating females, in either Pa or AC/ADP (Figure 28). Thus, the number of nonapeptidergic cells in dams was not significantly different from that in godmothers, and the number of OT-ir cells was not different between dams, godmothers or pup-naïve virgin female mice.

Although we did not find differences between the number of OT-ir cells in the AC/ADP in lactating vs non-lactating females, some data suggest that the functionality of those cells might be modified in dams. First, Tsuneoka *et al*., (2013) showed that OTergic (neurophysin I-expressing) cells in the AC/ADP are strongly activated, as measured by c-fos expression, in parturient females (processed 2h after delivery). This strongly suggests that OT cells in the preoptic hypothalamus and surrounding areas have an intense activity during delivery, maybe in association with hypophyseal OT release during parturition.

On the other hand, OT cells of the AC/ADP were not activated by pup stimuli in virgin females, but showed a specific increase in c-fos expression in postpartum females that were briefly exposed to foster pups 7 days after delivery. This suggests two possible changes induced in the brain of female mice as a consequence of pregnancy, parturition and lactation. One possibility is that pup-sensitive cells that did not express OT at histochemically detectable levels in the brain of virgin mice start expressing OT after delivery. Alternatively, pup-insensitive, OT-expressing cells in the brain of virgin females might become sensitive to pup stimuli after delivery. Our results showing that the number of OT-ir cells is not increased in dams support the second possibility.

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

In the light of a previous study that suggested an increase in co-localization between OT and AVP mRNA levels in the SO of rats (Mezey and Kiss, 1991), we hypothesised that, even if the total number of nonapeptidergic cells did not vary during lactation, the hormonal background of dams could still affect the percentage of co-localization of OT and AVP-ir in AC/ADP and Pa. The immunostaining of the rostral Pa and AC/ADP showed high OT-ir plus weak AVP-ir, a pattern which

fits the results reported by Otero-García *et al*., (2015), who suggested the AC/ADP population of nonapeptidergic cells as a distinct group in the mouse brain. Even so, we did not observe differences in the number or percentage of AVP+OT-labelled cells between dams and godmothers in this region (Figure 30), or in the Pa, which mostly contains neurosecretory cells (Figure 31). Thus, our results are in apparent disagreement with those by Mezey and Kiss (1991) in the SO in rats. It should be noted, though, that in the study by Mezey and Kiss no statistical analysis was conducted due to the small sample used (virgin females, n=2; lactating females, PPD2, n=3; PPD5, n=2; PDD9, n=2). Thus, they merely reported an increase in the percentage of cells co-localizing mRNAs coding for OT and AVP as measured by in situ hybridization. Data showed that this increase was 7-fold in PPD2 dams with respect to virgin controls, 5-fold in PPD5 dams and 3.5-fold in PPD9 dams. In fact, our results in PPD5 dams show an apparent average 2-fold increase in the percentage of doubly-stained cells in the two most rostral levels of Pa (Table 6) but this apparent increase does not resist a statistical test. Moreover, the fact that Mezey and Kiss analysed mRNA and we analysed the protein, and the fact that we analysed different nuclei could account for the different outcome of both studies.

Despite our findings, it is known that both OT and AVP might modulate maternal behaviour and anxiety during the peripartum period in rats and mice (Bosch and Neumann, 2012; Bosch, 2011; Lonstein, 2007; also see the review by Bosch, 2013). Perhaps there are differences in the nonapeptidergic system at the level of neurotransmitter release (which should be assessed by means of

microdialysis experiments), or mRNA transcription of the nonapeptides or their receptors (that can be checked with real-time quantitative PCR). In fact, in the lactating rat, the expression of mRNA for OT receptors is increased during lactation in the central amygdala, and so it is the OT release from Pa (Bosch *et al*., 2005). Thus, lactation might induce plastic responses in the brain that do not alter the number of nonapeptidergic cells but rather their physiology (Stern *et al*., 2000). These plastic changes might remain to anticipate future lactation periods, since the first episode of parturition improves the expression of maternal behaviours in following exposures to pups.

Maternal experience also enhances the OT gene expression in virgin female mice (Stolzenberg *et al*., 2012). Thus, in godmothers, pup-experience could be modulating gene expression, independently on hormone influence as it has been previously demonstrated (Akbari *et al*., 2013; Kuroda *et al*., 2007; Stolzenberg *et al*., 2012). Anyhow, our results in virgin pup-inexperienced females showed that, at least in the number of OT-ir cells, there were no differences between dams, godmothers and inexperienced virgins. In future experiments, we should incorporate a pup-inexperienced group of females to test whether exposure to pups might modulate the number of AVP-ir and OT+AVP-ir cells.

In conclusion, neither pregnancy nor pup-sensitization seem to affect the number of nonapeptidergic cells in the AC/ADP or Pa. Future experiments are required to investigate whether there might be changes in the abundance of OT and AVP fibres and receptors in other brain regions during the postpartum period, in nuclei of interest such as the nucleus accumbens and central amygdala (Otero-García *et al*.,

2015), which might be implicated in motivation towards pups and maternal aggression, respectively.

An additional possibility is that the co-expression of AVP and OT in our nuclei of interest is sexually dimorphic. Otero-García *et al*., (2015) published a detailed description of the co-labelling of both nonapeptides in males (see the summary in Table 7). In comparison to our results, males do not seem to differ in the relative number of nonapeptidergic cells that only express AVP in AC/ADP (1.6% in males vs. 0.92% in godmothers) or the proportion of cells that co-express both nonapeptides (40.3% in males *vs*. 54.34% in females). Conversely, in the Pa, the percentage of nonapeptidergic somata that express only AVP could be sexually dimorphic in favour of females, which show twice as much AVP-ir cells as males, whereas the co-labelling of OT and AVP seems to be more frequent in the Pa of males, which show between three and four times more double-labelled cells than females do. These possible sexual differences in the expression of AVP in favour of females contrast with the classical increase in expression of AVP in in the bed nucleus of the stria terminalis of males. In fact, Otero-Garcia *et al*., (2014) described increased AVP-ir somata and fibres in males in most of the AVP-ir nuclei except in the AC/ADP, where the number of cells did not differ bewteen males and females. In light of our results, the possible differences in the co-expression of both AVP and OT in the Pa should be reassessed.

	Godmothers		
	% Nonapeptide-ir cells expressing only OT	% Nonapeptide-ir cells expressing only AVP	% Nonapeptide-ir cells expressing OT + AVP
AC/ADP	44.74	0.92	54.34
Pa (-0.70mm)	52.92	41.82	5.26
Pa (-0.94mm)	39.73	52.43	7.83
	Males*		
	% Nonapeptide-ir cells expressing only OT	% Nonapeptide-ir cells expressing only AVP	% Nonapeptide-ir cells expressing OT + AVP
AC/ADP	58.1	1.6	40.3
Pa (-0.70mm)	59.2	16.5	24.3
Pa (-0.94mm)	51	28.6	20.4

Table 7.Comparison of the distribution of nonapeptidergic cell bodies immunoreactive for OT, AVP or both in the AC/ADP and Pa in virgin males and females (godmothers).

*Data from males are taken, with permission, from Otero-García *et al*., 2015.

5.5 THE NUMBER OF OXYTOCINERGIC CELLS IN AC/ADP CORRELATES WITH MATERNAL AGGRESSION

Our results suggest that OT-ir cells in the AC/ADP might play a role in maternal aggression. We have observed that the number of OT-ir cells in the AC/ADP, as measured by means of DAB immunostaining, significantly and positively correlates with higher rates of maternal aggression exclusively in dams (Figure 29). In our fluorescent material, the linear regression analysis revealed the same trend, albeit this analysis did not reach statistical significance (Figure 33). We would like to note that in the immunofluorescence material we had lower number of animals (n=6) than in the DAB material (n=9), so

it is possible that we could replicate the result in DAB and to reach significance by increasing sample size.

By contrast, we did not find any correlation or trend between OT in the Pa and aggressive behaviour, or between AVP-ir and double-stained cells in AC/ADP or Pa and aggressive behaviour. Moreover, we did not find any correlation in non-lactating females, which showed virtually null level of aggressiveness toward male intruders. Thus, our results suggest that OT in the AC/ADP, but not AVP, might be related to maternal aggression.

Previous studies have investigated the role of OT and AVP in maternal aggression, but this question is still poorly understood. Bosch *et al*., (2005) observed that OT release during exposure to an intruder positively correlated with the amount of aggressive behaviour that was displayed by dams. In that study, the authors analysed local release of OT in Pa and central amygdala, so that their data probably reflect the activity of OTergic efferent fibres from the Pa innervating the Ce. In this sense, recent studies in rats using viral tracers under the control of the OT promoter have demonstrated that neurosecretory cells (e.g. projecting to the posterior pituitary) in the Pa and anterior nucleus send collaterals to the Ce and NAcc (Knobloch *et al*., 2012). By contrast, the number of OT cells in Pa did not show any correlation with maternal aggression in our mice. It is likely that the activity of OT rather than the number of cells per se is regulated by maternity, but future studies in mice should address this possibility.

Within the MPOA-vBST interface, which anatomically includes the AC/ADP, there are conflicting data as to the modulation of

nonapeptidergic receptors by lactation. The expression of receptors for oxytocin and vasopressin (AVP1aR) has been shown to increase coinciding with the highest levels of maternal aggression (Caughey *et al*., 2011). However, recent studies have reported that the expression of mRNA of AVP1aR and AVP1bR do not differ between lactating dams and virgin rats in MPOA, medial posterior part of BNST and Pa (Bayerl *et al*., 2016, 2015). On the one hand, blockade of AVP1bR in MPOA and medial posterior part of BST increase pup retrieval and decrease licking/grooming behaviour during maternal aggression test, respectively (Bayerl *et al*., 2015). Moreover, blocking of V1bR in both regions reduced arched back posture, but it did not affect maternal aggression (Bayerl *et al*., 2015). Since both OT and AVP are able to bind AVP receptors (see Introduction), future studies are needed to clarify the role of both nonapeptides in maternal aggression.

Nonapeptides might also indirectly affect maternal aggression by means of the regulation of anxiety levels. Thus, dams display a reduced anxiety phenotype that allows them to defend their pups, especially in the presence of an infanticide intruder. In fact, higher levels of maternal aggression are correlated with lower anxiety in mice (Maestripieri and D'Amato, 1991) and higher exploratory activity (Bridges, 2015 and our own observations). Nonapeptides could act synergistically with norepinephrine reducing anxiety in dams of both species mice and rats (Smith *et al*., 2013, 2012). Recently, Scotti and collaborators (2011) have shown that anxiety-related behaviours and maternal aggression are dissociated in the lateral septum. They reported that injections of agonists of norepinephrine in this nucleus alter maternal aggression but not anxiety in lactating mice. Thus,

further studies are needed to clarify the relationship between anxiety and maternal aggression in mice.

5.6 OTHER HORMONES AND NEUROTRANSMITTERS INVOLVED IN MATERNAL AGGRESSION

Other hormones and neuropeptides, acting in concert with nonapeptides, are also involved in the regulation of the expression of this type of behaviours during pregnancy and postpartum period. One of the classical neurotransmitters that has been involved in aggression is serotonin. Serotonin is strongly linked to intermale aggression, with low levels of serotonin related to high levels of aggression (Nelson and Chiavegatto, 2001). Recent studies have related brain serotonin also to maternal aggression (Angoa-Pérez *et al*., 2014; Heiming *et al.*, 2013). Thus, Angoa-Pérez *et al*., (2014) showed that deficiencies in brain serotonin abolish both maternal care and maternal aggression. They used a null mutation for the gene tryptophan hydroxylase-2 (TPH2) that synthesizes neural serotonin. In spite of their abnormal maternal care, these $TPH2^{-/-}$ dams apparently nursed their pups, as milk bands were present in the body of new-borns suggesting normal lactation (Angoa-Pérez *et al*., 2014). The authors discuss that the maternal neglect observed in $TPH2^{-/-}$ dams could be due to alterations of circuitry modulating maternal motivation and reward. The lack of serotonin in those mutant mice or other serotonin-related signals in the brain maternal regions could responsible of the alterations of maternal behaviours, as Lerch-Haner *et al*., (2008) suggested in its mouse model of decreased brain serotonin.

Estrogenic signalling is also involved in maternal aggression. Thus, female mice deficient specifically for the estrogen receptor α (ERα), but not the estrogen receptor β (ERβ) gene (ERKO), not only showed dramatically reduced levels of maternal care, but also higher levels of maternal aggression against female intruders than wild type dams. ERKO females also showed aberrant behaviours such as infanticide behaviours during parental behaviours and male-like sexual behaviours e.g. mounts, when they are exposed to male intruders (Ogawa *et al*., 1998). Although these results must be taken with caution, some years later Lonstein *et al*., (2000) reported the importance of ERα in maternal behaviour responsive cells in MPOA. They reported that a wide proportion of the MPOA and vBST cells that express Fos during maternal behaviour also contain ERα.

Other evidences of the estrogen influence in maternal aggression were recently published. Aromatase is a key enzyme for the biosynthesis of oestrogens that catalyses the aromatization of androgens into oestrogens. Unger *et al*., (2015) demonstrated that females lacking the aromatase in medial antero-posterodorsal amygdalar neurons show maternal aggression impairment, but no impairment of other maternal behaviour components such as pup retrieval.

Finally, prolactin (PRL) might be involved in both maternal behaviour and aggression. Prolactin is involved in lactation, secretion of ovarian hormones, parturition (Nephew *et al*., 2007), embryo implantation and formation of corpora lutea (Grosdemouge *et al*., 2003), among other functions. The release of PRL is controlled (inhibited) by three hypothalamic dopaminergic subpopulations

(Freeman *et al*., 2000). This neuroendocrine neurones are the tuberoinfundibular dopaminergic neurones (TIDA), which are located in the arcuate nucleus and project to the median eminence, the tuberohypophyseal dopaminergic neurones, originate in the rostral arcuate nucleus and innervate the intermediate and neural lobes of the pituitary, and the periventricular hypophyseal dopaminergic neurones, which send their projections from the periventricular hypothalamic nucleus to the intermediate lobe of the pituitary. Oestradiol, mating and lactation supress the activity of TIDA neurons and therefore disinhibit (increase) the release of PRL. Recently, Ribeiro *et al*., (2015) reported that TIDA neurones express ERα in rats, and kisspeptin increases PRL secretion through inhibition of TIDA neurones in an Erα dependent manner.

In mice, there is conflicting evidence on the possible role of PRL in the development of maternal behaviour. PRL activates both long (PRLR-l) and short (PRLR-s) isoforms of the receptor generated by alternative splicing, differing in the structural features and docking specificity of their intracellular domain (Freeman *et al*., 2000). The PRLR-l is the most studied form of the PRL receptor. When PRL binds to the PRLR-l it activates, among others, the JAK/STAT signalling pathway. The proteins from the signal transducer and activator of transcription (STAT) family operate downstream the JAK/STAT pathway as signal transducers and transcription factors when activated through phosphorylation process (Freeman *et al*., 2000). The PRLR-l is exclusively associated to one member of STAT family in the brain, STAT5 (Brown *et al*., 2010). Previous data have shown that the expression of full maternal behaviours in mice depends on prolactin

(PRL), since general germ line null mutation of the PRLR gene impaired pup-induced maternal care (Lucas *et al*., 1998). By contrast, recent studies have demonstrated that the neuronal ablation of STAT5, a key component of PRL transduction signalling pathway, does not alter pup-evoked maternal behaviour (Buonfiglio *et al*., 2015). These surprising results suggest that maternal behaviour would not influenced by PRL via phosphorylated STAT5 (pSTAT5) –the active form of STAT5- pathway, but maybe PRL could act using an unknown PRLR-s pathway.

The study published by Buonfiglio and collaborators (2015) is in agreement with those results that have already described in this doctoral thesis and others (Alsina-Llanes *et al*., 2015; Stolzenberg and Rissman, 2011), suggesting that maternal care in mice is independent of endocrine factors, e.g. oestrogen (Stolzenberg and Rissman, 2011) and PRL (Buonfiglio *et al*., 2015). Noteworthy, Buonfiglio and collaborators (2015) focused only on proactive voluntary responses – latency to contact, latency to retrieve all pups and latency to group-related with motivation but no other components of maternal behaviours. Although motivational responses have been classically linked to dopaminergic system, a previous study have also referred the role of PRL in maternal responses to pup stimuli (Hashimoto *et al*., 2001). In this case, the PRL production is increased in rat dams in response to pup ultrasonic vocalizations (Hashimoto *et al*., 2001). However, our godmothers and pup-sensitized females displayed other maternal behaviours, such as crouching and licking/grooming in absence of hormonal-lactation influence.

Despite contradictory evidences describing the role of PRL in mediating pup-directed behaviours in mice, there are robust data that suggest an indirect but crucial role of PRL in maternal care. Shingo *et al*., (2003) reported that during pregnancy –even in pseudopregnancy conditions-, PRL could promote neurogenesis in the subventricular zone. The neural progenitors migrate to the olfactory bulb, generating new interneurons. This new input could be related with olfactory pup-discrimination. This process is critical for recognition of the offspring, avoiding maternal infanticide that it has been observed in some anosmic models (Fleming and Rosenblatt, 1974b; Lorenz, 1963; Sato *et al*., 2010, but see also Seegal and Denenberg (1974) on prevention of pup-killing in multiparous anosmic females). Unfortunately, these studies did not assess maternal aggression, so the influence of PRL in the aggressive component of maternal behaviour remains understudied (Buonfiglio *et al*., 2015; Lucas *et al*., 1998; Shingo *et al*., 2003).

Importantly, a recent study from our lab discovered a link between OT and PRL in the maternal brain. In this work, Saláis-López *et al*., (2016) characterized the OT-ir neurons that expressed the marker pSTAT5 and found that double-labelling was increased in the AC/ADP of pregnant and lactating mice, as compared to virgins. Thus, even if the number of OT-ir cells does not change (our results), OT-ir cells might indeed change their response under PRL influence during pregnancy and lactation. It is feasible that this is one of the mechanisms by which OT-ir neurons in the AC/ADP region change their responsiveness to pup stimuli, as was shown by Tsuneoka *et al*. (2013) and we discussed above. Thus, it will be interesting to check

whether OT-pSTAT5 neurons are involved in maternal aggression, maternal care or both.

5.7 NEUROBIOLOGY OF MATERNAL MOTIVATION

Our behavioural results in lactating and virgin females are in line with previous evidence about the existence of distinct neural pathways and mechanisms for the control of pup care and maternal aggression (reviewed by Gammie, 2005; Lonstein and Gammie, 2002; Numan and Woodside, 2010; Numan and Insel, 2003).

The circuits involved in both components of maternal behaviour share at least two important neural centres with differential roles, namely the lateral septum and medial preoptic area plus the ventral part of bed nucleus of stria terminalis (MPOA-vBST). Numan and Stolzenberg (2008) proposed that MPOA-vBST is the site where pregnancy hormones act to prime neural circuits that regulate proactive voluntary maternal responses in rats. Thus, the MPOA-vBST continuum seems to be the effector structure for maternal care. Lesions of the MPOA-vBST with fibre-sparing neurotoxic drugs dramatically reduce pup retrieval and nursing (Numan and Numan, 1996; Numan *et al*., 2005b), leading to severe pup weight loss. Noteworthy, MPOA-vBST also includes the AC/ADP region, which might be involved in maternal aggression and might show some degree of sexual dimorphism according to our data and those by Otero-García *et al*. (2015).

Although most studies examine the effects of MPOA lesions in maternal care, there are indirect evidences suggesting that this centre is also involved in maternal aggression. In fact, maternally driven

aggression increases neuronal activity in MPOA (Gammie and Nelson, 2001; Hasen and Gammie, 2005). Additionally, treatment with nitric oxide synthase inhibitor impaired both maternal care and aggression, and supressed Fos activity in the MPOA (Popeski and Woodside, 2004). The data suggest that MPOA-vBST is an effector for maternal care and a modulator of maternal aggression: thus, the MPOA-vBST could be the central node where both components of maternal behaviour are coordinated.

The MPOA efferent projections could affect maternal motivation by modulating mesolimbic dopamine system, oxytocin system and others in dams (Champagne, 2004; Hansen *et al*., 1993; Numan and Stolzenberg, 2009; Pedersen *et al*., 1994; Shahrokh *et al*., 2010). In fact, the MPOA, together with the ventral pallidum (VP) and nucleus accumbens (NAcc) receives dopaminergic afferents from the mesolimbic pathway, which is considered the key circuitry underlying reward and motivation. A wide number of studies showed that dopamine (DA) in this pathway is essential for the expression of normal maternal behaviour (Numan and Stolzenberg, 2009; Numan *et al*., 2005a; Numan and Stolzenberg, 2008). As we indicated in the introduction, DA is released into de NAcc during maternal behaviour and dysfunction of this system causes deficits in maternal responsiveness. Microinjections of antagonists of both dopamine D1 and D2 receptors into NAcc impair retrieval and pup-licking, suggesting that D1 and D2 are involved in the proactive voluntary aspects of maternal behaviour (Keer and Stern, 1999). However, Numan *et al.*, (2005a) revealed that D1 could be playing a more important role in the regulation of the proactive maternal behaviours

than D2. In this case, lactating female rats were injected with either a D1 antagonist or D2 antagonist into NAcc. They observed different effects. Nursing was not affected with either D1 or D2 antagonists and D1 antagonist was more effective in the blockade of pup retrieval.

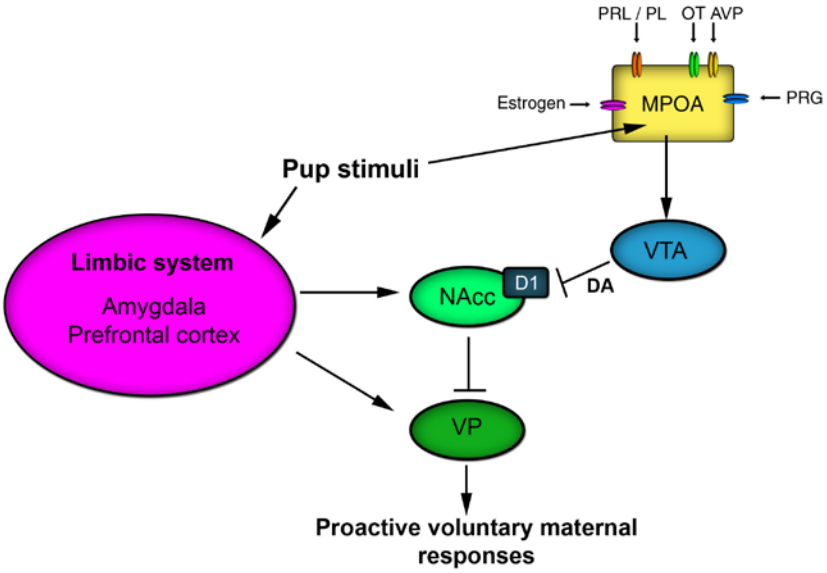

Figure 35. Neural model of mesolimbic dopaminergic system interaction with MPOA.

This sketch shows how MPOA could regulate the mesolimbic system. Pup stimuli might activate the DA release trough activation MPOA via VTA. In this case, DA binding to D1 in NAcc will disinhibit the VP to respond to pup stimuli to show proactive voluntary maternal behaviours. Abbreviations: AVP, arginine-vasopressin; DA, dopamine; MPOA, medial preoptic area; NAcc, nucleus accumbens; PL, placental lactogens/placental lactogens ; PRG, progesterone; OT, oxytocin; VTA, ventral tegmental area. Adapted and modified from Numan and Stolzenberg, 2008.

In mice, some proactive maternal behaviours seem to be regulated by the dorsal striatum. Dopamine release in this region is implicated in pup retrieval but not in licking/grooming behaviours

(Henschen *et al*., 2013). Females with restricted DA deficiency in dorsal striatum showed longer pup-retrieve latencies but displayed normal licking/grooming and nursing behaviours. On the other hand, severe DA depletion in all striatal areas impaired not only pup-retrieval but also licking/grooming behaviour, leaving nursing unaffected (Henschen *et al*., 2013).

Maternal motivation is essential for the proper expression of maternal care, which in turn is critical not only for the survival, but also for the future physical and mental well being of the offspring. In the present doctoral thesis, we have characterized two different components of maternal behaviour, namely maternal care and maternal aggression, in female mice of the strain CD1. We have shown that the former, but not the latter, is hardwired in the brain of virgin females, whereas the latter is expressed most likely as a consequence of the hormonal changes of pregnancy and lactation. In addition, we have demonstrated that darcin, a male sexual pheromone that is attractive for non-lactating females, elicits maternal aggression in lactating dams. Finally, we have shown that the number of OT and AVP cells in two brain nuclei related to maternal behaviour is not altered by maternity, albeit functional changes on these cells remain to be studied. In all, the results of the present thesis are a starting point for the use of CD1 mice as a model to investigate the neurobiology of motivated maternal behaviour and aggression, key components of the behavioural repertoire of social mammalian species, including humans. Understanding the neurobiology of these behaviours might help devise strategies to tackle their dysfunction, to ensure the mental and physical health of future generations.

6. CONCLUSIONS

1. Virgin female mice of the outbred strain CD-1 express maternal care in a near-spontaneous manner.
2. Virgins that were continuously exposed to pups, which we name *godmothers*, were indistinguishable from dams in the pup-retrieval test from the first testing day (on PPD 2).
3. Virgins that were exposed to pups in two-hour daily sessions (pup-sensitized females) took at least two sensitization sessions to express similar levels of maternal-like behaviour to those of dams.

4. Pup-sensitized females overexpress pup licking/grooming behaviour as compared to dams and godmothers. These high levels of licking/grooming might reflect an increased investigative behaviour induced by novelty.
5. In our tests for maternal care, godmothers showed higher frequency of crouching than dams. We speculate that, in these conditions *godmothers* might be more anxious than dams, which in turn might be more willing to leave the nest to explore the surroundings.
6. Pup sensitisation with either protocol (*godmothers* and regular daily exposure to pups) is not able to promote maternal aggression in virgin females. This suggests that endocrine agents acting on the brain of females during pregnancy, parturition or lactation might be key for the induction of maternal aggression.
7. Maternal aggression is a territorial behaviour, since it only occurs near the nest, and dams do not attack unknown adult males if encounters occur in an environment different than the dam's home cage where the nest is located.
8. Lactating dams display high levels of maternal aggression toward intact males, and low levels of aggression toward castrated male intruders. Thus, testosterone-dependent chemosignals emitted by males

promote maternal aggression, as it occurs in intermale aggression

9. Dams attack castrated males sprayed with either intact male urine or the male pheromone darcin alone. Thus, the male pheromone darcin is a sign stimulus that triggers maternal aggression.
10. Since the same male pheromone (darcin),is responsible for attracting non-lactating females and eliciting maternal aggression, it is likely that changes in the response of females to males during motherhood are due to transient alterations in the brain of the females, rather than to changes in the sensory organ.
11. The number of oxytocin immunoreactive cells in the AC/ADP and Pa is not affected by pup-sensitization or lactation.
12. Oxytocin might modulate maternal aggression in female mice, since the number of OT-ir cells in the AC/ADP correlates positively with parameters of maternal aggression exclusively in dams.
13. The number of AVP-ir or OT+AVP-ir cells in the Pa and AC/ADP was not different between dams and godmothers. Thus, lactation does not affect either the number of nonapeptidergic cells or the proportion of co-localization of both neuropeptides in these nuclei of the maternal brain.

RESUMEN EN CASTELLANO

7. INTRODUCCIÓN

El comportamiento parental se da en una gran amplia variedad de especies de vertebrados e invertebrados, pero es especialmente importante en mamíferos y aves (Numan and Insel, 2003). Numan e Insel definieron este comportamiento como "*cualquier comportamiento de un miembro de una especie hacia un conespecífico reproductivamente inmaduro que incrementa las probabilidades de que el receptor sobreviva hasta la madurez*" (Numan e Insel, 2003). Aunque, especialmente en mamíferos, la mayor parte del cuidado lo llevan a cabo las hembras, en algunas especies los machos muestran comportamiento parental, lo que se conoce como *comportamiento paternal*. Por otro lado, en algunas aves y mamíferos, incluida la especie humana, también se da el comportamiento aloparental, en el cual un adulto dirige sus cuidados hacia una cría

con la cual no está relacionado genéticamente (Riedman, 1982). Esto sugiere que mientras en unas especies el comportamiento maternal está estrictamente asociado al estado fisiológico del adulto (comportamiento maternal estricto), en otros (comportamiento paternal, comportamiento aloparental) el cuidado de las crías se da con cierta independencia de la situación fisiológica del animal.

El comportamiento maternal, tiene una profunda influencia sobre el desarrollo de los recién nacidos. Tanto la calidad como la cantidad de este comportamiento afectan al desarrollo de la prole. En este sentido, el comportamiento maternal es sumamente importante porque afecta a algunos aspectos fenotípicos de las crías (Li *et al*., 2015; Meaney, 2001; Pan *et al*., 2014; Pedersen *et al*., 2011). Muchos estudios, algunos de ellos llevados a cabo en humanos, revelan que un trastorno en las relaciones madre-hijo durante la vida postnatal temprana, como el provocado por la separación materno-filial, altera la regulación neuroendocrina y provoca retrasos psicomotores y cognitivos en los hijos (Mehta *et al*., 2009; Rutter *et al*., 2012). Uno de los sistemas que se ve afectados en mayor medida es el eje hipotalámico-pituitario-adrenal (HPA), que controla las respuestas a estrés.

De hecho existe evidencia clara de que la alteración de la relación materno-filial durante este periodo puede alterar las respuestas al estrés en la vida futura de las crías. Debido a que el eje HPA de roedores comparte muchas características moleculares y neurobiológicas con el eje HPA humano (Gilles *et al*., 1996; Walker *et al*., 1986), el roedor se usa como un buen animal modelo para el estudio de este fenómeno.

Este importante papel del comportamiento parental (sobre todo del maternal) en la salud presente y futura de los hijos justifica sobradamente la investigación de la biología del comportamiento maternal, con el fin de optimizarlo y combatir de forma efectiva sus alteraciones y patologías.

En este sentido, nuestro conocimiento de las bases neurales y endocrinas del comportamiento maternal está basado principalmente en estudios llevados a cabo en ratas en los últimos 50 años. Durante las últimas décadas, sin embargo, ha ido creciendo el interés por el ratón como especie de estudio debido a las grandes ventajas del uso de individuos genéticamente modificados en los estudios de la neurociencia. Estos estudios han revelado importantes diferencias entre el comportamiento maternal de ambas especies, rata y ratón, siendo el ratón más semejante en este sentido a los primates, incluyendo la especie humana, que las ratas.

La principal diferencia entre ambas especies es que las ratas poseen un comportamiento maternal exclusivamente asociado a la fase de maternidad, ocurriendo espontáneamente sólo durante el período de lactancia. De hecho, una rata no lactante -especialmente si es nulípara- tiende a evitar las crías que le generan una cierta ansiedad, y sólo la exposición reiterada a crías de corta edad durante 5-7 días, acaba por provocar primero una habituación y tolerancia a la presencia de las mismas, y después una exploración de las crías seguida de un comportamiento casi completo de cuidado de las crías (ver Numan e Insel, 2003). A este fenómeno se lo conoce como *sensibilización maternal*. Esta situación contrasta con la observada en primates no humanos (y evidentemente en humanos). Así, las

hembras vírgenes de macaco muestran un comportamiento maternal espontáneo (Maestripieri y Wallen, 1995). Por esta razón, la rata no parece ser la mejor especie para comprender algunos aspectos de la neuroendocrinología del comportamiento maternal de los primates, incluyendo al humano.

Por el contrario, como veremos, las hembras vírgenes de ratón de cepas de laboratorio sí que exhiben un comportamiento maternal casi espontáneo, sin apenas necesidad de sensibilización maternal. De ser esto cierto, el comportamiento maternal de ratones se asemejaría al de los primates. Mientras en las ratas el comportamiento maternal parece estar regulado por la fisiología de la maternidad, con un previsiblemente importante papel de la regulación endocrina del comportamiento, en ratones -como en primates- sería relativamente independiente de hormonas. Por ello, vamos a centrarnos en la neurobiología y neuroendocrinología del comportamiento maternal del ratón, que es la especie en la que hemos llevado a cabo este estudio.

7.1 BASES HORMONALES Y NO HORMONALES DEL COMPORTAMIENTO MATERNAL DEL RATÓN

En roedores, los investigadores han diferenciado entre los comportamientos maternales que son dirigidos hacia las crías de aquellos que no (Numan e Insel, 2003). Así, los comportamientos maternales incluyen respuestas dirigidas a las crías como recogerlas y agruparlas en el nido, cubrirlas, protegerlas y abrigarlas con el propio cuerpo, acicalarlas y limpiarlas, así como amamantarlas. Las respuestas no dirigidas a las crías incluyen construir y mantener el

nido, ingerir alimentos con mayor fruición (hay un cambio claro de requerimientos nutricionales), así como la llamada agresión maternal (Numan e Insel, 2003). Se trata de un comportamiento agresivo asociado a la defensa del nido (Vom Saal *et al*., 1995) frente a adultos intrusos, potencialmente infanticidas. Además, las repuestas no dirigidas a las crías incluyen una disminución en la ansiedad relacionada con un incremento en la actividad exploratoria (Bridges, 2015). Durante el periodo preparto, las hembras preñadas ya muestran altos niveles de agresión hacia intrusos -a menudo se habla de agresión preparto como una entidad comportamental independiente, a nuestro juicio erróneamente- y comienzan a construir el nido (Caughey *et al*., 2011).

Los estudios sugieren que existe una motivación intrínseca que promueve un comportamiento maternal casi espontáneo en las hembras de ratón (Alsina-Llanes *et al*., 2015; Gandelman *et al*., 1970; Stolzenberg y Rissman, 2011), debido a que las hembras vírgenes de ratón no evitan las crías cuando son expuestas a ellas por primera vez, sino que las recogen y agrupan en un nido improvisado (Alsina-Llanes *et al*., 2015; Gandelman, 1973). Estos datos sugieren que el comportamiento maternal de ratones es independiente de los eventos endocrinos relacionados con la preñez, como por ejemplo, los niveles de esteroides o prolactina.

Uno de los objetivos de este trabajo es caracterizar el estos aspectos del comportamiento maternal en ratones usando la cepa no consanguínea CD-1. Para ello compararemos el comportamiento de las madres con el de dos tipos de hembras vírgenes que serán expuestas a crías mediante diferentes procedimientos. Las *comadres*

son vírgenes que comparten con la madre el cuidado de las crías desde el momento del parto. Las hembras sensibilizadas por el procedimiento tradicional, son expuestas a crías en su caja durante períodos diarios de 2 horas. Queremos comprobar cómo se desarrollan en estos animales los comportamientos maternales, usando las madres como el control positivo.

7.1.1 Hormonas y comportamiento maternal

Los estudios llevados a cabo en ratas indican que la sensibilización maternal (que permite expresar comportamientos maternales a hembras vírgenes) es facilitada por esteroides y prolactina (Bridges y Ronsheim, 1990; Bridges *et al*., 1990).

Por el contrario, como hemos visto, en ratones el cuidado maternal muestra cierta independencia hormonal, si bien los resultados no son claros. Por un lado, la deleción del gen del receptor de la prolactina elimina por completo la expresión del cuidado maternal por parte de la madre (Lucas *et al*., 1998). Por otro lado, estudios recientes usando mutantes dirigidos de una de las moléculas de señalización de la acción de prolactina contradicen esta idea (Buonfiglio *et al*., 2015).

Uno de los aspectos más estudiados del comportamiento maternal en el ratón es la agresión maternal, dado que los ratones son en general muy agresivos (más que las ratas). Para su estudio, la hembra lactante actúa como residente y se suele utilizar como intruso a un macho. En comparación con la agresión entre machos, la

agresión maternal es cualitativamente diferente, ya que es menos ritualizada y es más violenta (Ervin *et al*., 2015; Parmigiani *et al*., 1998).

Dado que la agresividad en hembras está restringida al período maternal, se especula cual puede ser el mecanismo fisiológico de inducción de agresión maternal. Aunque parece lógico pensar que el cóctel de hormonas que caracterizan el período de la maternidad pudieran tener un papel muy relevante, Garland y Svare (1988) propusieron que la agresión maternal podría ser independiente de hormonas, y ser inducida únicamente por el contacto con crías, específicamente por estímulos somatosensoriales producidos por la succión de los pezones durante la lactancia. De hecho, estos autores y su grupo realizaron estudios en hembras lactantes de ratón en los que les extirparon la hipófisis o las trataron con fármacos que inhiben la producción de prolactina, sin que se viera aparentemente alterada de la agresión maternal (Mann *et al*., 1980). Igualmente McDermott y Gandelman (1979) fueron capaces de promover agresión maternal en hembras vírgenes mediante exposición a crías y estimulación endocrina para el crecimiento de los pezones. Sin embargo, los altos niveles de agresión maternal preparto (Caughey *et al*., 2011; Mann *et al*., 1984), cuando aún no ha habido contacto con las crías, contradicen esta visión.

Así pues, la regulación endocrina o no endocrina de la agresión maternal es un tema abierto al debate que vamos a estudiar en este trabajo. El segundo objetivo de nuestro trabajo es analizar esta cuestión comprobando si hembras vírgenes que muestran un comportamiento de cuidado maternal completo y poseen un nido que defender, las *comadres*,

7.1.2 Olfacción y comportamiento maternal en ratones

Una de las maneras de comenzar el estudio de la neurobiología de un comportamiento es conocer qué estímulos regulan su expresión. En este sentido los ratones son animales macrosmáticos y sus comportamientos están guiados en gran medida por las señales químicas, entre las cuales hay feromonas. En roedores, el sentido del olfato está compuesto por dos sistemas, el olfatorio y el vomeronasal u olfativo accesorio (para mayor detalle ver Munger *et al*., 2009). El órgano sensorial del sistema olfativo accesorio es el órgano vomeronasal (VNO) que detecta feromonas que pueden estimular comportamiento sociales y respuestas endocrinas (Wysocki y Lepri, 1991). Por el contrario, el sistema olfativo principal detecta miles de moléculas volátiles (odorantes) mediante el epitelio olfativo. La detección de estas sustancias puede influir en todo tipo de comportamientos incluyendo sociales, anti-predatorios y alimentarios entre otros. En el sistema nervioso central, las vías de ambos sistemas quimiosensoriales, el vomeronasal y el olfatorio, discurren en paralelo (Boehm *et al*., 2005; Halpern y Martínez-Marcos, 2003) y están parcialmente segregadas. A pesar de eso, ambas convergen en ciertos núcleos amigdalinos (Cádiz-Moretti *et al*., 2014), pudiendo así controlar comportamientos tan complejos como el cuidado de las crías y la agresión maternal.

Las señales olfativas que provienen de las crías guían los comportamientos maternales. Los primeros estudios indicaban que las lesiones del epitelio olfativo con $ZnSO_4$ o las lesiones de los bulbos olfativos principales tienen un efecto suave y severo en la

construcción del nido y comportamiento maternal, respectivamente. (Vandenbergh, 1973). De hecho, la extirpación del bulbo olfativo elimina el comportamiento maternal (Gandelman *et al*., 1971), y promueve ciertos comportamientos anti-maternales como el canibalismo de las propias crías (Gandelman *et al.,* 1972).

Otros estudios más recientes también apoyan la importancia de las señales olfativas de las crías en la regulación del comportamiento maternal de las madres. Así, la mutación nula del gen de la adenilil ciclasa de tipo 3 ($AC3^{-/-}$), necesaria para la transducción olfativa, genera anosmia (Wong et al., 2000) y una disrupción completa de la expresión del comportamiento maternal, tanto en hembras vírgenes como en lactantes (Wang y Storm, 2011).

Por otro lado, el sistema accesorio parece jugar un papel menor en las respuestas maternales, ya que las lesiones del VNO no afectan en la expresión de la construcción del nido y el cuidado maternal (Bean y Wysocki, 1989), mientras que mutaciones nulas del canal TRPC2, implicado en la transducción vomeronasal, apenas afecta a estos comportamientos (Hasen y Gammie, 2011, 2009; Kimchi *et al*., 2007). Pese a ello, como este sistema es crucial para la detección de las señales químicas relevantes en el contexto de las relaciones socio-sexuales, no es de extrañar que mutaciones en el canal TRPC y proteínas acopladas a los receptores de feromonas, sí supriman la agresión maternal (Hasen y Gammie, 2011; Kimchi *et al*., 2007; Leypold *et al*., 2002; Stowers *et al*., 2002).

7.1.3 Feromonas masculinas: de la atracción sexual a la agresión maternal

En ratones la mayoría de feromonas que median las interacciones socio-sexuales son proteínas urinarias (MUPs) de la familia de las lipocalinas. Éstas contienen una cavidad para unir moléculas hidrofóbicas y volátiles de bajo peso molecular (Novotny *et al*., 1999). Existe un claro dimorfismo en la producción de las proteínas urinarias en ratones de laboratorio (Hurst y Beynon, 2013; Hurst, 1987), ya que los machos adultos son capaces de secretar de dos a ocho veces más que las hembras (datos de la cepa C57; Cheetham *et al*., 2009).

Trabajos previos de nuestro laboratorio han demostrado que la hembra de ratón se ve atraída de forma innata por las feromonas masculinas no volátiles que contiene el lecho ensuciado por machos (Moncho-Bogani *et al*., 2002) y que estas feromonas son detectadas por el VNO (Martínez-Ricós *et al*., 2008). Trabajos posteriores de otro laboratorio consiguieron aislar la feromona responsable de la atracción sexual, siendo ésta una MUP específica de machos llamada *darcina* o Mup 20 (Roberts *et al*., 2010). La darcina por sí misma (la forma recombinante libre de odorantes, r-darcina) atrae a hembras y genera en ellas aprendizaje asociativo (Lanuza *et al*., 2014; Roberts *et al*., 2012) confiriendo atracción a odorantes con los que se asocia.. Además, se ha visto que no sólo promueve atracción, sino la agresión entre machos (Kaur *et al*., 2014).

7.2 NÚCLEOS NONAPEPTIDÉRGICOS Y COMPORTAMIENTO SOCIAL

Los comportamientos sociales dependen de un circuito cerebral bien conservado en la evolución llamado la red sociosexual. Los nonapéptidos vasopresina (AVP) y oxitocina (OT) participan en el control de la mayoría de estos comportamientos (Insel y Young, 2000) y difieren en estructura únicamente en dos aminoácidos situados en la 3ª y 8ª posición.

La OT y la AVP se conocen por su función como neurohormonas periféricas, secretadas al torrente sanguíneo en la neurohiopófisis por las neuronas neurosecretoras magnocelulares del hipotálamo. La OT promueve la eyección de leche durante la lactancia (Nishimori *et al*., 1996) y la contracción uterina durante el parto, mientras la AVP o también conocida como hormona antidiurética, incrementa la presión sanguínea también por su actividad vasoconstrictora. Pese a que hay dudas de que crucen la barrera hematoencefálica de vuelta al sistema nervioso central (Neumann y Landgraf, 2012), estos nonapéptidos se encuentran en el cerebro donde actúan como neurotransmisores o neuromoduladores en determinadas neuronas y conexiones de la red socio sexual cerebral.

Las funciones de los neuropéptidos se llevan a cargo al unirse a sus receptores. Mientras la OT tiene un receptor específico (OTR), las vasopresina cuenta con tres tipos, de los cuáles dos (AVP1aR y 2R) se expresan en el sistema nervioso central (Neumann y Landgraf, 2008). Sin embargo la unión a estos receptores no es específica del

todo. La OT se une al OTR pero este receptor también puede unirse a AVP con 100 veces menos de afinidad que por la OT (Manning *et al*., 2012), haciendo difícil la tarea de interpretar las funciones de cada nonapéptido.

La distribución central de AVP está diferenciada en dos subpoblaciones, una de ellas constituida por las células magnocelulares hipotalámicas y otra, por neuronas dispersas en el polo caudal núcleo del lecho de la *stria terminalis* (BST) (Otero-Garcia *et al*., 2014; Rood *et al*., 2013). Éstas últimas muestran un claro dimorfismo sexual a favor de los machos, en los que la producción de AVP depende de testosterona (Otero-Garcia *et al*., 2014; Rood *et al*., 2013). Las proyecciones de estas neuronas a los centros de la red socio-sexual del cerebro son igualmente dimórficas.

En la parte rostral del núcleo paraventricular hay células que están doblemente marcadas por OT/AVP. Éstas células se extienden desde la comisura anterior hasta el núcleo anterodorsal preóptico (AC/ADP; Otero-García *et al*., 2015). Forman así un grupo celular situado entre el MPOA y la parte ventral del BST (vBST), una región crucial para la expresión del comportamiento maternal en ratas (Numan y Numan, 1996; Numan *et al*., 2005b; Olazábal *et al*., 2002) y ratones (Tsuneoka *et al*., 2013). Además, Numan y Woodside, (2010) postulan que los comportamientos maternales podrían ser modulados por OT en el MPOA/BSTv. Esto sugiere que la región involucrada en el comportamiento maternal podría incluir al AC/ADP junto a MPOA-vBST.

8. OBJETIVOS

1. Caracterizar dos modelos diferentes de sensibilización maternal en hembras vírgenes de ratón de la cepa CD-1.

2. Investigar si el contacto íntimo y prolongado con las crías es suficiente para inducir agresión maternal en hembras vírgenes de ratón.

3. Investigar si las señales químicas masculinas, incluida la feromona atractiva sexual de macho *darcina*, promueven agresión maternal.

4. Investigar posibles cambios en el cerebro de la madre ocurridos durante la lactancia, específicamente en la expresión y co-localización de los neuropéptidos AVP y OT en los núcleos del cerebro supuestamente implicados en el comportamiento maternal

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9. MATERIAL Y MÉTODOS

9.1 ESTUDIO 1: CARACTERIZACIÓN DEL COMPORTAMIENTO MATERNAL EN RATÓN

9.1.1 Modelos de sensibilización maternal

Hemos caracterizado el desarrollo del comportamiento maternal en hembras que han sido sometidas a dos protocolos diferentes de sensibilización en hembras vírgenes de ratón.

A. Hembras sensibilizadas (protocolo tradicional). Hemos adaptado al ratón el procedimiento utilizado en ratas (Fleming y

Luebke, 1981). Para ello expusimos hembras vírgenes que nunca habían tenido contacto con crías, a crías de una madre donante, 2h diarias durante 3 (en Sección 3.1.3.) o 4 días consecutivos (en Sección 3.1.4).

b. Comadres. Estas hembras fueron estabuladas con hembras preñadas a partir del día de gestación 5-7, y compartieron con ellas la gestación, parto y lactancia. Así, las comadres fueron expuestas a las crías desde el momento del parto y compartieron con las hembras lactantes el cuidado de las crías (Figura 8).

9.1.2 Test de comportamiento dirigido a las crías

El test de comportamiento dirigido a las crías fue realizado diariamente en la caja de las propias hembras del día postparto (PPD) 2 a 4. Las hembras fueron testadas tras retirar de la caja la hembra acompañante.

En el grupo de hembras lactantes y comadres, las ocho crías fueron retiradas con una espátula y posteriormente esparcidas por la caja. En el caso de las hembras sensibilizadas, recibieron ocho crías de una madre donante esparcidas de igual modo (Figura 10). El test, acabó 40 minutos después y los comportamientos se grabaron para su análisis posterior. Los grupos experimentales (y sus tamaños muestrales) de los experimentos están resumidos en la Tabla 1.

9.1.3 Test de agresión maternal

Para evitar la interferencia de la realización de un test en el resultado de otro, para analizar este comportamiento usamos

animales diferentes de los anteriores, en experimentos separados. En este experimento utilizamos como hembras experimentales madres, comadres, hembras sensibilizadas y hembras vírgenes inexpertas (sin contacto con crías), estabuladas como en el experimento anterior.

El test de agresión maternal se llevó a cabo en la propia caja de las hembras (Figura 11) el PPD5 Las hembras fueron llevadas a las habitaciones de test y se retiraron, tanto las crías como las hembras acompañantes.

Para el test, se introdujo un macho dentro de la caja por el lado contrario al nido y los grabamos su comportamiento durante 5 minutos para su posterior análisis. Tras ello, el intruso fue retirado, y se intercambió a la hembra para ser testada. Los grupos de animales experimentales (y el tamaño muestral) están recogidos en la Tabla 1.

9.1.4 Efecto de la presencia de las crías durante el test de agresión con comadres

Para evaluar si la presencia de las crías promueve agresión maternal en las comadres, se llevaron a cabo tests de agresión maternal siguiendo el protocolo del experimento anterior, tanto en ausencia como en presencia de las crías, los días PPD4 y 5 respectivamente, utilizando un macho intacto como intruso.

9.2 ESTUDIO 2: ESTÍMULOS QUE PROMUEVEN LA AGRESIÓN MATERNAL

9.2.1 Maternal aggression test

El test de agresión maternal fue realizado como en el estudio 1. El objetivo es conocer la capacidad de los diferentes estímulos de macho como promotores de agresión maternal, utilizando dos condiciones diferentes de intruso, machos castrados y machos intactos. El tipo y tamaño de los grupos experimentales están recogidos en la Tabla 2.

9.2.2 Análisis de las proteínas urinarias de ratón

Analizamos el contenido de proteínas urinarias de la orina de hembras, machos intactos y machos intactos, utilizando una electroforesis simple siguiendo el protocolo de Lanuza *et al*., (2014). Para ello recogimos orina de un grupo de ejemplares de cada grupo, homogeneizamos la muestra y separamos las proteínas de la orina con el sistema PhastGel (General Electrics).

9.2.3 Propiedades promotoras de agresión de darcina recombinante en hembras lactantes

Para conocer las propiedades promotoras de la agresión de la darcina, medimos los ataques de las hembras lactantes y las comadres (control negativo) hacia machos castrados cuya zona urogenital y cuello se roció con solución salina, darcina recombinante (r-darcina) u orina de macho intacto. El protocolo de agresión maternal

fue idéntico al utilizado en el estudio 1 y el diseño experimental está resumido en la Tabla 2.

9.2.4 Agresión maternal en una arena neutral

Hasta nuestro conocimiento, la agresión maternal se ha descrito como un comportamiento de protección del nido, ante la presencia de posibles depredadores. En este experimento evaluamos el nivel de agresión de las hembras lactantes y comadres en un ambiente neutro, fuera de su caja, en donde fueron introducidas antes que el macho intruso. Nuestra hipótesis es que las hembras solo serían agresivas hacia un macho intruso en las cercanías de su nido, cuando estuvieran en su propio territorio. Para esto, evaluamos la agresión maternal en unas cajas de 220 x 220 x 145 h mm. Tras 2 minutos de habituación de la hembra a la caja, introdujimos durante 5 minutos un macho castrado (n=7) al que habíamos aplicado orina de macho intacto. Los comportamientos se grabaron y posteriormente fueron medidos por alguien ajeno al experimento. El tamaño de los grupos y los tipos de animales experimentales utilizados están recogidos en la Tabla 2.

9.3 ESTUDIO 3: DISTRIBUCIÓN DE NONAPÉPTIDOS EN NÚCLEOS DEL CEREBRO MATERNAL

9.3.1 Perfusión, fijación y corte

Los animales fueron sacrificados mediante sobredosis de pentobarbital y perfundidos a través del ventrículo cardíaco izquierdo con solución salina (5.5 ml) seguida de paraformaldehído al 4% en tampón fosfato 0.1M pH 7.4 a razón de 5.5 ml/min durante 12

minutos. Los encéfalos fueron extraídos cuidadosamente y sumergidos en sacarosa al 30% en tampón fosfato para su crioprotección. Acto seguido, los cortamos en un micrótomo de congelación y recogimos los cortes en 4-5 series paralelas, en función del experimento. Para la detección de AVP y OT por inmunofluorescencia usamos 2 de las 4 series obtenidas. Para la detección con inmunoperoxidasa, usamos dos (series 1 y 3) de las cinco series obtenidas. Las series restantes se congelaron (-18ºC) para uso futuro.

9.3.2 Inmunohistoquímica para la detección de oxitocina

Para la inmunohistoquímica simple (OT) usamos el método indirecto del complejo avidina-biotina-peroxidasa (ABC). Para ello, preincubamos en 1% peróxido de hidrógeno (H_2O_2, solución de bloqueo, anticuerpo primario (1:25000, rabbit anti-oxytocin IgG, Millipore Cat#AB911), anticuerpo secundario (1:200, goat anti-rabbit IgG, Vector Labs, BA-1000) y ABC. Finalmente, las preparaciones fueron reveladas con diaminobenzidina, montadas y cubiertas para hacerlas permanentes.

9.3.3 Análisis de la co-localización de OT+AVP

Para el marcaje simultáneo de oxitocina y vasopresina combinamos la immnuofluorescencia de ambos péptidos en 2 series de hembras lactantes y no lactantes (6 por grupo). Brevemente, incubamos las series en borohidruro sódico -para reducir la autofluorescencia-, incubamos en solución de bloqueo, la mezcla de

anticuerpos primarios (rabbit anti-vasopressin IgG, 1:2500; Millipore, AB1565; mouse anti-oxytocin, monoclonal IgG 1:200; Dr. Harold Gainer, NIH, PS38), y de anticuerpos secundarios marcados con fluoróforos diferentes (Alexa Fluor 488-conjugado Goat anti-rabbit IgG, 1:250; Jackson ImmunoResearch, 111-545-003; Rhodamine Red X-conjugado goat anti-mouse IgG, 1:250; Invitrogen R6393). Los cortes se lavaron cuidadosamente entre pasos. Para revelar la citoarquitectura en las secciones, contrateñimos con DAPI. Finalmente, montamos los cortes en portaobjetos gelatinizados y cubrimos con medio de montaje para fluorescencia.

Analizamos las preparaciones con un microscopio confocal Olympus FV1000 invertido, escaneando secuencialmente los canales para identificar el 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 separadas entre 1.5 y 4 micras, a x100, x200 y x600 aumentos, en las regiones de interés. Guardamos las imágenes en formato TIF y OIF.

Procesamos los *stacks* (pilas de imágenes) obtenidos con ImageJ para el recuento de las células en cada una de las secciones ópticas, en cada uno de los canales, para identificar las células marcadas con sólo-OT, sólo-AVP y con ambos nonapéptidos (Figura 15A, B y C).

10. DISCUSIÓN DE LOS RESULTADOS

10.1 LA EXPOSICIÓN A CRÍAS PROMUEVE EL CUIDADO MATERNAL EN HEMBRAS VÍRGENES DE RATÓN

Hemos caracterizado y validado dos protocolos de sensibilización maternal en hembras vírgenes de ratón de la cepa CD1. Para ello, hemos adaptado el protocolo clásico en el cuál hembras vírgenes de ratón fueron expuestas 2h diarias a crías. Además, hemos diseñado un procedimiento más natural, en el que hembras vírgenes, llamadas comadres, compartían el cuidado de las crías con las lactantes desde el momento del parto. Este contacto

continuo con las crías, induce una expresión rápida y completa del comportamiento maternal dirigido a las crías desde el día postparto 2.

Ambos protocolos de sensibilización maternal indujeron con éxito, aunque a diferentes ritmos, comportamientos dirigidos a las crías. Así las comadres se comportaban de modo indistinguible a las madres en la mayoría de los aspectos desde el primer día de test (PPD2), mientras que las hembras sensibilizadas por el procedimiento tradicional necesitaron al menos dos sesiones de sensibilización antes de alcanzar niveles de comportamiento maternal similar al de las restantes hembras. Éstos resultados son consistentes con los obtenidos en otras cepas de ratón (Stolzenberg y Rissman, 2011). Por otro lado, estos autores demostraron que mientras las hembras inexpertas necesitan solo dos días de exposición a crías para recoger las crías en su propia caja, este periodo no es suficiente para responder a las crías en un ambiente nuevo, como un laberinto en T. Por esta razón, la elección del ambiente del test es tan crucial para observar las respuestas maternales a crías en hembras nulíparas de ratón.

Las comadres expuestas a crías desde el momento del parto, fueron tan rápidas como las madres en el test de recogida de las crías (Figura 17). Sin embargo, las hembras sensibilizadas fueron más lentas que las madres durante tanto durante el primer día de test – en el que aún no habían tenido contacto con crías previamente- y el segundo. A lo largo de los tests, las hembras sensibilizadas adquieren experiencia con las crías y aprenden a recogerlas antes. Así, durante el tercer día de test, tras dos sesiones de 2h de exposición diaria, las

hembras sensibilizadas consiguen ser tan rápidas como las madres en recoger a las crías.

Por otra parte, las hembras sensibilizadas sobreexpresaron el acicalamiento/limpieza de las crías (Figura 19). Nuestra interpretación es que los altos niveles de este comportamiento se deberían al efecto novedad de las crías para las hembras sensibilizadas (Rinaldi *et al*., 2010). De este modo, las madres y comadres estarían reconociendo a sus propias crías, y tras reagruparlas en el nido, expresarían un menor nivel de comportamiento de acicalamiento/limpieza. Esta hipótesis concuerda con los resultados obtenidos por Stolzenberg y Rissman (2011), que observaron que las hembras expertas fueron más rápidas en recoger las crías pero presentaban una menor tasa del comportamiento de lamida/acicalamiento (ver también Alsina-Llanes *et a*l., (2015)).

Estos resultados contrastan con el número de veces que las hembras se acurrucan sobre las crías, puesto que las comadres sorprendentemente lo realizan con mayor frecuencia que las lactantes (Figura 18). Interpretamos esta observación como que, en las condiciones de test en el que no hay amenazas visibles, las lactantes, tras poner a salvo a la crías en el nido, podrían estar más dispuestas a dejar el nido y explorar los cambios en los alrededores. De hecho, de acuerdo con nuestra hipótesis, Bridges, (2015) propone que los niveles de ansiedad en las lactantes disminuye y ello les lleva a incrementar la actividad exploratoria. Por el contrario, las comadres, estarían más ansiosas por tratarse de la primera vez que están a solas con las crías, sin la presencia de la madre, por lo que estarían más motivadas para reagrupar las crías y permanecer en el nido con ellas.

10.2 LA SENSIBILIZACIÓN MATERNAL NO INDUCE AGRESIÓN MATERNAL

En nuestros experimentos, ni el contacto constante con las crías ni una exposición de 2h diarias a las mismas fueron capaces de promover agresión maternal en hembras vírgenes de ratón, a pesar de que éstas expresaban comportamiento maternal dirigido a las crías con niveles similares a los de las madres. La falta de agresión de las hembras sensibilizadas en el Experimento 3.1.4 podría atribuirse a la falta de un nido para defender. Pero el hecho de que cuando se dejó el nido a las comadres éstas no expresaran agresión, parece demostrar que, un periodo de contacto íntimo con crías (5 días) *per se* no es capaz de provocar agresión maternal en hembras vírgenes (Figura 21), tal y como se ha visto en ratas (Erskine *et al*., 1980b). Así, a diferencia del comportamiento maternal, la agresión maternal parece ser que requiere cambios fisiológicos asociados a la maternidad.

Además, la agresión maternal solo se da cerca del nido, puesto que las hembras lactantes no atacan a un macho adulto desconocido si se encuentran en un ambiente diferente al de la propia caja de la hembra (Figura 25; Experimento 3.2.7). Así, todas las hembras lactantes de la presente tesis expresaron agresión maternal cuando el encuentro sucedía en sus propias cajas. Sin embargo, en una caja neutra sólo 1/10 hembras lactantes atacaron al macho intruso (Tabla 5), pese a que esta agresión duró menos de 1 segundo por lo que podríamos concluir que en general, la agresión maternal sucede específicamente dentro del territorio de la madre. Este hecho demuestra que la agresión maternal es un comportamiento territorial,

como la agresión entre machos (Miczek y O'Donnell, 1978; Miczek *et al*., 2001).

10.3 DE LA ATRACCIÓN A LA AGRESIÓN: LA LACTANCIA CAMBIA EL VALOR EMOCIONAL DE LAS FEROMONAS

Los resultados del estudio 2 nos llevan a dos conclusiones principales. Primero, sólo las hembras lactantes atacaron a los intrusos y más importante aún, las comadres y las hembras vírgenes inexpertas no mostraron respuesta agresiva frente a machos intactos (0.76±0.36s, 0.26±0.14 s tiempo de ataque; comadres y vírgenes inexpertas, respectivamente; Figura 22). Este hecho sugiere que sólo las hembras lactantes responden de modo agresivo ante machos conespecíficos cuando son introducidos en la caja de las hembras.

Además de atacar con mayor fiereza, las hembras lactantes fueron mucho más rápidas en iniciar el ataque que las no lactantes (ver Resultados). Sin embargo, cuando las hembras lactantes se enfrentan a machos castrados, no se diferenciaron de las no lactantes en términos de tiempo total de ataque y latencia al primer ataque. Estos resultados indican que las señales químicas de masculinidad, cuya producción es testosterona-dependiente, generan la agresión de las hembras lactantes (agresión maternal), como sucede en la agresión entre machos (Kaur *et al*., 2014; Stowers *et al*., 2002). Por lo tanto, la agresión maternal observada parece ser inducida de forma por feromonas sexuales masculinas.

Otra de las implicaciones de nuestro trabajo es haber demostrado que la darcina es una señal química promotora de la agresión maternal en hembras lactantes. De hecho, cuando aplicamos r-darcina sobre los machos castrados, ésta induce en hembras lactantes los mismos niveles de agresión maternal y con la misma latencia que la orina de macho intacto (ver Figura 24). Cabe destacar que la inducción de agresión maternal por parte de r-darcina ocurre en ausencia de odorantes de macho (la r-darcina sólo podría unirse a olores de macho castrado). En otras palabras, las hembras lactantes podrían reconocer la misma señal masculina tanto en orina como en r-darcina. Por esta razón, la darcina contenida en ambas soluciones –orina y tampón salino- es la promotora de agresión maternal.

Así pues, si la misma molécula –darcina- induce diferentes respuestas comportamentales en vírgenes –atracción- y en lactantes -agresión-, el cambio en la respuesta hacia los machos alrededor del parto no debe implicar modificaciones en el órgano vomeronasal –como por ejemplo cambio en el patrón de expresión de receptores- sino que las hormonas circulantes durante la preñez deben cambiar el valor emocional de este estímulo, regulando así la respuesta a darcina. Nuestra hipótesis es que deben darse cambios a nivel del circuito socio-sexual del cerebro. Por ello, empezamos a explorar posibles cambios a nivel de las células nonapeptidérgicas, en dos núcleos relacionados con el comportamiento maternal. Debido a que los nonapéptidos OT y AVP están implicados tanto en el comportamiento social como en el procesamiento de las señales químicas, estos nonapéptidos podrían ser los candidatos modulados

por los factores que actúan durante la preñez y el periodo cercano al parto.

10.4 EN RATONES CD1 LA PRODUCCIÓN DE DARCINA ES TESTOSTERONA DEPENDIENTE

La electroforesis para la separación de la orina de los machos –castrados en intactos- y de hembras, reveló dos bandas presentes en todas las muestras, junto con una banda que excepcionalmente se observaba en la orina de machos (Figure 23). Esta banda corresponde a una proteína urinaria cuya producción es dependiente de testosterona y fue previamente descrita como darcina por Roberts *et al*., (2010). Darcina es una MUP de 18893Da de peso, con una movilidad inusual ya que aparece como una banda equivalente a 16kDa. Nuestra electroforesis reveló la presencia de esta MUP en ratones machos de la cepa CD-1, pero no en los machos castrados.

10.5 EL NÚMERO DE CÉLULAS NONAPEPTIDÉRGICAS NO ESTÁ AFECTADO DURANTE LA MATERNIDAD NI SENSIBILIZACIÓN POR CRÍAS

Tras la caracterización del cuidado de las crías y agresión maternal en hembras de ratón CD-1, investigamos las posibles adaptaciones en el cerebro maternal que pudiesen explicar el aumento de la motivación por los estímulos de las crías y la defensa del nido. Nuestra hipótesis es que modificaciones de los sistemas nonapeptidérgicos del cerebro de las hembras lactantes podrían explicar los cambios comportamentales asociados a la maternidad, entre otros la agresión maternal.

Para explorar esta idea cuantificamos el número de células que expresaban OT, AVP o ambos nonapéptidos en el AC/ADP y Pa, dos núcleos que parecen estar implicados en el comportamiento maternal. Nuestros resultados tanto con técnicas de inmunoperoxidasa como inmunofluorescencia coincidieron en no revelar diferencias en el número de células OTérgicas y AVPérgicas –inmunofluorescencia sólo- entre hembras lactantes y no lactantes, ni en el Pa ni en el grupo del AC/ADP (Figura 28). Así, el número de células nonapeptidérgicas fue idéntico en madres y comadres, y el número de células inmunoreactivas para OT no difirió entre madres, comadres y hembras vírgenes sin experiencia con crías.

Pese a que no encontramos diferencias en las células OT positivas en el AC/ADP entre ambos grupos, otros estudios sugieren que sí hay modificaciones funcionales de estas células en lactantes (Tsuneoka *et al*., 2013). Estos autores demostraron que células OT positivas en el AC/ADP no fueron activadas por los estímulos de las crías en hembras vírgenes, pero mostraron un incremento muy significativo de actividad c-fos en hembras lactantes cuando fueron expuestas a crías ajenas 7 días tras el parto. Dado que nuestros resultados indican que no hay un incremento en células OTérgicas en esta región el cerebro de las madres, todo sugiere que las células OTérgicas del AC/ADP son insensibles a estímulos de las crías en hembras vírgenes pero se vuelven sensibles a estos estímulos tras el parto.

A la luz de resultados previos que sugerían un aumento de la co-expresión entre los niveles de ARN mensajero de OT y AVP en el núcleo supraóptico en ratas (Mezey y Kiss, 1991), partimos de la

hipótesis de que, aunque el número total de células nonapeptidérgicas no cambiaban durante la lactancia, el sustrato hormonal de las madres podría afectar al porcentaje de la co-localización de OT y AVP en el AC/ADP y el Pa. Sin embargo, no observamos diferencias en este porcentaje entre madres y comadres, tanto en el AC/ADP (Figura 30) como el Pa, que contiene mayormente neuronas neurosecretoras (Figura 31).

Por lo tanto, nuestros resultados podrían contradecir los obtenidos por Mezey y Kiss (1991). Sin embargo, este estudio no aportó ningún análisis estadístico de los datos y utilizaron un tamaño muestral pequeño (n=2 por grupo). De hecho, sólo aportaron un mero recuento e incremento de porcentajes de co-localización. Nuestros resultados en hembras lactantes de día PPD5, muestran un aparente incremento de dos veces más en el porcentaje de células doblemente marcadas en las dos regiones más rostrales del Pa (Tabla 6), pero estos datos no superan un test estadístico, probablemente debido a la alta variabilidad en la muestra.

Otra posibilidad interesante es que la población celular que co-expresa AVP y OT en el AC/ADP sea sexualmente dimórfica. Otero-García *et al*., (2015) publicaron la descripción detallada de la co-expresión de ambos nonapéptidos en machos (ver Tabla 7). En comparación con nuestros resultados, los machos no parecen diferir en la proporción de células nonapeptidérgicas que sólo expresan AVP en el AC/ADP (1.6% en machos *vs*. 0.92% en comadres) o en la proporción de células que co-expresan ambos nonapéptidos (40.3% en machos *vs*. 54.34% en hembras). Por otro lado, en el Pa, el porcentaje de los somas nonapeptidérgicos que expresan sólo AVP

pueden ser sexualmente dimórfico en favor de las hembras, mientras que la co-localización de AVP y OT parece ser más frecuente en el Pa de machos, por mostrar entre 2 y 4 veces más de células doblemente marcadas

	Comadres		
	% Células Nonapeptidérgicas expresando solo OT	% Células Nonapeptidérgicas expresando solo AVP	% Células Nonapeptidérgicas expresando AVP+OT
AC/ADP	44.74	0.92	54.34
Pa (-0.70mm)	52.92	41.82	5.26
Pa (-0.94mm)	39.73	52.43	7.83
	Machos*		
	% Células Nonapeptidérgicas expresando solo OT	% Células Nonapeptidérgicas expresando solo AVP	% Células Nonapeptidérgicas expresando AVP+OT
AC/ADP	58.1	1.6	40.3
Pa (-0.70mm)	59.2	16.5	24.3
Pa (-0.94mm)	51	28.6	20.4

Table 8.Comparison of the distribution of nonapeptidergic cell bodies immunoreactive for OT, AVP or both in the AC/ADP and Pa in virgin males and females (godmothers).

*Los datos de machos han sido tomados con el permiso de Otero-García *et al*., 2015

10.6 EL NÚMERO DE CÉLULAS OXITOCINÉRGICAS EN AC/ADP CORELACIONA CON LA AGRESIÓN MATERNAL

Nuestros resultados sugieren que los somas OT positivos en el AC/ADP podría jugar un papel importante en la agresión. Hemos observado que el número de células OTérgicas en el AC/ADP, con la técnica de inmunoperoxidasa, correlaciona positivamente con altas

tasas de agresión maternal exclusivamente en hembras lactantes (Figura 29). En nuestro material de inmunofluorescencia, el análisis de regresión lineal reveló la misma tendencia, que no llegaba a ser estadísticamente significativa (Figura 33). Cabe indicar que para el material de inmunofluorescencia teníamos un menor número de animales (n=6 por grupo) que en el de DAB (n=9 por grupo), por lo que es probable que los resultado obtenidos en la inmunodetección con peroxidasa pueda replicarse incrementando el tamaño muestral.

Por el contrario, no encontramos ninguna correlación o tendencia en la expresión de OT en el Pa y comportamiento agresivo, o entre el número de somas AVPérgicos y la agresión. Además, no encontramos correlación en las hembras no lactantes, que no mostraron apenas ataques hacia los machos intrusos. Por lo tanto, nuestros datos sugieren que las células OTérgicas del AC/ADP, pero no las AVPérgicas, podrían estar implicadas en el control de la agresión maternal

11. CONCLUSIONES

1. Las hembras vírgenes de ratón de la cepa CD-1 expresan cuidados maternales de un modo casi espontáneo.

2. Las hembras vírgenes que fueron expuestas a las crías de modo continuado (comadres) mostraron una velocidad de recogida de las crías en el test similar al de las madres desde la primera sesión de test (el día postparto 2).

3. Las hembras que fueron expuestas a crías durante dos horas diarias (hembras sensibilizadas) necesitaron al menos dos días de sensibilización para expresar niveles de comportamiento maternal similares a los de las hembras lactantes.

4. Las hembras sensibilizadas a crías sobreexpresaron el comportamiento de limpieza/acicalamiento en comparación con las hembras lactantes y las comadres. Nuestra hipótesis es que estos niveles de limpieza se podrían deber a un

incremento en el comportamiento de investigación debido a un efecto novedad de las crías.

5. Las comadres cubrieron a las crías con su cuerpo con mayor frecuencia que las lactantes. Especulamos que bajo las condiciones experimentales, las comadres podrían estar más ansiosas que las hembras lactantes, las cuales pueden estar más dispuestas a dejar el nido y explorar los alrededores.

6. La sensibilización maternal de hembras vírgenes por cualquiera de los dos procedimientos empleados, induce la expresión completa del repertorio de comportamientos dirigidos a las crías, pero no es suficiente para promover agresión maternal. Esto sugiere que los agentes endocrinos que actúan sobre el cerebro de las hembras durante la gestación parto deben ser clave para la inducción de la agresión maternal.

7. La agresión maternal es un comportamiento territorial, dado que sólo ocurre cerca del nido y las madres no atacan a machos adultos desconocidos si el encuentro ocurre en un ambiente diferente al de la propia caja de la hembra lactante.

8. Las hembras lactantes de ratón expresan altos niveles de agresión maternal hacia los machos intactos, y bajos frente a intrusos castrados. Por lo tanto, como ocurre con la agresión entre machos, la agresión maternal es promovida por señales químicas dependientes de testosterona secretadas por los machos.

9. Las madres atacan a machos castrados a los que se roció con orina de machos intactos o con la feromona masculina darcina sola. Así pues, la feromona sexual masculina darcina es un estímulo clave que desencadena la agresión maternal.

10. Dado que la misma feromona masculina, darcina, desencadena atracción en hembras no lactantes y agresión maternal en lactantes, los cambios en la respuesta de las

hembras hacia los machos durante la maternidad se deben a alteraciones reversibles del cerebro de las hembras, más que del órgano sensorial.

11. El número de células immunoreactivas para oxitocina en el AC/ADP y en el Pa no se ve afectado por la sensibilización maternal ni por la lactancia.

12. La oxitocina podría estar modulando la agresión maternal en hembras de ratón, ya que el número de células inmunoreactivas para la oxitocina en el AC/ADP correlaciona positivamente con los parámetros de agresión, exclusivamente en madres.

13. El número de células immunopositivas para AVP o para AVP+OT en el Pa y AC/ADP no difirió entre hembras lactantes y comadres. Por lo tanto, la lactancia no afecta ni al número de células nonapeptidérgicas ni a la proporción de co-localización de ambos neuropéptidos en estos núcleos del cerebro maternal.

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