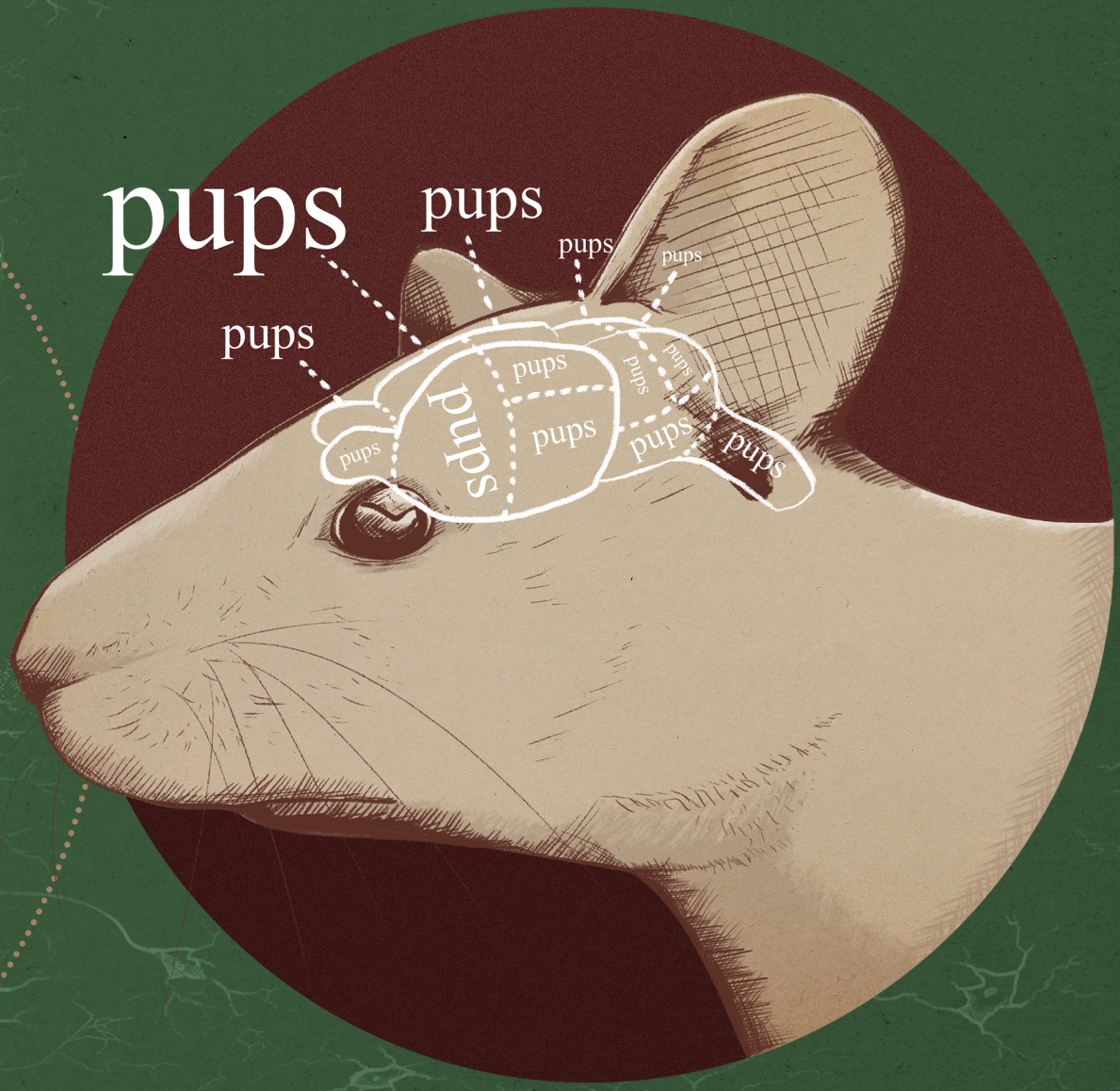
Ready for motherhood

Chemosensory, socio-sexual and motivational brain networks adjust their activity during pregnancy



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Título de la tesis

Ready for motherhood. Chemosensory, socio-sexual and motivational brain networks adjust their activity during pregnancy.

Lista para la maternidad. Los sistemas quimiosensorial, socio-sexual y motivacional del cerebro ajustan su actividad durante el embarazo.

Memoria presentada por Cinta Navarro Moreno para optar al grado de doctora por la Universitat Jaume I

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"Yo creo que estoy así de mala

porque he tenido muchos hijos"

Rosa

Quizás no es lo más ortodoxo, pero dada la temática del manuscrito que tenemos entre manos quiero comenzar los agradecimientos haciendo mención a mi madre. Conocí a Rosa a mediados de los 80. Cuando me tuvo, ella ya sumaba 45 años y 6 hijos criados, compaginaba varios trabajos de enfermera y asumía buena parte del cuidado de su padre. Por cruel que parezca, durante mis primeros años mis hermanos me llamaban "error" con la potestad que otorga el tener toda la razón del mundo. La cuestión es que entre pitos y flautas - una de las expresiones favoritas de mi madre - Rosa nunca dejó de cuidar, ni siquiera cuando ya le tocaba un descanso. Dedicó su vida al trabajo, a sus 7 hijos y después a sus 9 nietos, e inevitablemente tuvo muy poco tiempo para explorar sus propios intereses y desarrollar otras facetas de sí misma más allá del ámbito laboral o familiar. Así que cuidó, cuidó y cuidó hasta que los nietos fueron mayores. Y entonces, antes de que a ella le diese tiempo a reconciliarse con ese nuevo tiempo libre, la demencia hizo aparición en su vida. Cuando empezó a mostrar los primeros signos de déficit cognitivo, los médicos le aconsejaron centrarse en sus hobbies y encontrar "actividades que le motivaran", pero ella llevaba tantos años dedicados a cuidar a los demás que no tenía adquirido el hábito de dedicarse tiempo a sí misma. -A estas alturas el lector puede pensar que me estoy yendo por las ramas, pero más adelante, con el manuscrito leído, entenderá por qué le cuento esta historia-. Estos primeros signos de su enfermedad

comenzaron más o menos cuando yo empecé esta tesis, y conforme yo iba leyendo e investigando sobre la neurobiología de la conducta maternal, mi madre iba empeorando poco a poco. Hace un par de años, cuando yo estaba inmersa en el análisis de los cambios que sufre el cerebro durante la gestación, mi madre dentro de su confusión acertó a decirme: "¿sabes qué? Yo creo que estoy así de mala porque he tenido muchos hijos". Y, de una forma casi poética, esa afirmación tuvo todo el sentido del mundo para mí. Así que impepinablemente -que es otra de las palabras favoritas de mi madre- esa debía ser la frase que abriera este manuscrito. Y mis primeros agradecimientos han de ser para ella: gracias mamá. Muchas gracias. Y perdón.

"And now, for something completely different": los agradecimientos de toda la vida. En primer lugar, he de agradeceros a mis dos directores de tesis, Ferran y Maria José, las innumerables horas que habéis invertido en formarme como científica y la dedicación con la que habéis guiado mi trabajo, especialmente teniendo en cuenta que buena parte de esas horas las habéis sacado de vuestro tiempo libre. Cuando todo esto termine, estoy segura de que lo más satisfactorio para vosotros será darle click a "salir del grupo" en ese grupo de whatsapp "tesis Cinta" que suena a las horas más intempestivas.

Ferran, a ti quiero agradecerte varias cosas, pero las voy a agrupar en dos porque esta es la única sección en la que no me puedes reñir por usar frases demasiado largas. En primer lugar, lo obvio; la oportunidad de hacer una tesis tan bonita en un tema apasionante y poder aprender constantemente de tu mano y de la mano del resto del equipo de NeuroFun. Ha sido un gustazo. En segundo lugar, las facilidades que me has dado para compaginar mi vida personal con mi vida científica, sobre todo en momentos muy difíciles en los que tu gestión y tu acompañamiento me permitieron no tener que añadir a mi drama más preocupaciones por cuestiones laborales. Siempre te estaré especialmente agradecida por eso.

María José, llegaste tarde pero llegaste a tiempo. Sin ti, sin tu apoyo y tus ánimos, probablemente no habría continuado con el proceso de escritura. Así que no te miento cuando te digo que para la elaboración de esta tesis todos hemos sido contingentes, pero tú has sido necesaria. Me enorgullece mucho ser tu primera doctoranda, espero que a los que vengan detrás los quieras menos.

No quiero dejar las altas esferas del laboratorio sin agradecerte a ti, Quique, la oportunidad que me brindaste al acogerme en el grupo dirigiéndome el TFM. Lo a gusto que me hiciste sentir y lo mucho que aprendí en pocos meses fueron la chispa que encendió mis ganas de continuar por esta vía. De alguna forma todo esto es culpa tuya, así que espero que te guste el

manuscrito final y no te arrepientas de aquella decisión de hace 7 años. De lo que sí espero que te arrepientas es de mandarnos un gigoló a las 3 de la mañana a la habitación del hotel en la SENC de 2017. No espero que lo admitas, pero sabemos que fuiste tú.

Carmen, mi "casi co-directora de TFM" y "casi co-directora de tesis", creo que a ambas se nos ha quedado la espinita de poder trabajar juntas en algún momento. A pesar de eso, en muchos momentos has sido un gran apoyo y he aprendido mucho contigo en lo científico, en lo personal y en lo reivindicativo. Y piensa que a lo mejor el universo tiene sus motivos para no volvernos a juntar en un laboratorio, sobre todo en base a la única inmuno que hicimos juntas en 2017 en la que casi morimos asfixiadas por mezclar agua oxigenada con lejía y DAB. Sea como sea, siempre nos quedará twitter.

El proceso de elaboración de una tesis es muchas veces tedioso y frustrante, se suele decir que está lleno de altibajos pero la realidad es que abundan más los "-bajos" que los "alti-". Según mi experiencia, las personas que te acompañan diariamente en el laboratorio son lo que marca la diferencia entre disfrutar del proceso o vivir al borde del colapso aparentando estar bien mientras tu retrato va ajándose lentamente en una buhardilla polvorienta. Los próximos párrafos van dedicados precisamente a esas compañeras que han inclinado la balanza hacia el disfrute a lo largo de mi doctorado. Gracias a todas.

María, ¿qué decirte que no te haya dicho ya en medio de una tormenta de exabruptos? Eres mi persona favorita del doctorado y probablemente estés entre mis primeras 100 personas favoritas del mundo. Que a lo mejor parece poco pero el número mejora si contamos solo gente viva. No te digo esto mucho porque da como grima, pero allá va: te quiero mucho. Espero que lleguemos a viejóvenes juntas y hagamos playbacks de las spice girls en muchos, muchos, muchos escenarios más.

Manoli, trabajar contigo es un gustazo. Aunque al principio nos costase, siempre he podido hablar contigo de todo y creo que al final conseguimos compenetrarnos casi a la perfección. Hoy por hoy no solo te considero una de las mejores compañeras que se puede tener, sino también una buena amiga. Además, quiero que sepas que esta tesis es tanto mía como tuya y me gustaría que así la considerases. Tus fantásticas ideas están por todo el manuscrito y lo que he aprendido de ti me lo llevo conmigo para siempre. Muchísimas gracias.

Hugo, Ceci y Sergio, de los comienzos de mi andadura en la neurociencia me llevo vuestra amistad, sois ese trio de amables fans de Nicolas Cage que toda persona necesita en su vida. Os agradezco mucho vuestra constante celebración de la tontería, hay capítulos de Roboberto

y Alejandroide que son directamente culpa vuestra. Gracias por no dejar nunca que la vergüenza me ganase la batalla.

De los intermedios y finales de mi andadura me llevo a muchos compañeros. Personas que ni siquiera erais de mi grupo, pero tuvisteis a bien acogerme como a una más de la pandilla de predoctorales que poblaba la facultad. Noemí, yo aún no sé qué he hecho en mi vida para merecer una amiga tan bonita como tú. Eres todo bondad y alegría, y me salvaste el día en muchas ocasiones. Espero que sigamos juntas mucho tiempo más. Héctor, con tanta ida y venida al final te convertiste en uno de mis imprescindibles, y míranos ahora, hablando cada 2 o 3 años como si fuésemos best friends. Espero no perder nunca la amistad que nos une, aunque nos veamos una vez al lustro. Esther, Sandra, Cris, Nuno, Ernestina, Bárbara, Alberto; ¿cómo no voy a agradecer a la vida que os pusiera en mi camino? Erais la mezcla perfecta de risas, caos, mala baba y buena gente. Sois los personajes perfectos para montar una sitcom, pero no voy a entrar en detalles porque tengo un plan secreto para hacerme rica escribiendo una sobre vosotros y no quiero daros ningún crédito. Pero que sepáis que os adoro a todos.

Y bueno, como solo llevo 35 páginas de agradecimientos voy a explayarme un poquito más con las personas que me han sostenido durante estos años fuera del laboratorio.

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A todas esas amigas del cole, de la universidad, del IIAMA... Sé que os he desatendido durante eones, os pido disculpas y os agradezco la paciencia. En poco tiempo volveré a vivir en el

mismo plano dimensional que vosotras y conseguiré que echéis de menos cuando no estaba. Palabra.

A toda mi familia (a los veintipico): Gracias. No os digo que os quiero porque lo mismo os sale una erupción, pero tengo que agradeceros el apoyo que me habéis dado y las facilidades que me habéis puesto para poder terminar este proyecto. Y quiero agradecérselo especialmente a mi padre. Si de mi madre heredé la tozudez que me ha permitido terminar de escribir esta tesis, de mi padre heredé la capacidad de sobrellevar los malos momentos con el peculiar sentido del humor que caracteriza a los Navarro. Hoy por hoy sé que sin tanta tontería yo no sería yo, ni me caería especialmente bien. Por culpa vuestra, y en contra de todas las recomendaciones, estos agradecimientos son casi más largos que los capítulos de la tesis. Lector, si tiene cualquier queja puede dirigirse directamente a mi familia; la próxima reunión es a las 7.00 en la cala. Buena suerte.

Y por último, Sergio, quiero agradecértelo todo. Has vivido a mi lado todo el proceso y sé que no ha sido fácil para ti. Porque cuando empecé en esto de la neurociencia ya me gustabas (un poquito), pero durante todo este tiempo has ido convirtiéndote en mi hogar. Tanto que nos hemos casado y todo por el camino. No sabía cómo explicar todo lo que te quiero y todo lo que te debo, pero si quieres hacerte una idea puedes coger todos los agradecimientos que he dedicado al resto de personas y multiplicarlos por tropochorrocientos mil millones de trillones. Si 1 es -6 y 10 es un narciso, te quiero un gargamélido. Y prepárate porque ahora tengo tiempo y vamos a volver a grabar webseries, a organizar festivales de cortos y a viajar lejos para chupar castillos. Y ojalá sea así durante el resto de nuestras vidas.

So long, and thanks for all the fish

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Abbreviations List

3V: 3rd ventricle
ac: anterior commissure
aca: anterior commissure: anterior part
AcbSh nucleus accumbens (shell)
AcbC nucleus accumbens (core)
acp: anterior commissure: posterior
AC/ADP: the region of the nucleus of anterior commissure/anterodorsal preoptic
ADP: anterodorsal preoptic nucleus
AHC: anterior hypothalamic area: central part
AHiPM: amygdalohippocampal area: posteromedial part
AI: agranular insular cortex
AOB: anterior olfactory bulb
AON: anterior olfactory nucleus
APir: anterior lobe of pituitary
Aq: aqueduct (Sylvius)
Astr: amygdalostriatal transition area
bl: basal layer of the VNO
BLA: basolateral amygdaloid nucleus: anterior part
BLP: basolateral amygdaloid nucleus: posterior part
BM: basomedial amygdaloid nucleus
BMA: basomedial amygdaloid nucleus: anterior part
BST: bed nucleus of the stria terminalis
BSTIA: bed nucleus of the stria terminalis: intraamygdaloid division
BSTMPI: bed nucleus of the stria terminalis: posterointermediate part
BSTMPL: bed nucleus of the stria terminalis: posterolateral part
CA3: field CA3 of the hippocampus
cc: corpus callosum
CeC: central amygdaloid nucleus: capsular part
CeL: central amygdaloid nucleus: lateral division
Cl: caudal interstitial nucleus of the medial longitudinal fasciculus
cp: cerebral peduncle: basal part
CPu: caudate putamen (striatum)
D3V: dorsal 3rd ventricle
DAB: diaminobenzidine tetrahydrochloride
DEn: dorsal endopiriform nucleus
dlo: dorsal lateral olfactory tract
DM: dorsomedial hypothalamic nucleus
ec: external capsule
EPI: external plexiform layer of the olfactory bulb
EW: Edinger-Westphal nucleus
f: fornix
fmi: forceps minor of the corpus callosum
gcc: genu of the corpus callosum
Gl: glomerular layer of the olfactory bulb
Gr: granular layer of the olfactory bulbs
ic: internal capsule
IPC: interpeduncular nucleus: caudal subnucleus
IPl: inner plexiform layer of the olfactory bulbs
IPR: interpeduncular nucleus: rostral subnucleus
ir: immunoreactive
LH: lateral hypothalamic area
lo: lateral olfactory tract
LS: lateral septal nucleus
LSD: lateral septal nucleus: dorsal part

LSS: lateral stripe of the striatum
LSV: the ventrolateral septum
LV: lateral ventricle
MB: mushroom body of the VNO
MePV: medial amygdaloid nucleus: posteroventral part
mcl: mitral cell layer of the accessory olfactory bulb
mdl: midline; mv: microvillar layer
MePD: medial amygdaloid nucleus: posterodorsal part
MePV: medial amygdaloid nucleus: posteroventral part
Mi: mitral cell layer of the main olfactory bulb
ml: medial lemniscus
MOB: main olfactory bulb
MPO: the medial preoptic area
MS: medial septum
NSE: non-sensory epithelium of the VNO
OB: olfactory bulb
OE: olfactory epithelium
opt: optic tract
PaA: the anterior portion of the paraventricular nucleus
PAG: the lateral column of the periaqueductal grey
PB: phosphate buffer
PBS: phosphate saline buffer
pe: external plexiform layer of the accessory olfactory bulb
Pe: periventricular hypothalamic nucleus
PFA: paraformaldehyde
Pir: piriform cortex
PLCo: posterolateral cortical amygdaloid nucleus
PP: peripeduncular nucleus
PRh: perirhinal cortex
PrL-IL: the infralimbic cortical areas
PVA: paraventricular thalamic nucleus: anterior part
Re: reuniens thalamic nucleus
RMC: red nucleus: magnocellular part
RPC: red nucleus: parvicellular part
Rt: reticular thalamic nucleus
SCh: suprachiasmatic nucleus
SE: sensory epithelium of the VNO
sm: stria medullaris of the thalamus
SN: substantia nigra
SO: supraoptic nucleus
st: stria terminalis
TB: tris buffer
TBS: tris saline buffer
Vd: vomeronasal duct
VDB: nucleus of the vertical limb of the diagonal band
VEn: ventral endopiriform nucleus
VMHVL: the ventrolateral portion of the ventromedial hypotalamic nucleus
Vn: vomeronasal nerve
VP: ventral pallidum
VTAa anterior part of the ventral tegmental area
VTAa posterior part of the ventral tegmental area
Vv: vomeronasal vein

GENERAL INTRODUCTION

I.1. Parental behaviours: adaptive value and importance in mammals

Parental behaviours include a variety of conducts performed by adult individuals towards youngsters in order to ensure their survival until their reproductive age. It is one of the most important socio-sexual behaviours, since it is essential for the survival and adequate development of the offspring in a wide range of animal species. In mammals, alongside with birds and some other animals, individuals are born with very immature features so parental behaviour becomes especially needed to keep offspring alive. A good parental care assures thermoregulation, nurturing and hygiene of the youngsters, and is crucial for their correct neurodevelopment and maturation. In fact, parental neglect has devastating consequences on bodily and mental health of the offspring (Haller et al., 2014; Boccia & Pedersen, 2001; Branchi et al., 2013; Caldji et al., 1998; Fairbanks, 1996; Francis & Meaney, 1999; Maestripieri & Carroll, 1998). In humans, Spitz (1945) explored the differences between children raised in a foundling house, without contact with any parental figure (just nurses taking care of their basic needs), and children raised in the nursery of a prison, with daily contact with their loving mothers (Spitz comments on the reasons why imprisoned young women are exceptionally dedicated mothers). The study revealed that the lack of contact with a caring mother induced delayed psychomotor development and resulted in poor language and emotional expression. In addition "all these children … showed a seriously decreased resistance to disease, and an appalling mortality" (Spitz, 1945). Furthermore, studies in different species have shown that maternal behaviour not only influences the offspring health but also determines their own performance as parents (Champagne & Curley, 2009; Fairbanks, 1996; Gonzalez et al., 2001).

The study of parental behaviours and its underlying mechanisms is key to understanding the development of the offspring, but also to promote the well-being of parents, particularly of the mother. In vertebrates, parental labour is not always restricted to the female progenitor; in fact, male-only care (paternal care) is present in many organisms, including some fish and amphibian species. In birds, the main type of offspring care is biparental, both male and female cooperate in the breeding of their brood (Dulac et al., 2014), as it happens in a few mammalian species. Nevertheless, in the present Doctoral Thesis, we mainly focus on the neurobiological mechanisms involved in the parental behaviour of the female (maternal behaviour) in mice, since mammalian gestation and nurturing depend exclusively on the mother, and consequently females are usually more implicated in offspring care than males. In fact, during motherhood females undergo major changes involving endocrine and

neurobiological systems that ensure an adequate maternal behaviour (Dulac et al., 2014), and important allostatic adaptations of their physiology to cope with the burden of nurturing their offspring. Therefore, a better understanding of such adaptations would be very useful to help both mother and offspring well-being.

Sometimes, a non-progenitor individual takes care of youngsters that are not their own offspring. At times, the cared youngsters are not even genetically related to them, as occurs frequently across human societies (Martin et al., 2020). This so-called "alloparental care" is associated to altricial pups, often hairless, blind, immobile and helpless, which suggests possible adaptive reasons (see Numan & Insel, 2003). We share the display of alloparental care with several species, like some primates, prairie voles and laboratory mice (Kenkel et al., 2017), which is an advantage for our research in maternal behaviour. The study of maternal care in both dams and virgin laboratory female mice provides an excellent model to explore the underlying mechanisms of maternal behaviour, including those affecting non-pregnant females, along with the specific effects of pregnancy in the maternal brain.

So, in order to better understand the complexity of the onset and maintenance of maternal behaviour, in the next section I will further develop the different factors influencing matenal behaviour in some model species, with special emphasis in the endocrine changes related to pregnancy.

I.2. The main factors influencing maternal behaviour

Maternal behaviour depends on several factors, the main one being the presence of pups and their derived stimuli. Other factors have a strong influence on its expression, such as the hormonal state of the female (prepuberal, adult, pregnant or postpartum) and previous experience with pups (Figure I.1; Numan & Insel, 2003). The relative role of each one of these factors varies across species, but endocrine pregnancy-related changes are essential in most of them. Very likely, the reason for this hormonal dependence is that maternal behaviour requires such a huge investment of time and energy that restricting it to postpartum (when their own pups are present), by coupling behaviour to hormonal changes associated to motherhood, is strongly adaptive as a reproductive strategy for the female (Hrdy, 1999).

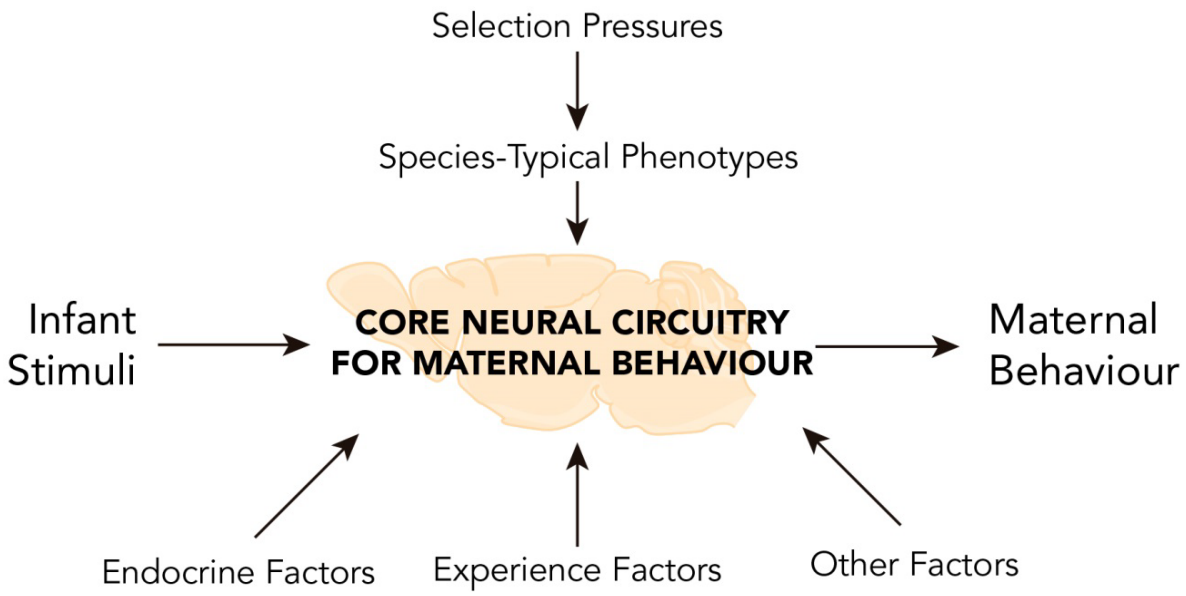


Figure I.1. Factors influencing the expression of maternal behaviour in the mammalian species, adapted from Numan & Insel (2003).

The pattern of pregnancy-related hormonal changes is very similar among mammals. Since rodents are probably the most studied group of animals concerning maternal behaviour, here we show a brief introduction to the rodent endocrine changes during pregnancy, which can be summarized as consisting of three major adjustments that prepare the female body to parturition and maternal behaviour (Bridges, 2015) (Figure I.2). The first two changes affect gonadal steroid hormones, oestradiol and progesterone. Oestradiol, which has been proved to play a major role in the induction of maternal behaviour of numerous mammals including rodents, sheep and primates, increases through the second term of pregnancy, reaching its maximum prior to birth. Progesterone, which promotes pregnancy or gestation by facilitating embryo implantation in the uterine endometrium, plays an important role in priming the gestating brain to elicit adequate response to pups' stimuli, and its levels increase through pregnancy, but decline prior to parturition or at birth. The last major endocrine change involves the pituitary hormone prolactin (PRL) and placental-derived lactogens (PL), which have also been proven to play a role in the onset and induction of maternal behaviours. For the first term of pregnancy, pituitary gland secrets prolactin following a twice-daily regime, but close before mid-gestation, while gonadal steroids rise, the placenta starts producing placental lactogens, which inhibit hypothalamic production of PRL. So, secretion of PRL suddenly drops, but it is substituted by lactogens (binding the same receptors as hypophysial PRL), produced by the placenta, which constitutes an important endocrine gland during the second half of pregnancy, by secreting two placental lactogens, PL-I first and PL-II until delivery. Then, the

pre-term sudden drop in progesterone elicits a new increase in PRL production in the day prior parturition(Salais-López, 2017).

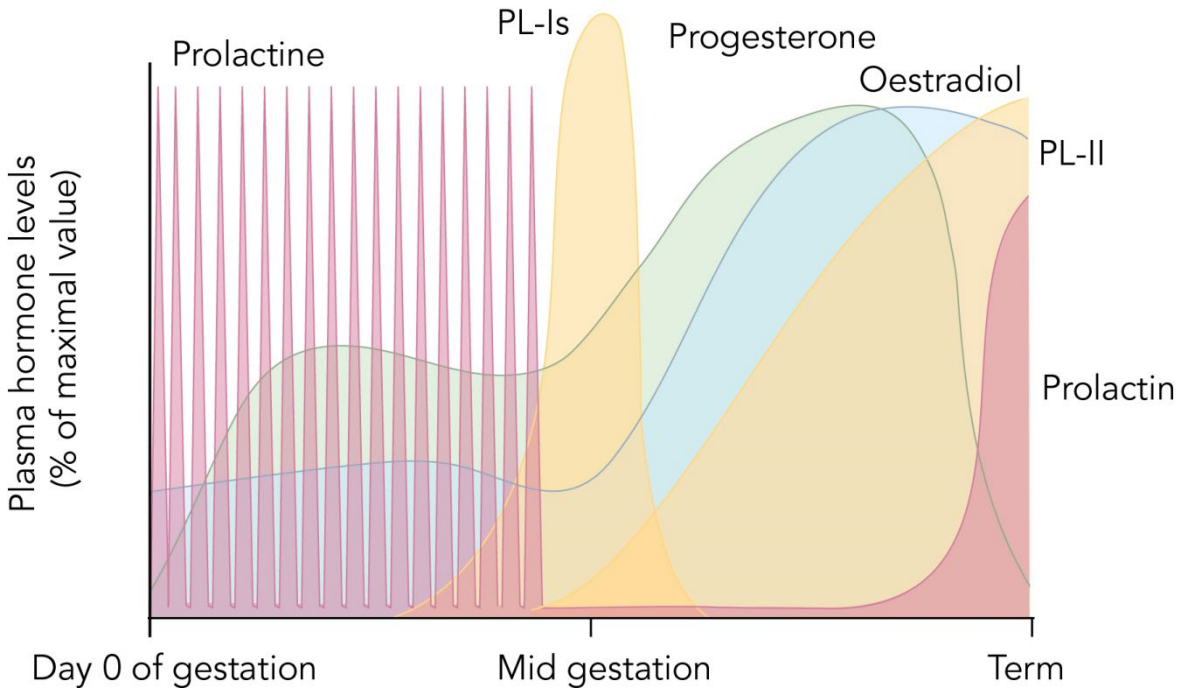


Figure I.2. Schematic representation of hormone profiles along pregnancy in mice. X axis represents time, while Y axis represents plasma hormonal levels, expressed as a percentage of their maximal value along pregnancy. Hormones are shown by colours, being red for prolactin, yellow for placental lactogens Is and II, green for progesterone and blue for oestradiol. Adapted from Salais-López (2017) and Soares (2004).

Although all mammals undergo similar hormonal changes during pregnancy in order to prepare their bodies for parturition and lactation, the expression of maternal behaviour does not depend only on those endocrine changes, and can be influenced by other factors depending on the species.

In rodents, extensive literature in the rat (*Rattus norvegicus*), used for decades as a model for the study of the neurobiology of maternal behaviour, sheds light on the complexity of the onset and maintenance of maternal behaviour. Pregnancy hormones play, indeed, a critical role in the onset of pup care in rats; whereas postpartum female rats show immediate care of their own and even of alien pups, pup-naïve virgin adult females initially avoid pups. But research has shown that there is another factor involved in the expression of maternal behaviour in dams: the pup-derived cues. The research conducted by Beach and Jaynes in the 50's concluded that the retrieving of pups to the nest in lactating rats depends on multisensory stimulation, including at least visual, chemical, tactile and thermal cues, although none of them seems essential by its own. If the female lacked two or more of these sensory cues, the maternal behaviour was impaired, but not abolished (Beach & Jaynes, 1956; Herrenkohl &

Rosenberg, 1972). However, later research showed that chemosensory cues are key stimuli for maternal care in rats, since main olfactory lesions affect the maternal behaviour (Fleming & Rosenblatt, 1974a; Fleming et al., 1992) and the lesions of the vomeronasal system decrease the amount of maternal ano-genital licking displayed by dams towards pups (Brouette-Lahlou et al., 1999). Furthermore, Brouette-Lahlou and colleagues described a chemical compound found in the prepuberal rat preputial gland, dodecyl propionate, that regulates ano-genital licking from dams (Brouette-Lahlou et al., 1991). Hence, pup derived cues seem to play a role in the expression or modulation of maternal behaviour in lactating dams. By contrast, in non-maternal virgin female rats pup derived cues initially induce pup avoidance, very likely due to the anxiogenic properties of distal pup-derived stimuli. However, after a process of "maternal sensitization" consisting of repetitive exposure to pups (Rosenblatt, 1967), virgin females become habituated to the anxiogenic pup-derived distal stimuli and start approaching pups. Then, they get access to their proximal stimuli, pick up the youngs, group them together and begin maternal behaviour similar to that of mothers.

Sensitization studies not only shed light about the role of pup-derived cues, but they were also very useful for the study of other factors influencing the onset of maternal behaviour. For instance, Harding and Lonstein in 2016 described that experience influences the onset of maternal behaviour, since juvenile allomaternal experience reduces the time of maternal sensitization and diminishes pup-induced anxiety in virgin females, maybe by upregulating serotonin (Harding & Lonstein, 2016). Furthermore, the length of the sensitization process, e.g. the number of days of exposure to pups required for a virgin female to start exhibiting maternal care, has been used to further understand the role of specific hormones in facilitating maternal behaviour, after the finding that transfusion of blood from parturient dams shortened maternal sensitization in virgin females (Terkel & Rosenblatt, 1972). In a series of elegant pioneer experiments, the group of Robert Bridges demonstrated that oestradiol plus prolactin (or placental lactogens) following progesterone withdrawal, facilitated maternal behaviour in ovariectomized virgin female rats (Bridges & Ronsheim, 1990; Moltz et al., 1970), confirming the major role of pregnancy-related endocrine changes in maternal behaviour in rats.

In some other mammals, the hormonal dependence of maternal behaviour might be even more pronounced. Ewes, for instance, need to undergo through late-pregnancy changes and parturition-related vagino-cervical stimulation in order to show maternal behaviour towards their lambs. Immediately after parturition, they have a short time window (a few hours) to establish a bond with their offspring by intensely licking them, thus acquiring a memory that

allows taking care of their own lambs while rejecting alien cubs. If the ewe is deprived of contact with her offspring during this period, she is not able to recognize a lamb as her own, and does not take care of any lamb but rudely rejects it instead (Lévy et al., 1996). Non-pregnant ewes can only accept a foster lamb if they are stimulated with late-pregnancy hormones and undergo vagino-cervical stimulation simulating parturition (Keverne et al., 1983). But this particular allomaternal behaviour only occurs in multiparous females, so experience may also be an important factor in the expression of this behaviour.

Previous social experience with youngsters seems to be more relevant for the onset of maternal behaviour in primates than in non-primate mammalian species, which are more dependent on hormonal state (Numan & Insel, 2003). Nonetheless, in primates the hormonal changes associated with pregnancy also influence the onset of maternal behaviour (Pryce, 1996), as it occurs even in humans (Hrdy, 2000). For instance, in rhesus monkeys, experienced multiparous females show immediate maternal behaviour towards alien infants, while nulliparous females do not accept alien infants even after a week of previous daily presentations of the infants (Holman & Goy, 1980). But when those same nulliparous females become mothers, they display full maternal behaviour with their own offspring. Therefore, despite experience being a major factor for the maternal behaviour, hormones are also playing a role. In this regard, Maestripieri showed that, in pigtail macaques, high serum oestradiol/progesterone ratio during pregnancy is correlated to the amount of interactions with infants during the last weeks of pregnancy and, accordingly, oestrogen treatment also increase the interaction with pups in ovariectomized females (Maestripieri & Zehr, 1998). Furthermore, Pryce et al. (1993) performed an operant paradigm in common marmosets showing that maternal motivation towards infants appears to be maximal in their late pregnancy, when oestradiol-progesterone ratio in blood is high, and when virgin females where treated with oestrogens to mimic the late-pregnancy hormone state they also increased their operant maternal behaviour (Pryce et al., 1993).

Maternal behaviour in humans also seems affected by the hormonal state of the mother. The change in oestradiol/progesterone ratio from early to late pregnancy correlates to the attachment with the future infant reported through the last days of pregnancy, and to the attachment they feel during lactation period towards their baby (Fleming et al., 1997). Furthermore, human postpartum mothers rate the infant body odours arousal and valence higher than nulliparous women do, so that pregnancy may be also affecting the sensitivity towards infant cues (Fleming et al., 1993).

In summary, the expression of maternal behaviours can be influenced by many factors, and all of them should be taken into account when studying the maternal brain response towards pups. As we have discussed, hormones are likely the most relevant factors to timely and reversibly boosting maternal behaviours. However, besides inducing behavioural changes, hormones also mediate physiological changes related to motherhood that facilitate the adaptation to being a mother. Due its relevance, those adaptations should also be considered when studying the neurobiology of motherhood and, therefore, they are discussed in the next section.

I.3. Maternal allostasis: adaptation of female physiology to being a mother

For a mammalian female, motherhood constitutes a true challenge that entails a whole bunch of physiological changes beyond those involving behaviour. Thus, pregnancy entails metabolic and physiological adjustments that females must undergo in order to ensure reproduction. During motherhood, female homeostasis has to be maintained while providing nurturing, protection and a good environment for offspring development. Hence, motherhood challenged the concept of homeostasis, leading Sterling & Eyer (1988) to propose the term "allostasis", which they defined as "stability through change", considering that the aim of biological regulation is not the maintenance of the internal milieu, but its modification in order to adjust to different conditions, thus promoting survival and reproduction. In their study about maternal obesity, Power & Schulkin (2012) pointed out that the allostasis vision perfectly fits with what we know about pregnancy and motherhood, meaning there is a "maternal allostasis" that comprehends a great amount of changes in biological parameters in order to "achieve viability through change". Furthermore, they define pregnancy as an "allostatic state" with sustained activation of regulatory systems, which confers pregnant females vulnerability to "allostatic overload". In 2019, Russell & Brunton further developed this idea and explored the cost of pregnancy-related biological changes: the allostatic load of maternal adjustments. Traditionally, allostatic load had been studied in the context of chronic or high levels of stress as a factor for the development of diseases (McEwen & Seeman, 1999). But, according to Russel & Brunton, pregnancy multi-system adaptations can also be seen as a metabolic or regulatory load, therefore it can be considered an "allostatic load".

The assessment of this allostatic load opened a new approach to understand the consequences of pregnancy in the female well-being and its offspring. In fact, some studies

found that in human beings this allostatic maternal load, measured as a combination of different biomarkers, correlate negatively with gestational age at birth, but more importantly it correlated positively with poor sleep in pregnancy and incidence of pre-eclampsia, severely affecting maternal well-being (Hux & Roberts, 2015; Sterling & Eyer, 1988).

One major allostatic mechanism of pregnancy is the metabolic adjustment aiming to gain weight. During pregnancy effectiveness of signals indicating hunger are increased, while satiety signals have less effect that in non-pregnant females. It has been shown that there is an inhibition of the central actions of leptin, which includes satiety regulation in ventromedial hypothalamic nucleus (VMH) via oxytocinergic neurons (Ladyman et al., 2012). This inhibition seems related to increased PRL levels during pregnancy (Ladyman et al., 2010). Furthermore, PRL increase also raises the production of insulin by the Langerhans islets of the pancreas, but the pregnant brain seems resistant to its central anorexic actions. In fact, pregnancy also increases peripheral insulin resistance, and blood glucose raises with the risk of gestational diabetes (Power & Schulkin, 2012). On the other side, the increased levels of oestrogen in pregnancy seem responsible for a pregnancy-specific profile of reduced fatty acid metabolism in the hypothalamus, which stimulates appetite (Martínez De Morentin et al., 2015). Besides, the usual gestational weight gain may increase adipose tissue, which, along with the placenta, secretes humoral factors like leptin, cytokines/adipokines and steroids. The production of cytokines and adipokynes by fat stores may lead to an inflammatory state that can affect the maternal brain. This adjustments can also lead to allostasis error, resulting in a risk of binge eating (Micali et al., 2018).

Another allostatic mechanism of pregnancy is the reduction of neuroendocrine stress response, mediated by an inhibitory action of pregnancy hormones on the maternal hypothalamus-pituitary-adrenal (HPA) axis (Slattery & Neumann, 2008). During pregnancy, the neural circuitry controlling the HPA axis shows hypo-responsiveness to stressors that is maintained during lactation, and this decrease seems related to changes in the hypothalamic paraventricular nucleus (Pa), that influences the production of the adrenocorticotropic hormone (ACTH) by the pituitary gland, which in humans controls the production of cortisol, the stress-related hormone (Russell & Brunton, 2019). This effect in the Pa is apparently related to a reduced secretion of the neurotransmitter vasopressin (AVP) and to an opioid endogenous inhibition of magnocellular oxytocin neurons (but see Douglas et al., 2003), which ultimately depends on the progesterone-related fall occurring by the end of pregnancy.

Therefore, overall, pregnancy entails behavioural and hormonal changes, such as metabolic and physiological adjustments that females must undergo in order to ensure reproduction, and this allostatic load implies a significant cost for the female well-being, which should not be underestimated in the studies of neurobiology of maternal behaviour.

I.4. The mouse as a model for the study of motherhood

Traditionally, the neurobiology of maternal behaviour has been studied in the rat model (*Rattus norvegicus*), since this species presents many benefits as small size, easy manipulation, low cost, short gestational period and numerous litters and pups per litter. But rats difer in many aspects of maternal behavior from primates, such as the lack of spontaneous maternal behaviour and pup-induced anxiety observed in virgin female rats (Maestripieri & Wallen, 1995; Numan & Insel, 2003). By contrast, in laboratory mice strains, pup-naïve virgin females do display nearly spontaneous allomaternal care (Noirot, 1972), thus resembling primates more than rats. Hence, for the last two decades, the mouse model (*Mus musculus*) has been gaining ground in the neuroscience of maternal behaviour, since it presents the same advantages described for the rat model but it better mimics the primate maternal behaviour. In addition, the mouse was the first mammalian species whose genome was sequenced (Waterston et al., 2002). As a consequence, genome bioengineering started in mice long time ago, and there are currently thousands of lines of genetically modified mice and databases that constitute useful tools for the study of brain-behaviour relationships in mice.

Therefore, for this thesis we are using the Swiss-CD1 laboratory strain of *Mus musculus* to further understand the changes occurring in the brain of females during motherhood.

Like in other rodents, maternal behaviours in mice can be classified into two different categories, as shown in Fig. I.3: pup-directed behaviours and non-pup-directed behaviours. Among the pup-directed behaviours we can find the retrieval of displaced pups into the nest (used as a common test of maternal behaviour), nursing pups, crouching over the litter (assuring good thermoregulation) and licking-grooming the pups (providing sensory stimulation – *caressing* - and some hygiene). The non pup-directed behaviours consist of nest building and maintenance, as well as *maternal aggression*, a well-known behaviour shown by lactating dams and pregnant females when their litter or nest is being threatened by an adult conspecific intruder.

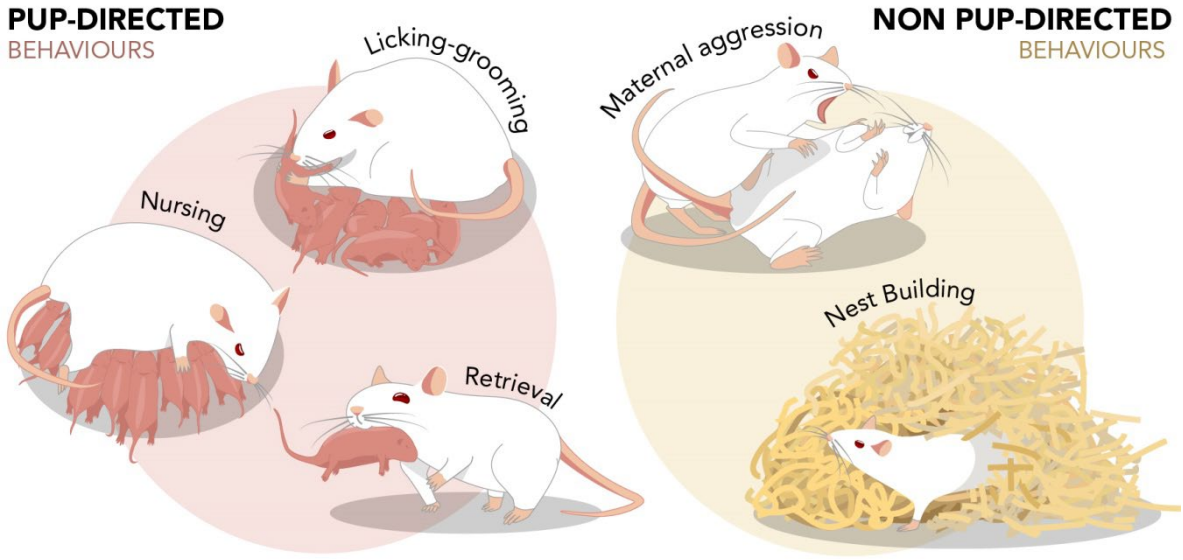


Figure I.3. Components of maternal behaviour in rodents, adapted from Salais-López (2017). Pup-directed behaviours are shown in the pink circle, while non pup-directed ones are shown in the yellow circle.

As commented before, contrary to rats, laboratory nulliparous virgin female mice show spontaneous allomaternal pup-directed behaviours; they quickly retrieve pups that have been scattered in the cage and immediately crouch over those pups and become engaged in intense licking/grooming (Calamandrei & Keverne, 1994; Noirot, 1972). Further research has shown that in all laboratory mouse strains, whether they are outbred (e.g. Rockland-Swiss), inbred (C56BL/6J) or hybrid (129/Sv), most of the virgin females are able to show a quick maternal response towards pups, without a long period of sensitization (Lucas et al., 1998; Numan & Insel, 2003). In our model, the Swiss-CD1 mice strain, allomaternal behaviour appears almost immediately in virgin nulliparous pup-naïve females (Martín-Sánchez et al., 2015b), which lack pregnancy-related hormonal changes. This indicates a key role of pup's sensory cues in the onset of maternal behaviour in Swiss-CD1 strain, which makes it the perfect model for our research in chemosensory signals.

Although pup cues play a significant role in maternal behaviour of laboratory mice, there are other factors involved that we need to take into account in our approaches to understand the mechanisms underlying maternal behaviour. For instance, when compared to wild strains in which virgin female mice usually kill alien pups (McCarthy & Vom Saal, 1985), laboratory mice show an enhanced maternal behaviour that, very likely, has been artificially selected to increase breeding success (Jakubowski & Terkel, 1982; Kohl et al., 2017). This implies a significant role of genetic factors in the maternal responsiveness of females which should not be underestimated. Another factor to consider in the analysis of mouse maternal behaviour is previous experience with pups, since it has been shown that in most strains consecutive

exposures can improve virgin females maternal behaviour (Alsina-Llanes et al., 2015; Stolzenberg & Rissman, 2011).

Of course, along with all the other factors, the role of pregnancy-related hormones in the facilitation of maternal behaviours should be also taken into account in CD1 strain since, as described in section I.2, it has been proved to be a major key for several mammalian species including *Mus musculus* (Bridges, 2020). Pregnancy hormones seem to be responsible for some maternal behaviours that non-pregnant females fail to accomplish. This is the case for the "maternal aggression"; slightly before parturition and after delivery females behaviour towards adults conspecifics switches from sexual or affiliative to aggressive, as they fiercely attack any adult approaching the nest in order to defend their offspring, especially in the first postpartum week (Gandelman, 1972). Virgin females do not ever become aggressive towards conspecifics, not even those who have permanent contact with pups. In our group, we have studied the maternal behaviour of what we call "godmothers" or "co-mothers", which are virgin females that have been cohabitating with a dam through her pregnancy, delivery and postpartum, and therefore also cohabitate with her pups. Godmothers display almost the same pup-directed maternal behaviours as their dam partners, even sometimes more frequently, but they fail to show maternal aggression towards an intruder (Martín-Sánchez et al., 2015a). It seems that the mere presence of pups is not enough to engage in more risky or demanding maternal behaviours, and only the hormonal changes related to pregnancy prepare female to display such highly motivated behaviours. Even the retrieval of the pups, which godmothers perfectly perform in standard conditions, is affected when these nulliparous females face a physical barrier that forces them to climb back and forth to collect the pups (Salais-López et al., 2021). In those cases, dams continue to display retrieval while virgins avoid the extra effort and godmothers show little motivation. Our research with godmothers ties in with previous literature pointing out that pups are a powerful reward for dams, but not for virgins, in both rats and mice (Hauser & Gandelman, 1985; Mattson et al., 2001). Thus, apparently, in mice, maternal behaviours that require an extra effort depend on pregnancy hormones. Hence, pregnancy-related variations in progesterone, oestradiol, prolactin and placental lactogens elicit changes in some circuits of the brain involved in the control of socio-sexual behaviours (which include maternal behaviours) and motivational aspects of behaviour, thus leading to an increase in motivated maternal behaviours, the so-called maternal motivation. However, it is not clear yet how and where in the brain these hormone-mediated changes occur, neither how these changes affect the activity of those brain centres and circuits to increase the salience of pups and modulate behaviour. Although our group has recently

analysed some of the effects of pregnancy hormones in the brain (Salais-López et al., 2017, 2018, 2021), there is a surprising lack of information on how the activity of the brain in response to pup-derived stimuli may change during pregnancy. Therefore, one of the objectives of this doctoral thesis is to explore how the mouse pregnant brain responds to pups and whether that response differs from that of the virgin female brain.

I.5. Chemosensory communication in mice: the role of chemical cues in maternal behaviour

As it has been described in the previous section, along with hormonal changes and experience, pup derived cues play a major role in eliciting maternal behaviour in CD1 mice. Although pups display a wide range of stimuli (vocalizations, shape, temperature), chemosensory cues are likely to be involved in eliciting maternal behaviour, since rodents are macrosmatic animals and rely on chemosensory detection for many behaviours.

In fact, chemosensory signalling between conspecifics has been largely studied in mice, which are usually the chosen model for this purpose (Tirindelli et al., 2009). Traditionally, the term used for social-communication-related chemical cues is "chemosignal". More specifically, the term *pheromone* applies to specific chemical compound(s) secreted or excreted by an individual that elicits a stereotyped response in its conspecifics (Karlson & Lüscher, 1959), either behavioural (trigger pheromones) or endocrine/developmental (prime pheromones). For instance, the detection of pheromones allow mice to recognize potential partners (Roberts et al., 2010) or even illness in conspecifics (Lanuza et al., 2014). Moreover, some of the most known effects in hormonal state in female mice are dependent on pheromones, as the Lee-Boot effect (modification of the oestrous cycle when females are grouped together; Ma et al., 1998), and many of them are dependent on male urine detection, as the Bruce effect (preventing implantation in a recently mated female; Bruce, 1960), the Whitten effect (restoring and synchronizing the oestrus cycle; Whitten, 1958) or the Vandenbergh effect (early onset of puberty; Vandenbergh, 1969). The detection of compounds on male urine has been also associated to the display of some socio-sexual behaviours, as sexual attraction to males in virgin females (Martínez-Ricós et al., 2007; Moncho-Bogani et al., 2002; Roberts et al., 2010), aggression/mating in males (Leypold et al., 2002; Stowers et al., 2002) and the onset of maternal aggression in maternal females (Martín-Sánchez et al., 2015a).

Taking into account the major role of chemosignals in socio-sexual behaviours in mice, it is likely that pups may produce chemosensory cues to become recognizable to their conspecifics

and elicit an innate caring response, particularly in females. This is, indeed, one of the hypotheses of this thesis; the existence of a pup-derived pheromone involved in the maternal behaviour in mice. Further in this work we will explore this hypothesis and the detection of pup chemosignals by maternal chemosensory systems. Hence, in this section I will briefly introduce the mice chemosensory systems of *Mus musculus* and the role of each one of them in the chemosensory function, and finally, I will concisely expose what is known so far about the role of chemosignals in maternal behaviour of mice.

I.5.1. Chemosensory systems: main and accessory olfactory systems

Terrestrial vertebrates have four sensory organs for the detection of chemical compounds in their environment: the main olfactory epithelium (MOE), the vomeronasal organ (VNO), also called Jacobson's organ (Trotier & Døving, 1998), the septal organ of Masera, and the Grueneberg ganglion (Fig. I.4). Traditionally, the study of chemosensory function has been centred in the MOE and the VNO, which define two different olfactory systems. The first one is the main olfactory system (MOS), consisting on the MOE and its projections to the main olfactory bulb (MOB), and it is present in all terrestrial vertebrates. The second one is known as the "accessory olfactory system" (AOS) because it is absent or unfunctional in some animals, including humans and other primates (Stoyanov et al., 2018; Witt & Wozniak, 2006). The AOS involves VNO and its projections to a specialized region of the olfactory bulbs known as the accessory olfactory bulb (AOB) (Fig. I.4).

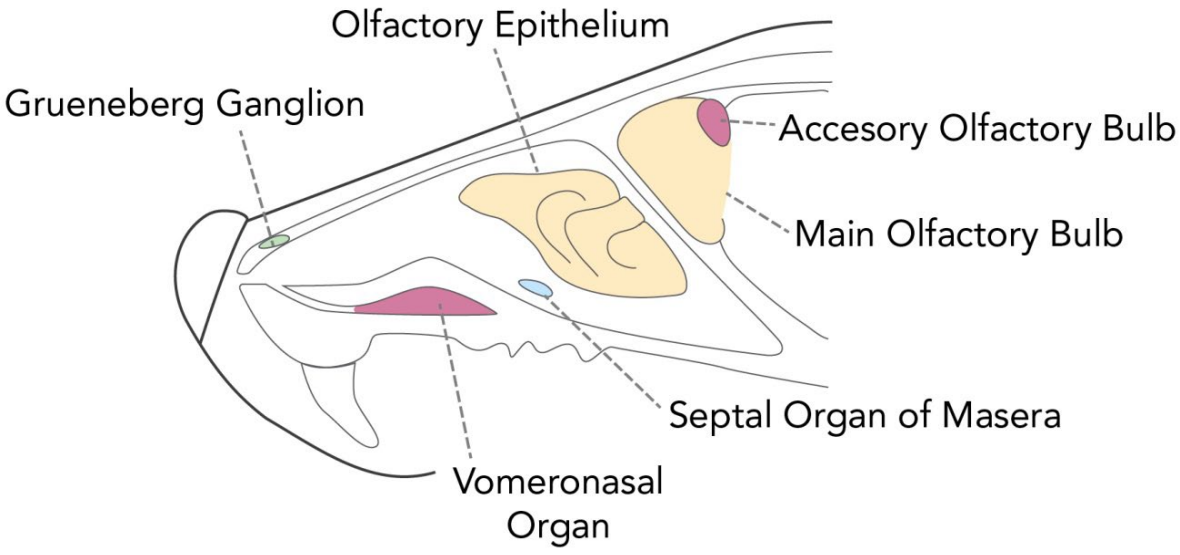


Figure I.4. Schematic representation of the main olfactory organs and the olfactory bulbs in *Mus musculus.*

Rodents are macrosmatic animals, meaning that they rely on their olfactory systems for many behaviours and, consequently, display enlarged olfactory organs and systems. Thus, the structure and function of their olfactory organs and their central pathways have been widely studied, especially in the mouse. In this section, I will briefly present the main characteristics of the two principal chemosensory systems in our animal model, *Mus musculus*, and the main projections of both MOS and AOS.

A. The main olfactory system

The MOE of mammals consist of a few million olfactory sensory neurons (OSNs) lining the cartilaginous lamellae in the posterior nasal cavity, called turbinates. OSNs are bipolar cells that project their single dendrite to the epithelial surface, where their abundant and long cilia penetrate the nasal mucus allowing contact with the environmental odorants. The interaction between OSNs and odorants depends on olfactory receptors (ORs) in the ciliary membrane. These ORs constitute a large family of G-protein-coupled receptors that are considered generalists: each receptor binds a wide range of compounds, while a single odorant can be recognized by several OR types. There are about 1300 ORs genes, but each OSN express only one of them.

In mice, which have been investigated in depth, the MOE essentially expresses genes from the olfactory receptor family (OR) detecting general odours, but some cells also express trace amine-associated receptors (TAAR) and transient receptor potential channels of the TRPM5 family. The OR family includes the OR37 subsystem, which seems involved in detection of predator odours (Brennan, 2018). In the ventrolateral zones of the olfactory epithelium there is a subset of olfactory sensory neurons expressing the ion channel TRPM5. Their axons project to the ventral region of the MOB which is related to responses to social chemosignals, although the specific ligand(s) detected by TRPM5-expressing cells is/are still unknown (Brennan, 2018; Tirindelli et al., 2009). Trace amine-associated receptors (TAARs) are a family of G protein-coupled receptors, which are expressed in the olfactory epithelium at similar levels as OR, without co-expression with OR. This suggests that TAARs are expressed in a subpopulation of neurons recognizing several amines in the mouse urine which may be giving information about the gender and social status of an individual (Tirindelli et al., 2009). TAARs-expressing neurons are also present in the Grueneberg ganglion.

Additionally, a subset of olfactory neurons in the dorsal MOE presents the olfactory-specific Guanylyl Cyclase Type D Receptor (GC-D). In mice, they have been proven to respond to some urine and intestinal peptide hormones, allegedly related to hunger, satiety or thirst (Tirindelli

et al., 2009). Some literature relates GD-D function to the detection of environmental levels of CO_2 and O_2 (Brennan, 2018).

In the olfactory bulb, the axons of those MOE neurons expressing the same OR converge onto an individual glomerulus that, accordingly, is activated by multiple odorants. This organization creates a chemospecific map in the MOB which encodes the combinatorial processing of molecular entities that ultimately leads to odorant recognition (Breer et al., 2006).

B. The accessory olfactory system: vomeronasal organ and central pathways

In mice, the VNO consist of a blind tube located in the nasal septum which contains nasal mucus and houses a crescent-shaped sensory epithelium in its medial wall, and a large blood vessel running lateral to the lumen (Breer et al., 2006). Since the vomeronasal sensory neurons (VSNs) are enclosed in this blind-ending tube, a pumping mechanism is needed to empty and fill the the vomeronasal lumen (Meredith, 1994). This is achieved thanks to repetitive constriction of the vomeronasal blood vessel, a pumping mechanism (vomeronasal pumping) that draws air and particles dragging even non-volatile compounds, thus allowing individuals to detect them along with volatile molecules.

The vomeronasal epithelium has a layered organization, defined by two main sensory cells subpopulations characterized by expressing distinct membrane receptors and different intracellular transduction cascades (Tirindelli et al., 2009). The apical layer is defined by sensory cells expressing V1R receptors, which are coupled to the G protein subunit Gαi2, and the axons of these VSNs project to the anterior part of the accessory bulb. These apical V1R-expressing cells detect small hydrophobic volatile molecules with a high sensitivity and specificity for individual compounds, contrary to the OSNs of the main olfactory epithelium. On the other side, the basal layer of the VNO epithelium (VNE) consists of sensory cells expressing V2R receptors that are strictly coupled to the G protein subunit Gαo. These basal V2R-expressing cells project to the posterior part of the accessory bulb, and detect peptidic, nonvolatile components, such as Major Histocompatibility Complex (MHC) peptides and major urinary proteins (MUPs) (Breer et al., 2006; Tirindelli et al., 2009). A V2R receptor has been also detected in the Grueneberg ganglion, which makes it a good candidate for alarm signal detection since Grueneberg ganglion seems to be related to predator odours and to alarm pheromones, inducing freezing behaviour (Fleischer, 2021; Tirindelli et al., 2009).

Furthermore, VNE also contains apical cells expressing the formyl peptide receptor family (FPR), also G protein-coupled receptors, which apparently play a role in the defence against

immunological diseases, or even transmitting information about the presence of pathogen chemosignals in conspecifics urine and faeces, e.g. formyl peptides (Brennan, 2018; Tirindelli et al., 2009).

Along with these subpopulations, the VNE also contains sensory neurons expressing olfactory receptors that are typically expressed in the MOE (Breer et al., 2006; Nakahara et al., 2016), and at least some of them project to the anterior region of the olfactory bulb (Lévai et al., 2006).

I.5.2. Projections of the MOS and the AOS

As described above, in *Mus musculus* MOE and VNO project to the main olfactory bulb (MOB) and the accessory olfactory bulb (AOB) respectively, which are separated zones of the olfactory bulbs that have a similar organization but project to different brain areas. The Grueneberg ganglion also innervates the MOB, specifically a necklace-shaped chain of glomeruli surrounding the AOB (Matsuo et al., 2012), but the coding of the Grueneberg ganglion input in the MOB has not been completely assessed yet (Fleischer, 2021).

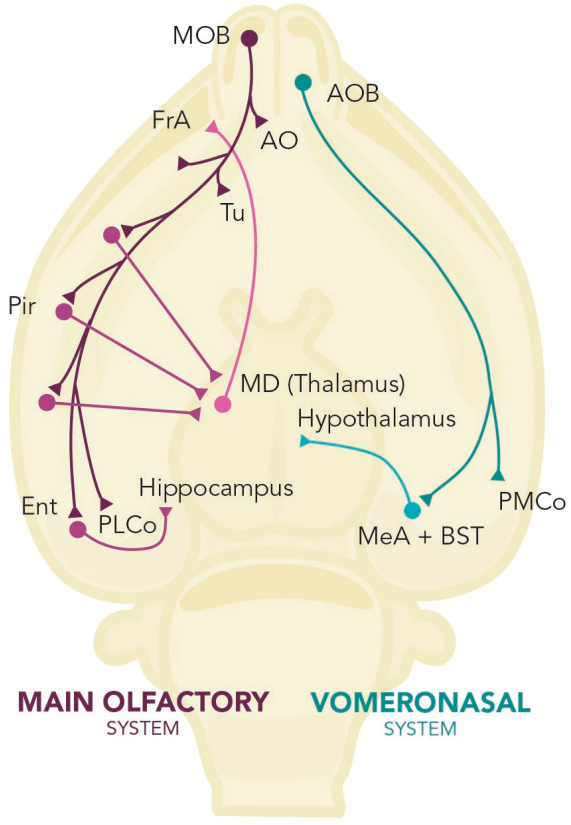


Figure I.5. Neuroanatomical pathways from the MOB (left) and the AOB (right) delineating the main (purple) and accessory (blue) olfactory systems respectively. Neuronal pathways are represented by individual cell bodies (circles), axons (lines) and synaptic terminals (triangles). Nomenclature from Paxinos & Franklin (2004). In the main olfactory system; AO: Olfactory anterior nucleus, Ent: entorhinal cortex, FrA: frontal association cortex, MD: mediodorsal thalamic nucleus; MOB: Main Olfactory Bulb, Pir: piriform cortex, PLCo: posterolateral cortical amygdaloid nucleus, and Tu: Olfactory tubercle. In the vomeronasal system; AOB: Accessory olfactory Bulb, BST: bed nucleus of the stria terminalis, MeA: medial amygdaloid nucleus and PMCo: posteromedial cortical amygdaloid nucleus.

The MOB mainly projects to the anterior olfactory nucleus, the piriform cortex (Pir), and some cortical amygdaloid nuclei (Fig.I.5). On the other hand, the AOB mainly innervates the medial amygdala (Me), the posteromedial cortical (PMCo) amygdaloid nucleus and parts of the

posteromedial bed nucleus of the stria terminalis (BST) (Pardo-Bellver et al., 2017) (Fig. I.5). Although the pathways arising from MOB and AOB had been traditionally described as parallel, reaching non-overlapping areas, in the first decade of the millennia several studies found that, along with olfactory and vomeronasal areas, there are secondary chemosensory centres receiving convergent inputs from both olfactory bulbs (Martinez-Marcos, 2009)(Mohedano-Moriano et al., 2012). This, together with further convergence due to connections between the MOS and AOS, ensure common processing of olfactory and vomeronasal stimuli in several brain loci including the olfactory bulbs, several nuclei of the amygdala (Cádiz-Moretti et al., 2013, 2016; Gutiérrez-Castellanos et al., 2010) and of the hypothalamus (Mohrhardt et al., 2018).

I.5.3. The role of the main and accessory olfactory systems in olfactory function

As described above, the last 20 years of research have challenged the "dual olfactory hypothesis" proposed by Scalia and Winans in 1975, which considered that the MOS and the AOS were anatomically and functionally segregated systems, and detected different sets of chemosensory cues that were related to different behaviours. This traditional view proposed that the MOS detects multiple airborne volatile chemical compounds, thus mediating chemical analysis of the environment for general purposes (e.g. foraging), whereas the vomeronasal system, which is more suitable for detecting large non-volatile compounds, was typically associated with the specific detection of biologically relevant social cues as pheromones or kairomones (e.g. predator-derived chemosignals). Nowadays, several studies point to a more complicated scenery. For instance, there are innate behaviours strictly dependant on the main olfactory system, as nipple search in rabbit pups (Hudson & Distel, 1986). A particularly interesting case is sexual attraction towards male in female mice, which seems to depend on the detection of a non-volatile compound (Moncho-Bogani et al., 2002) by the vomeronasal system (Martínez-Ricós et al., 2008) that is reinforcing for females (Martínez-Ricós et al., 2007), and was identified as a male urinary protein called darcin (Roberts et al., 2010). So, apparently, this particular behaviour matches the traditional view. But the tricky part is that the main olfactory system also plays a facilitator role in attraction towards males. Social experience can lead to odorant associative learning, so attraction towards male chemosignals can occur after the removal of the VNO, relying on the detection of secondarily attractive male odorants detected by the MOS (Moncho-Bogani et al., 2002). In this regard, in 2008 Ramm et

al. showed that, although females preferred male to female urine, attraction to airborne urinary volatiles only occurred if females had contacted the urine of that specific male before.

In brief, literature is conflicting with the idea of functional separated systems, since there seem to be pheromonal effects dependant on olfactory but not vomeronasal function (Wang et al., 2006), volatiles can be perceived by both VNO and olfactory epithelium, and the MOB and the AOB are simultaneously activated by odours and pheromones (Martinez-Marcos, 2009; M. Spehr et al., 2006b). For instance, the Major Histocompatibility complex peptide ligands (MHC), non-volatile cues related to individual recognition in mice, can be detected by both the MOE and the VNO (Spehr et al., 2006a). In fact, in 2017 Pardo-Bellver et al. suggested that exposure to chemical stimuli, whether conspecific-derived or non-social odorants, involved the synchronicity of the MOB and the AOB, likely due to several factors including coupling between sniffing and vomeronasal pumping.

In 2006, Spehr et al. reviewed the parallel processing of social signals by the MOS and the AOS. Briefly, he showed that the MOE and the VNO can be simultaneously activated by the same compounds, but their sensory neurons use different signalling pathways and may be using different strategies for coding information. Furthermore, the simultaneous processing of the same ligand by both systems is not redundant, since the behavioural outcomes seem to differ.

Taken together, evidence points to an integration of the information carried by both kinds of chemical stimuli in higher centres of the brain, such as amygdala and hypothalamic nuclei, which are highly dependent on hormonal regulation.

I.5.4. The role of chemosignals on maternal behaviour

Pups display a variety of features which could induce the onset of maternal behaviour in females, like shape, vocalizations, specific movement patterns or odours. As expected from a macrosmatic species, interaction with pups activates the olfactory processing areas along with maternal behaviour-related nuclei in female mice, e.g. the medial preoptic area, but depletion of noradrenaline in the olfactory bulbs does not abolish the activation of this nucleus of the maternal brain (Calamandrei & Keverne, 1994). Some literature points out that maybe different type of cues act synergistically in order to elicit maternal behaviour, especially pup vocalizations and chemosensory cues (Okabe et al., 2013). Some researchers hypothesized that each type of stimulus may be facilitating a different aspect of maternal behaviour. For instance, Noirot (1969) found that dams previously exposed to olfactory cues spent more time licking the pups, while those previously exposed to auditory cues spent more time building

their nest. Other studies focused on the possible interactions between both stimuli, as pup odours apparently modulate the response of the primary auditory cortex to pups vocalizations (Cohen et al., 2011). However, some research points to chemosignals as the principal cues fo pup retrieval in a Y maze, and vocalizations as mere facilitators of the behaviour (Smotherman et al., 1974), e.g. ultrasonic vocalizations alone do not elicit pup retrieval (similar approaches to the arm containing a loudspeaker playing pup vocalizations than to an empty arm).

During gestation females undergo major hormonal changes that may lead to modifications in their chemosensory function. For instance, oxytocin has been proven to modulate VNO sensory activity or brain sensory processing of olfactory/vomeronasal cues, thus influencing socio-sexual behaviour (Liu et al., 2017; Nakahara et al., 2020), while steroids can rapidly modulate responsiveness in olfactory receptor neurons (Kanageswaran et al., 2016). There is also abundant research about the effects of lesions of chemosensory organs on maternal performance, which suggest that chemosensory cues are the main input for the onset of maternal behaviour in mice. For instance, virgin or postpartum female $AC3^{-/-}$ mutants, which do not express type 3 adenylyl cyclase involved in olfactory transduction, fail to retrieve pups, to build a nest and to attack intruders (Wang & Storm, 2011). Olfactory bulbectomy diminishes or even eliminates maternal behaviour (Gandelman et al., 1972; Gandelman et al., 1970; Sato et al., 2010; Zarrow et al., 1971), even causing dams to systematically eat their own pups (Gandelman et al., 1971a, 1971b). Central and peripheral anosmia disrupt maternal behaviour (Vandenbergh, 1973), and peripheral anosmia induced by $ZnSO_4$ intranasal administration in primiparous females induces pup-killing of the litter, although previous maternal experience prevents this from happening (Seegal & Denenberg, 1974).

On the other hand, vomeronasal impaired $Trpc2^{-/-}$ females, which lack the Trpc2 ion channel expressed in sensory neurons of the VNO crucial for sensory transduction, also show a mild deficiency in maternal care (Kimchi et al., 2007), with mutant dams spending significantly reduced time in nest (in contact with pups) as compared to wildtype females. Furthermore, the surgical removal of the VNO in adult females also resulted in a mild but significant reduction in maternal retrieval (Lepri et al., 1985). But vomeronasal stimuli seem to be especially critical for some non-pup-directed maternal behaviours such as nest defence. Maternal aggression towards intact male intruders is more intense than towards castrated males, and it depends on the vomeronasal detection of the darcin, a male urine protein acting as sexual pheromone, as it is also related to sexual attraction in females (Martín-Sánchez et al., 2015a). Furthermore, knockout (KO) mice for Trpc2 show no aggression towards intruders at all, either inter-male or maternal aggression (Hasen & Gammie, 2009; Hasen & Gammie, 2011;

Kimchi et al., 2007; Leypold et al., 2002). Null mutants for Gαo protein, coupled to the sensory transduction of V2R receptors of the VNO (detecting urinary proteins including darcin) also show lack of aggression (Chamero et al., 2011; Leinders-Zufall et al., 2014; Norlin et al., 2003). This clearly indicates that maternal aggression critically depends on the detection of darcin through V2R receptors in the VNO of females.

Interestingly, in *Mus musculus,* vomeronasal signalling seems also related to paternal behaviour. Males only display parental behaviour after mating and cohabiting with the dam during pregnancy and lactation (McCarthy & Vom Saal, 1986). In contrast, virgin males are typically aggressive towards pups, and vomeronasal signalling is crucial for this type of aggression (Isogai et al., 2018; Nakahara et al., 2016; Wu et al., 2014). Apparently, when a male become paternal, the shift from attack to parenting is facilitated by the down-regulation of the activation of vomeronasal system (Tachikawa et al., 2013). In fact, experimental ablation of the VNO makes virgin males paternal.

In spite of all this research, there is still a surprising lack of evidence of whether changes in sensory processing in the olfactory and/or vomeronasal systems, specifically in the perception of pup-derived chemical cues, are part of the hormone-induced changes that facilitate motivated maternal behaviours in female mice. One of the aims of this thesis is to explore the possible existence of a pup-derived pheromones and to study how maternal chemosensory systems respond to pups, compared to pup-naïve virgin females systems.

I.6. Neurobiology of maternal behaviour: How pup stimuli and hormones activate the maternal brain

So far, we have described that the expression of maternal behaviours depends on several factors, which vary between species. For these factors to induce a behavioural response, they have to somehow affect the activation of brain nuclei that are, ultimately, responsible for behavioural outcomes, which in the case of maternal behaviours would be the “core circuit for maternal behaviour” illustrated in Figure I.1. Identifying this core circuit is one of the aims of this Doctoral Thesis.

After her pioneer two-decade research on the neuronal substrate of social behaviours, Sarah Winans Newman (1999) coined the term socio-sexual brain network (SBN) to name a group of 6 brain nuclei which are profusely and reciprocally interconnected (thus constituting the nodes

of a true neural network), express receptors for sexual steroids (Simerly et al., 1990), and are innervated by or contain nonapeptidergic cells (Fig. I.6; Newman, 1999). The SBN is highly conserved among vertebrate species (Numan & Insel, 2003). The SBN, schematized in Fig. I.6, includes the medial extended amygdala (MeA) including the medial nucleus of the amygdala together with the posteromedial bed nucleus of the stria terminalis (BSTMP), the lateral septum (LS), the midbrain periaqueductal gray (PAG), the ventromedial hypothalamus (VMH), the anterior hypothalamus (including the paraventricular nucleus, Pa) and the medial preoptic area (MPO). As Newman describes, there is not a discrete function for each node, but the activity of the full network determines the probability of each possible behavioural outcome. It is possible that pregnancy hormones change the interaction with pups by altering the socio-sexual brain activation pattern in order to elicit maternal behaviours (pup care, maternal aggression), instead of other social interactions, in response to appropriate social stimuli.

In spite of this view on the neuroscience of social behaviours, apparently there is a certain consensus in the literature attributing a pivotal role to a single one of the nodes of the SBN, the medial preoptic area (MPO), in the control of the expression of maternal behaviours. Evidence supporting this view relies on different kinds of studies. Thus, fibre-sparing lesions of the MPO and adjoining ventral BST (BSTv) in rats resulted in a sharp decrease in maternal behaviours (Numan et al., 1988; 1990). These data also lead Numan to suggest that projections from the MPO region to the ventral tegmental area (VTA) (Numan & Numan, 1996) would mediate motivational aspects of maternal behaviours.

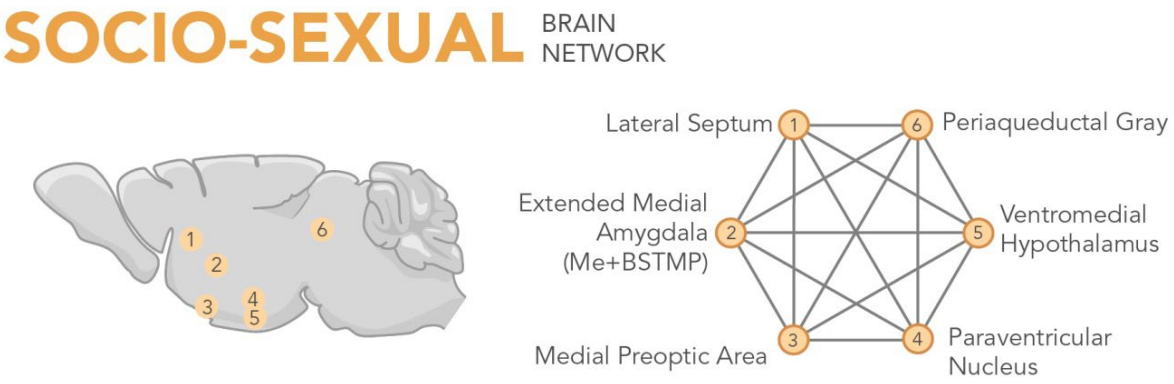


Figure I.6. Main nuclei in the socio-sexual brain network adapted from Newman, (1999).

In parallel, M. Numan et al. (1990) performed knife cuts dorsolateral to the MPO and the adjoining ventral aspect of the bed nucleus of the stria terminalis (BSTv), but not involving the region itself, and observed similar devastating effects on maternal behaviour. This was interpreted as an evidence of the critical role of unknown neural inputs to the MPO/BSTv

reaching the nucleus from the stria terminalis and amygdala in the expression of maternal behaviours. But this can be alternatively interpreted as a proof of the role of a circuit or network (such as the SBN), instead of a single brain centre, in the control of maternal or other social behaviours, as proposed by Winans Newman (1999).

More recent studies on the neural activity in response to pup stimuli also highlight a role of the MPO in maternal behaviour. Using cFos expression, Tsuneoka et al. (2013) showed that a central region of the MPO, where GABAergic and/or peptidergic neurons are abundant, is activated during exposure of females to pups (Tsuneoka et al., 2013), although activity does not change between virgins and postpartum dams. In 2014, Wu et al. (2014) described that, apparently, parenting specifically activates a population of galaninergic cells (Gal+) of the MPO and specific lesions or inactivation of this cell population abolished parental care in both females and males. By contrast, optogenetic activation of these Gal+ MPO cells in males suppressed attack and promoted pup grooming and care. But recent work has described that there are two different populations in the MPO related to parental behaviours; neurons expressing oestrogen receptor alpha (Esr1+), related to pup retrieval and sexual behaviours, and the Gal+, related to pup grooming and parental motivation (Fang et al., 2018; Kohl, 2020; Wei et al., 2018; Zilkha et al., 2021).

In 2017, Okabe et al. demonstrated that there is an enhanced activity of the MPO region in virgins exhibiting maternal behaviour due to previous repeated exposure to pups, as compared to virgins with low exposure to pups, and that this facilitation of allomaternal behaviour seems to be related to oxytocinergic organizational role in the medial and lateral preoptic area (Okabe et al., 2017). In this regard, several studies in rodents also endorse this key role of the MPO in maternal behaviours. According to Numan et al (2016), pregnancy hormones would increase the response of MPO/BST to oxytocin (OXT) and to inputs from the cortex conveying pup stimuli (Numan & Young, 2016). In fact, injections of OXT, AVP or opioid receptors antagonists in the MPO reduced maternal behaviours, while injections or implants of oestradiol, PRL or placental lactogens into the MPO facilitate maternal responses (Bridges & Freemark, 1995; Brown et al., 2017; Gammie, 2005). In mice, our group has also shown a significant correlation between PRL signalling in MPO and maternal motivation, although this correlation also occurs in other socio-sexual nuclei (Salais-López et al., 2021). Furthermore, some literature suggests that a weak maternal behaviour can be due to impaired serotonegic neurotransmission in the MPO and BNST (Dulac et al., 2014).

In 2018, Kohl & Dulac proposed an entire circuit underlying parental behaviours with the Gal+ neurons of MPO as the pivotal elements on which all neural and endocrine stimuli would converge and from which the pathways controlling all responses related to maternal behaviour would emerge (Kohl et al., 2018; Kohl & Dulac, 2018). An idea that may be derived from this vision is that the mere pattern of activation in the MPO could explain all behavioural responses towards pups, but this challenges Newman's idea of the socio-sexual brain network. According to Newman, the behavioural response towards conspecific stimuli would depend on the pattern of activation of several socio-sexual nuclei acting as a network, meaning the activation of a single node (e.g. the MPO) would not explain the resulting behavioural response (Newman, 1999).

In this regard, Olazabal et al. (2013) analysed the literature on the maternal neural substrate in different species, and proposed a hypothetical model for the circuit underlying facilitation and inhibition of maternal behaviour. This hypothetical model includes mechanisms mediating a temporary switch in behaviour, which would depend on the species but also on the physiological and social context. Although they emphasize the critical role of the MPO, they consider that its function changes under different physiological and social conditions, and highlight the implications of other brain areas, such as cortical regions and the mesolimbic dopaminergic system.

This systemic approach, which avoids focusing just on the MPO but gives more value on the role of other nuclei, has been useful to unravel the different parental responses towards pups. For instance, the lactating dam's engagement in risky behaviours like maternal aggression or pup retrieval in a novel, threatening environment, is probably facilitated by the attenuation of anxiety and the decreased stress in risky situations, which has been described in several species during motherhood. As described in section I.3, Slattery & Neumann (2008) proposed that the mechanism underlying this behavioural change is a decreased activity of the paraventricular nucleus (Pa), which leads to a sustained inhibition of the HPA axis. In fact, several studies in rats suggest that maternal aggression is particularly related to OXT and AVP signalling in different brain nuclei, including Pa, the central amygdala (CeA), the lateral septum (LS) and the bed nucleus of the stria terminalis (BNST) (Bosch, 2013). Although in mice the OXT pathway does not seem particularly involved in the anxiety decrease in maternal behaviour (Douglas et al., 2003), in rats it has been proposed as a modulator of maternal motivation and olfactory preference towards pups (Munetomo et al., 2016). Traditionally, central OXT effects during motherhood include modulation of nurturing behaviour in several species (Burbach et al., 2006), although the role of oxytocin in mice seems more related to the initiation but not

the maintenance of maternal behaviour, since oxytocin receptor KO female mice display a higher rate of pup abandonment than normal female mice, but once they achieve to initiate maternal care, they do not show further impairments (Rich et al., 2014).

In the same line, systematic approaches have shed light on the neurobiological basis of the increase in motivation towards pups in pregnant females, which has been described in section I.4 for female mice. Studies in rats have shown that this reinforcement of pups as salient stimuli depends on a "motivational" dopaminergic system (Fig. I.7), which includes the nucleus accumbens (Acb), the basolateral amygdala (BLA), the ventral pallidum (VP) and the ventral tegmental area (VTA) (Numan & Young, 2016). The VTA contains the cell bodies of dopaminergic cells which mainly project to Acb (core, AcbC; and shell, AcbSh) and prefrontal cortex (PFC; specifically the limbic cortex), and this pathway has been commonly recognised as the brain reward system (Kelley & Berridge, 2002; Wise, 2002). In the maternal context, it has been described that the MPO projections to VTA may increase the activity of the tegmental-striatal pathway in response to pups (Fang et al., 2018; Numan & Smith, 1984; Numan & Stolzenberg, 2009; Numan & Woodside, 2010; Numan & Young, 2016). In the Numan's model, this MPO-mediated dopamine release in the targets of the tegmento-striatal pathway would activate the GABAergic input on VP (from Acb), eliciting higher excitability of the VP to glutamatergic inputs from BMA/BLA and PFC, conveying information on pup-derived stimuli. This would facilitate that sensory cues from pups elicit goal-directed appetitive maternal responses (Numan & Woodside, 2010). This model is supported by experimental evidence indicating that maternal proactive responses need the activation of the dopamine system (Pereira & Morrell, 2011). Moreover, electroencephalographic recordings in the rat indicate that activation of the VTA in response to sniffing pup-derived chemical cues increases in lactating females (Hernandez-Gonzalez et al., 2005), whereas lesions of VTA projections or dopamine depletion impair the pup retrieval in post-partum rats (Hansen et al., 1991b, 1991a). Otherwise, lesions of the ventral striatum, and specifically in AcbSh, induce neglect of pups by dam rats (Hansen, 1994; Li & Fleming, 2003), and dopamine receptor blockade by infusion of cis-flupenthixol (FLU) in the Acb impair pup retrieval and pup licking in dams (Keer & Stern, 1999).

Overall, these studies suggest a key role of the dopamine brain regions in maternal motivated behaviour toward pups, being the hormonal state and the maternal experience relevant to dopamine system functioning (Afonso et al., 2008, 2009). Although, the majority of studies on motivation and parental behaviour have been performed in female rats, recent research in male mice also sheds light on the complexity of the responses towards pups, and suggest that

a circuit including the MPO, the VTA, the Acb and the VP is also involved in paternal behaviour (Zhong et al., 2014).

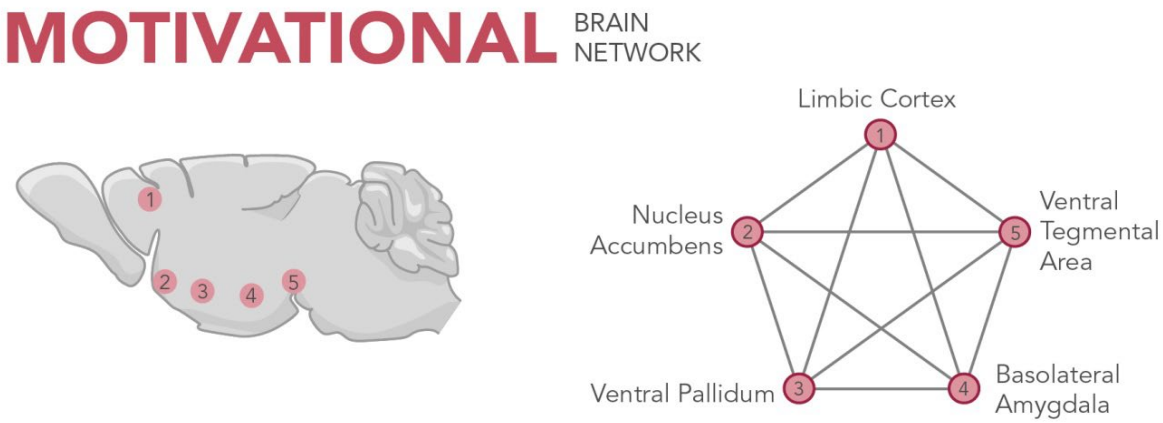


Figure I.7. Main nuclei in the motivational brain network. Adapted from (Newman, 1999).

So, although there are claims for a specific role of the MPO in the control of parental behaviours, the possible interplay of this nucleus with other nuclei of the SBN (making up a true network) and with centres controlling motivational aspects of behaviour, needs to be investigated to fully understand the neural and neuroendocrine mechanisms of maternal behaviours. This is, indeed, one of the aims of this work.

I.7. Objectives and structure of the Doctoral Thesis

The goals of the present work are:

Objective 1. To analyse the response of females to pup-derived odours and the changes occurring during motherhood.

To do so, in **Chapter 1** we expose females, postpartum dams, pup-naïve virgins and pup-experienced virgins, to different kind of substrates containing pup-derived chemosignals. We assess the best way of obtaining neonatal pup-soiled material and we explore the female chemo-investigational response towards the collected stimuli.

Objective 2. To analyse how pregnancy changes the perception and processing of pup chemosensory cues.

To do so, in **Chapter 2** we expose late-pregnant females (prepartum dams) and virgin females, both pup-naïve, to either 3-day old pups or to control non-social objects of about the same

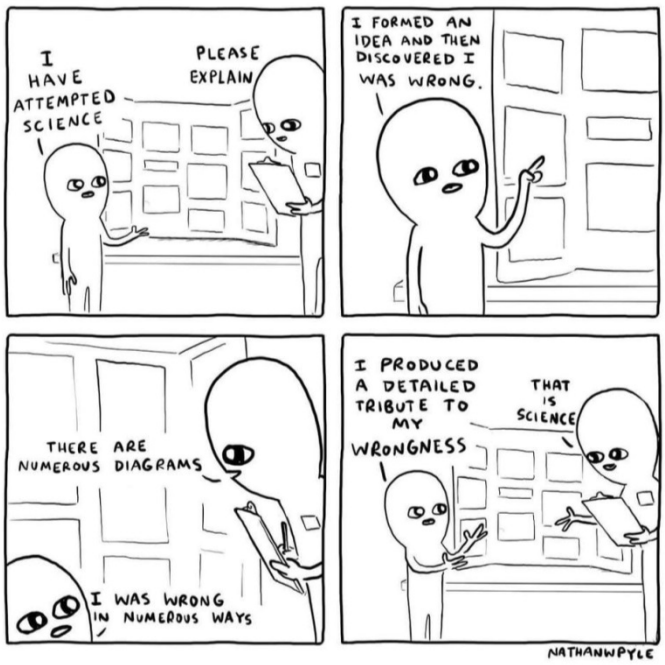
size, e.g. plastic buttons. We measure different aspects of maternal and chemo-exploratory behaviour of the females exposed to pups, to assess changes related to pregnancy. Finally, we process the brain and the vomeronasal organ of the females and analyse markers of neural activity, e.g. immediate early gene (IEG) expression, in their olfactory and vomeronasal systems, including the vomeronasal organ. This allows us relating brain activity with chemoexploratory and maternal behaviours to evaluate pregnancy-related changes.

Objective 3. To study how the response of socio-sexual and motivational brain systems differs between pregnant and virgin females during interaction with pups.

In **Chapter 3**, we use brain sections of the same animals as in Chapter 2, to analyse brain activity in the nuclei composing the socio-sexual brain network and motivation brain circuitry. Once again, having measured the behaviour of females elicited by pups, we are able to relate brain activation with maternal behaviour and to assess the pregnancy-related changes in the activity patterns of those systems.

CHAPTER 1

Female exploration of chemosensory pup-cues in mice



1.1. Introduction

Pheromones are chemicals secreted or excreted by an individual that are detected by conspecifics, in which they elicit stereotyped responses (Karlson & Lüscher, 1959). Consequently, at least in macrosmatic species, pheromones are fundamental for the control of social behaviour. The mouse is an excellent model for the research of the neural substrate of socio-sexual behaviours, which are extremely important in humans. These include sexual attraction and behaviour, aggression and its control mechanisms and parental behaviours, the latter having a huge impact in the development of offspring and its future physical and mental health. Our group is mainly focused on maternal behaviour in the mouse, which comprises two kinds of behaviours. On one hand, pup-directed maternal behaviours, such as pup retrieval, licking-grooming and nursing. On the other hand, non pup-directed behaviours, such as nest building and maintenance and nest defence towards putatively infanticide intruders, e.g. maternal aggression.

In previous works, our group has shown that maternity entails behavioural changes that depend on the effects of pregnancy, parturition and lactating hormones on the brain of the dams (Martin-Sanchez et al., 2015; Salais-López, 2017). According to Numan and Insel (2003), especially during motherhood, when these hormones are acting upon the brain of females, cues emitted by pups and other conspecifics, especially chemosignals, would play a relevant role in eliciting both aspects of maternal behaviour (Corona & Lévy, 2015; Liberles, 2014) by activating specific circuits of female's brain involved in expression of pup care (pup-derived chemosignals) or non pup-directed maternal behaviours. For instance, regarding maternal aggression our group demonstrated that darcin, the same pheromone that induces sexual attraction in virgin females, elicits maternal aggression towards male intruders in dams (Martín-Sánchez et al., 2015a). Although virgin females cohabitating with dams during gestation and lactation (which our group calls "godmothers" or "comothers") apparently share pup care with dams and display full maternal care, they are unable to attack male intruders approaching the nest. Since maternal aggression seems to be dependent on detection of the male urinary pheromone darcin (Martin-Sanchez et al., 2015b), a process abolished by knocking out the channel Trpc2 (Kimchi et al., 2007) involved in vomeronasal transduction (Hasen & Gammie, 2011), the changes in females' physiology occurring with pregnancy, parturition and lactation may probably include altered perception and/or processing of conspecific chemosensory cues.

In contrast to maternal aggression, females do not need to undergo pregnancy, parturition and lactation to show pup-directed maternal behaviours, as virgin females show nearly spontaneous maternal care when exposed to alien pups (Alsina-Llanes et al., 2015; Martín-Sánchez et al., 2015b; Stolzenberg & Rissman, 2011). However, recent work of our group has shown that there are differences in the motivational value of pups between virgin females and dams, suggesting that there are changes in the brain motivational circuits related to motherhood (Salais-López et al., 2021). It has also been shown that pup-directed maternal behaviours depend on olfactory function, since anosmic females do not show maternal care (Vandenbergh, 1973; Wang & Storm, 2011) and bulbectomized females frequently show infanticidal behaviour (Gandelman et al., 1971a, 1971b, 1972), although previous maternal experience may prevent pup-killing in anosmic mice (Seegal & Denenberg, 1974). This link between pup cues and the shift between infanticidal behaviour and parental care is clear in male mice. In paternal males, behavioural transition from attacking pups in virgin males to displaying paternal behaviour in sires, seem to rely on changes in vomeronasal function, since usually infanticidal virgin males display paternal behaviour when their vomeronasal organ (VNO) is removed (Tachikawa et al., 2013). Likewise, Nakahara et al. (2016) demonstrated that pup-derived cues activated neurons expressing a specific olfactory receptor (Olfr692+) in the VNO of infanticidal virgin males, but not in parental fathers and mothers, neither in virgin females that also show maternal care.

Overall, these data in the literature strongly suggest that pup-derived chemosensory cues are essential for an adult mouse to recognize a pup as conspecific and express adaptive parental or infanticidal behaviours in the appropriate physiological conditions. However, pup-derived pheromones have not been identified yet, and we still ignore how the neural networks of the female brain involved in maternal care respond to pup-derived chemosignals. In the experiments of the present Chapter, we try to clarify if female mice respond to pup-derived chemosignals, and if the response of dams and virgin females differ. To this end, our first approach has been to test whether dams, godmothers or virgins are attracted by pup-derived odours, as expected if these odours contained pup pheromones eliciting motivated maternal behaviour at least in dams. Following Noirot (1969), in this first experiment we use pup-soiled nest material, as opposed to pre-partum female-soiled nest material. In the first experiment, therefore, we performed **two-choice preference tests** between nest material obtained from a lactating dam three days after parturition (postpartum day 3 nest material, soiled by a dam and her offspring), as compared to nest material from a late-pregnant female (postconception

day 17). Both stimuli share the presence of chemosignals of an adult female, but differ in the presence of pup-derived secretions/excretions only in the former case.

Given the similar response of females to both kinds of nest material, we performed a second experiment, in which we tried to check whether both kinds of nest material were distinguishable to female mice, by means of regular **habituation-dishabituation tests**. To do so, we used the same stimuli as in the previous experiment (late-pregnant female-soiled nest material; nest material soiled by a lactating dam and her offspring), but also other substrates supposedly containing only chemosignals derived from pups of different ages (neonatal pups, pups at the age of weaning).

Finally, we evaluated how adult females (dams or virgins) **chemoinvestigated bedding that had been soiled by pups** for 90 minutes. We compared the behaviour of the females when exploring bedding soiled by neonatal pups, with their investigation of bedding soiled by pups at the age of weaning. To avoid interference between chemosignals derived from different conspecifics, each female had only a source of conspecific chemosignals, namely, contrary to the two-choice tests, here the female had no choice.

1.2. Experiment 1: Two-choice preference tests between E17 and P03 soiled nest material

For this experiment we tried to collect a good source of neonatal pup chemosignals, as neonatal pups elicit more intense maternal care than elder ones (Gandelman, 1973; Londei, 1983). In this regard, literature points to urine as a reliable source of pup chemosignals (Londei et al., 1989; Wang & Storm, 2011), but we found that young pups (postpartum day 3 to 4) do not produce such quantity of urine to properly collect it in the laboratory conditions. Therefore, we collected nest material of a nest with young pups (pospartum day 3, P03), which surely contained many chemosignals from the pups. However, P03 nest material also contained many molecules derived from the dam, an adult female. Therefore, as a control we employed nest from late-pregnant females (post-conception day 17, E17). The difference between both kinds of samples would be the presence of pup chemosignals in P03 nest material but its absence in E17 nest material.

We performed preference tests between these two stimuli in dams, virgins and godmother females. We wanted to explore whether females preferred one to the other and if this preference was altered (presumably increased) during motherhood due to the action of

pregnancy hormones (dams vs virgins/godmothers) or experience and interaction with pups (dams/godmothers vs virgins), as it had been shown with maternal motivation (Salais-López et al., 2021).

1.2.1. Material and methods

A. Animals

For this experiment, we used CD1 adult female mice (Janvier, France) that were randomly distributed into 3 experimental groups: 8 dams, 8 godmothers and 8 virgins. All females were 9 weeks old at the beginning of the experiments. In addition, we used 12 same-age donor females for both E17-E18 nest material and P03 nest material.

Following the quarantine week after their arrival to the animal facility of the UJI (SEA, *Servei d'Experimentació Animal*), experimental females were housed in pairs in rat cages (45 x 24 x 20 cm) in standard conditions (24°C and 12 hr light/dark cycle), with water and food ad libitum. Each dam was paired with a virgin female through pregnancy parturition and postpartum (this female is called a godmother), and virgins were paired. A few minutes before the test, the females were transferred to the test room in an empty cage. After the test, females were returned to their home cages.

All procedures were approved by the Committee of Ethics and Animal Welfare of the Universitat Jaume I and Conselleria de Agricultura de la Generalitat Valenciana (Spain), in agreement with directive 86/609/EEC of the European Community on the protection of animals used for experimental and other scientific purposes (2015/VSC/PEA/00055; 2019/VSC/PEA/0049).

B. Stimuli

Stimuli were collected from female donors prior the test. First, we made nest material by shredding filter paper, which we aliquoted and sterilized in individual hermetic plastic bags containing 6 gr of nest material each. When donor dams were about prepartum day E14-E15, we put an aliquot of nest material in their cages and let it there for 3 days. After 3 days, on prepartum days 17 or 18 (E17-E18), we collected the soiled nest in individual plastic bags and replaced it with new nest material, which we collected again in postpartum day 3 (P03). After collection, soiled nest material was frozen at -80°C. Clean aliquots of shred filter paper were used as control stimuli and for the habituation phase.

Each day, prior to the test we thawed a number of soiled samples at room temperature, mixed all E17-E18 collected material to homogenize it, and we made 0.35 gr aliquots to use them for the tests. We did the same for the P03 collected nests.

In order to allow females to contact the stimulus samples during the test, which is necessary for the detection of non-volatile stimuli, we filled the jars as in Figure 1.1. The bottom of the jars was filled with clean glass marbles, covered with clean cotton wool. On top of the cotton wool we put 0.35 gr of the stimulus nest material, and then closed the jar with a metal screw cap which had a hole in the centre covered by a mesh wire. This allows the animals to contact the sample, thus supposedly having access to non-volatile compounds, but avoids that they can remove and spread the nest material.

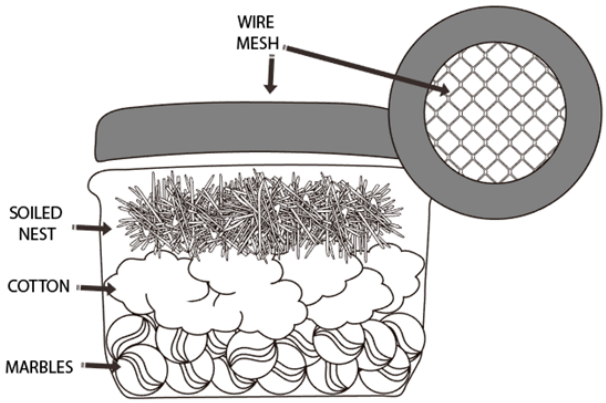


Figure 1.1. Schematic figure showing the different layers inside the stimulus jar for the Experiment 1.

C. Test cages and experimental setup

Two-choice tests were performed in a cage sized 88.5x13.3x29 cm, with 3 compartments (Fig. 1.2). The middle compartment was smaller and contained two openings to communicate it with the lateral compartments, which were double–size and contained a glass jar filled with the stimulus. During choice tests, in one of the lateral compartments the jar contained nest collected from donor females and their offspring on day postpartum 3 (P03), and on the opposite side the jar contained nest collected from day 17-18 of gestation from donor pregnant females (E17-E18).

Figure 1.2. Capture of a SMART recording of an Experiment 1 trial. Picture shows 2 females performing the preference test in two adjacent experimental three-chamber cages.

D. Experimental design

The test was performed around postpartum day 3 of the dams, as close as possible to the date of pup-soiled nest collection.

Experimental design includes 4 days with two 5-minute tests per day (Fig. 1.3), one in the morning (9:00–11:00 a.m.) and the other in the afternoon (03:00-05:00 p.m.). On day 1 and 2, habituation sessions consisted of allowing females to explore the test cage while both jars contained clean nest material (on top of marbles and clean cotton wool). On the morning of day 3 we performed and recorded control sessions with the same conditions as habituation phase, and on the afternoon, we performed and recorded the preference tests. In the preference test females were able to explore the cage which had a jar of E17-E18 stimulus in one compartment of the cage and another jar of P03 stimulus in the opposite compartment. Finally, on day 4 we performed and recorded two different preference tests; on the morning females were able to explore a cage in which one jar presented P03 stimulus while the other jar contained clean nest. On the afternoon females were able to explore a cage in which one jar presented E17-E18 stimulus while the other jar contained clean nest.

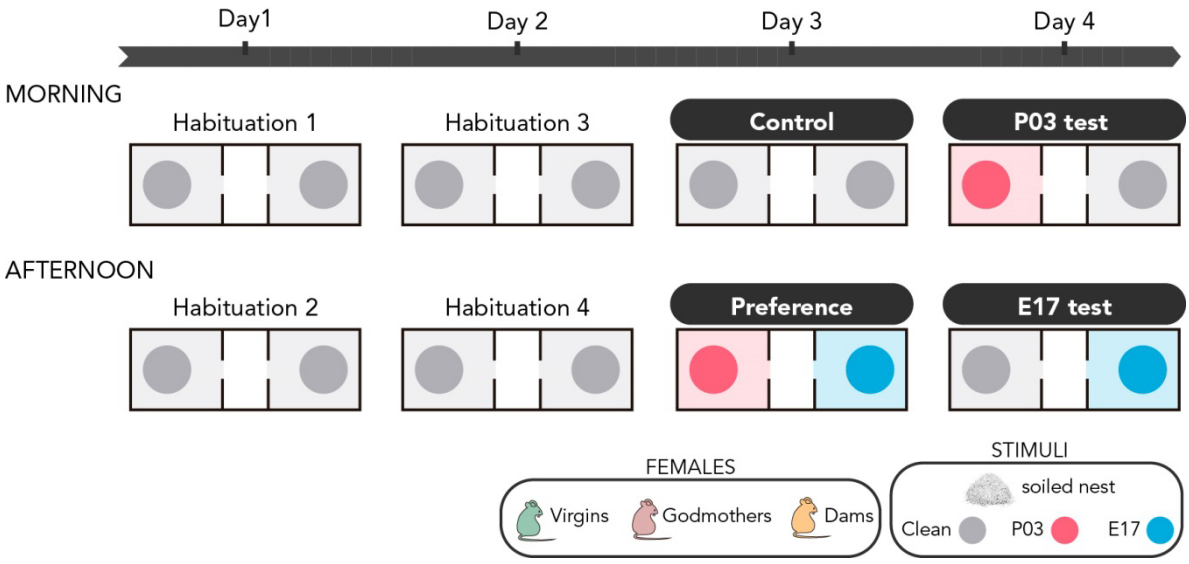


Figure 1.3. Experimental design and schedule of sessions. Each box represents a trial, showing the testing cage with the corresponding compartments and stimuli jars. Pink circles correspond to jars containing P03 soiled nest (P03stim), while blue circles correspond to jars containing E17 soiled nest (E17stim), and grey circles to jars containing clean nest. Consequently, compartments are lightly coloured based on the contained stimulus (P03surr and E17surr respectively), and the empty central compartment remains white. The colours of the mice in the legend correspond to the colour code for the three groups used in this experiment, being green for virgins, garnet for godmothers and yellow for dams.

E. Behaviour analysis

The tests were video-recorded using a digital video camera (Panasonic V180 HC-V180) and the behaviour of the animals analysed with SMART VIDEO TRACKING (Panlab, Cornellà, Barcelona, Spain). Using this software, we delimited several zones of the test cage to automatically measure the time spent by the females in each one. Briefly, for each trial and female we registered the time spent on or around each jar (Fig. 1.3, darker circles) and in the adjoining area of the compartment where the jar was located (Fig. 1.3, light coloured areas). We analysed data from tests in days 3 and 4, including the control trial, the preference trial, the P03 test trial and the E17 test trial.

Throughout all trials, P03 and E17 stimuli were systematically located in the same compartments that we name as P03comp and E17comp, whether it contained the actual stimulus or clean nest material. So, for each trial in P03comp we acquired the time spent in the jar area (P03stim), and in its surroundings (P03surr), and in the opposite side (E17comp) we acquired the time spent in the jar area (E17stim) and its surroundings (E17surr). Afterwards, we calculated the time spent in the whole P03 Compartment (P03comp) as the summatory of P03stim plus P03surr, and the same for the E17 compartment (E17comp).

To analyse the preference for P03 over E17 stimulus, for the control (morning of day 3) and preference test (afternoon of day 3) we calculated stimulus and compartment ratios:

- Stimulus Ratio:

$$\frac{P03stim}{E17stim}$$

- Compartment Ratio:

$$\frac{P03comp}{E17comp}$$

F. Statistical analysis:

We began analysing the data of the preference test (Fig. 1.3: afternoon of day 3) as compared to the previous control test (Fig. 1.3: morning of day 3). First, we analysed separately the time of investigation of P03 (stimulus or compartment) and E17. When data accomplished normality and homoscedasticity, we explored the differences between females on the time spent in each area in both trials (control and preference test) by means of a two-way repeated measures ANOVA, with the GROUP (virgin, godmother or dam) as between-subjects factor,

and the TEST (control vs test) as within-subjects factor. When data failed to accomplish normality or homoscedasticity, we performed a Wilcoxon rank-test for paired data to assess if there are differences between trials considering all females together. In those cases, we also analysed the three groups of females separately, performing a Wilcoxon rank-test for paired data for each group to assess if there were differences between trials. Finally, in those cases, we also performed a Kruskal-Wallis one-way analysis for independent samples between groups considering only the data of the preference test.

Second, we compared between P03/E17 ratios in control vs preference trial. As ratios did not accomplish normality, we performed a logarithmic transformation of the data and analysed transformed data with a two-way repeated measures ANOVA, considering again GROUP (virgin, godmother or dam) as between-subjects factor and the TEST (control vs test) as within-subjects factor. Additionally, we performed a Student's t test (or a Wilcoxon signed rank test in non-normal data) against fixed value one (1=no preference) for control trial and for preference trial separately, but considering all females together to evaluate if there was global preference for one of the stimulus.

Finally, we analysed the differences between groups in two separated tests in which females were exposed either to P03 or to E17 stimulus respectively, in both cases opposed to clean stimulus (Fig. 1.3: morning and afternoon of day 4, respectively). To do so, we used a one-way ANOVA (between females) to compare the time that different females spent exploring the P03 in the P03 vs clean test (Fig. 1.3: day 4, morning); independently, we also analysed the time females spent exploring the E17 stimulus in the E17 vs clean test (Fig. 1.3: day 4, afternoon). When data did not display normal distribution, we performed a Kruskal-Wallis one-way analysis for independent samples.

All statistical analyses were performed with the IBM SPSS 22 Statistics Software and the level of significance used was $p<0.05$.

1.2.2. Results and discussion

In this section, we analyse the exploratory behaviour that females exhibited in the preference test (Fig. 1.3; day 3, afternoon) and the control test (Fig. 1.3; day 3, morning). In the control test, both jars contained clean nest material, while during preference test jars contained P03 and E17 nest respectively. For the comparison between trials, in control trial we considered the zones as "P03 and E17", by assigning them the same stimulus that was present in the compartment in the preference trial. For instance, if P03 was located in the right compartment

in the preference test, we also considered the right compartment of the control trial as the P03 compartment, although the jar contained clean nest material. This approach allowed us to avoid misinterpreting results in the preference test due to preference for a given side of the cage.

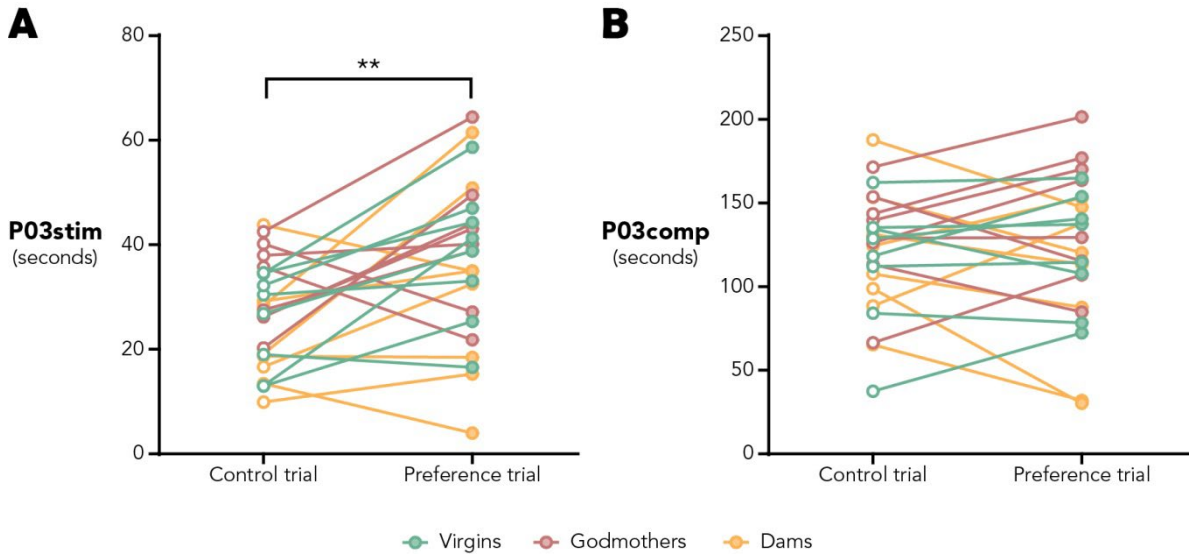


Figure 1.4. Time that females spent exploring the side of the cage corresponding to P03 stimulus, on control trial (left) and on preference trial (right) (Experiment 1). The colour code is yellow for dams, garnet for godmothers and green for virgins. Open circles represent the value of each female in the control test, while filled dots represent the value in the preference test. The values corresponding to the same female are linked with a line. In both figures (A and B) X axis reflects the trial (control vs preference test). **A**. Y axis represents the amount of time (seconds) spent in the P03 stimulus area (P03stim), near the jar. **B**. Y axis represents the amount of time (seconds) spent in the whole P03 compartment (P03comp). Statistical significant differences are indicated by asterisks (** indicates a p-value below 0.01).

A repeated measures two-way ANOVA, with GROUP (virgin, godmother, dam) as between-subjects factor and TEST (control trial, preference trial) as inter-subject factor, was first applied to the time exploring the P03 stimulus zone (Fig. 1.4A; P03stim). This analysis rendered a significant effect of the TEST ($F_{1,24}$=12.099, p=0.002), with more investigation of the jar when P03-soiled nest material was present than in the control test, when the jar contained clean nest material. By contrast, there were not global differences between females (GROUP; $F_{1,21}$=1.709, p=0.205), neither TESTxGROUP interaction ($F_{2,21}$=0.162, p=0.851). Therefore, females detect the presence of an odour in the P03 soiled-nest material, and chemoinvestigate it irrespective of the physiological status of the female (virgin, godmother or dam).

When the time in the whole compartment was analysed (instead of just the stimulus zone around the jar) repeated measures two-way ANOVA concerning the P03 stimulus (Fig. 1.4B; P03comp) failed to show any significant difference between TEST ($F_{1,21}$=0.032, p=0.859), GROUP ($F_{1,21}$=1.134, p=0.341) or significant TESTxGROUP interaction ($F_{2,21}$=2.121, p=0.145).

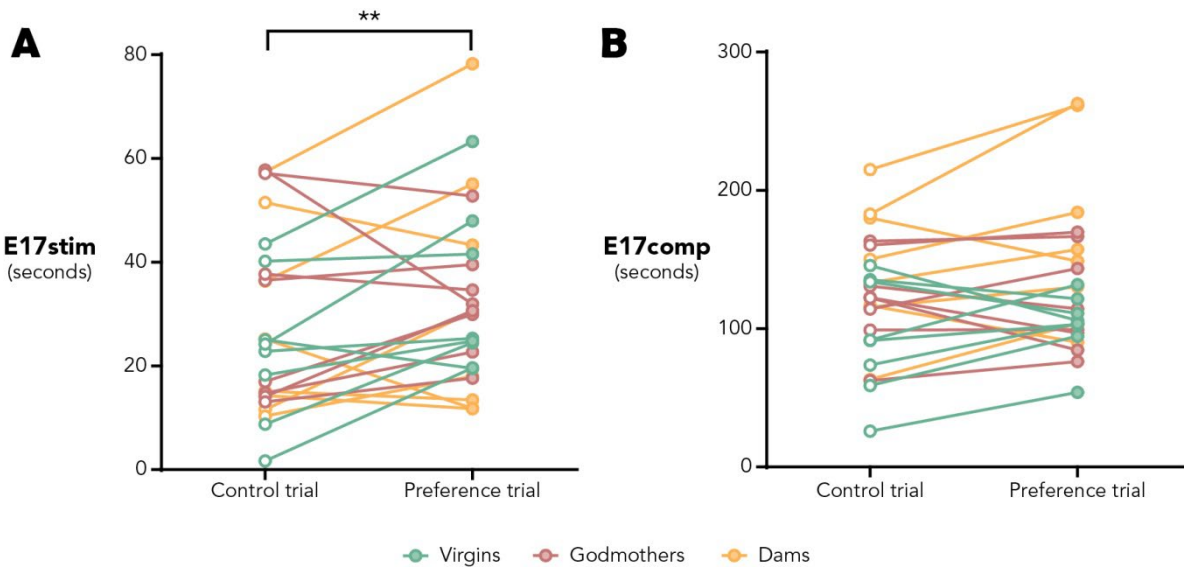


Figure 1.5. Time that females spent exploring the side of the cage corresponding to E17 stimulus, on control trial (left) and on preference trial (right) (Experiment 1). The colour code is yellow for dams, garnet for godmothers and green for virgins. Open circles represent the value of each female in the control test, while filled dots represent the value in the preference test. The values corresponding to the same female are linked with a line. In both figures (A and B) X axis reflects the trial (control vs preference test). **A**. Y axis represents the amount of time (seconds) spent in the E17 stimulus area (E17stim), near the jar. **B**. Y axis represents the amount of time (seconds) spent in the whole E17 compartment (E17comp). Statistical significant differences are indicated by * (** indicates a p-value below 0.01).

Regarding the E17stim, we also found a significant effect of the TEST ($F_{1,21}$=4.874, p=0.039; see Fig. 1.5A), with E17-soiled nest material being more explored than the equivalent jar with clean nest material. Like in the previous case, with E17stim neither GROUP ($F_{1,21}$=0.093, p=0.912) nor TESTxGROUP interaction ($F_{2,21}$=1.014, p=0.380) were significant, e.g. the odour of the nest soiled by a pregnant female attracted equally virgins, godmothers and dams. In relation to the time spent exploring the whole E17 compartment, E17comp failed to show homoscedasticity so we could not explore the repeated measures ANOVA, although Wilcoxon rank test showed there were no differences between trials considering all females jointly (Z_{23}=1.400, p=0.162, see Fig. 1.5B). The Wilcoxon rank tests for each group separately also failed to show differences between trials in any group (Dams Z=1.540, p=0.123; Godmothers Z=-0.140, p=0.889; Virgins Z=0.840, p=0.401). We also explored the differences between groups in just the preference test trial, but Kruskal-Wallis test did not show significant results (H_2=4.805, p=0.090).

As a conclusion, both P03- and E17-soiled nest materials seem attractive to adult females when presented in a three-compartment cage in simple two-choice tests. In addition, there seems to be no differences in that respect between dams, virgins or godmothers, namely all of them are equally attracted by odours of an adult female or a dam with her pups.

Next, we decided to calculate the ratio of time spent on P03 zones and E17 zones in each trial, in order to explore possible additional attraction of P03 nest material over E17 nest material, due to the presence of pup-derived chemosignals in the former. To do so, we calculated the ratio between the time exploring both stimuli areas, P03stim/E17stim, and the ratio between exploration of the whole compartment areas, P03comp/E17comp. As commented before, ratios did not accomplish normality so we transformed the data with a logarithmic transformation. Then, we compared the ratios between TEST (control, morning of Day 3; preference test, afternoon of Day 3) and GROUP of females (virgins, godmothers and dams) and explored the TESTxGROUP interaction, by means of a two-way ANOVA of repeated measures.

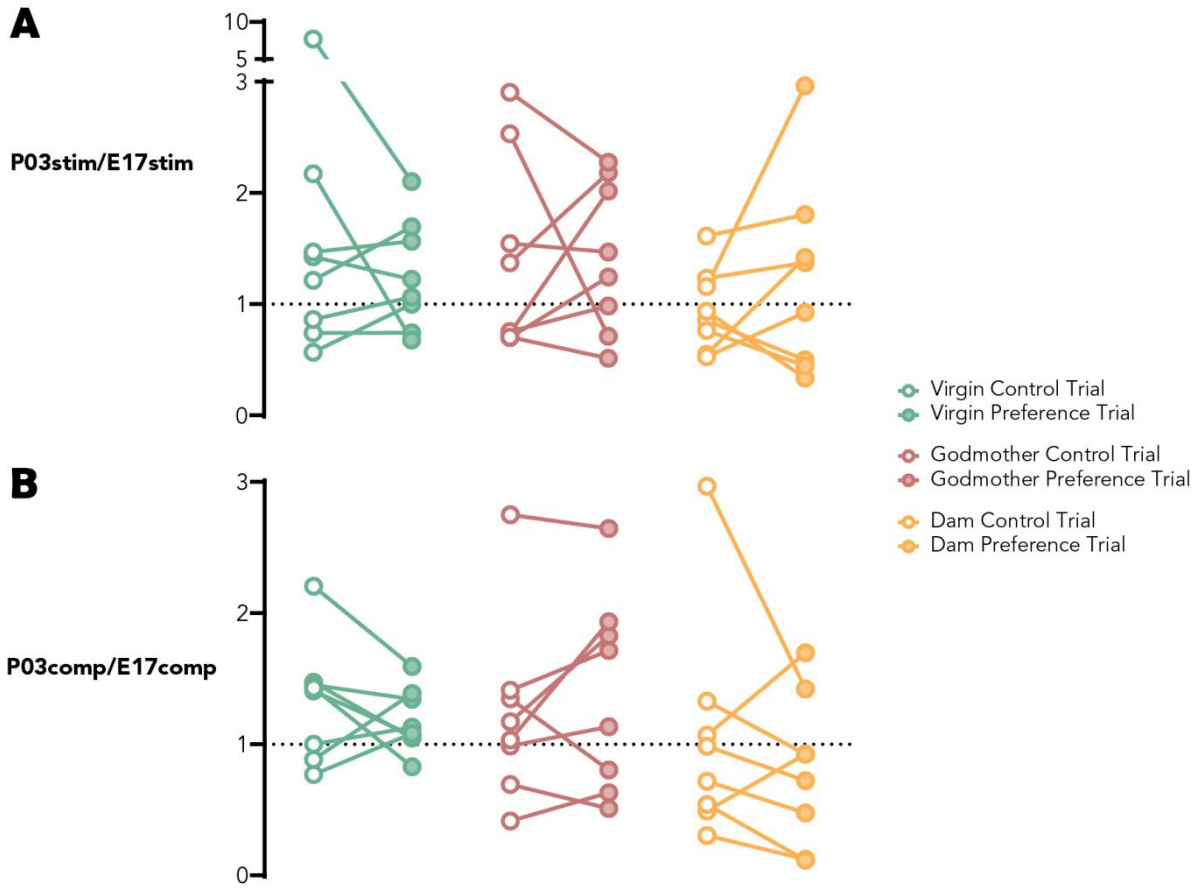


Figure 1.6. Ratios between P03 and E17 zones (Experiment 1). The groups are represented along the X axis marked with the colour code, being yellow for dams, garnet for godmothers and green for virgins. Open circles represent the value of the ratio for each female in the control test, while filled dots represent the value of the ratios in the preference test. The values corresponding to the same female are linked with a line. Dotted lines mark ratio values of 1 (equivalent to spending the same amount of time in both areas). **A.** Y axis represents the ratio between the time the female spent exploring the P03 stimulus near the jar and the time spent exploring the E17 stimulus near the jar. **B.** Y axis represents the ratio between the time the female spent in the P03 compartment and the time spent in the E17 compartment.

Results failed to show significant effects for both ratios. The ANOVA for P03stim/E17stim did not result in significant differences between TEST ($F_{1,21}$=0.030, p=0.863), neither between GROUP ($F_{1,21}$=851, p=0.441), nor TESTxGROUP interaction ($F_{1,21}$=0.715, p=0.500) (Fig. 1.6). The ANOVA for P03comp/E17comp showed similar results, with no differences between TEST ($F_{1,21}$=541, p=0.470), neither GROUP ($F_{1,21}$=1.651, p=0.216), nor TESTxGROUP interaction ($F_{1,21}$=1.470, p=0.253) (Fig. 1.6). Therefore, odours contained in nest material soiled by either a pregnant female (E17) or a dam in postpartum day 3 with her offspring (P03) are attractive to adult females, but this attraction seems similar for both stimuli, as there is no significant effect of the TEST.

Nevertheless, the comparison of the ratios with a fixed value (1) showed that, globally, P03stim/E17stim ratio is equal to 1 (no preference) for the control test (Z_{23}=1.086, p=0.278), but it is significantly higher than 1 during the preference test (t_{23}= 2.181; p=0.04). This suggests that pup-derived odours contained in P03-soiled nest material might be attractive to females, although this attraction would not differ between females. When analysing the ratio of whole compartments, P03comp/E17comp ratios did not differ significantly from 1 either in control trial (Z_{23}=0.971, p=0.331) or in preference trial (t_{23}=1.059, p=0.301).

To further investigate how females explored each one of the stimuli, we decided to analyse their behaviour when a single odorous stimulus (either P03 or E17) was confronted to the control stimulus (clean nest material). To do so, we first measured the time spent exploring the stimulus in the P03 test (Fig. 1.3; day 4, morning) and compared the results between groups (virgin, dams and godmothers). Afterwards, we repeated the same approach for the E17 test (Fig. 1.3; day 4, afternoon).

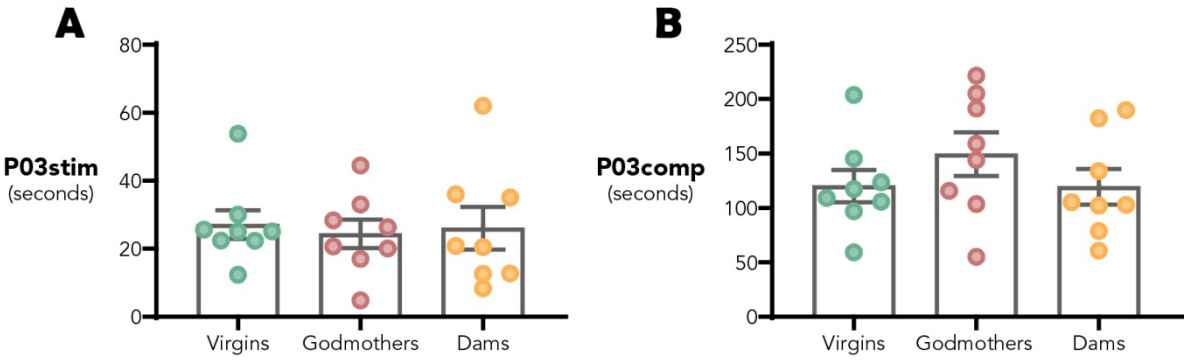


Figure 1.7. Time spent exploring the P03 and in the P03 compartment during P03 test trial (Experiment 1). In both figures (A and B) X axis reflects the female group, with the assigned colour: yellow for dams, garnet for godmothers and green for virgins. **A.** Y axis shows the amount of time (seconds) spent exploring P03 stimulus. **B**. Y axis shows the amount of time (seconds) spent in the P03 compartment.

When analysing the investigation of the P03 in the P03 vs clean trial (Fig. 1.7), we found no differences between groups in the time females spent exploring the P03 jar area (Fig. 1.7A; Kruskal-Wallis; K2=0.315, p=0.854), neither the whole compartment (Fig. 1.7B; one-way ANOVA; F2,21=0.996, p=0.386).

The same occurred in the E17 vs clean trial (Fig. 1.8), with no significant differences in the time exploring the E17 jar area (Fig 8A; one-way ANOVA; $F_{2,21}$=0.705, p=0.506) neither the whole compartment (Fig 8B; one-way ANOVA; $F_{2,21}$=0.015, p=0.985).

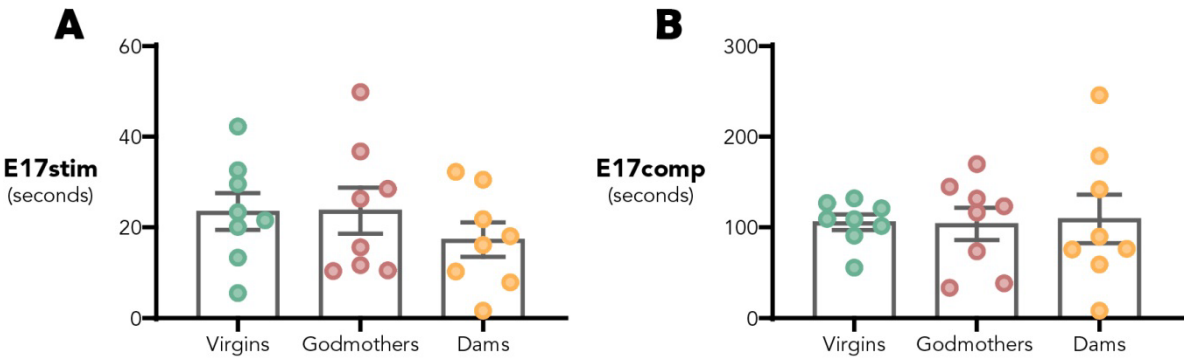


Figure 1.8. Time spent exploring the E17 and in the E17 compartment during E17 test trial (Experiment 1). In both figures (A and B) X axis reflects the female group, with the assigned colour: yellow for dams, garnet for godmothers and green for virgins. **A.** Y axis shows the amount of time (seconds) spent exploring E17 stimulus. **B.** Y axis shows the amount of time (seconds) spent in the E17 compartment.

Overall, there were no significant differences between groups, nor a clear preference towards pup chemosignals in any of the groups, when presented in soiled nest material, but just hints of some general preference of females for P03 nest material, suggestive of (but not demonstrating) some attraction induced by pup-derived odours.

Given these somewhat contradictory results, we wondered if the collected stimuli were adequate for our purpose. In fact, the nest collected in P03 probably contained peripartum-related chemical stimuli, including blood from the partum and postpartum dam's odours, which maybe masked the pup-derived odours. If that was the case, the P03 nest collection would not be the best way to present chemosensory pup-cues. Another possible interpretation of the results is that maybe females did not show preference for P03 nest material because the prepartum E17 nest was also attractive to them. In that case, a preference test between these two stimuli may not be the best test to begin with.

Taking those considerations into account, in the following experiments we decided to explore these possibilities, using different ways to collect and present the pup-derived chemosensory cues.

1.3. Experiment 2: Habituation-dishabituation tests using different kinds of pup-derived odour samples

The results of experiment 1 indicate that E17 and P03 soiled nest material are equally attractive to females. We assumed that P03 nest material contained perceptible pup-derived chemicals that made this stimulus discriminable from E17 nest material. However, an adult female (e.g. a pregnant/postpartum female) undoubtedly produces a large amount of chemosignals that may mask pup-derived odours present in P03 nest material. To check this, we used habituation-dishabituation paradigm with virgin females to evaluate if adult females could discriminate E17 and P03 nest material.

The habituation-dishabituation test is based on the assumption that subjects explore a novel stimulus, but after repeated presentations they become habituated and ignore it. Therefore, habituation phases consist of the repeated presentation of a stimulus to the subject, in which theoretically the interest towards the stimulus gradually declines. Habituation phase is followed by the presentation of a different, odorous novel stimulus. If the subject detects the new odour and discriminates it from the previous stimulus, it will probably display interest for it compared to the old one, showing dishabituation (increased exploration). This process can be repeated with a third stimulus and so on.

Therefore, we used habituation-dishabituation first to check whether odours derived from P03 and E17 nest material could be detected and discriminated by female mice. Then, using the same procedure, we also checked detection of pup-derived odours and discrimination with other social odours, using different ways to collect and expose chemosensory pup cues to adult females, besides soiled nest material. On one hand, we tried to collect pup chemosignals by rubbing a cotton-swab around the ano-genital area and the facial area of the pups, since previous work described lacrimal age-dependent peptides as pheromones, one of them acting like a juvenile pheromone protecting young mice from sexual behaviours of adult males (Ferrero et al., 2013). On the other hand, we tried to collect pup-derived odorants by letting pups rest on a beaker with bedding for 90 minutes at 37-40°C (comfort temperature), and then used that soiled bedding as a source of pup-derived odours (Okabe et al., 2013). We also tried different ways of presentation of the samples to the females during the habituation dishabituation tests.

1.3.1. Material and methods

A. Animals

We used eight 10-weeks-old virgin CD1 female mice per test (Janvier, France) (total N=34). Following the quarantine after their arrival to the animal facility, animals were housed by pairs in rat cages (45 x 24 x 20 cm) to avoid isolation stress, with water and food *ad libitum* and standard conditions (24°C and 12 h light/dark cycle; lights ON at 8:00, lights OFF at 20:00).

All procedures were approved by the Committee of Ethics and Animal Welfare of the *Universitat Jaume I and Conselleria de Agricultura de la Generalitat Valenciana* (Spain), and were in agreement with directive 86/609/EEC of the European Community on the protection of animals used for experimental and other scientific purposes (2015/VSC/PEA/00055; 2019/VSC/PEA/0049).

B. Experimental design and stimuli

We performed four consecutive habituation-dishabituation tests that consisted on repeated 1-minute presentations of the stimuli, with 15 seconds intervals between presentations.

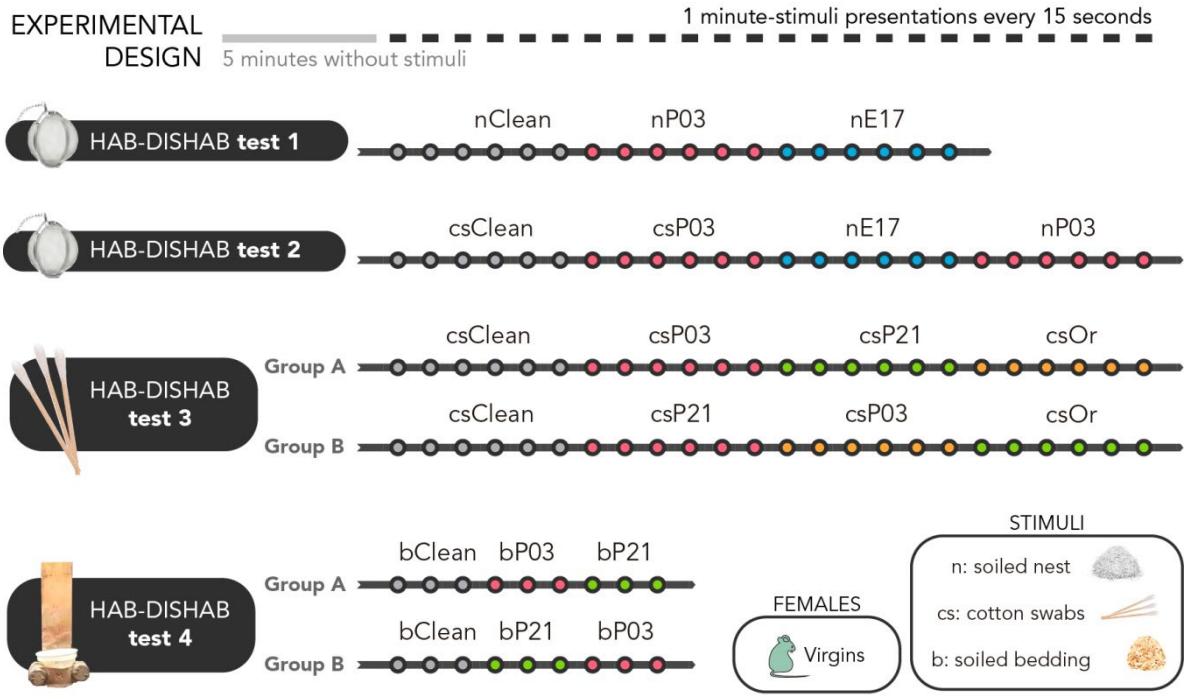


Figure 1.9. Experimental design of habituation-dishabituation tests. All tests consisted on repeated 1 minute presentations of the stimuli, with 15 seconds intervals between presentations. The photos on the left represent the way of presentation of stimuli for each one of the tests; the hanging tea infuser, the nude cotton swab, and the immovable laboratory well. On the right there are schematic representations of the experimental design of each one of the tests. Grey circles represent clean stimuli, pink ones represent P03 stimuli, blue ones represent E17 stimulus, green ones correspond to P21 stimulus and orange ones represent orange stimulus. The right inferior

square sum up the stimuli legend; those stimuli names starting with n correspond to nest collected ones, those starting with cs correspond to soiled cotton swabs and those starting with b correspond to soiled bedding.

The four habituation-dishabituation tests differed in the nature, collection and/or presentation of the stimuli, and in the number of presentations for each stimulus, as described below and illustrated in Figure 1.9.

B.1. Habituation-dishabituation test with nest material (HD1)

a) Stimuli

In order to assess whether or not females could discriminate between P03 nest (nP03) and E17 nest (nE17), we performed a habituation-dishabituation were both stimuli were successively presented to them. The stimuli used in this experiment came from remaining aliquots of the same donors from experiment 1, whose collected nests had been stored at -20°C (see section 1.2.1.B). Prior to the test, same-stimulus aliquots were mixed and thawed at room temperature, and then distributed in 0.40 gr aliquots, which is the amount of nest used during each presentation in the test. We also used clean nest aliquots (nClean; made of shred paper) as a control stimulus.

The stimuli were presented to the females in tea infusers hanging by one side of the cage as the image 10 shows, at 8-9 cm from the floor. The presentation allowed females to sniff and touch the hanging tea infuser.

Figure 1.10. Capture from the videos of the first and second habituation-dishabituation tests (HD1 and HD2), showing the set up for the presentation of the stimulus. Brown area corresponds to the region of interest.

b) Experimental design

Eight virgin females were moved to a room located next to the test room a few hours before the test. By the time of the test, each female was transferred to the test room in an empty cage and introduced into her corresponding test cage. Usually, two females performed the test simultaneously in two different cages that were recorded simultaneously. The test consisted of (Fig. 1.9):

1. Habituation to the box for 5 minutes.

2. Six 1-minute presentations of the tea infuser with 0.40 grams of clean nest alternated with resting periods of 15 seconds.
3. Six 1-minute presentations of the tea infuser with 0.40 grams of nP03 alternated with resting periods of 15 seconds.
4. Six 1-minute presentations of the tea infuser with 0.40 grams of nE17 alternated with resting periods of 15 seconds.

B.2. Habituation-dishabituation test with cotton swabs and nest material (HD2).

a) Stimuli:

In order to avoid collecting dam chemosignals, which may mask pup-odours, we tried to collect only chemosignals from pups by using soiled cotton swabs as stimuli. Briefly, we performed a habituation-dishabituation test in which females were able to explore P03 chemosignals collected in a cotton swab (csP03), P03 nest material (nP03), and finally E17 nest material (nE17) (Fig. 1.9).

For the collection of P03 chemosignals in a cotton swab (csP03), we gently rubbed about 150 postnatal-day-3 pups with swabs (one swab per pup) for about a minute, trying to cover all areas of the skin, especially the mouth and ano-genital zones. These pup-soiled cotton swabs were saved together in a hermetic bag and stored at -20°C until the experiment, when they were left to thaw at room temperature for 5 minutes before the test.

Stimuli were presented to the females inside a closed tea infuser hanging from the wall of one side of a test cage, similar to the one used in the previous habituation-dishabituation test (Fig. 1.10). For each presentation we used either 3 cotton swabs inside the infuser, or an aliquot of nest material. The presentation allowed females to sniff and touch the hanging tea infuser.

b) Experimental design:

A few hours before the test, 8 virgin females were moved to a room located next to the test room. By the time of the test, each female was transferred to the test room and introduced into her corresponding test cage (usually 2 females perform the test simultaneously in 2 different cages). The test consisted of:

1. Five minutes of habituation to the cage.
2. Six 1-minute presentations of the tea infuser with 3 clean end pieces of cotton swab alternated with resting periods of 15 seconds.

3. Six 1-minute presentations of the tea infuser with csP03 (cotton swab rubbed against the skin of P03 pups) alternated with resting periods of 15 seconds.
4. Six 1-minute presentations of the tea infuser with 0.40 grams of nE17 (soiled nest) alternated with resting periods of 15 seconds.
5. Six 1-minute presentations of the tea infuser with 0.40 grams of nP03 (soiled nest) alternated with resting periods of 15 seconds.

B.3. Habituation-dishabituation test with only cotton swabs (HD3)

a) Stimuli

For this experiment we used soiled cotton swabs from P03 (csP03) and a new kind of control stimulus: soiled cotton swabs from postnatal day 21 juveniles (csP21), which were around the age of weaning. We decided to use juvenile cues because previous literature described that younger pups elicit maternal behaviour, which decreases over time until weaning (Gandelman, 1973; Londei, 1983), thus suggesting that females could identify the age of a pup by its odour. We also added a third stimulus from orange skin (csOr), which has a powerful odour, to be sure that this modality of presentation worked.

For csP03 stimulus we used the remaining cotton swabs collected in the prior habituation-dishabituation test, which had been frozen. To collect the csP21 stimulus we rubbed the skin of about 150 postnatal day 21 juveniles with a cotton swab per pup, for about a minute trying to cover all areas, especially the mouth and ano-genital regions. Finally, we rubbed the skin of an orange with some cotton swabs (csOr). Afterwards, all same kind cotton swabs were stored in a hermetic bag and preserved in the freezer at 20°C. Prior to the test we let them for 5 minutes at room temperature.

To present the stimuli to the females, we used a different procedure to the one used in previous experiments, which is shown in Figure 1.11. This time the cotton swab was hanging directly from a chain at 8-9 cm from the floor on one side of the cage, thus, females were able to sniff and even touch the cotton swab during presentations.

Figure 1.11. Capture from the videos of the third habituation-dishabituation test with only cotton swabs (HD3), showing the set up for the presentation of the stimulus. Brown area corresponds to the region of interest.

b) Experimental design

Two groups of 8 virgin females were randomly selected for this experiment, so overall we used 16 virgin females. A few hours before the test, females were moved into a room located next to the test room. For the test, the experimental females were transferred to the test room and introduced into the corresponding test cage, one at a time.

For Group A the test consisted of:

1. Five minutes of habituation to the test cage
2. Six 1-minute presentations of a clean cotton swab alternated with resting periods of 15 seconds.
3. Six 1-minute presentations of csP03 alternated with resting periods of 15 seconds.
4. Six 1-minute presentations of csP21 alternated with resting periods of 15 seconds.
5. Six 1-minute presentations of csOr alternated with resting periods of 15 seconds.

For Group B the test consisted of:

1. Five minutes of habituation to the box
2. Six 1-minute presentations of a clean cotton swab alternated with resting periods of 15 seconds.
3. Six 1-minute presentations of csP21 alternated with resting periods of 15 seconds.
4. Six 1-minute presentations of csP03 alternated with resting periods of 15 seconds.
5. Six 1-minute presentations of csOr alternated with resting periods of 15 seconds.

In sum, females were presented clean swabs, and then csP21 and csP03, the order of presentation being counterbalanced. Finally, they were exposed to csOr.

B.4. Habituation-dishabituation tests with bedding (HD4)

a) Stimuli and stimulus presentation:

In the previous habituation-dishabituation tests, the stimulus object (nest material or cotton swab) was presented to the females hanging from one of the walls of the cage using a chain. The females were able to move the stimulus, which could be a distraction itself masking the exploration of the stimulus. To avoid this, in this experiment stimuli were presented within a laboratory plastic well that was fixed to the wall during the habituation-dishabituation tests, as illustrated in Figure 1.12, and was immobile.

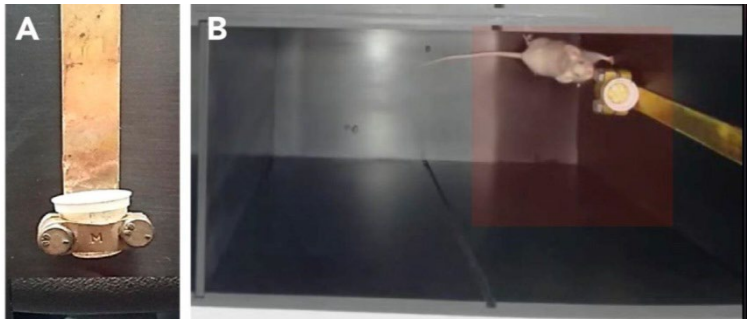


Figure 1.12. Setup for the fourth habituation-dishabituation tests (HD4) using a metallic holder. **A.** Snapshot of the device: the laboratory plastic well rested on a holder attached to the cage wall at 8-9 cm from the floor. **B.** Capture from the video of a HD4 test. Brown area corresponds to the region of interest.

For this purpose, we built a metal holder that set the well on one side of the cage at approximately 8-9 cm from the floor, allowing the females to sniff the stimulus through the bottom mesh without being able to move it. This new device had been successfully tested in a prior pilot study in which we were able to detect the habituation and dishabituation of virgin females towards soiled male bedding, which contains male urine as a powerful attractive stimulus for virgin females (data not shown).

We also used a new type of collection of the stimuli: this time we used bedding of P03 and P21 mice (bP03 and bP21 respectively). For the collection of bedding we put pups into beakers with bedding for 90 minutes. For P03 soiled bedding we put seven P03 to P06 pups on 100 ml of bedding (18 gr of bedding), and since younger pups do not properly regulate temperature, we maintained the beakers inside an incubator at 35°C during the collection (Fig. 1.13). For P21 soiled bedding we put three P21 juveniles in a beaker containing 36 gr of bedding. After the incubation time, we returned the subjects to their original cages, collected the bedding of the beakers and stored it at -20°C in properly labelled, hermetically closed plastic bags.

Figure 1.13. Picture of the setup for collection of P03 soiled bedding. Seven 3-day old pups were maintained inside each beaker for 90 minutes at 35°C.

b) Experimental design

For this experiment we used 16 female virgins, 2 groups of 8 females. A few hours before the test females were moved to a room located next to the test room. For the test, the

experimental females were transferred to the test room and introduced in the test cage, one at a time.

For group A the test consisted of:

1. Five minutes of habituation to the cage.
2. Three 1-minute presentations of clean bedding alternated with resting periods of 15 seconds.
3. Three 1-minute presentations of bP03 alternated with resting periods of 15 seconds.
4. Three 1-minute presentations of bP21 alternated with resting periods of 15 seconds.

For group B the test consisted of:

1. Five minutes of habituation to the cage
2. Three 1-minute presentations of clean bedding alternated with resting periods of 15 seconds.
3. Three 1-minute presentations of bP21 alternated with resting periods of 15 seconds.
4. Three 1-minute presentations of bP03 alternated with resting periods of 15 seconds.

To sum up, in this experiment females were presented clean bedding, and then bP21 and bP03, the order of presentation being counterbalanced (8 animals each order).

C. Behavioural analysis

In all four habituation-dishabituation tests we filmed the behaviour of the females, and behavioural analysis was performed by an experimenter who ignored the identity of the female and stimuli, and the details of the experimental design. We used Smart software (Panlab, Cornella, Spain) to delineate a region of interest by dividing the cage into three areas, and selecting the one included the stimulus (brown shaded areas in Fig. 1.10-1.12). The duration of all events of sniffing towards the stimuli showed by the female within that region were scored. To assess if there was habituation, we checked the differences in the time that the females spent exploring the first and last presentations of each stimulus, while to ascertain if there was a dishabituation we explored the differences between the last presentation of a stimulus and the subsequent first presentation of the next one.

D. Statistical analysis

For all habituation-dishabituation tests, statistical analysis was performed with the software IBM SPSS Statistics 22. When data accomplished normality, we performed a paired t-test for

related samples, whereas when data did not follow a normal distribution, we used the Wilcoxon signed-ranks test for paired data. Level of significance was $p<0.05$.

1.3.2. Results and discussion

Statistical analysis revealed some significant effects in the four habituation-dishabituation tests. The first one was a dishabituation from clean nest to P03 soiled nest in HD1 (Fig. 1.14A; Z=2.38, p=0.017), suggesting that virgin females could detect and discriminate P03 soiled nest material from clean one. However, P03 soiled nest material seems indistinguishable from E17 soiled nest (Fig. 1.14A; Z=0.105, p=0.917). Apparently, nP03 and nE17 were too similar to be discriminated, possibly because of the presence of peripartum dam chemosignals in both kinds of soiled nest material, which may be masking the pup-derived chemosignals.

Regarding HD2 test (Fig. 1.14B) in which swabs rubbed onto the skin of P03 pups were tested immediately after clean cotton swabs, we did not observe any significant effect, neither between clean cotton swabs and csP03 cotton swabs (Z=0.734, p=0.463), nor between csP03 and n17 soiled nest (Z=0.314, p=0.753), or between nE17 and nP03 soiled nest (Z=-0.338, p=0.735). These negative results might be attributed to a large variability of the behaviour of the animals, maybe because the tea infuser moved during exploration by the animal, thus unpredictably altering the ensuing behaviour of the female. In fact, females did not show any habituation either and some of the females displayed playful behaviour towards the hanging ball, which may alter the exploration towards the stimulus inside.

Taking into account the results from HD1 and HD2, we considered to use a different control stimulus in HD3. We changed the E17 stimulus for P21 stimulus, to avoid the effect of peripartum female odours, and used another way to present the stimuli by hanging a cotton swab that had been rubbed against the skin of the pups. Moreover, we performed 2 experimental groups with different order of presentation of the stimuli. In group A we found a habituation towards the clean cotton swab (Fig. 1.14C; Z=-1.992, p=0.046), although it was not replicated in the order B (Fig. 1.14D; Z=-0.338, p=0.735). In general, this approximation also failed to show the predicted discriminating effects: clean cotton swab vs csP03 (Fig. 1.14C; Z=0.980, p=0.327), csP03 vs csP21 (Fig. 1.14C; Z=0.560, p=0.575), clean cotton swab vs csP21 (Fig. 1.14D; Z=0.338, p=0.735) or csP21 vs csP03 (Fig. 1.14D; Z=-1.483, p=0.138). So females did not display dishabituation, not even when we presented a cotton swab soiled with a powerful odour (orange) after the last stimuli presentation, whether it was csP21 (Fig. 1.14C; Z=0.314, p=0.753) or csP03 (Fig. 1.14D; Z=1.342, p=0.180). The lack of any kind of dishabituation

suggests that a swab is not an appropriate substrate for this kind of studies, at least if it is presented hanged from a wall, e.g. it can be moved by the female. Therefore, global results from HD1 to HD3 suggest that hanging a mobile stimulus does not seem a good way to present the stimuli in habituation-dishabituation tests.

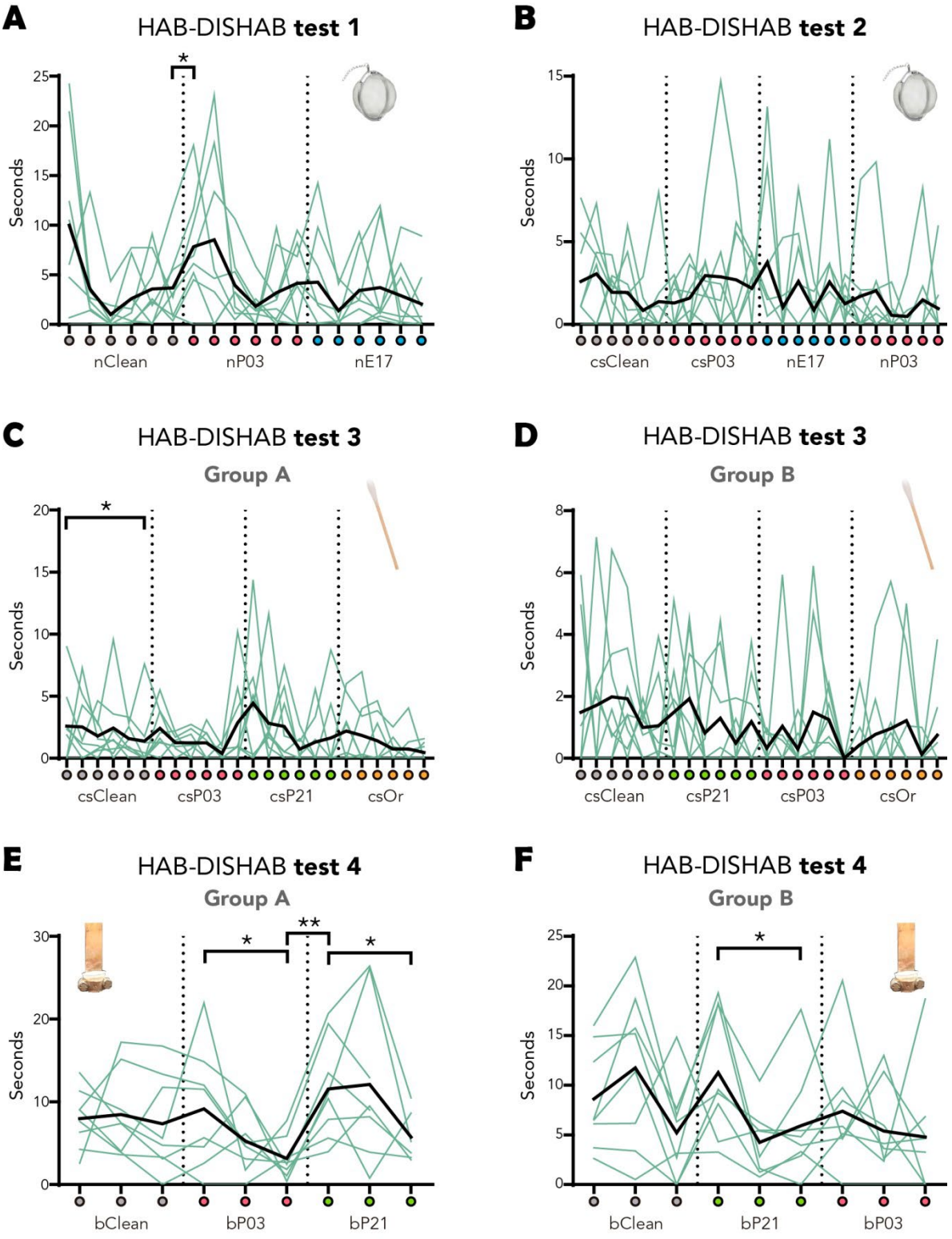


Figure 1.14. Results of all habituation-dishabituation tests. In all representations, X axis represents the consecutive 1 minute presentations of the stimuli to the females while encoding the nature of it: grey circles represent clean stimuli, pink ones represent P03 stimuli, blue ones represent E17 stimulus, green ones correspond to P21 stimulus and orange ones represent orange stimulus. Stimuli names starting with n correspond to soiled nest, those starting with cs correspond to soiled cotton swabs and those starting with b correspond to soiled bedding. Y axis shows the amount of time (seconds) each female spent exploring the stimulus of each presentation. Green lines correspond to individual virgin females, while the black one corresponds to the arithmetic mean of all females. **A**. Habituation-dishabituation test 1. **B**. Habituation-dishabituation test 2. **C and D**, Habituation-dishabituation test 3, groups A and B respectively. **E and F**. Habituation-dishabituation test 4, groups A and B respectively. Statistical significant differences are indicated by * (* indicates a p-value below 0.05 and ** indicates a p-value below 0.01).

Therefore, since the previous approaches did not seem suitable for the aim of this study in habituation-dishabituation test 4 (HD4; Fig.1.14E-F), we tested the soiled bedding as a new way to collect the stimuli and the laboratory well as a new kind of presentation. We also used two experimental groups with different order of stimulus presentation. In group A (Fig. 1.14E) we found a statistically significant dishabituation from bP03 to bP21 (t=-5.095, p=0.001), and habituations to both bP03 stimulus (t=2.666, p=0.032) and bP21 stimulus (t=3.010, p=0.030).

Contrarily, in order B (Fig. 1.14F) we could not replicate all the effects from order A since we only found the habituation towards bP21 (t=2.381, p=0.049), and a trend towards dishabituation from clean to bP21 (t=-2,058; p=0.079). Considering this, a possible interpretation is that bP21 emits a detectable odour that induces a dishabituation when presented after habituation to bP03 (Fig. 1.14E) and nearly so when presented after clean bedding (Fig. 1.14F). By contrast, bP03 seems to be not odorous enough as to induce dishabituation either after clean bedding (Fig. 1.14E; t=-0.825, p=0.437) or to bP21 (Fig. 1.14F; t=-0.569, p=0.587).

As a conclusion, considering together the results of all the habituation-dishabituation tests, they failed to reveal an effective way to collect and present the stimulus containing pup-derived odours, although fixing a plastic well to the wall seemed the best presentation format so far. Concerning the stimuli, the pup-derived odorants contained in the nest are probably masked by the high concentration of adult-female chemosignals it surely contains. On the other hand, rubbing a cotton swab against the skin of pups for a minute seems an inefficient procedure to collect pup odours. Finally, putting pups onto bedding for 90 minutes seems to be a good way of collecting chemosignals from P21 but not P03 pups. This is probably related to urination in elder pups, and the lack of it in neonatal ones, which seems to need stimulation by dams' licking the ano-genital area of the pups (Londei et al., 1989).

1.4. Experiment 3: Simple exposure to pup-derived chemosignals

Since habituation-dishabituation tests pointed to soiled bedding as the best way to present the stimuli, we decided to expose virgins and dams to P03-soiled bedding to see if there are differences in the exploration between groups. We also exposed females to P21-soiled bedding as a control conspecific stimulus, partially because of the restraints we had about E17 stimuli since the preference test suggested it was not an appropriate control stimulus. In addition, this time we allowed full contact with the bedding, so that females could detect both pup-derived volatile and non-volatile compounds.

1.4.1. Material and methods

A. Animals

For this experiment, we used 14 CD1 adult female mice (Janvier, France), 7 dams in their postpartum day 3 and 7 virgins, both about 9 weeks old. All females were housed in pairs in rat cages (45 x 24 x 20 cm) with water and food *ad libitum* and standard conditions (24°C and 12 hr light/dark cycle). Dams were housed with a "godmother" virgin female after the mating and during all the pregnancy, parturition and post-partum (Martín-Sánchez et al., 2015b), while virgins were also housed in pairs with another virgin.

All procedures were approved by the Committee of Ethics and Animal Welfare of the Universitat Jaume I and *Conselleria de Agricultura de la Generalitat Valenciana* (Spain), and were in agreement with directive 86/609/EEC of the European Community on the protection of animals used for experimental and other scientific purposes (2015/VSC/PEA/00055; 2019/VSC/PEA/0049).

B. Stimuli

For this experiment we used remaining aliquots of P03 and P21 soiled bedding, collected for the prior experiment (see section 1.3.1.B.4.a). For the presentation we introduced 6 gr of stimulus in an open glass jar, so females were able to explore the stimulus, sniff it and even get inside the jar and dig the bedding.

C. Experimental design

The test consisted of 5 minutes in which females could freely explore a cage containing a single, open jar full of bedding. Usually, females performed the test in pairs, in two adjacent

cages that were video-recorded together (Fig. 1.15). Prior to the test, all females were isolated in individual cages for one hour, so dams were separated from their pups to avoid the initial separation stress (Demarchi et al., 2021). During this period, pups remained in their original cage with the godmother female, which had been demonstrated to show maternal care (Martín-Sánchez et al., 2015b). After the test, the experimental females were returned to their housing cages.

Figure 1.15. Capture from Smart software during a habituation trial. Jar was placed in the centre of the experimental cage and the female was free to explore the whole compartment and the jar, and direct contact with the stimulus was allowed.

In order to avoid an unwanted effect of novelty in the first presentation of any stimuli, we counterbalanced the order of presentation. Experimental females were randomly assigned to one of two groups that differed in the order of the stimulus presentation, namely group A (4 dams and 4 virgin females) and group B (3 dams and 3 virgin females). For group A the first stimulus to explore was P03, while group B started with the exploration of P21.

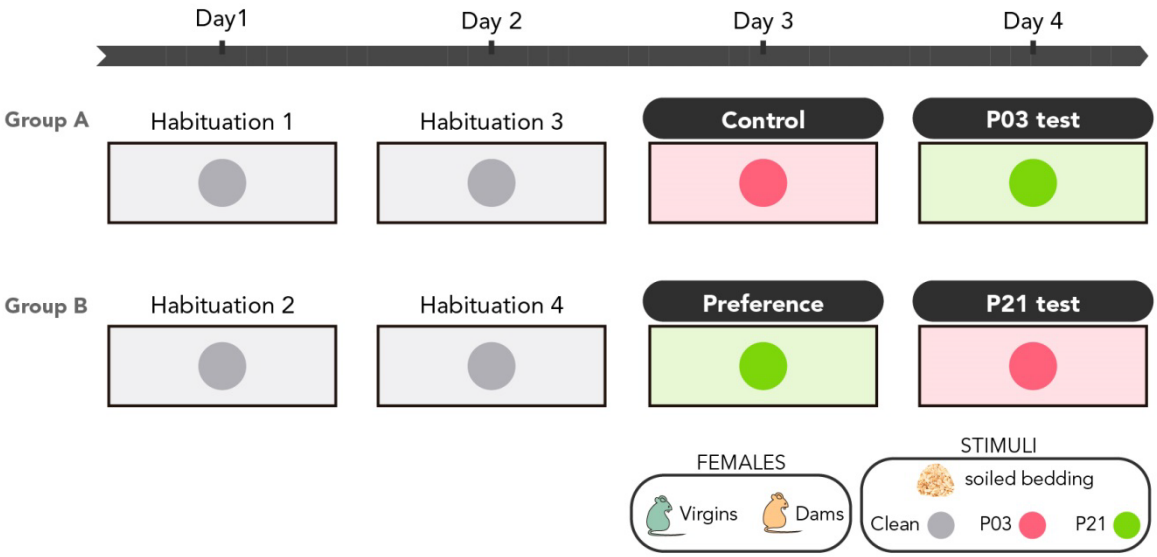


Figure 1.16. Experimental design of the simple exposure test. Pink circles correspond to jars containing P03 soiled bedding, while green circles correspond to jars containing P21 soiled bedding, and grey circles to jars containing clean bedding. Consequently, compartments are lightly coloured based on the contained stimulus. The colours of the mice in the legend correspond to the colour code for the 2 groups used in this experiment, being green for virgins, and yellow for dams.

The experimental design included 4 days of stimulus presentation: on day 1 and 2 females were able to explore the cage with 6 gr of clean bedding inside the jar (Habituation 1-4). On day 3 group A was exposed to a jar containing 6 gr of P03 bedding while group B was exposed to 6 gr of P21 bedding inside the recipient. On day 4 we repeated the exposition only in this case group A was exposed to the P21 stimulus and group B to the P03 stimulus (Fig. 1.16).

D. Behaviour assessment and statistical analysis

We video-recorded the tests with the Smart Software (Panlab, Cornellá, Spain) and later we measured the time spent by the female sniffing at and digging the stimulus, an also the time spent inside the jar. We also calculated the latency to the first episode of those three behaviours in each trial. Finally, we created a new variable "SJD" by summing up the duration of all 3 behaviours.

First, we analysed whether the order had any impact in the results. To do so, using a two-way ANOVA, for each variable we compared the ORDER (groups A and B) and the GROUP (virgins and dams). No significant differences appeared due to ORDER, neither GROUP, nor ORDERxGROUP interaction. Therefore, data from the same stimuli from groups A and B were collapsed and processed together for further analysis.

Then we performed a repeated measures ANOVA with GROUP (virgins vs dams) as the inter-individual factor and STIMULUS (P03 and P21, irrespective of the group) as the intra-individual factor. When data did not follow a normal distribution, we log-transformed them ($Log_{10}[n + 1]$). If, once transformed, they still failed to accomplish normality or homoscedasticity, we explored the differences between stimuli with the original non-transformed data, using a non-parametric Wilcoxon signed-rank test for dams and virgins independently. Statistical analyses were performed with IBM SPSS statistics 22 software. Level of significance was set at $p<0.05$.

1.4.2. Results and discussion

Concerning the duration of events, the first effect we found is that virgins spent more time sniffing the stimuli than dams (Fig. 1.17A; SniffDur), whether they faced P03 or P21 soiled bedding (GROUP; $F_{1,12}$=10,63, p=0.007). On the contrary, there were no differences in SniffDur between stimuli (STIMULUS; $F_{1,12}$= 3.423, p=0.089), nor an interaction STIMULUSxGROUP ($F_{1,12}$=0.506, p=0.490).

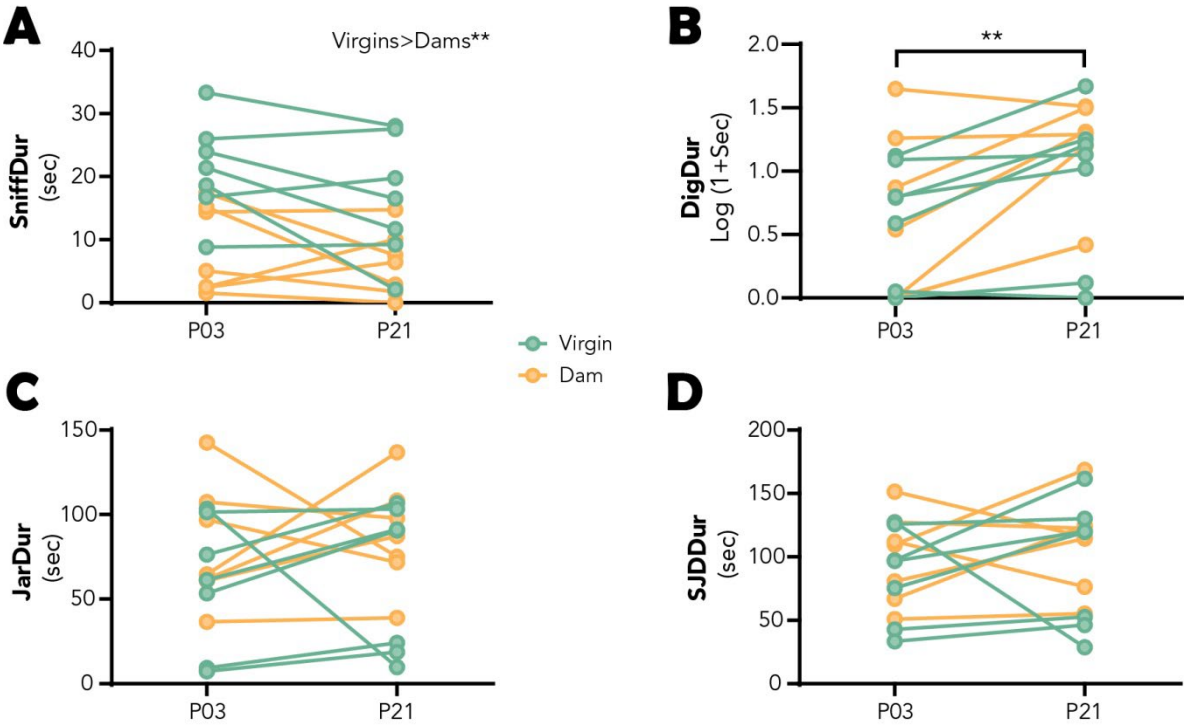


Figure 1.17. Behavioural response of females faced with one isolated stimulus (Experiment 3). Y axis represent the time (seconds or logarithmic transformation of seconds) every female spent sniffing (**A**), Digging (**B**), standing inside the jar (**C**) and all those behaviours combined (**D**) during the P03 and P21 trials. Pairs of dots connected with a line show the performance of the same female in both trials. Green dots and lines correspond to virgin females, while yellow ones correspond to dams. Statistical significant differences are indicated by * (** indicates a p-value<0.01).

Concerning the duration of the digging (Fig. 1.17B; DigDur), we found that females spent more time digging the P21 stimulus than the P03 one (STIMULUS; $F_{1,12}$=14,56, p=0.002), but it occurred equally in both groups (GROUP; $F_{1,12}$=0.082, p=0.780), and there was no interaction between factors (STIMULUSxGROUP; $F_{1,12}$=1.007, p=0.335).

In relation to the time females spent inside the jar (Fig. 1.17C; JarDur), we did not find significant differences between groups (GROUP; $F_{1,12}$= 2.802, p=0.120), either between stimuli (STIMULUS; $F_{1,12}$= 0.200, p=0.663) or an interaction between factors (STIMULUSxGROUP; $F_{1,12}$<0.001, p= 0.990). Finally, when combining the 3 measures (Fig. 1.17D; SJDDur) there were no differences between stimuli (STIMULUS; $F_{1,12}$=0.643, p=0.438), either between GROUP ($F_{1,12}$=0.783, p=0.393) or an interaction (STIMULUSxGROUP; $F_{1,12}$=0.011, p=0.918).

To sum up; the analysis of the duration of events showed that virgins sniffed both stimuli more than dams, and that, globally, females spent more time digging the P21 than the P03 stimulus.

Regarding the latencies, repeated measures ANOVA showed that the latency to sniff (Fig. 1.18A; SniffLat) was different between females (GROUP; $F_{1,12}$=5.413, P=0.038) since virgins displayed the sniffing behaviour earlier than dams. Nevertheless, results were similar between

stimuli (STIMULUS; $F_{1,12}$=0.309, p=0.588) and the ANOVA showed there was no interaction between factors (STIMULUSxGROUP; $F_{1,12}$<0.001, p=0.983).

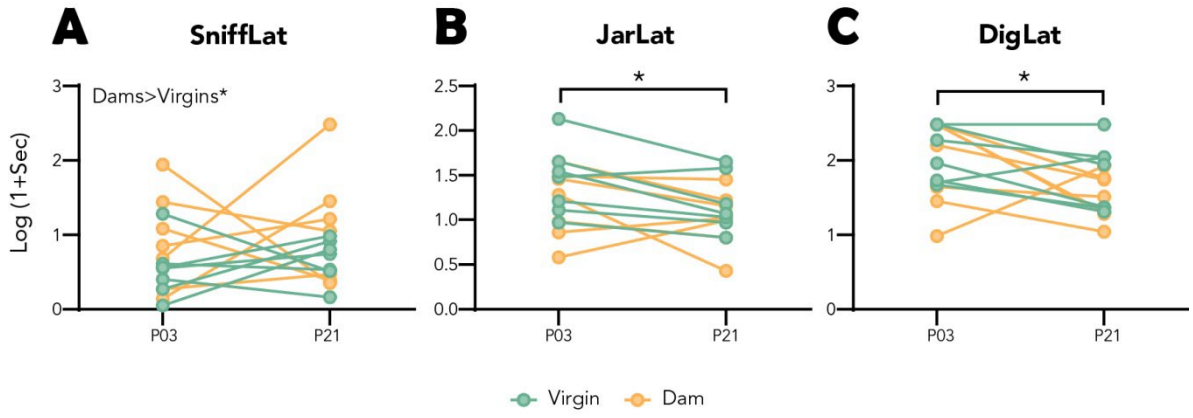


Figure 1.18. Latency to show each measured behaviour of the females faced with one isolated stimulus (Experiment 3). Y axis represent the logarithmic transformation of the latency (seconds) to show: sniffing (**A**), Digging (**B**) and standing inside the jar (**C**) during the P03 and P21 trials. Pairs of dots connected with a line show the performance of the same female in both trials. Green dots and lines correspond to virgin females, while yellow ones correspond to dams. Statistical significant differences are indicated by * (* indicates a p-value below 0.05).

In the case of the latency to explore the jar (Fig. 1.18B; JarLat), both groups of females displayed less latency when it was filled with P21 stimulus than when filled with P03 (STIMULUS: $F_{1,12}$=6,08, p=0.030). Nonetheless, there was no effect of the GROUP ($F_{1,12}$=1.606, p=0.229), nor STIMULUSxGROUP interaction ($F_{1,12}$=0.218, p=0.649).

The latency to dig the stimuli (Fig. 1.18C; DigLat) showed the same results as JarLat: both groups of females showed less latency to dig when the jar was filled with P21 soiled bedding that when filled with P03 soiled bedding P03 (STIMULUS: $F_{1,12}$=5.385, p=0.039). In this case there was not effect from the GROUP ($F_{1,12}$=0.985, p=0.341), neither STIMULUSxGROUP interaction ($F_{1,12}$=0.453, p=0.514).

In sum, results showed that dams display a delay to sniffing the stimuli as compared to virgins, and they also spent less time sniffing them. Furthermore, females spent more time exploring P21 than P03 stimulus, although the differences are only significant in the time digging the bedding and in the latency to be inside the jar. Moreover, the lack of significant STIMULUSxGROUP interaction suggests that dams do not show an increased interest towards P03 stimulus when compared to virgins, at least in the conditions of our experiment.

1.5. DISCUSSION

Overall, our data indicate that social odorous stimuli are attractive to females, irrespective of their physiological status. In a two-choice test between nest material soiled by a pregnant female (day 17 after conception; E17) and nest material soiled by a postpartum dam (day 3 after birth; P03) undoubtedly containing pup-derived chemosignals, both stimuli were more investigated than the control (clean nest material) in different conditions. However, females apparently did not show a clear preference of P03 over E17 nest material, although indirect data suggest that some attraction may exist.

Since E17 and P03 nest material surely include large amounts of odorous compounds derived from the pregnant/postpartum female owning the nest, we wondered if this might have masked the pup-derived odorants contained in the P03 nest material. We tried to test this by means of habituation/dishabituation tests. The first one (HD1) directly compared both stimuli and demonstrated that P03 odour was detected by virgin females, but was not distinguished from E17 odour, thus confirming a dominant adult female odour of both kind of samples.

Therefore, on a second and third set of HD experiments, we tried to check if P03 pups (postnatal pups eliciting intensive maternal care) and P21 pups (at the age of weaning) produced chemosignals that could be detected and distinguished by adult females. Cotton swabs that had been rubbed onto the skin of the pups did not provide a good substrate for HD tests, and rendered inconclusive results, mainly due to a high variability. The use of bedding (sawdust) on which pups of both ages had rested comfortably for 90 minutes rendered better results if the stimulus was fixed to the wall of the test cage. Nonetheless, the results indicate that P21-soiled bedding contains odorants in an amount enough as to be detected by adult females (very likely due to pups urinating onto the bedding), but P03 does not elicit chemoinvestigation. Nevertheless, our last experiment showed that, although P21 seem to elicit more intensive chemoinvestigation that P03 bedding (e.g. digging and remaining inside the jar), females spent equal time sniffing both stimuli, so results are contradictory. Unfortunately, these unclear results also discard bedding as a good substrate to study the response of females to neonatal pup chemosignals.

Altogether, our findings fit previous studies suggesting that dams do not show a strong preference towards chemosensory cues alone (but see Goterris-cerisuelo et al., 2021), meaning that they could need other cues in order to display maternal behaviour, such as visual features (including movement), sounds (pup vocalisations), or tough (e.g. detecting body

temperature). In this regard, Gandelman (1973) showed that virgin females constructed larger and more maternal nests when presented with inaccessible alive pups than recently killed ones, and although females were not able to contact the pups, the corpses allegedly conserved volatile chemosensory cues and only lacked movement, temperature and vocalizations. Smotherman and colleagues (Smotherman et al., 1974) explored the choice response of lactating female mice towards different stimuli consisting of pups, taped vocalizations and chilled pups. They found that although females chose chilled pups (not emitting any sound) over vocal cues, they chose the intact pup over the chilled one, meaning there might be a synergy between cues eliciting maternal behaviour. However, this experiment was conducted between 9 to 13 postpartum days, so one may argue that the non-chemosensory cues grow in importance over time. That is debatable, since, although Noirot & Pye (1969) reported pup distress calls to fall dramatically from P14, P03 pups seem to emit a higher number of vocalizations than older pups when they are in presence of an adult female (Ostermeyer & Elwood, 1983). More recent work by Cohen et al (2011) found that exposition to P04 pup odours increased the response of auditory cortex towards pup sounds (postnatal day 4 and 5) in dams but not in virgins, which suggest a synergy between stimuli dependent on previous experience. They also found that retrieval behaviour in dams decreased after washing the pups, reinforcing the role of chemosensory cues in maternal behaviour. The synergy between stimuli was also explored by Okabe et al (2013) by two-choice tests with different stimuli, as hypothermic pups (emitting ultrasonic vocalizations), anesthetized pups, pup-derived recorded ultrasounds and collected chemosensory cues (cotton soiled by 6-day-old pups in a glass beaker for 3 hours). First of all, dams spent more time exploring hypothermic pups than anesthetized ones, highlighting the role of vocalizations. However, neither the recorded ultrasounds nor the chemosensory cues alone elicit preference on the dams towards those stimuli. Curiously, compared to the sole presentation of pup odour, the simultaneous presentation of pup-derived ultrasonic vocalizations and odours induced a specific approach response, suggesting again a synergy between stimuli.

Thus, above all, although chemosensory cues play a significant role in maternal behaviour, finding the behavioural approach to test the power of these chemosensory cues seems more challenging than we had thought. This is mainly due to the difficulties of obtaining samples enriched with enough amounts of pup-derived odours and with no other interfering social odours, to be sure that females are responding selectively to pup-derived chemosignals. Therefore, for the rest of the present thesis, we used alive pups to explore the response of the maternal brain towards pup-derived chemosensory cues, by analysing the primary and

secondary olfactory and vomeronasal centres of the brain (Chapter 2), where the influence of other stimuli are minor (even negligible).

Nevertheless, the struggles of this Chapter to obtain samples of pup-derived odours opened new research lines in which our group is currently working on. On one side, preference test with anesthetized pups have been conducted and they have already reported interesting results suggesting a relevant role of pup-derived chemosignal in female's behaviour (data not published yet; see Goterris-cerisuelo et al., 2021). On the other side, we thought about a different approach to address the search of the "pup pheromone" eliciting maternal care. In this regard, we started a series of experiments with the Research Institute for Pesticides and Water (IUPA, Universitat Jaume I) in which we used an original strategy to try to identify relevant pup-derived volatiles. To do so, we adapted the dynamic headspace methodology to extract the volatolome of whole alive animals. Untargeted metabolomic methodology was then used to compare the volatolome of neonatal pups (4–6 days) with the one of elder ones, until the age of weaning (21–23 days old). Pup volatolome was analysed by gas chromatography (GC) coupled to single quadrupole mass spectrometry (MS) using automated thermal desorption for sample introduction. This has allowed us to identify 11 compounds that could be good candidates for being pup pheromones involved in maternal behaviour (Lacalle-Bergeron et al., 2021). Indeed, our team is currently testing those compounds behaviourally and the results will be part of another doctoral thesis within the group.

CHAPTER 2

Pregnancy changes the response of the vomeronasal and olfactory systems to pups in mice

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2.1. Introduction

Maternal behaviour can be defined as any interaction of an adult female with infant conspecifics that helps the latter to survive until their maturity (Numan & Insel, 2003). Maternal behaviour has therefore a strong impact on reproductive success, but it is also very beneficial for infant neurodevelopment (Curley & Champagne, 2016). Indeed, well-adapted mammalian dams are frequently engaged in devoted maternal care (pup-directed behaviours) consisting of retrieving the pups to the nest, crouching over the pups to keep them warm and nurturing them by means of lactation while frequently licking-grooming their bodies. In addition, dams also show an intense activity not directed to pups, such as building and maintaining the nest already before parturition and defending it against adult conspecific intruders that might constitute a threat for their pups (maternal aggression) (Numan & Insel, 2003).

The enormous investment of time and energy that these behaviours require may explain why females only exhibit fully motivated maternal behaviour during peripartum (Kohl et al., 2017; Salais-López et al., 2021). Although maternal behaviours are normally expressed after delivery, when pups are present, they are already facilitated during pregnancy. Thus, pregnant females already show nest building (Lisk, 1971) and maternal aggression (prepartum aggression, Mann et al., 1984). In addition, it was shown that primigravid female rats that were hysterectomized before parturition, also displayed facilitated pup-directed behaviours (Bridges et al., 1978; Rosenblatt & Siegel, 1975). By contrast, it has been reported that pregnancy also facilitates pup attacks in both rats (Mayer & Rosenblatt, 1984; Peters & Kristal, 1983) and mice (McCarthy & Vom Saal, 1985). Although this may seem a contradiction in terms, infanticide may constitute an adaptive behaviour during motherhood in some circumstances (Hrdy, 1979; Kuroda & Tsuneoka, 2013; Latham & Mason, 2004) and this may include late pregnancy.

The most likely mechanism underlying this timely, temporary enhancement of maternal responses to pups is their facilitation by hormones associated to pregnancy, as indeed it has been demonstrated for several mammalian species (Bridges, 2020). For instance, sexual steroids together with prolactin and/or placental lactogens (Bridges & Freemark, 1995; Bridges & Ronsheim, 1990), acting onto centres of the socio-sexual brain network (singularly the medial preoptic area, Brown et al., 2017), accelerate the onset of maternal behaviours in virgin rats.

Therefore, the current view of the neurobiology of motherhood assumes that hormonal events of late pregnancy prime specific brain circuits mediating maternal behaviours (the socio-sexual brain network), so that parturient females react properly to infant-derived stimuli, but once maternal behaviour is initiated, it continues without the need of further hormonal regulation (Numan & Insel, 2003).

In this context, it is important to understand what sensory channels are involved in the detection of the relevant pup stimuli. Although the identity of specific pup chemosignals has not been elucidated yet, since rodents are macrosmatic animals it is likely that pup-derived chemosignals have a critical role in eliciting maternal behaviour. In fact, altered chemosensing has dramatic consequences on maternal responses in rodents. Thus, bulbectomy (Gandelman et al., 1971a, 1971b; Vandenbergh, 1973) and nasal epithelium lesions (Seegal & Denenberg, 1974), result in nearly systematic pup-killing by lactating females. Moreover, null mutations of genes encoding critical molecules for olfactory transduction not only result in anosmia, but also lead to maternal neglect of pups and deficient nest maintenance (Belluscio et al., 1998; Wang & Storm, 2011). By contrast, null-trpc2 mice, whose vomeronasal organ (VNO) is not functional (Leypold et al., 2002; Stowers et al., 2002), show just reduced maternal care (Kimchi et al., 2007), as well as deficient nest maintenance but complete lack of maternal aggression (Hasen & Gammie, 2011; Leypold et al., 2002). In addition, (Lepri et al., 1985) reported reduced pup retrieval after VNO ablation. Together, these findings suggest a key role of chemosensory olfactory stimuli in maternal care. By contrast, vomeronasal stimuli seem to play a clear role in aggression, including maternal nest defence, but there is conflicting evidence on its function in pup-directed maternal behaviours.

Conversely, the VNO is critical for the response of males to pups. First, VNO ablation reduces infanticide in sexually naïve males (Tachikawa et al., 2013). Also, targeted mutations abolishing VNO function (trpc2 knockout; Nakahara et al., 2016) provoke paternal behaviour, inducing pup care similar to that of lactating dams. Surprisingly, the VNO of mice possess a population of cells that express non-canonically a receptor of the olfactory family (olf692) that has been related to pup-derived odour detection. In sex-naïve males, which are infanticidal, a high proportion of olf692-expressing VNO cells are activated by pup odours. In contrast, in paternal males and in females (irrespective of their status, virgins or dams) a much smaller proportion of these cells are activated following pup exposure (Nakahara et al., 2016).

All these data indicate that pup chemosignals are important in the response of adult rodents to infants, and in females this is especially critical during motherhood, when altered

chemosensing has a strong impact on maternal behaviours. However, the specific role of each sensory channel, e.g., olfactory and vomeronasal, in this communication is still unclear. In addition, there is a surprising lack of information on possible functional changes in these systems induced by pregnancy hormones, which might explain, at least in part, the enhanced reinforcing properties of pups for females during motherhood (Hauser & Gandelman, 1985; Salais-López et al., 2017, 2021).

Thus, to study the possible changes in both main and accessory olfactory systems during pregnancy, we recorded and scored the behaviour of late-pregnant (LP) (post-conception day 18) and virgin female mice in response to pups' exposure. Afterwards, in these females, we assessed activation of the VNO by means of immunohistochemical detection of Egr1 expression, and the primary and secondary olfactory and vomeronasal brain centres by means of cFos detection. As a control stimulus, we used a non-social object (buttons) of approximately the same size as pups. Since both variables (behaviour and brain activation) were measured in the same animals, we were able to analyse possible correlations between brain activity and specific aspects of maternal behaviour. The results confirm the presence of pup-derived chemosignals activating the VNO of females and suggest changes in stimulus processing in both chemosensory systems during late pregnancy. In addition, these findings suggest the existence of two distinct pathways in the vomeronasal system of females related to pup care and pup-directed attacks, respectively.

2.2. Materials and methods

2.2.1. Animals

For the present study, we initially used 10-weeks-old virgin female mice (n=16) and late-pregnant female mice (n=14) of the CD1 strain (total n=30). Late-pregnant mice (LP) were bred in the animal facilities and parturition (usually occurring at gestational day 19) was expected 1–2 days after behavioural testing. Females were housed in homologous pairs, in order to avoid isolation stress. Pairs of same condition females were housed together at least 20 days before the experiment (pairs of LP females were mated by the same male) in polypropylene cages with a controlled temperature of 24°C and a 12-hr light/dark cycle (lights on at 08:00 h) with *ad libitum* water and food supply. The pregnant day was considered as the one in which a pair of LP females were mated with a male (housed together overnight).

Later on, we required some supplemental experimentation for which we also used 10-weeks-old virgin female mice (n=8) and late-pregnant female mice (n=8) of the CD1 strain (total n=16), with the same characteristics and same housing conditions as those described above for the main experiment.

Experimental procedures were approved by the Committee of Ethics and Animal Experimentation of the Universitat Jaume I and treated throughout according to the European Union Council Directive of June 3rd, 2010 (6106/1/10 REV1). A license was issued by the *Direcció General de Producció Agrària i Ramaderia de la Generalitat Valenciana* (code 2015/VSC/PEAI00055 type 2).

2.2.2. Experimental design and behaviour analysis

A. Initial experimental design and analysis of maternal behaviour

Experimental design is summarize in Figure 2.1. In the day of the test, females were exposed to either pups or buttons, plastic and round objects from similar size than pups, which constitute socially neutral stimuli. Pups in postnatal day 4 were obtained from different female donors. Thus, we used four female groups: (1) LP-P, late pregnant females exposed to pups, (2) V-P, virgins exposed to pups, (3) LP-B, late pregnant females exposed to buttons, and (4) V-B, virgins exposed to buttons.

Two days prior the behavioural testing, females underwent a habituation phase. Eight glass marbles were deposited in the females' home cage once per day for 2 days at the time in which experiments were scheduled to be performed, in order to habituate the animals to the procedure. In the test day, pairs of virgins and of pregnant female mice were exposed to eight buttons or eight pups, placed in distal areas of the home cage relative to the nest (consisting on pieces of shredded paper). Buttons (Fig. 2.1) were round, white, with four holes and made of plastic. Two different sizes (13 and 20mm in diameter) but similar weights (0.69 and 0.63 g, respectively) were used. Four buttons of each class were introduced in each cage. The behaviour of the females exposed to pups was video recorded for 90min, although observation of maternal behaviour was restricted to the first 8 min since it is mainly expressed immediately following pup introduction (Martín-Sánchez et al., 2015b) and may better reflect the expression of immediate early genes (IEGs) observed, which reaches its maximum 60–90min after stimulation occurred (Hoffman et al., 1993).

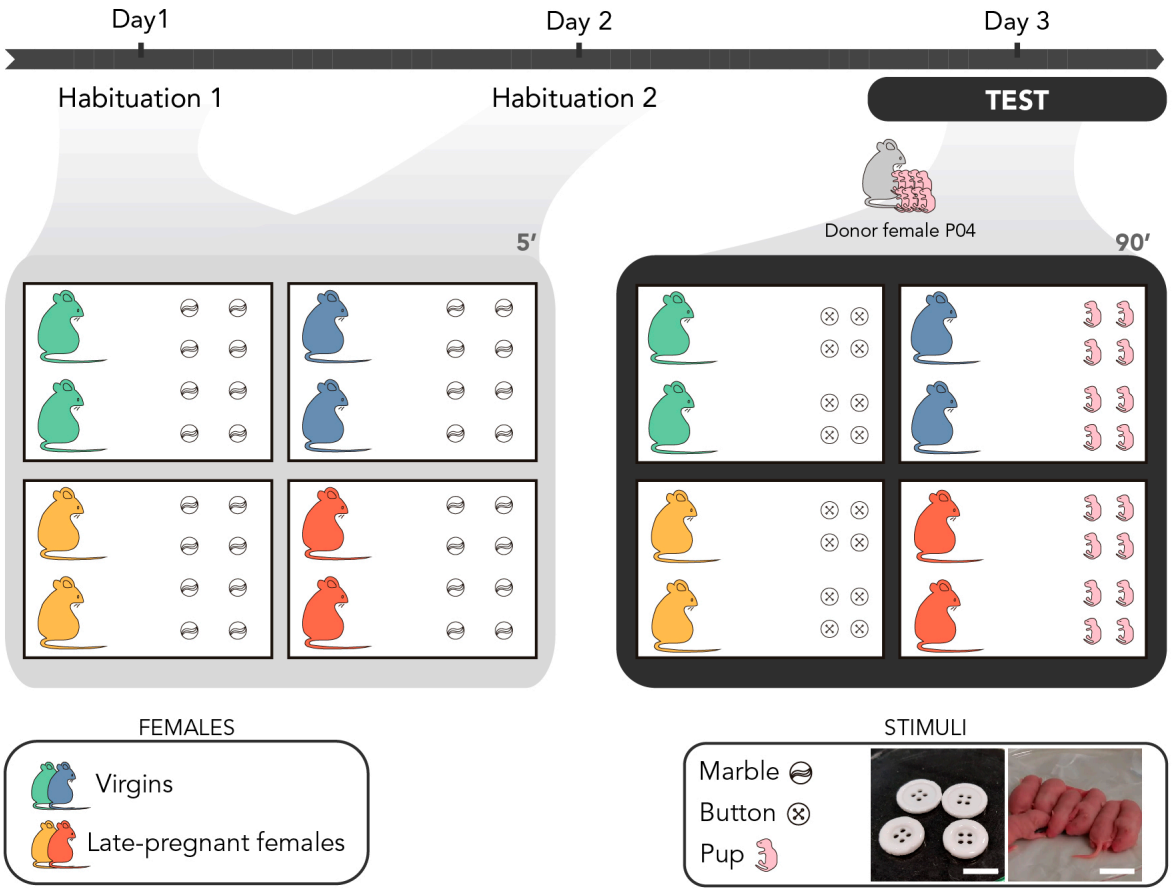

Figure 2.1. Experimental design of the exposure/behavioural test. Procedure consisted on 2 days of habituation in which females were exposed to marbles (grey background), plus the experimental test conducted on day 3 (black background). The test consisted on exposure of pairs of virgins and pairs of late-pregnant females to either 8 buttons or 8 pups, approximately the same size (see STIMULI, scale bars represent 2 cm). Groups are represented using a colour code (see FEMALES); virgins are represented with cool colours (green for those exposed to buttons, blue for those exposed to pups) and late-pregnant females with warm colours (yellow for those exposed to buttons, orange for those exposed to pups).

Within these 8 minutes, thirty-two 5-seconds periods were analysed (four 5-seconds periods per min, separated by 10-seconds intervals). For each 5-seconds period, an observer blind to the experimental conditions (and the design of the experiment altogether) registered the most maternal behaviour exhibited by the female, according to the following hierarchy: pup **retrieval**, females carried the pups to the nest; **in nest**, females stayed inside the nest in close contact with pups; **nest building**, females gathered pieces of nest material; **on nest**, females were located on the nest, near the pups but not in contact with them; **approach and retreat**, olfactory exploration of pups out of the nest, not followed by retrieval; and **off nest**, females were out of the nest and show no interaction with pups.

Then, 32 behavioural events were registered for each animal, distributed among the items described above. Nests were big and well-organized so that during in-nest periods the female

and the pups could not be observed. Therefore, specific pup-care items occurring within the nest (licking grooming, arch-back posture of the female) were not assessed.

Moreover, those behavioural items were used to calculate maternal and chemosensory scores for each animal. The maternal score is a weighted sum of those episodes in which female's behaviour reflects a maternal state (pup retrieval, nest building, in nest, and on nest):

$$Maternal\ Score = 5\ x\ Retrieval\ +\ 5\ x\ In\ Nest\ +4\ x\ Nest\ Building\ +\ 2\ x\ On\ Nest$$

In the same way, the chemosensory score is composed of a weighted sum of episodes in which the females are likely interacting and sniffing at pups:

$$Chemosensory\ Score = 5\ x\ In\ Nest\ +\ 3\ x\ Retrieval\ +3\ x\ Approach\ To\ Pups\ +\ 1\ x\ On\ Nest$$

At the end of experiment, we observed 1–3 pups killed, sometimes partially mutilated, in the cages of LP females. Then, we revised the video/audio-recordings and identified those moments in which pup-directed attacks occurred, which were easy to recognize as they always occurred while the female was out of the nest, licking-grooming a pup, which suddenly started emitting strong distress vocalizations which stopped after a few seconds. We measured the latency to each attack to a pup and assigned it to the female that displayed pup-directed aggression. For each female we calculated a pup aggression score:

$$Pup\ Aggression\ Score = \sum_{i=1}^{i=8}(25\ -\ latency\ to\ attack\ pup\ i)$$

A latency of 25min was assigned for those females not attacking pups (all pup attacks occurred during the first 24min). This way, pup aggression score was zero for the females not expressing any pup-directed aggression, and it was higher for those females attacking more pups and/or attacking pups with a lower latency.

Finally, the interaction between females in the same cage was also measured for each of these 32 5-s periods as present (1) or not present (0), considering an interaction when a female sniffed the other.

B. Observation and quantification of behaviour of a second set of females exposed to buttons

Since we were mainly interested in pup-directed behaviours, we did not record behavioural responses of the females exposed to buttons in our initial experiment. Nevertheless, the results revealed differences in brain activation between virgins and late-pregnant females exposed to buttons. This lead us to evaluate how virgins and late-pregnant females interacted with buttons, so we replicated the exposure to buttons in a second set of females (n=16). To do so, virgins (n=8, 4 pairs) and late pregnant (n=8, 4 pairs) females were treated exactly as those in the previous experiment, e.g. they were housed in same-condition couples and were habituated for two days to introducing 8 objects (glass marbles) in their home cage at the same time of the day (see Fig. 2.1). The next day, when pregnant females were in postconceptional day 17, 8 buttons were deposited in their home cage and their behaviour was video-recorded for 20 min.

Then, using BORIS (Behavioural Observation Research Interactive Software, Friard and Gamba, 2016), a person blind to the experimental conditions observed the videos and measured the number and duration of interactions of the females with buttons (sniffing at them) and inter-female interactions for the first 5 min after the introduction of the buttons.

These females were only used for behavioural analysis and did not undergo perfusion or tissue processing, so the next methodological sections only refer to the initial experiment using 30 females.

2.2.3. Tissue processing and immunohistochemistry

Following 90min of stimulus introduction, females from the initial experiment were overdosed with an intraperitoneal injection of sodium pentobarbital (Vetoquinol, Madrid, Spain; 0.02 mg/g of body weight, Shipley & Adamek, 1984) and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB), pH 7.4. Brains were dissected from the skull, snouts were separated from the skull and muscles removed in order to obtain a block with the VNO. Both brains and snouts were post-fixed overnight in 4% PFA at 4°C. After fixation, snouts were washed 3 × 10’ in 0.01M phosphate buffer (PB) with 0.9% NaCl (PBS), decalcified using 250mM EDTA in 0.1M PB during 5 days at 4°C and washed 3 × 10’ in 0.05MTris Buffer (TB) with 0.9% NaCl (TBS), pH 7.6. Then they were placed into a cast of warm 15% gelatine in 0.05MTB, kept at 4°C overnight, trimmed and placed in 4% formaldehyde in 0.1M PB for 2 h at 4◦C.

Brains and snout blocks were cryoprotected in 30% sucrose in 0.01M PB at 4°C until they sank, and then coronal sections (snouts 30 µm-thick; brains 40 µm-thick) were obtained using a freezing microtome (Microm HM-450, Walldorf, Germany), collected in five parallel series in 30% sucrose in PB and stored at −20◦C. One series of snout sections of each animal was processed for free-floating immunohistochemistry of Egr-1 protein, in order to assess the activity of VNO sensory neurons (Isogai et al., 2011). To do so, sections were (a) rinsed 4 × 5min in TBS; (b) immersed in 1% H_2O_2 and 0,3% Triton X-100 in TBS solution for 30min for endogenous peroxidase inhibition; (c) rinsed 3 × 5min in TBS; (d) immersed for an hour in a blocking solution containing 4% normal goat serum and 0.3% Triton X-100 in TBS 0.01 M, pH 8; (e) incubated overnight at room temperature with the primary antibody (rabbit anti-Egr1, no. 4153S; Cell Signalling Technology) diluted 1:500 in the blocking solution; (f) rinsed 5× 5min in TBS; (g) incubated in 1:400 dilution of biotinylated goat anti-rabbit secondary antibody (Vector BA1000) in the blocking solution for 2 h; (h) rinsed 5 × 5min in TBS; (i) transferred to 1:50 avidin-biotin-peroxidase complex (Vectastain-Elite, Vector Laboratories) in TBS for 90min; (j) rinsed 3 × 5min in TBS and 3 × 5min in 0.05M TB, pH 7.6; and finally, (k) the peroxidase activity was revealed with diaminobenzidine tetrahydrochloride (DAB) reaction (0.025% DAB and 0.01% H_2O_2 in TB). The reaction was stopped by successive rinsing of sections in TB. Sections were mounted on slides and coverslipped in DPX (Scharlau Laboratory).

In parallel, a series of brain free-floating sections were processed for cFos immunohistochemistry. Sections were (a) rinsed 3 × 10min in TBS; (b) immersed in 1% H_2O_2 in TBS solution for 30min for endogenous peroxidase inhibition; (c) rinsed 3 × 10min in TBS; (d) immersed for an hour in a blocking solution containing 3% normal goat serum, 3% bovine serum and 0.3% Triton X-100 in 0.01M TBS, pH 8; (e) incubated overnight at room temperature with the primary antibody (rabbit anti-cFos n◦. 226003; Synaptic Systems) diluted 1:5,000 in the blocking solution; (f) rinsed 3 × 10min in TBS; (g) incubated in 1:200 dilution of biotinylated goat anti-rabbit secondary antibody (Vector BA1000) in the blocking solution for 2 h; (h) rinsed 3 × 10min in TBS; (i) transferred to 1:50 avidin-biotinperoxidase complex (Vectastain-Elite, Vector Laboratories) in TBS for 90min; (j) rinsed 2 × 10min in TBS and 2 × 10min in TB (Tris Buffer 0.05M pH 7.6); and finally, (k) the peroxidase activity was revealed with diaminobenzidine tetrahydrochloride (DAB) reaction (0.025% DAB and 0.01% H2O2 in TB). The reaction was stopped by successive rinsing of sections in TB. Sections were mounted on slides and coverslipped in DPX (Scharlau Laboratory).

For each immunohistochemistry (Egr1 and cFos), sections of animals of the different groups (LP and virgin females exposed to buttons and pups) were processed simultaneously using the

same batches of reagents and antibodies, in order to minimize inter-individual variability and to avoid inter-group bias.

2.2.4. Image analysis

For the assessment of Egr1 expression, images of all VNO sections of a series (1 in 5) were acquired at 10× using a digital camera (DFC495) attached to a microscope Leitz DMRG (Leica, AG, Germany) and evaluated with ImageJ (NIH) (see Fig. 2.4A-D). Acquisition conditions included gamma=1 and a level of exposure just high enough as to avoid white saturation in void areas of the image. For each picture VNO Egr1 immunoreactive cells (Egr1-ir cells) were manually counted (cell counter tool, ImageJ) by a person who was blind to the experimental conditions of the samples. VNO area was calculated on Image J software (NIH). Then, for each animal, Egr1 density (Egr1-ir cells/mm^2) was calculated by dividing the total number of Egr1-ir cells counted in all the VNO sections by the total area of these sections.

Expression of cFos was assessed in a selection of brain nuclei involved in chemosensory processing, including nuclei from both vomeronasal and olfactory systems. For the vomeronasal system we sampled the accessory olfactory bulb (AOB, mitral cell layer) and its main synaptic targets, the posteromedial cortical amygdaloid nucleus (PMCo), the medial amygdala (posterodorsal division, MePD) and the medial part of the posteromedial division of the bed nucleus of the stria terminalis (BSTMPM). Concerning the olfactory system, we analysed the main olfactory bulb (MOB, granular cell layer) and the anterior and posterior divisions of the piriform cortex (PirAnt and PirPost, respectively). The expression of IEGs in the granular layer of the MOB is a good estimator of the activity of the centre and reflects the activity of the projection neurons (mitral cells; see Bepari et al., 2012).

For each nucleus, we sampled specific frames at particular anteroposterior levels, as indicated in Figures 2.6 y 2.7 (Paxinos & Franklin, 2004). We acquired images of both hemispheres as described above and, in the case of the MePD, selected a triangle-shaped region of interest to exclude the optic tract (Figure 2.6.C). Image processing and analysis were conducted on ImageJ software (NIH). Briefly, the RGB colour image was converted to grayscale by selecting the green channel. Images were then binarized setting the threshold at 75% of the mode of the grey histogram, so that every pixel below this threshold was considered labelled. The resulting binary images were further filtered using commands “fill holes,” “open” (3 iterations), and “watershed.” Then, particles were automatically counted, discarding those smaller than half

the average size of the cells from that specific nucleus (calculated in turn by measuring the average area of six randomly selected intensely labelled cells in the nucleus).

For most nuclei the density of cFos-ir cells (cells/mm^2) was calculated by dividing the total number of particles in both hemispheres, by the sum of the areas of the regions of interest. In the AOB and the MOB the high density of cells and intensity of immunostaining made it difficult to separate single cells using the image analysis procedure described above. Therefore, we simply measured the area fraction occupied by labelling after thresholding (immunoreactive area/total area).

2.2.5. Statistical analysis

A. Behaviour

We first compared the behaviour of the females (virgins and LP females) exposed to pups. To do so, after testing for normality with the Kolmogorov-Smirnov test, data derived from most of the behavioural events did not follow a normal distribution or showed normality but not homogeneous variance. Then, behavioural differences (behavioural events or scores) between virgin and LP females exposed to pups were evaluated using a two-sample t test for non-homogenous variances (for samples showing normality) or a Wilcoxon test for those displaying no normal distribution. For the behavioural test concerning the second set of females exposed to buttons, we also applied a Wilcoxon test since data did not accomplish normality even after logarithmic transformation.

B. Intra-nucleus comparison of Egr-1 and cFos-ir cell density

Regarding the analysis of Egr-1 and cFos expression, when data accomplished normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene test), a two-way ANOVA was performed, with “FEMALE” (virgin or LP) and “STIMULUS” (buttons or pups) as factors. Significant FEMALE×STIMULUS interactions were explored by post-hoc pairwise comparison with Bonferroni corrections. If data did not fulfil normality and homoscedasticity, we applied the two-way ANOVA after logarithmic transformation ($Log_{10}[n + 1]$). If the transformation failed to render normality and/or homoscedasticity, a two-sample t-test for non-homogenous variances (for samples showing normality) or a Wilcoxon test (for those displaying no normal distribution) was performed with non-transformed data to assess the differences between FEMALE (virgin vs LP) and between STIMULUS (buttons vs pups).

C. Heterogeneity of the VNO

When inspecting the VNO sections, we realized that cross-sections through the centre of the VNO showed few Egr-1 positive cells whereas, very often, small sections through the tips of the VNO were rich in labelled cells (see Fig. 2.4A-C). Hence, we tried to analyse if this difference was significant or anecdotic. We first performed a Spearman non-parametric correlation study of the density in each section and the area covered by the VNO sensory epithelium in that section, since data did not accomplish normality. Then, we performed a second Spearman correlation analysis separately for each group of females: Virgins/buttons, Virgins/pups, LP/buttons, LP/pups.

Second, we tested if specific populations located at the ends of the VNO were sensitive to pup-derived stimuli. To check further this possibility we selected the two largest (central) and two smallest sections (through the tip of the VNO) of each animal and compared the density of Egr1-positive cells between females (virgins, LP) and stimuli (buttons, pups). To do so, we performed a logarithmic transformation of the data which resulted in normality and homoscedasticity, so we used a three-way ANOVA with an intra-subject factor (LEVEL; large vs small sections) and two inter-subject ones (FEMALE, virgins vs LP; and STIMULUS, buttons vs pups).

D. Brain-behaviour correlation analysis

After that, we explored Spearman correlations between behavioural data and IEGs (Egr1-ir for the VNO; cFos-ir for the brain) separately in LP and virgin females exposed to pups. This allows investigating the relationship between activity in specific olfactory and vomeronasal nuclei with the expression of specific maternal behaviours and exploring this relationship during pregnancy.

E. Inter-nuclei correlation analysis

Finally, we performed Spearman correlation analyses between IEGs expression in the VNO and the different chemosensory brain centres in both groups of pup-exposed females. This allows investigating patterns of neural activity in the centres of the olfactory and vomeronasal systems during interaction with pups and exploring whether hormones acting during late pregnancy may change these patterns of brain activity.

All statistical analyses were performed using SPSS software package (IBM). The significance level was set at $p<0.05$.

2.3. Results

2.3.1. Behaviour of late-pregnant and virgin female mice exposed to pups

During exposure to pups, we scored several of the behavioural events displayed by females: pup retrieval, in nest, nest building, on nest, approach and retreat, off nest and pup-directed aggression and interfemale interaction.

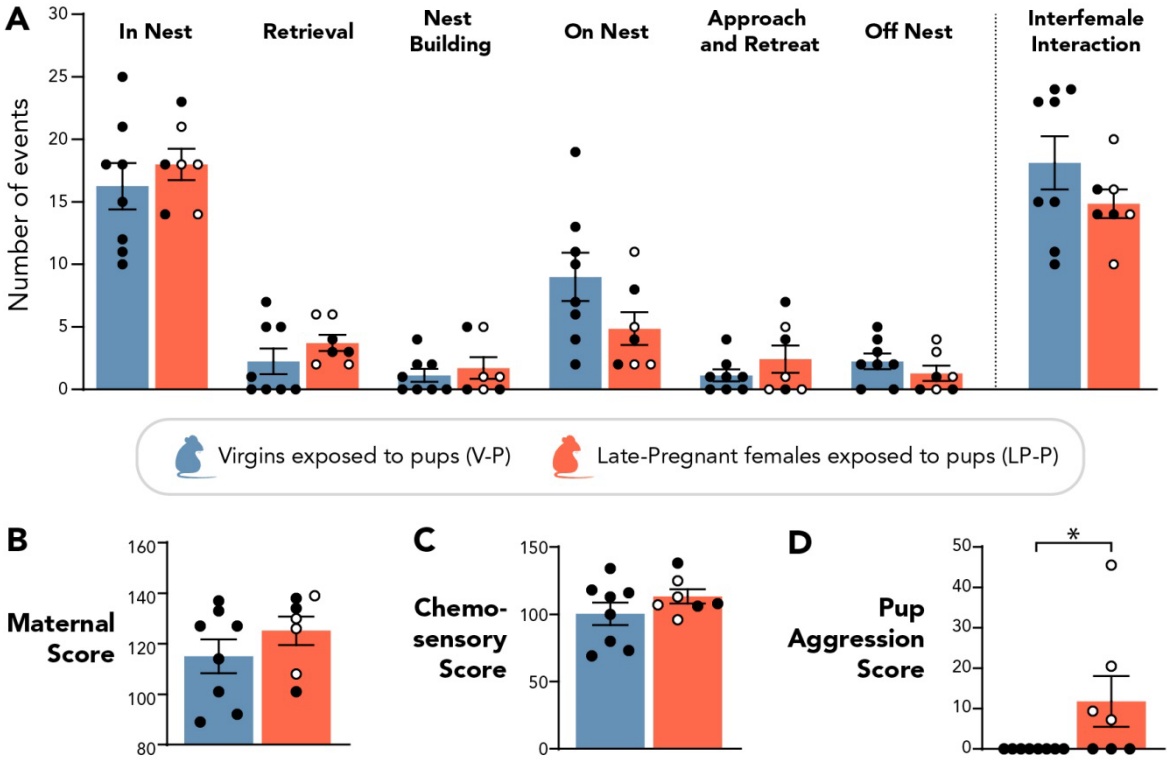

Figure 2.2. Behaviour of virgin and late pregnant (LP) female mice following exposure to pups. Colour legend is shown in the grey rectangle; blue bars correspond to virgins, orange bars correspond to late-pregnant females. **A.** Histogram showing the occurrence of maternal behaviours and interfemale interactions (mean ± SEM), scored as number of events during the first 8min after pup introduction (in nest, retrieval, nest building, on nest, approach and retreat and off nest; see text), did not show significant differences between female groups. **B.** Similarly, the maternal score and **C.** chemosensory score were similar in virgins and late-pregnant females. **D.** Finally, the pup aggression score was significantly different between female groups, since only some of the LP females performed pup-directed aggression (*p<0.05). Individual data are also shown, with empty circles corresponding to the LP females displaying pup-directed aggression, and black filled ones corresponding to those females not displaying aggression.

Statistical analysis revealed nonsignificant differences for most behaviours between LP and virgin females (see Figure 2.2A; pup retrieval, Z =−1.289, p=0.197; in nest, t =−0.760, p=0.461; nest building, Z=-0.368, p=0.713; on nest, t=1.722, p=0.109; approach and retreat, Z=-0.544, p=0.587; off nest, t=1.105, p=0.289). In a similar way, there were no differences between females concerning the maternal score (Fig. 2.2B; t=−1.141, p=0.274) and the chemosensory

score (Fig. 2.2C; t=−1.252, p=0.233). Four out of 7 LP females displayed pup aggression: one attacked three pups, one attacked two pups, and two attacked one pup each. By contrast, virgin females did not attack pups. Accordingly, comparison of pup aggression score rendered significant differences between females (Fig. 2.2D; Z=-2.376, p=0.017). Finally, interfemale contact did not differ between the LP and virgin females (Fig. 2.2A; Z=-1.108, p=0.268).

Overall, these results show that maternal behaviour does not differ substantially between LP and virgin females. Also, possible differences in the activity of chemosensory brain centres between females (or the VNO) cannot be attributed to differences in interaction with pups, since with exception of pup-directed aggression, LP and virgin females displayed similar behaviour. Moreover, differences in IEGs-ir between females cannot be attributed either to interfemale interactions.

2.3.2. Behaviour of late-pregnant and virgin female mice exposed to buttons

Since we did not record the buttons-exposed groups in the initial experiment, independently, we performed a replica of the original procedure but with a different set of females and only with exposure to buttons, not pups.

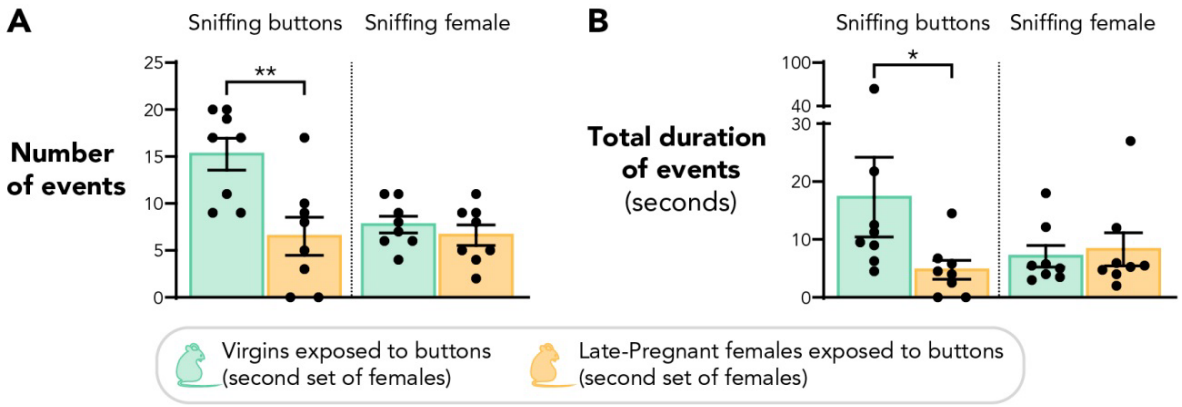

Figure 2.3. Interaction with buttons and inter-female interactions of the second set of animals exposed to buttons. Colour legend is shown in the grey rectangle; green bars correspond to virgins, yellow bars correspond to late-pregnant females. **A.** Number of sniffing bouts (mean ± SEM) **B.** Total investigation time (mean ± SEM). Bar histograms at left of the dividing dotted vertical line illustrate differences in the investigation of buttons and bar histograms at right of the dividing dotted vertical line illustrate differences in the investigation of partner-female. Statistical significant differences are indicated by * (* indicates a p-value below 0.05 and ** indicates a p-value below 0.01).

This second experiment allowed us to measure if there were differences in the exploration of the non-social stimuli between V and LP. Wilcoxon test for independent samples showed that the two groups differed significantly in both the number of events (Z=-2.645, p=0.007) and the

total time of sniffing (Z=-2.366, p=0.015), as shown in Figure 2.3. By contrast, females did not differ in the time they spent sniffing at their cage mate either in number of events (Z=-0.742, p=0.505) or total duration of sniffing (Z=-0.210, p=0.878). Therefore, our results suggest that pregnancy changes specifically the investigation of non-social objects.

2.3.3. Response of the vomeronasal system to pup-derived stimuli

A. Response of the vomeronasal organ

One of the aims of this work is to explore the response of the vomeronasal system to possible pup-derived chemosignals detected by the VNO, and the possibility that adult females change their sensitivity to these stimuli and/or their sensory processing mechanisms during late pregnancy. To do so, we analysed the neuronal response of the VNO and the primary and secondary vomeronasal brain centres by using quantification of IEGs expression in LP and virgin female mice.

First, we analysed the response of the VNO to pups or buttons exposure in LP and virgin females. A two-way ANOVA of log-transformed Egr1-ir cell density detected a significant main effect for STIMULUS ($F_{1,24}$=10.259, p=0.004), but no significant differences for FEMALE ($F_{1,24}$=1.249, p=0.275) and no FEMALE×STIMULUS interaction ($F_{1,24}$=1.223, p=0.280). As expected, pups induced a higher Egr1-ir cell density in the VNO compared to buttons in both LP and virgin females (Fig. 2.4.E). This suggests that pups secrete chemosignals that are detected by the VNO of adult females.

When exploring the distribution of Egr1-positive cells in the frontal sections of the VNO in our material, it seemed that labelled cells were concentrated in small sections, corresponding to the tip of the organ, and were less frequent in large, central sections (compare Figs. 2.4B and 2.4C). Therefore, we explored a possible non-homogenous expression of Egr1 in the VNO by performing a correlation analysis between Egr1-ir cell density and section area. Spearman's correlation test results indicated a significant global negative correlation (rho=-0.187, p=0.003): the larger the section, the lower the Egr1-positive cell density. A second Spearman correlation analysis for each group of females threw some interesting results. No significant correlation appeared between both variables in virgins (exposed to either pups or buttons; p>0.4) and LP exposed to buttons (p=0.076) but a highly significant negative correlation appeared in the group of LP exposed to pups (rho=-0.327; p<0.005). These data suggested that

response of VNO cells to pup chemosignals showed a different distribution in LP females and virgins.

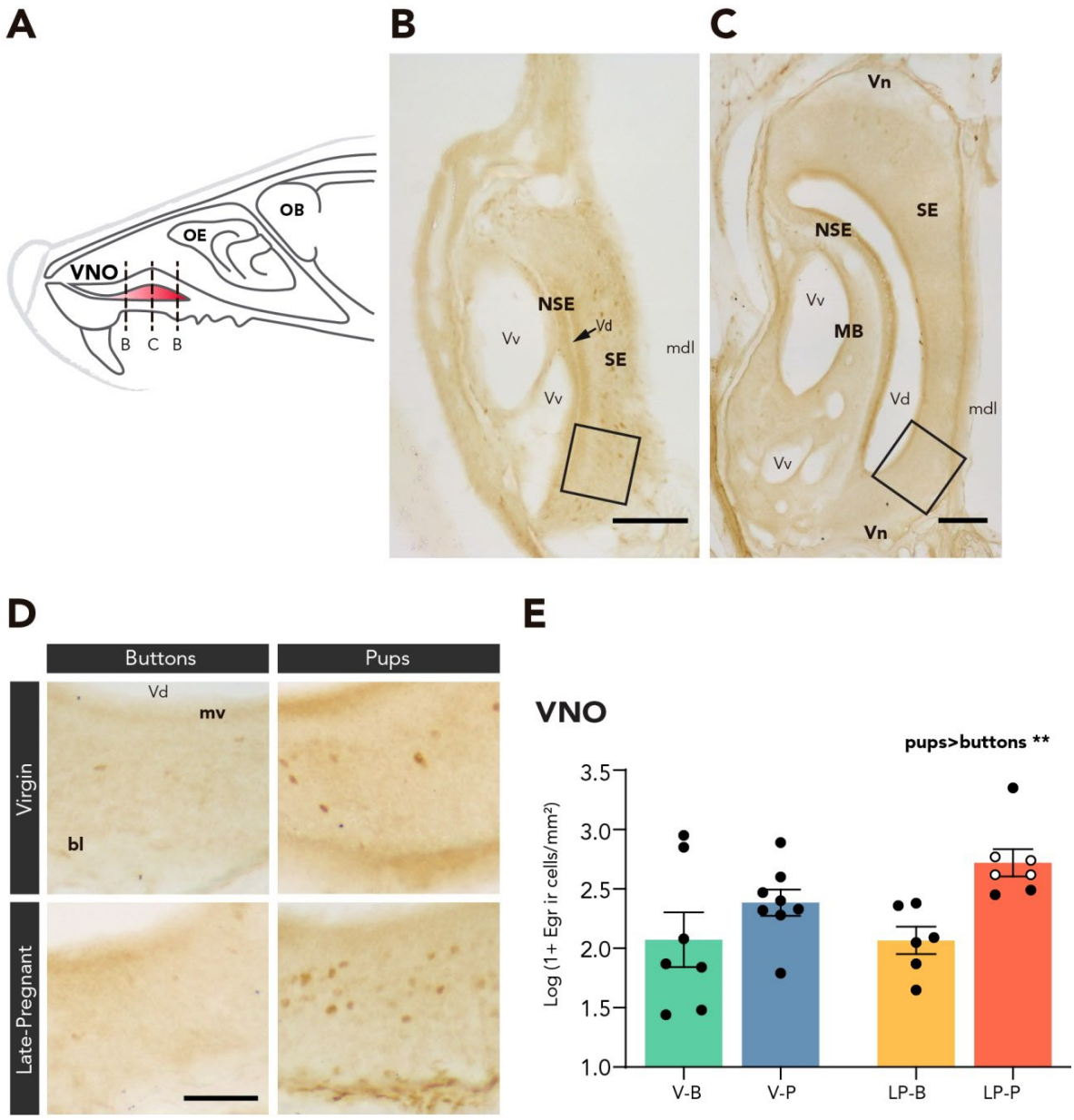

Figure 2.4. Expression of Egr1 in the vomeronasal organ (VNO) of virgins exposed to buttons (green bar; V-B), virgins exposed to pups (blue bar; V-P), late-pregnant females exposed to buttons (yellow bar; LP-B) and late-pregnant females exposed to pups (orange bar; LP-P). **A.** Diagram of the VNO illustrating the levels of the sections shown in B-C low-power photographs of the VNO. **B**. Tip of the VNO and its main anatomical landmarks. **C**. Centre of the VNO and its main anatomical landmarks. **D.** Examples similar to the framed areas in B-C at higher magnification for each experimental group. **E.** A bar histogram of raw data (mean ± SEM) of Egr1-positive cells in the VNO of the different groups is shown, where individual data are also plotted. The empty circles correspond to those females displaying pup-directed aggression. Statistical analysis of the density was performed on the log transformed values to achieved normality and homoscedasticity (see text). Egr1 expression is increased in response to pups (**p<0.01) as compared to a socially neutral stimulus (buttons), but there is no difference between virgin and late-pregnant females. Scale bars: B-C 100µm; D 50µm. Other abbreviations: bl, basal layer of the VNO; MB, mushroom body of the VNO; mdl, midline; mv, microvillar layer; NSE, non-sensory epithelium of the VNO; OB, olfactory bulb; OE, olfactory epithelium; SE, sensory epithelium of the VNO; Vd, vomeronasal duct; Vn, vomeronasal nerve; Vv, vomeronasal vein.

To further explore this finding, we tested whether this might be due to heterogeneous distribution of specific cell population responding to pups. To do so, we selected the two largest (central) and the two smallest sections (tip) of each animal and analysed whether they showed different density of Egr1-ir cells in Virgin and LP females exposed to pups and buttons, using a three-way ANOVA (see Fig. 2.5). The results confirmed a strong effect of the stimulus (pups rendered higher density of Egr1-ir cells than buttons; p=0.013) and the level (tip sections having significantly higher density of Egr1-ir cells than central sections; p<0.001). Interactions between factors, LEVEL×FEMALE, LEVEL×STIMULUS, STIMULUS×FEMALE, or LEVEL×STIMULUS×FEMALE were not significant (p>0.6 in all cases). Therefore, although expression of Egr1 was dependent on the stimulus and the level or the VNO (heterogeneous distribution), this was not dependent on the female (does not change with pregnancy).

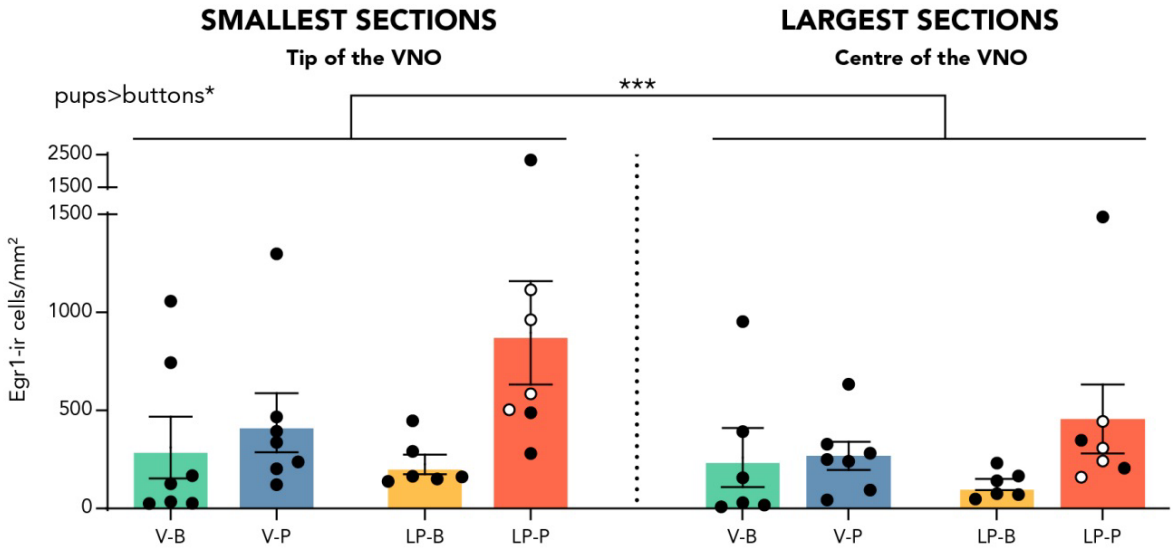

Figure 2.5. Heterogeneous distribution of Egr1-positive cells in the VNO of females exposed to pups and buttons. The figure plots the number of Egr1-immunoreactive cells/mm^2 found in the smallest (left) and largest (right) sections of the VNO for each group. Different groups are shown by colours and labelling; virgins exposed to buttons (green bar; V-B), virgins exposed to pups (blue bar; V-P), late-pregnant females exposed to buttons (yellow bar; LP-B) and late-pregnant females exposed to pups (orange bar; LP-P). Individual data are also plotted. The empty circles correspond to those females displaying pup-directed aggression. Statistical significant differences are indicated by * (*** indicates a p-value below 0.001).

B. Response of the vomeronasal brain centres

Then, we explored cFos expression in primary and secondary vomeronasal brain centres. For the AOB (Fig. 2.6), a two-way ANOVA of Log cFos-ir area fraction revealed a significant main effect for STIMULUS ($F_{1,19}$=4.527, p=0.047), but no significant differences for FEMALE ($F_{1,19}$=0.272, p=0.608) neither FEMALE×STIMULUS interaction ($F_{1,19}$=1.288, p=0.270). Thus, pups evoked a higher expression of cFos in the AOB as compared to buttons in both groups of females (Fig. 2.6A–A'').

However, for the secondary vomeronasal brain centres, e.g., the PMCo (vomeronasal cortex), the MePD and the BSTMPM, statistical analysis revealed further significant differences. Thus, the two-way ANOVA of Log cFos-ir cell density in the PMCo (Fig. 2.6B-B'') showed a significant main effect for STIMULUS ($F_{1,26}$=51.313, p<0.001) and significant FEMALE×STIMULUS interaction ($F_{1,26}$=21.597, p<0.001), but no differences were found for FEMALE factor ($F_{1,26}$=2.346, p=0.138). Post-hoc pairwise comparisons revealed that pups elicited higher response in LP than in virgin females (p=0.037), whereas buttons raised higher response in the virgin group than in pregnant females (p<0.001). In the LP group, pups elicited higher cFos response than buttons (p<0.001), whereas this difference did not reach significance in virgin females (p=0.077) (Fig. 2.6B–B'').

Likewise, the two-way ANOVA of Log cFos-ir cell density in the MePD showed a significant main effect of STIMULUS ($F_{1,26}$=81.312, p<0.001) and FEMALE×STIMULUS interaction ($F_{1,26}$=15.27, p=0.001), but no main effect of FEMALE ($F_{1,26}$=1.143, p=0.295). Post-hoc analysis of these effects revealed that pups elicited higher cFos response than buttons in both LP (p<0.001) and virgins (p=0.001) (Fig. 2.6C–C''). On the other hand, exposure to buttons elicited a higher level of cFos in virgins than LP (p=0.002), but interfemale differences in pup-induced cFos-ir cell density did not reach significance (p=0.055). Overall, our results revealed that pup exposure induced a higher neuronal response in the PMCo (not significant for the MePD) of LP vs virgin females, whereas buttons, used as neutral vomeronasal stimulus, induced a lower neuronal response in LP than in virgin females in both brain areas.

Concerning the BSTMPM, Wilcoxon test comparing stimuli rendered significant differences (Z=-2.426, p=0.015), with pups eliciting higher cFos levels than buttons, whereas comparison of females did not reveal significant differences (Z=-0.725, p=0.469) (Fig. 2.6D–D''). The pattern of activity in the BSTMPM (cFos expression) in the different females exposed to pups and buttons, was similar to the one observed for VNO and AOB, and different to the one seen in PMCo and MePD.

In sum, the pattern of cFos expression observed in response to pups and buttons differs between LP and virgin females in some secondary vomeronasal centres (PMCo and MePD), whereas both groups of females show similar response in the VNO, AOB, and BSTMPM. In general, pups elicit more activation than buttons, thus suggesting that buttons are a good control stimulus for vomeronasal stimulation.

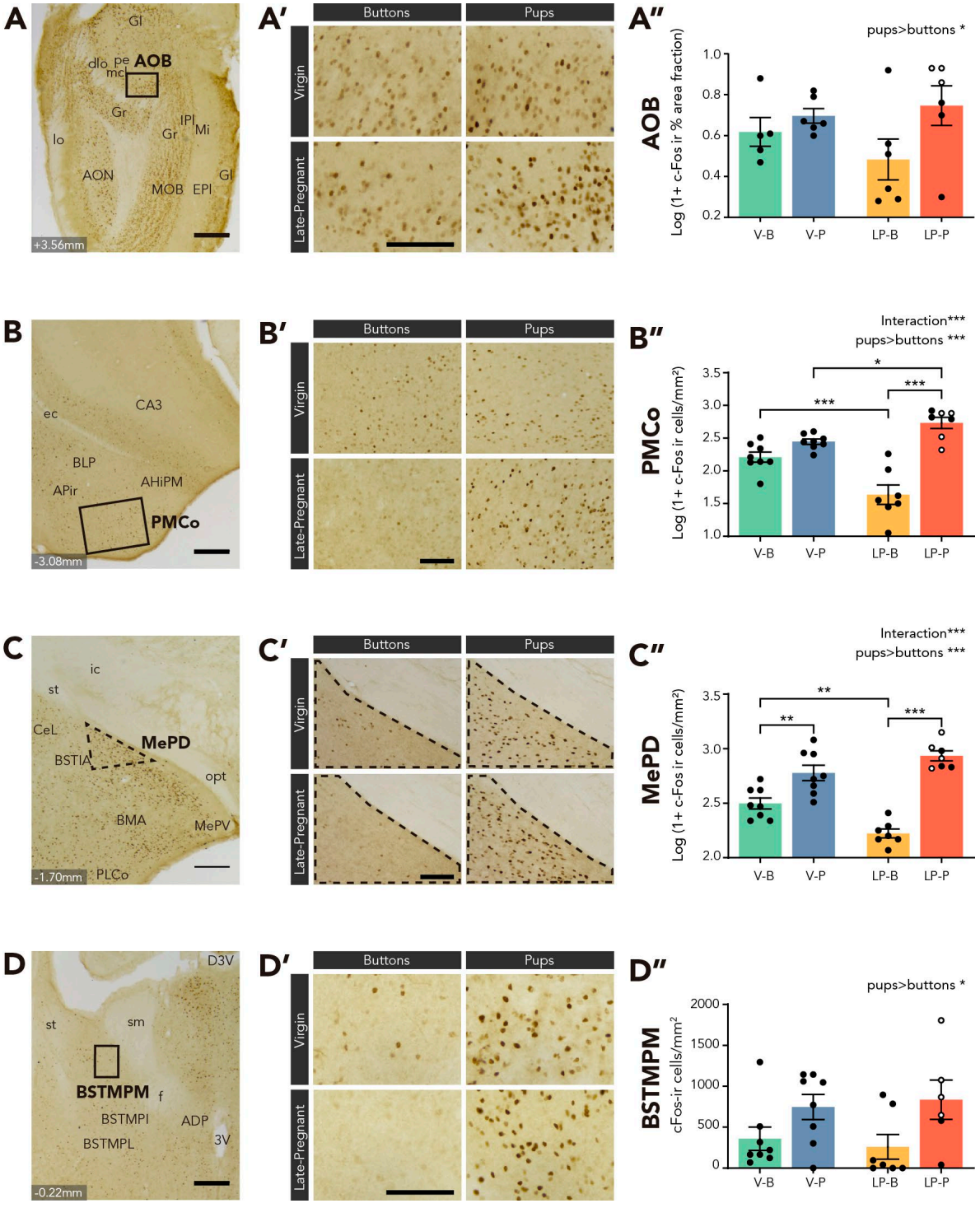

Figure 2.6. Expression of cFos in brain areas of the vomeronasal system following exposure to pups or non-social control stimulus. A-D. Low power photomicrographs showing cFos expression in (A) the anterior olfactory bulb (AOB), (B) the posteromedial cortical amygdaloid nucleus (PMCo), (C) the posterodorsal medial amygdala (MePD), and (D) the posteromedial part of the bed nucleus of the stria terminalis (BSTMPM). The numbers in the lower left corner of the images indicate the approximate anteroposterior coordinate of the sections relative to bregma (Paxinos and Franklin, 2004). Framed or dotted areas (regions-of-interest, ROI) indicate the specific zones where cFos expression was analysed. **A'-D'.** Example of photomicrographs of the brain regions analysed for each experimental group: virgin/buttons, virgin/pups, late-pregnant/buttons, and late-pregnant/pups. Images correspond to the AOB (A'), the PMCo (B'), the MePD (C'), and the BSTMPM (D'). Scale bars, 250µm (A–D) and 100µm (A'-D'). **A"-D".** Bar histogram showing the cFos positive cell density (mean ± SEM) in the vomeronasal

system. Different groups are shown by colours and labelling; virgins exposed to buttons (green bar; V-B), virgins exposed to pups (blue bar; V-P), late-pregnant females exposed to buttons (yellow bar; LP-B) and late-pregnant females exposed to pups (orange bar; LP-P). Individual data are also indicated, with empty circles corresponding to the females displaying pup-directed aggression. Significant main effects revealed by the statistical analysis are indicated for each histogram. When FEMALE×STIMULUS interaction is observed, the results of post-hoc pairwise comparisons are indicated using asterisks: ***p<0.001, **p<0.01, and *p<0.05. Other abbreviations: 3V, 3rd ventricle; ADP, anterodorsal preoptic nucleus; AHiPM, amygdalohippocampal area, posteromedial part; AON, anterior olfactory nucleus; APir, anterior lobe of pituitary; BLP, basolateral amygdaloid nucleus, posterior part; BMA, basomedial amygdaloid nucleus, anterior part; BSTIA, bed nucleus of the stria terminalis, intraamygdaloid division; BSTMPI, bed nucleus of the stria terminalis, posterointermediate part; BSTMPL, bed nucleus of the stria terminalis, posterolateral part; CA3, field CA3 of the hippocampus; CeL, central amygdaloid nucleus, lateral division; dlo, dorsal lateral olfactory tract; D3V, dorsal 3rd ventricle; EPI, external plexiform layer of the olfactory bulb; ec, external capsule; f, fornix; Gl, glomerular layer of the olfactory bulb; Gr, granular layer of the olfactory bulbs; ic, internal capsule; IPl, inner plexiform layer of the olfactory bulbs; ir, immunoreactive; lo, lateral olfactory tract; mcl, mitral cell layer of the accessory olfactory bulb; MePV, medial amygdaloid nucleus, posteroventral part; Mi, mitral cell layer of the main olfactory bulb; MOB, main olfactory bulb; opt, optic tract; pe, external plexiform layer of the accessory olfactory bulb; PLCo, posterolateral cortical amygdaloid nucleus; sm, stria medullaris of the thalamus; st, stria terminalis.

2.3.4. Response of the main olfactory system to pup-derived stimuli

Regarding the main olfactory system, we studied the cFos response in the MOB and the olfactory cortex, PirAnt and PirPost, following pups or neutral stimulus exposure in LP and virgin mice. The statistical analysis for Log cFos-ir area fraction in the MOB showed significant differences for FEMALE ($F_{1,19}$=5.611, p=0.029) in favour of virgins, and a FEMALE×STIMULUS interaction effect ($F_{1,19}$=4.678, p=0.044), whereas no differences were found for STIMULUS condition ($F_{1,19}$=0.027, p=0.872). Post-hoc analysis of the interaction revealed that interfemale differences are mainly due to a significantly higher response of virgins to buttons as compared to LP females (p=0.006) (Figures 2.7A–A''), as females do not differ in their response to pups. Those results evidence that pups do not evoke a different response between female groups, but buttons raised a MOB response higher in virgins than in LP mice.

On the other hand, the ANOVA for the cFos-ir cell density in the PirAnt showed significant differences for STIMULUS ($F_{1,26}$=11.425, p=0.002; pups elicit more cFos-ir than buttons) and a FEMALE×STIMULUS interaction effect ($F_{1,26}$=21.748, p<0.001), but no significant main effect of FEMALE factor ($F_{1,26}$=0.358, p=0.555). Post-hoc comparisons showed that pups elicited higher cFos-ir density than buttons in LP females (p<0.001) but not in virgins (Figures 2.7B–B''). In fact, when comparing animals exposed to pups, LP females displayed significantly higher cFos-ir than virgin animals (p=0.001), and conversely, buttons elicited higher cFos expression in virgin than in LP females (p=0.008).

For the PirPost a two-way ANOVA showed a significant FEMALE×STIMULUS interaction ($F_{1,26}$=32.209, p<001), but no significant main effects for either FEMALE ($F_{1,26}$=0.620, p=0.438) or STIMULUS ($F_{1,26}$=1.436, p=0.242) (Figures 2.7C–C''). Post-hoc analysis showed that pups elicited higher response in LP than in virgin females (p=0.002), while buttons induced higher response in virgins compared to LP females (p<0.001). Moreover, differential effects of the stimuli were found within each female condition. Thus, LP females showed higher response to pups than to buttons (p<0.001), whereas virgin females had a higher response to buttons than to pups (p=0.003). Altogether, our results of neuronal activation in centres of the main olfactory system prompt to differential stimulus discrimination, with higher activation by buttons in virgin females and by pup-derived stimuli in LP females.

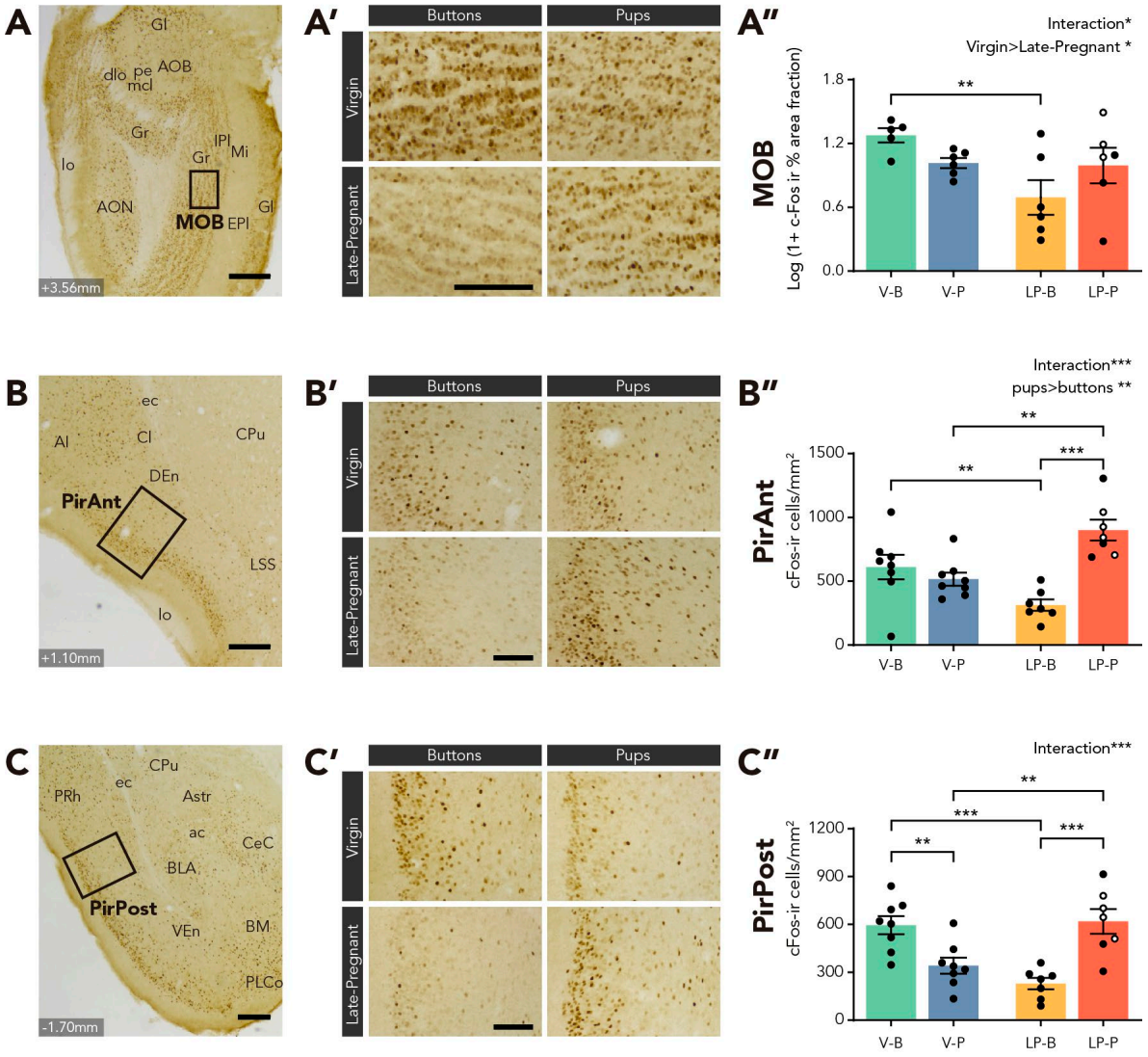

Figure 2.7. cFos response in brain areas of the main olfactory system following exposure to pups or non-social control stimulus. A-C. Low power photomicrographs showing the cFos immunohistochemistry in the (A) main olfactory bulb (MOB), (B) the anterior piriform cortex (PirAnt), and (C) the posterior piriform cortex (PirPost). The numbers in the lower left corner of the images indicate anteroposterior levels of the sections relative to bregma (Paxinos and Franklin, 2004). Framed areas indicate the specific zones where cFos expression was analysed. **A'-C'.**

High-power photomicrographs of the brain centres analysed for each experimental group: virgin/buttons, virgin/pups, late-pregnant/buttons, and late-pregnant/pups. Images correspond to the MOB (A'), the PirAnt (B'), and the PirPost (C'). Scale bars, 250µm (A–C) and 100µm (A'-C'). **A''-C''.** Bar histogram showing the density (mean ± SEM) of cFos-expressing cells in the main olfactory system. Different groups are shown by colours and labelling; virgins exposed to buttons (green bar; V-B), virgins exposed to pups (blue bar; V-P), late-pregnant females exposed to buttons (yellow bar; LP-B) and late-pregnant females exposed to pups (orange bar; LP-P). Individual data are also indicated, with empty circles corresponding to the females displaying pup-directed aggression. Significant main effects revealed by the statistical analysis are indicated for each histogram. When FEMALE × STIMULUS interaction is observed, the results of post-hoc pairwise comparisons are indicated using asterisks: ***p<0.001, **p<0.01, and *p<0.05. Other abbreviations: ac, anterior commissure; AI, agranular insular cortex; AOB, anterior olfactory bulb; AON, anterior olfactory nucleus; Astr, amygdalostriatal transition area; BLA, basolateral amygdaloid nucleus, anterior part; BM, basomedial amygdaloid nucleus; CeC, central amygdaloid nucleus, capsular part; Cl, caudal interstitial nucleus of the medial longitudinal fasciculus; CPu, caudate putamen (striatum); DEn, dorsal endopiriform nucleus; dlo, dorsal lateral olfactory tract; ec, external capsule; EPl, external plexiform layer of the olfactory bulb; Gl, glomerular layer of the olfactory bulb; Gr, granular layer of the olfactory bulbs; IPl, inner plexiform layer of the olfactory bulbs; lo, lateral olfactory tract; LSS, lateral stripe of the striatum; mcl, mitral cell layer of the accessory olfactory bulb; Mi, mitral cell layer of the main olfactory bulb; pe, external plexiform layer of the accessory olfactory bulb; PLCo, posterolateral cortical amygdaloid nucleus; PRh, perirhinal cortex; VEn, ventral endopiriform nucleus.

2.3.5. Correlation analysis of female behaviour and IEGs expression

Finally, we performed correlation analysis between behavioural measures and the levels of IEGs expression in the vomeronasal and olfactory systems of virgin and LP female mice exposed to pups. Spearman analysis revealed a different pattern of correlation in LP and virgin females (see Table 2.1). Thus, LP, but not virgins, displayed a significant positive correlation between "off nest" behaviour and the cFos response in the AOB (p=0.020) and the MOB (p=0.001). On the contrary, the behaviour "approach and retreat," consisting in sniffing a pup far from the nest without retrieving it afterwards, showed a significant positive correlation to cFos response in the AOB (p=0.011) and the MePD (p=0.017) in virgins, but not in LP females. Moreover, "nest building" was significantly and positively correlated with cFos response in the PirAnt (p=0.011) in LP females, but not in virgins.

The behaviour "pup retrieval" was the only one displaying a significant positive correlation to neuronal response in the PMCo of both virgin females (p=0.036) and in LP females (p=0.012). In addition, in LP but not virgins, maternal score showed a significant positive correlation with the response in the PMCo (p=0.023) and chemosensory score to the response in the MePD (p=0.023). Finally, pup-directed aggression score correlated to the neural response in the AOB (p=0.005) and in the BSTMPM (p<0.05) only in LP females. The behaviours "on nest," "in nest," and "interfemale interaction" did not correlate with IEGs expression in any vomeronasal/olfactory brain region analysed. In fact, expression in the VNO did not correlate with any behavioural item or score. Overall, those positive correlations showed that in LP

females, pup-directed and non-directed behaviours are mainly correlated to signal processing in some of the vomeronasal-related nuclei.

Table 2.1. Correlation analysis of IEGs expression in the VNO and centres of the chemosensory systems with behaviour in virgin (V-P) and late-pregnant females (LP-P) exposed to pups. Statistically significant correlations are indicated by bold values in group-specific coloured cell background (blue for virgins, orange for late-pregnant females) and asterisks *p<0.05; **p<0.01.

			VNO	AOB	PMCo	MePD	BSTMPM	MOB	PirAnt	PirPost
Off Nest	V-P	Correl. Coef.	0,307	0,617	0,172	0,061	-0,278	0,370	-0,098	0,049
		Sig. (bilateral)	0,460	0,192	0,684	0,885	0,505	0,470	0,817	0,908
		N	8	6	8	8	8	6	8	8
	LP-P	Correl. Coef.	0,561	**0,883***	0,112	0,393	0,559	**0,971****	0,449	0,374
		Sig. (bilateral)	0,190	**0,020**	0,811	0,383	0,249	**0,001**	0,312	0,408
		N	7	**6**	7	7	6	**6**	7	7
Approach and Retreat	V-P	Correl. Coef.	-0,200	**0,912***	0,350	**0,801***	0,164	-0,441	0,626	0,175
		Sig. (bilateral)	0,634	**0,011**	0,395	**0,017**	0,699	0,381	0,097	0,678
		N	8	**6**	8	**8**	8	6	8	8
	LP-P	Correl. Coef.	-0,482	-0,116	-0,556	0,148	0,030	-0,290	-0,148	-0,704
		Sig. (bilateral)	0,274	0,827	0,195	0,751	0,954	0,577	0,751	0,077
		N	7	6	7	7	6	6	7	7
On Nest	V-P	Correl. Coef.	-0,119	-0,657	-0,476	-0,143	0,263	-0,086	-0,143	-0,238
		Sig. (bilateral)	0,779	0,156	0,233	0,736	0,528	0,872	0,736	0,570
		N	8	6	8	8	8	6	8	8
	LP-P	Correl. Coef.	0,000	-0,152	-0,185	-0,630	-0,290	-0,395	0,185	0,037
		Sig. (bilateral)	1.000	0,774	0,691	0,129	0,577	0,439	0,691	0,937
		N	7	6	7	7	6	6	7	7
Nest Building	V-P	Correl. Coef.	0,651	0,555	-0,243	0,089	0,373	-0,123	0,153	-0,128
		Sig. (bilateral)	0,080	0,252	0,563	0,833	0,363	0,816	0,717	0,763
		N	8	6	8	8	8	6	8	8
	LP-P	Correl. Coef.	0,624	0,525	0,208	-0,567	0,359	0,309	**0,869***	0,624
		Sig. (bilateral)	0,135	0,285	0,655	0,184	0,485	0,552	**0,011**	0,135
		N	7	6	7	7	6	6	**7**	7
Retrieval	V-P	Correl. Coef.	-0,192	0,541	**0,741***	0,294	-0,707	0,439	0,192	0,396
		Sig. (bilateral)	0,650	0,268	**0,036**	0,480	0,050	0,383	0,650	0,332
		N	8	6	**8**	8	8	6	8	8
	LP-P	Correl. Coef.	-0,165	0,088	**0,863***	-0,441	-0,206	0,000	-0,092	-0,165
		Sig. (bilateral)	0,723	0,868	**0,012**	0,323	0,695	1.000	0,845	0,723
		N	7	6	**7**	7	6	6	7	7
In Nest	V-P	Correl. Coef.	-0,383	0,029	0,108	-0,252	0,108	-0,348	-0,168	-0,120
		Sig. (bilateral)	0,349	0,957	0,799	0,548	0,798	0,499	0,691	0,778
		N	8	6	8	8	8	6	8	8
	LP-P	Correl. Coef.	-0,056	-0,324	0,150	0,636	0,093	0,000	-0,487	0,150
		Sig. (bilateral)	0,905	0,531	0,749	0,125	0,862	1.000	0,268	0,749
		N	7	6	7	7	6	6	7	7
Maternal Score	V-P	Correl. Coef.	-0,240	-0,029	0,192	-0,240	-0,145	0,203	-0,144	0,048
		Sig. (bilateral)	0,568	0,957	0,649	0,568	0,733	0,700	0,734	0,910
		N	8	6	8	8	8	6	8	8
	LP-P	Correl. Coef.	0,143	-0,086	**0,821***	0,000	-0,029	0,200	-0,143	0,321
		Sig. (bilateral)	0,760	0,872	**0,023**	1.000	0,957	0,704	0,760	0,482
		N	7	6	**7**	7	6	6	7	7
Chemo-sensory Score	V-P	Correl. Coef.	-0,452	0,086	0,238	-0,024	0,012	-0,143	0,000	-0,048
		Sig. (bilateral)	0,260	0,872	0,570	0,955	0,978	0,787	1.000	0,911
		N	8	6	8	8	8	6	8	8
	LP-P	Correl. Coef.	-0,393	-0,371	-0,214	**0,821***	-0,029	-0,086	-0,714	-0,357
		Sig. (bilateral)	0,383	0,468	0,645	**0,023**	0,957	0,872	0,071	0,432
		N	7	6	7	**7**	6	6	7	7
Pup Aggression Score	V-P	Correl. Coef.	-	-	-	-	-	-	-	-
		Sig. (bilateral)	-	-	-	-	-	-	-	-
		N	8	6	8	8	8	6	8	8
	LP-P	Correl. Coef.	0,371	**0,941****	-0,556	0,259	**0,812***	0,698	0,259	0,408
		Sig. (bilateral)	0,413	**0,005**	0,195	0,574	**0,050**	0,123	0,574	0,364
		N	7	**6**	7	7	**6**	6	7	7
Female Interaction	V-P	Correl. Coef.	0,024	0,120	0,048	0,436	0,122	0,000	0,509	0,097
		Sig. (bilateral)	0,955	0,822	0,909	0,280	0,774	1.000	0,197	0,819
		N	8	6	8	8	8	6	8	8
	LP-P	Correl. Coef.	-0,374	-0,463	0,019	-0,412	-0,030	-0,772	-0,337	-0,056
		Sig. (bilateral)	0,408	0,355	0,968	0,359	0,954	0,072	0,460	0,905
		N	7	6	7	7	6	6	7	7

Finally, we also performed a correlation analysis of the levels of IEGs expression between vomeronasal and olfactory structures of females exposed to pups (Table 2.2). Interestingly, this analysis revealed that VNO activation correlated with cFos expression in the MOB (p=0.042), the PirAnt (p=0.023), and the PirPost (p=0.003) of LP females, while no similar correlations were found in virgins. Moreover, in LP females (but not virgins) AOB response displayed a positive correlation with MOB (p=0.042) and with the BSTMPM (p<0.001), whereas MOB and BSTMPM display no mutual correlation. By contrast, in virgin females, the BSTMPM displayed a significant negative correlation to PMCo (p=0.040) and to MOB (p=0.036), whereas the PirAnt positively correlated to MePD (p=0.002) and the PirPost (p=0.047).

Table 2.2. Correlation analysis of IEGs expression between the VNO and centres of the chemosensory systems in virgins and late-pregnant females exposed to pups. Statistically significant correlations are indicated by bold values and darker colours in group-specific coloured cell background (blue for virgins, orange for late-pregnant females) and asterisks *p<0.05; **p<0.01.

		VNO	AOB	PMCo	MePD	BSTMPM	MOB	PirAnt	PirPost
VNO	Correl. Coef.		-0,200	-0,643	-0,381	0,335	0,486	-0,262	-0,214
	Sig. (bilateral)		0,704	0,086	0,352	0,417	0,329	0,531	0,610
	N		6	8	8	8	6	8	8
AOB	Correl. Coef.	0,600		0,486	0,714	0,145	-0,314	0,771	0,143
	Sig. (bilateral)	0,208		0,329	0,111	0,784	0,544	0,072	0,787
	N	6		6	6	6	6	6	6
PMCo	Correl. Coef.	0,071	-0,143		0,667	**-0,731***	0,143	0,643	0,690
	Sig. (bilateral)	0,879	0,787		0,071	**0,040**	0,787	0,086	0,058
	N	7	6		8	**8**	6	8	8
MePD	Correl. Coef.	0,071	0,200	-0,464		-0,240	-0,143	**0,905****	0,571
	Sig. (bilateral)	0,879	0,704	0,294		0,568	0,787	**0,002**	0,139
	N	7	6	7		8	6	**8**	8
BSTMPM	Correl. Coef.	0,257	**1.000****	-0,543	0,314		**-0,841***	-0,228	-0,707
	Sig. (bilateral)	0,623		0,266	0,544		**0,036**	0,588	0,050
	N	6	**5**	6	6		**6**	8	8
MOB	Correl. Coef.	**0,829***	**0,829***	0,029	0,486	0,700		0,029	0,657
	Sig. (bilateral)	**0,042**	**0,042**	0,957	0,329	0,188		0,957	0,156
	N	**6**	**6**	6	6	5		6	6
PirAnt	Correl. Coef.	**0,821***	0,543	0,036	-0,321	0,314	0,543		**0,714***
	Sig. (bilateral)	**0,023**	0,266	0,939	0,482	0,544	0,266		**0,047**
	N	**7**	6	7	7	6	6		**8**
PirPost	Correl. Coef.	**0,929****	0,543	0,143	0,036	0,143	0,771	0,679	
	Sig. (bilateral)	**0,003**	0,266	0,760	0,939	0,787	0,072	0,094	
	N	**7**	6	7	7	6	6	7	

Overall, those results suggest that when exposed to pups LP females display an associated activity of the olfactory and vomeronasal systems. By contrast, in virgins, correlations mirror somehow the connectivity within the vomeronasal and olfactory pathways.

2.4. Discussion

In the present study, we explored pregnancy-induced adaptations of the response of the chemosensory systems to pups in female mice. To do so, we analysed the expression of IEGs in the VNO, as well as in the main centres of the vomeronasal and olfactory systems of virgin and

LP female mice, in response to pups or to a non-social stimulus (buttons). This allows assessing changes in sensory processing of pup-derived chemosignals occurring by the end of pregnancy, most likely associated to the action of pregnancy hormones known to be relevant for inducing full maternal behaviour. Last, we ascertained possible correlations between patterns of brain activity and behaviour. Overall, our results confirm that pup-derived chemosignals activate the VNO and reveal changes in stimulus processing in chemosensory systems by the end of pregnancy. In addition, our data suggest that activation of different vomeronasal pathways are likely underlying pup care or pup attack in LP females.

2.4.1. Methodological issues and behavioural response to pups

Our experimental design has several advantages. First, it prevents a differential novelty effect of pups in late-pregnant and virgin females, since both female groups were completely pup-naïve. This is relevant since novelty has a strong impact on exploratory behaviours (Rinaldi et al., 2010). Second, the use of a non-social control stimulus (buttons) is another advantage, since using non-exposed animals as controls does not allow us to interpret IEGs expression as due to a specific stimulus (pups). Third, the females were housed in pairs at least 20 days before the experiment, and tested also in pairs, which avoided isolation stress along the procedure. Moreover, in order to avoid possible competition for stimuli between both females, we introduced a large number of pups/buttons into the test cage (eight), so that both females could interact with them simultaneously and independently. Fourth, when designing a IEGs experiment, using LP instead of postpartum females to check the activity induced by pups has the additional advantage that does not require mother-infant separation. This suppresses another potentially confounding factor for interpreting the expression of IEGs in brain centres, e.g., pup-separation-induced stress (Aguggia et al., 2013).

Moreover, we are aware of some caveats in our procedure. First of all, the lack of behavioural recordings of the initial buttons-exposed groups limited the interpretation of Egr1 and cFos results, although the replica with the second set of females exposed to pups threw some enlightening results, which we will discuss in the next section. Secondly, the presence of two females in the same cage during the experiment may have interfered in the procedure, as an adult female is a source of chemosignals. Nonetheless, we minimized this possibility as we paired same-condition females for a long period and objects used as stimuli were introduced in large number to avoid competition, as above described. In any case, there were no significant differences in interfemale interactions between groups exposed to pups, therefore,

differences in IEGs expression in the pup-exposed groups are unlikely due to this factor, as supported also by the behaviour-IEG expression correlation analysis.

When analysing the behaviour of virgin and LP females during exposure to pups, we observed no differences in any pup-directed or non pup-directed behaviour item, except for pup-directed aggression performed by some LP females (4 females out of 7) (see below). Our results on that issue agree with previous reports showing that virgin female mice having no previous experience with pups do not display pup aversion. Instead, pups constitute a highly attractive stimulus for pup-naïve females (Alsina-Llanes et al., 2015; Martín-Sánchez et al., 2015b; Stolzenberg & Rissman, 2011). In those previous reports, authors demonstrated that maternal females (lactating dams or pup-sensitized virgins) display faster pup retrieval compared to pup-naïve virgins; however, in those experiments maternal females had previous pup-experience, while control virgins did not. Therefore, the lack of differences in most of the measured behavioural items between LP and virgin females in our experiment is likely due to pups being an equally novel stimulus for both kinds of females.

Although expression of IEG by neurons in vomeronasal and olfactory centres is mainly driven by detection of chemical stimuli, pups also emit distress vocalizations that are relevant in the context of maternal behaviours (Smotherman et al., 1974), together with olfactory cues with multisensory integration occurring at the level of the primary auditory cortex (Cohen et al., 2011). Whether, and to what extent, these stimuli might contribute to IEG expression in secondary olfactory and vomeronasal centres is not known. However, an analysis of the response of neurons in the primary auditory cortex to pup vocalizations (Marlin et al., 2015) revealed very faint response in pup-naïve virgins, as compared to pup-experienced virgins and dams. Since our females, both LP and virgins, had no previous experience with pups before the trial, we can safely assume that most, if not all, of the activity (IEG expression) observed in the centres of the vomeronasal and olfactory systems is due to pup-derived chemosignals.

2.4.2. Olfactory function and behavioural response to buttons

As commented in the previous section, although we did not have the behavioural results of the initial groups exposed to buttons, the replica performed on a different set of females helped us to interpret the results of the cFos activation of the initial buttons-exposed groups.

The behaviour of the second set of females exposed to buttons clearly indicates that virgins explore buttons significantly more than LP females (more episodes of button sniffing; more

time engaged in sniffing buttons). Moreover, this does not seem a general effect of pregnancy on mobility or on interest on exploring objects in their environment, as inter-female interactions (sniffing to the other female in the same cage) do not differ between virgin and LP females.

Likely, this decrease in chemoinvestigation of buttons in LP females is related to the decrease we spotted in the cFos activation of several chemosensory nuclei of our initial experimental buttons-exposed LP-females, when compared to buttons-exposed virgins. This defect is pretty remarkable in the MOS, since all 3 studied areas share a significant difference in activation between virgins and LP females exposed to buttons (MOB, PirAnt and PirPost). Nonetheless, two of the secondary nuclei of the vomeronasal system also respond similarly (MePD and PMCo), but since buttons are non-social objects, we assume that they do not directly activate vomeronasal neurons, so that the differential activation of these nuclei between buttons-exposed LP and virgins may be due to other afferents rather than direct vomeronasal inputs, as it will be discussed later.

As a conclusion, it seems that pregnancy reduces specifically interest in non-social objects such as buttons, which might have resulted in reduced Egr1 in VNO and cFos activity in MOS and secondary vomeronasal nuclei of the brain in LP females exposed to buttons, as compared with buttons-exposed virgin females.

2.4.3. Vomeronasal system function and behavioural response to pups

There is solid evidence indicating that VNO-detected chemosignals are likely crucial for some pup-directed responses, both parental and infanticide (Isogai et al., 2018; Kimchi et al., 2007; Nakahara et al., 2016; Tachikawa et al., 2013). In that respect, our results on Egr1 expression in the VNO demonstrate that pups are a source of vomeronasal stimuli for adult females (Figures 2.4D-E and 2.5). Unlike other previous reports, we use a control, non-social novel stimulus, and compare the Egr1 expression in the VNO induced by buttons to that induced by pups. A role of VNO-detected stimuli in maternal behaviour was proposed by Lepri et al. (1985), who reported delayed pup retrieval in lactating dams that had undergone removal of the VNO, as compared to sham-operated dams. Moreover, mice with impaired VNO function (null-trpc2 mice) display reduced maternal care (Kimchi et al., 2007), deficient nest maintenance and reduced nursing (Hasen and Gammie, 2011). Taken together, these data strongly suggest that pups emit chemosignals that are detected by the VNO of females and mediate adult female-pup

interactions in the context of motivated maternal behaviour. However, our results indicate that pup exposure did not elicit differential Egr1 expression in the VNO of LP and virgin females (Figures 2.4D-E and 2.5), thus suggesting that hormone-induced changes in neurogenesis during pregnancy (Oboti et al., 2015) or hormone-induced changes in sensory transduction at the level of the VNO (Dey et al., 2015) might not be very relevant in the context of detection of pup chemosignals. By contrast, those changes may be relevant for the response of females to adult male chemosignals (e.g., major urinary proteins, Dey et al., 2015) perhaps in the context of nest defence (Martín-Sánchez et al., 2015a), which is displayed by LP females (Mann & Svare, 1982). This lack of differences in VNO response to pups between females makes very unlikely that changes in pup-directed behaviours associated to pregnancy are due to altered sensitivity of the VNO. Instead, they should be attributed to altered sensory processing in the central nervous system during pregnancy. In this respect, our results also indicate that although pups induced an increase in cFos expression in the AOB (as compared to buttons) of females, this response was indistinguishable between virgin and LP females exposed to pups and a similar situation is found in the BSTMPM (compare Figures 2.6A'' and D''). This suggests that, like sensory transduction of pup chemosignals in the VNO, response to pups in the AOB-BSTMPM pathway is not under strong influence of pregnancy hormones.

Importantly, in the vomeronasal cortex (PMCo) and the medial amygdaloid nucleus (MePD), both secondary vomeronasal nuclei, LP females show increased activation by pups as compared to buttons, whereas virgins only show this pup-specific increase in cFos expression in the MePD, but not in the PMCo (where both stimuli elicit a similar activation). Consequently, in the PMCo, pup-induced cFos expression is significantly higher in LP than virgins. This indicates that sensory processing in the AOB-PMCo and AOB-MePD is modified during pregnancy. In the PMCo, this differential discrimination results in significant pup-button differences occurring only in LP females, suggesting that pregnancy modifies the functioning of specific vomeronasal pathways resulting in pup-specific activation of the vomeronasal cortex. This may be associated to reported changes in gene expression of key genes for endocrine signalling (e.g., receptor for prolactin) in afferents to the PMCo, such as the AOB and medial amygdala, during peripartum period in mice (Canavan et al., 2011). In addition, PMCo neurons display oestrogen and progesterone receptors in rodents (Hagihara et al., 1992; Mitra et al., 2003; Shughrue et al., 1997). Thus, the important changes in steroid hormone levels occurring during late pregnancy (progesterone withdrawal, oestrogen rise) may affect neural processing in the PMCo, altering the response to pup chemosignals.

An interesting finding of this work is the highly significant correlation observed between pup retrieval and cFos activity in the PMCo in both, LP and virgins. This points to a previous unknown role of this neural structure in the control of maternal behaviour (the vomeronasal cortex, Gutiérrez-Castellanos et al., 2014), which fits the impact of VNO lesion in pup retrieval (Lepri et al., 1985). In addition, in LP females (but not virgins) the expression of cFos in the PMCo shows a remarkable positive correlation with the maternal score, a weighted sum of episodes in which female's behaviour reflects a maternal state (pup retrieval, nest building, in nest and on nest). Although this suggests a relationship between both phenomena, PMCo activity and maternal behaviour, the causal relationship is not clear, e.g., whether the PMCo becomes activated by pups' stimuli during LP female interaction with them, or the PMCo activation is part of the neural mechanism responsible of the induction of maternal behaviour. The PMCo projects to the BMA and to some extent to the BLA (Gutiérrez-Castellanos et al., 2014) and these nuclei of the basolateral amygdala are involved in goal-directed (e.g., pup-directed) behaviours via its projections to the accumbens-ventral pallidum (Numan & Woodside, 2010). Therefore, it is tempting to suggest that the PMCo may influence motivational aspects of maternal behaviour using intraamygdaloid pathways.

Concerning the MePD, its pattern of activation during exposure to pups/buttons looks rather similar to the one found in the PMCo, with some slight significant differences, as previously described. The MePD shows a very strong expression of steroid hormone receptors (Hagihara et al., 1992; Mitra et al., 2003; Shughrue et al., 1997) and, compared to virgins, pregnant females display a significant increase in pSTAT5-immunoreactive cell density, probably induced by placental lactogens (Salais-López et al., 2017). Thus, the influence of pregnancy hormones in LP females may underlie the correlation of cFos expression in the MePD with the chemosensory score, a weighted average of the episodes in which female-infant interactions are likely to include chemoinvestigation of pups. In contrast, in virgins, cFos expression in the MePD significantly correlates with the number of episodes in which the female exhibits "pup approach and retreat" a kind of risk-assessment behaviour directed to pups, in which the female approaches a pup but retreats afterwards without trying to retrieve it. Although indirect, these data suggest that activity in the MePD is mainly related to chemosensory stimulation in both kinds of females, but in LP this mainly occurs in the context of maternal approaches to pups, whereas in virgins it seems more related to pup-directed exploratory behaviour.

2.4.4 Vomeronasal function and pup-directed aggression

The role of vomeronasal stimuli in pup-directed aggression has been well-established in males. Thus, Tachikawa et al. (2013) demonstrated that infanticide in sexually naïve male mice is VNO-dependent, and accordingly, virgin infanticide males displayed much higher pup-induced activation (evaluated as cFos expression) in the VNO and AOB than sexually experienced, paternal males (Tachikawa et al., 2013). In line with this, (Nakahara et al., 2016) showed that pups induced activation of an atypical subpopulation of neurons in the VNO that expresses a specific gene in the OR family, Olfr692. More recently, Isogai et al. (2018) demonstrated the implication of V2R-expressing VNO cells in the detection of specific molecules covering pup's bodies during postpartum (salivary secretions from the dam; haemoglobin) that induce pup killing in virgin males. In contrast, Trouillet et al. (2019) suggested the involvement of V1R/Galphai2-detected volatiles in virgin male infanticide.

Although the number of LP females exhibiting and not exhibiting pup-directed aggression is not large enough to establish two groups and compare their brain activity using robust statistics tools, the correlations between occurrence of pup aggression and brain activity renders interesting results. Our data show a positive correlation of AOB and BSTMPM activation (cFos expression) with pup-aggression score in LP females (Table 2.1). This suggests that some vomeronasal-detected pup chemosignals might induce attacks in LP females, as it occurs in males, although the kind of VNO receptors involved is still unknown. In males transition from infanticide (sexually naïve males) to paternal care (sexually experienced males) seems associated to altered sensitivity of Olfr692-expressing VNO cells to pups (Nakahara et al., 2016) probably due to changes in sensory transduction in Galphai2/V1R-expressing cells (Trouillet et al., 2019). By contrast, our results suggest that in females other mechanisms seem to be at play. Thus, according to our results, LP and virgin females show similar Egr1-ir cell density in the VNO in response to pups, differences being observed only in some central vomeronasal centres, such as the PMCo. This again suggests that pregnancy-induced altered functioning of central circuits, rather than changes in vomeronasal sensory transduction, might mediate infant-directed aggression observed in some LP females.

A highly significant positive correlation was also observed in the group of LP females, between pup-induced cFos expression in the two nuclei whose activity is correlated with pup-aggression: BSTMPM and AOB (Table 2.2). Therefore, our data suggest that the AOB-BSTMPM pathway may be involved in pup-directed aggression. The effects of pregnancy hormones in

the BSTMPM or its afferents (Salais-López et al., 2017) might promote a pattern of activity that would facilitate pup-attack in LP females.

The medial posterior BST, as part of the medial extended amygdala, is a heterogeneous brain region (Dong & Swanson, 2006a, 2006b) involved in social behaviour (part of the socio-sexual brain network) including parenting, mating and aggressive behaviour (Fukui et al., 2019; Tsuneoka et al., 2015). Studies carried out in males suggest that caring of pups or attacking them results from the activity of a specific circuit within the BST-preoptic area (Tsuneoka et al., 2015), and also that the oestrogen receptor signalling in the BST is likely contributing to infanticidal behaviour (Fukui et al., 2019). In addition, this nucleus is involved in the regulation of inter-male aggression by VNO-detected male chemosignals (see also Trouillet et al., 2019). Our data suggests that in females, the BSTMPM may be part of an activated brain circuit associated to pup attack by the end of pregnancy, but future studies are needed to explore this possibility.

By contrast, virgin females did not show pup attacks, and this might be related to the significant negative correlation observed in virgins (but not in LP females) between the activity in the PMCo/MOB and the one in the BSTMPM (see Table 2.2). This suggests that tonic inhibition of the BSTMPM by these two nuclei inhibits pup attack in virgin females, whereas this inhibition may be reduced to a certain degree in some LP females, thus facilitating pup-directed aggression.

2.4.5. Olfaction and behavioural response to pups: vomeronasal-olfactory integration

Pup chemosignals are also detected by the main olfactory epithelium and processed by the associated brain pathway to trigger maternal behaviours (Belluscio et al., 1998; Fraser & Shah, 2014; Seegal & Denenberg, 1974; Wang & Storm, 2011). Our results suggest that, although pups are a source of olfactory stimuli, at the level of MOB there are no changes in olfactory sensitivity to pups associated to pregnancy (Fig. 2.7A''). In fact, we did not find a preferential activation of the MOB by either stimulus, but a differential response of the two kinds of females, with virgins showing globally a higher cFos density than LP females. This is mainly due to buttons inducing significantly higher cFos activation in virgins than LP females and indicates that buttons are not olfactory neutral. As shown by the behavioural results of the second set of buttons-exposed females (Fig. 2.3), LP females explore buttons to a lesser extent than virgins, which fits with the cFos results in the MOB of the females from the main and first experiment.

Very likely, top-down centrifugal projections within the olfactory systems (Aqrabawi et al., 2016; de Olmos et al., 1978; Mohedano-Moriano et al., 2012; Michael T. Shipley & Ennis, 1996; Wachowiak, 2010) change their activity during pregnancy resulting in a reduced chemoinvestigation of buttons, which constitute novel, salient stimuli for virgin females.

Concerning exploration of pups, even if pups induced similar levels of cFos expression in the olfactory bulbs of LP and virgin females, it is interesting to note that MOB and AOB cFos levels show a positive and very significantly correlation in LP but not in virgin females (Table 2.2). In addition, cFos-expressing cell density in both olfactory bulbs correlate with the number of periods that LP females were off nest (Table 2.1). These data suggest a coupled activation of both chemosensory systems during exploration of the cage, far from the nest, further reinforcing the view that the main and accessory olfactory pathways are not parallel systems, but they work in tandem and play complementary roles in chemical analysis of the environment (see Martínez-García et al., 2009). Indeed, there is anatomical evidence indicating that the olfactory and vomeronasal pathways converge on several secondary centres (Cádiz-Moretti et al., 2013). Moreover, our data suggest that, at least during pregnancy, both chemosensory systems are functionally interrelated already in their first central rely, the main and accessory olfactory bulbs, as pointed out by Pardo-Bellver et al. (2017) using an electrophysiological approach. This olfactory-vomeronasal functional relationship also results in correlation of pup-induced Egr1 expression in the VNO with the MOB and the piriform cortex of LP females (see Table 2.2).

Although the anatomical substrate of the reciprocal influence between vomeronasal and olfactory systems (probably consistent of multiple indirect connections) is currently unknown, our findings indicate that functional coupling of MOB and AOB is probably associated to specific behaviours, such as chemoinvestigation of the environment (off nest, rather than pup-directed conducts), as reported by Pardo-Bellver et al. (2017). Our data also suggest that behaviour-specific coupling is facilitated under some physiological circumstances, such as late pregnancy. A possible explanation for this could be an increased sniffing-induced vomeronasal pumping during investigation of the environment by LP females (Meredith & O'Connell, 1979), although more experiments are needed to test this hypothesis.

The pattern of cFos expression indicates that, in females, the activity of the olfactory cortex in response to the presence of pups or buttons is different to what we found in the main olfactory bulbs. Thus, whereas in LP the olfactory cortex is preferentially activated by pup odours, such higher activation does not occur in virgin females, which show a preferential

activation by button odours instead, at least in the PirPost (Fig. 2.7C''). Since the chemosensory score, likely related to detection of pup odours, does not differ between both kinds of females, these data suggest that processing of odorant stimuli through the olfactory pathway is altered during pregnancy favouring response to pup odours. This may reflect a role of the piriform cortex as an associative rather than a primary sensory cortex (see review by Haberly, 2001), where some neurons respond preferentially to rewarded odours (Choi et al., 2011; Schoenbaum & Eichenbaum, 1995). Functional changes induced in the brain of females by the action of pregnancy hormones might increase the rewarding properties of pup-derived stimuli (Londei et al., 1989) and, consequently, it would increase the response of Pir cells to this stimulus. Indeed, enhanced response to pups in the olfactory cortex of LP females might be caused by changes in gene expression observed during the peripartum in the Pir and other centres of the olfactory systems in mice, such as reduced expression of oxytocin receptor to less than a half or a 2- to 3-fold increase in the expression of prolactin receptor (Canavan et al., 2011).

Although the functional consequences of this pup-biased response of the piriform cortex in LP females are difficult to ascertain yet, it is tempting to suggest that pup-induced activity in the Pir may be related with the expression of both, pup-directed and to non pup-directed maternal behaviours. In fact, in LP females (but not virgins) there is a positive, significant correlation between nest building episodes and cFos expression in the PirAnt (see Table 2.1). This suggests that cFos-related activity in the olfactory cortex, at least in PirAnt, is not a mere consequence of pup chemoinvestigation, but probably has a causal role in the induction of maternal behaviours.

In summary, our results from Chapter 2 reveal that pups are a source of chemical signals detected by the VNO, as demonstrated using quantitative assessment of Egr1 in the VNO. In LP females, processing of these chemosignals involves co-activation of the olfactory and vomeronasal systems, already at the level of the olfactory bulbs. Our data also depict two different subsystems within the vomeronasal system. On the one hand, in the pathway from the AOB to the PMCo and MePD sensory processing seems to be altered during late pregnancy so that discrimination between pups and buttons is enhanced. In addition, in LP females the activity in this pathway seems associated to pro-maternal behaviours, including pup retrieval and nest building. On the other hand, the pathway from the AOB to the BSTMPM shows no evidence of differential sensory processing in LP and virgin females. Although globally, activity in these centres is higher in pup- as compared to button-exposed females, within the group of LP females activity in both centres is correlated to pup-directed aggression, thus suggesting a

role of vomeronasal stimulation of the BSTMPM in inducing pup attacks in females (during pregnancy), similar to what has been reported in virgin males for other portions of the BST.

CHAPTER 3

Becoming a mother shifts the activity of the social and motivation brain networks in mice

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3.1. Introduction

Maternal behaviours are strongly adaptive, as they promote survival of the offspring until reproductive age and ensure proper neurodevelopment of pups (Curley & Champagne, 2016). Conversely, parental neglect or mistreatment has devastating effects on development and health of the offspring (Mehta et al., 2021). Understanding the neuroendocrine mechanisms of maternal care would therefore help avoiding maternal neglect and ensuring proper maternal care, promoting mental and physical health of both mothers and future generations (Kundakovic & Champagne, 2015).

Motherhood constitutes a special period in the lifespan of a female mammal. As observed in rodents, by the end of pregnancy females radically change their behaviour, as they start building a nest where pups may be kept warm and safe (see Numan & Woodside, 2010), and furiously attack unknown adults approaching it. This phenomenon is known as maternal aggression (also called prepartum aggression in late-pregnant females; Mayer & Rosenblatt, 1984; Svare et al., 1982). After delivery, rodent females stop interactions with adult conspecifics and their social life becomes virtually restricted to dedicatedly taking care of their offspring. Thus, dams spend hours per day nurturing and licking-grooming pups while covering them with their own body to keep them warm. These changes in behaviour are not merely due to the presence of pups, as virgin females either avoid pups (rats; Fleming & Rosenblatt, 1974b) or show little motivation to take care of them (as in mice; Salais-López et al., 2021). In addition, even pup-sensitized virgin female mice displaying frequent pup care show no nest defence (Martín-Sánchez et al., 2015b). These data suggest that hormones acting on the brain of females during pregnancy and postpartum (steroids and lactogens; Salais-López et al., 2017) contribute to changes in their response to pup stimuli, including chemosignals (see Chapter 2), and other social cues, such as male pheromones (Martín-Sánchez et al., 2015a, 2015b). These changes promote a strong motivation to take care of pups and to defend the nest from putatively infanticide intruders. Where and how these changes in brain activity occur is still largely unknown.

Based mainly on early studies using lesion experiments in rats (Lee et al., 2000; Numan et al., 1990; Numan & Numan, 1996), there is a quite generalized view attributing a fundamental role in the expression of maternal behaviours during postpartum to the medial preoptic region and parts of the adjoining bed nucleus of the stria terminalis. Some later findings have further emphasized this view (Wu et al., 2014). In fact, pup-induced activity specifically occurs in galanin-expressing (Gal+) cells of the preoptic region in males and females expressing parental

care (but not in males attacking pups). In addition, MPO-Gal+ cell's ablation impairs maternal behaviour in virgin and lactating females and facilitates pup attack in (otherwise parental) virgin females and fathers. Finally, optogenetic activation of Gal+ preoptic cells promotes pup care and suppresses pup attack in virgin male mice.

Taken together, these pieces of evidence led to a ''linear view'' of the neuroanatomy of maternal care, according to which, activation of the medial preoptic area (MPO), or even a specific cell population within it, would generate parental care and suppress pup neglect or attack (Wu et al., 2014). The activity of the MPO as the ''maternal brain centre'' would be boosted by hormones (oestrogens and prolactin/lactogens) (Numan & Woodside, 2010; Numan & Young, 2016) during pregnancy and postpartum. Other nuclei might influence the activity of the MPO, such as serotonergic inputs from the raphe (Lu et al., 2001), vasopressinergic inputs from the paraventricular hypothalamus (Kohl et al., 2018), or dopaminergic afferents from anteroventral periventricular nucleus (Scott et al., 2015).

By contrast to this linear view, recent literature highlights that maternal allostasis depends on multiple systems affecting the activity of many brain centres, such as neuroendocrine systems involved in food intake, energy expenditure, and stress (Russell & Brunton, 2019), as well as oxytocin and endogenous opioid systems (Wallin et al., 2021). Based on this and other lines of evidence, Swain & Ho (2019) propose the concept of maternal behaviour neurocircuit, an evolutionarily conserved, widespread neural network controlling parenting under the influence of hormones and opioids; this recalls the socio-sexual brain network (SBN) concept, coined by Sarah Winans Newman two decades ago (Newman, 1999), a network of interconnected brain centres whose activity profile would allow expression of different social behaviours (sexual, agonistic, affiliative; we must add parental conducts) under the influence of steroid (and maybe other) hormones.

Motivational aspects of social behaviours have deserved specific attention (O'Connell & Hofmann, 2011), and this aspect of maternal behaviour has also been linked to the theory of the MPO as the maternal brain centre. Thus, it has been shown that, through descending projections to the ventral tegmental area (VTA), the MPO would enhance activity of the tegmento-striatal pathway in response to pups (Fang et al., 2018; Numan et al., 2009; Numan & Smith, 1984; Numan & Young, 2016), thus leading to pup-directed motivated behaviours typical of motherhood (Pereira & Morrell, 2011).

Despite this, several data support important participation of other nuclei (besides the MPO) in the control of motivated maternal behaviours. Thus, the basolateral division of the amygdala

has also been implicated in motivated maternal behaviours through direct amygdalo-striatal (Lee et al., 2000; Numan & Woodside, 2010). Recent work has also shown how increased motivation of females for pups during motherhood is associated with prolactin signalling in several locations of the brain, not just the MPO (Salais-López et al., 2021). In fact, mapping the action of prolactin and placental lactogens in the brain of female mice (Salais-López et al., 2017, 2021), as well as the distribution of oestrogen receptors in the brain of rodents (Mitra et al., 2003; Simerly et al., 1990) indicate that many centres, besides the MPO, are targeted by lactogens and steroids. Thus, the linear view of maternal care does not fit the widespread action of these hormones in the brain of females. In addition, results from Chapter 2 indicate that sensory centres not directly linked with the MPO also change their activity profile in response to pups during late pregnancy. However, how and where the combined action of lactogens (placental and hypophysial, e.g. prolactin) and steroids during pregnancy might change the response to pup-derived and other social stimuli during motherhood is still largely unknown.

Trying to fill this gap, in the present work we have exposed adult virgin and late-pregnant (LP) females to pups or to a non-social control stimulus (buttons), and we compare the activity of several socio-sexual and motivational centres of their brain by analysing the density of cells expressing cFos protein in these brain nuclei. The advantage of using LP instead of postpartum females to study the neurobiology of motherhood is that they have already undergone the effects of pregnancy hormones but they are pup-naïve, so that pups constitute an equally novel stimulus for LP and virgin females. Therefore, differences in pup-directed behaviours and pup-induced brain activity between females can be safely attributed to the effects of pregnancy hormones but not to previous experience with pups (important in postpartum females). In fact, pups induce similar behaviours in LP and virgin female mice, except for pup-directed attacks, which are exhibited by some LP but not virgin females (see Chapter 2; Figure 2.2).

Here, we focus on the brain centres and circuits directly involved in the expression of social behaviours, the socio-sexual brain network (Newman, 1999), as well as the main brain centres involved in motivated and goal-directed behaviours. Specifically, we aim to check whether changes in brain response to pups induced by pregnancy hormones are restricted to the MPO or, alternatively, a network view may better explain motherhood-associated changes in brain activity elicited by pups and parenting/pup attack. To do so, we combine classic statistics (ANOVA analysis of data from individual nuclei or equivalent nonparametric tests) with a

somewhat innovative statistical approach consisting of a global (data of all the brain nuclei under scrutiny) analysis of principal components and linear discriminant analysis.

Together, our results confirm important pregnancy-related changes in the response to pups and buttons in the SBN and, even more pronouncedly, in the motivation brain circuitry. Moreover, the activity of the MPO alone has a poor predictive power of whether a female is pregnant or not and whether it was exposed to pups or buttons. By contrast, considering the data of the whole system (SBN and motivation circuitry) allows classifying animals with a high accuracy and sensitivity. These findings strongly recommend the use of a network, rather than hierarchical perspective, to understand the neurobiology of social conducts, including maternal behaviours.

3.2. Material and methods

For the analysis of motivational and social brain circuits we employed the same females of the Chapter 2 (n=46): the 30 original ones for the initial experiment (for **animals characteristics and housing** conditions see section 2.2.1 from Chapter 2). Therefore, the **experimental design** is also the same (for the original experimental design and maternal behaviour analysis see section 2.2.2.A from Chapter 2). Since the present Chapter is related to social and motivational behaviours, for this analysis we did not use the Chemosensory Score. Likewise, the **tissue processing and immunohistochemistry** also correspond to the methodology described in Chapter 2 (see section 2.2.3).

3.2.1. Image analysis

Expression of cFos was quantified in a selection of 6 brain nuclei involved in the control of socio-sexual behaviours (see Fig. 3.1 and 3.2): lateral septum ventral portion (LSV), medial amygdala (posterodorsal division, MePD), medial preoptic nucleus (MPO), ventromedial hypothalamic nucleus (VMHVL), paraventricular hypothalamic nucleus (anterior part, PaA), anterior commissure nucleus/anterodorsal preoptic area (AC/ ADP) and lateral column of the periaqueductal grey (PAG).

In addition, we also sampled 6 nuclei/cortical regions belonging to the motivation brain circuitry (see Fig. 3.3), namely anterior and posterior ventral tegmental (VTAa and VTAp), accumbens shell and core (AcbSh and AcbC), basolateral amygdala (BLA) and medial prefrontal cortex (including prelimbic and infralimbic cortex, PrL-IL).

To reduce variability, for each one of these brain centres a specific anteroposterior, dorsoventral and mediolateral region of interest was selected using the mouse brain atlas (Paxinos and Franklin, 2004; see Fig. 3.1A-D, 3.2A-C and 3.3A-F). Pictures of both hemispheres were systematically taken using a Leica DFC450 digital camera attached to a Leica DM750 microscope. For each region an appropriate magnification was used to cover the region of interest and, when needed, a specific portion of the picture was manually selected as a region of interest (ROI) and the density of cFos-ir cells measured using image analysis techniques.

Image processing and analysis was performed as described in Chapter 2 (see section 2.2.4).

3.2.2. Statistical analysis

Statistical analysis was performed using SPSS software package (IBM) and R (R Core Team, 2020). The significance level was set at $p<0.05$.

A. Behaviour

Analysis of behavioural data was performed as described in Chapter 2 (see section 2.2.5).

B. Intra-nucleus comparison of cFos-ir cell density

Concerning cFos expression, when data accomplished normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene test), what sometimes required logarithmic transformation of data ($Log_{10}[n+1]$), a two-way ANOVA was performed, with FEMALE (V or LP) and STIMULUS (buttons or pups) as factors. Significant FEMALExSTIMULUS interactions were explored by post-hoc pairwise comparison with Bonferroni corrections.

Otherwise, in those nuclei in which one or more of the 4 groups (V-B, V-P, LP-B, LP-P) failed to accomplish normality or homoscedasticity, we assessed the differences in the main factors separately; FEMALE (all virgin vs all LP) and STIMULUS (all females exposed to buttons vs all females exposed to pups). A two-sample t-test (for samples showing normality), or a Mann-Whitney test for those displaying no normal distribution, was performed with non-transformed data.

C. Brain-behaviour correlation analysis

Since we had measured pup-induced behaviour and brain activity (density of cFos-ir cells in the nuclei of the socio-sexual and motivation brain networks) in the same animals, e.g. LP and V females exposed to pups, we explored the relationship between activity in these brain nuclei with the expression of pup-elicited behaviours, by means of a Spearman correlation analysis (most behaviours did not follow normal distribution). We did so separately in LP and virgin

females exposed to pups. This allows exploring changes in brain-behaviour relationship during pregnancy.

D. Principal components analysis (PCA)

Principal component analysis provides a way to achieving a global analysis of the data, which may shed light of the response of the whole system (the whole set or nuclei analysed) to the two stimuli in the two kinds of females. This statistical approach reduces a set of intercorrelated variables (cFos-ir cell density in the different nuclei of the SBN and motivation brain circuitry) into a few dimensions that gather a big amount of the variability (e.g. information) of the original variables. These dimensions are called principal components (PCs) and have the properties of collecting highly correlated variables within each component while being uncorrelated with each other. Therefore, PCA was performed after standardizing each variable to have mean zero and standard deviation one. The results reveal 13 principal components, and we focused on the two main principal components (PC1 and PC2) that, together, explain about 65% of the variance.

Each principal component is obtained using a linear combination of the original data (X_1 to X_p; where X_1, X_2, X_3, ..., X_p are the cFos-ir cell density in the p nuclei analysed; p=13 in our case), with a specific loading factor (φ; 1> φ >-1) for each nucleus. For instance, for the j-th principal component (PC_j):

$$PC_j = \varphi_{1j}X_1 + \varphi_{2j}X_2 + \cdots + \varphi_{pj}X_p, \text{ with } \sum_{i=1}^{p} \varphi_{ij}^2 = 1$$

Using this equation, we calculate the scores of each component for each female. We show the scores of each animal in the two first principal components as a biplot (PC1 vs PC2), in which each animal is represented as a dot with a colour code indicating the group it belongs to (LP-P, LP-B, V-P, V-B). In addition, the loading factors for PC1 and PC2 for each nucleus, p, are indicated as vectors (φ_{p1}, φ_{p2}) in Figure 3.4B. The orientation (direction/angle) of the vector indicates how much the variable contributes to the principal component: the more parallel to a principal component axis, the more it contributes only to that PC. The length of the vector indicates how well the two principal components explain the variability of the density of cFos expression in this nucleus. The angles between vectors corresponding to different nuclei show their correlation: small angles represent high positive correlation, right angles represent lack of correlation, opposite angles represent high negative correlation.

E. Linear discriminant analysis

Assuming that $X=(X_1,..., X_p)$ is the vector with the covariates/predictors measured for each individual, and that these observations in each group, (LP-P, LP-B, V-P, V-B), follow a multivariate Gaussian distribution, Bayes' theorem is used to flip these around into estimates for Pr(Y=LP-P|X), Pr(Y=LP-B|X), Pr(Y=V-P|X) and Pr(Y=V-B|X). Mathematically, the problem turns into the problem of finding the borders of the zones defined by the covariates, which maximize each probability. The linear discriminant analysis looks for linear borders, linear combinations of $X_1,..., X_p$, (discriminant functions) that allow to characterize or separate the zones that maximize the probability of each category. Once these discriminant functions are estimated, they can be used as classifiers for new individuals. Then, given a new individual with measurements $Xnew=(X_1new,..., X_pnew)$, it will be labelled as a LP-P if Pr(Y=LP-P|Xnew) is greater than the probabilities of the other groups. The number of discriminant functions in this technique is either Ng-1 where Ng is the number of groups (4 in our case), or p, the number of predictors, whichever is smaller. This renders three LD functions for the analysis using the full set of data and a single LD function for the analysis using MPO cFos density.

So, we used our data to first calculate the linear discrimination functions, each one characterised again by a set of coefficients applied to the original dataset. With this, the proportion of trace is calculated, e.g. the proportion of between-class variance that is explained by successive discriminant functions and, if considered enough (>75%) the two first discriminant functions are used for classification.

Usually, the classification methods split the dataset into a subset of observations used to get the discriminant functions, the so-called training data, and another subset of observations called test data, which is used to check the performance of these discriminant functions. In our case, due to the limited size of our dataset we are checking the performance of the discriminant functions by leave-one out Cross Validation. It is an iterative procedure where in each step we use as a training set the whole data set except one observation that is used as a test set. Then we compare the classification obtained by the iterative procedure with the real classification of the individuals to determine the global **accuracy** (proportion of individuals correctly classified), **sensitivity** of the classification for each class (number of individuals of a given class that are correctly classified), **specificity** of the classification for each group (number of individuals assigned to a group that actually belong to that group, not shown). We also calculate the **No Information Rate** (NIR; maximum accuracy if all individuals were assigned to the same group).

These data allow assessing the correctness of the classification based on the dataset employed, using Kappa (K=1 perfect classification; K=0 null correctness):

$$K = \frac{N \sum_{i=1}^{n} m_{i,i} - \sum_{i=1}^{n} (G_i C_i)}{N^2 - \sum_{i=1}^{n} (G_i C_i)}$$

Where:

i is the class number

N is the total number of classified values compared to truth values

$m_{i,i}$ is the total number of values belonging to the truth class ***i*** that have also been classified as class ***i*** (e.g. values found along the diagonal of the confusion matrix)

C_i is the total number of predicted values belonging to class ***i***

G_i is the total number of truth values belonging to class ***i***

An associated p-value contrasts whether classification is significantly better ($p<0.05$) than NIR.

To illustrate the results, instead of the traditional graphics showing the classification on the basis of the 13 original variables, which would render a high number of graphics, we use a single 3D graphic on the scores in the three linear discriminant functions (Link in Fig. 3.5A). For the case of the MPO, we represent the classification using the original data of the cFos density.

3.3. Results

In the present study, we analysed the density of cFos immunoreactive cells in specific frames of brain centres belonging to the socio-sexual brain network (Fig. 3.1 and 3.2) and the centres involved in motivated behaviours (Fig. 3.3) in the four initial experimental groups of females (see Chapter 2, section 2.2.2.A): virgins exposed to buttons (V-B) or pups (V-P), and late-pregnant females exposed to buttons (LP-B) or pups (LP-P). The data on behaviour of females exposed to pups (V-P and LP-P) has been reported in the previous Chapter (see section 2.3.1). Although the behaviour of the initial buttons-exposed groups was not recorded, we studied the behavioural response of a second set of females exposed to buttons, also reported in Chapter 2 (see section 2.3.2).

3.3.1. The SBN shows a differential response to pups in virgins and pup-naïve LP females

We compared the expression of cFos in four nuclei of the SBN of both virgin and LP pup-naïve female mice, e.g. lateroventral septum (LSV), medial amygdala (posterodorsal division, MePD), medial preoptic nucleus (MPO), ventromedial hypothalamic nucleus (ventrolateral division, VMHVL), and the lateral columns of the periaqueductal gray (PAG). Data on the MePD were already reported in our previous Chapter (see section 2.3.3.B) devoted to the impact of pregnancy on chemosensory processing.

In these nuclei, a two-way ANOVA with FEMALE (LP versus V) and STIMULUS (P versus B) as the main factors (raw or log-transformed data when needed to achieve normality), revealed a significant effect of STIMULUS, with exposure to pups eliciting higher levels of cFos expression than buttons in the MePD ($F_{1,26}$=81.312, p<0.001) (Fig. 3.1A"), LSV (U=15, p<0.001) (Fig. 3.1B"), MPO (t=-4,17, p<0.001) (Fig. 3.1D"), and the VMHVL ($F_{1,26}$=48.966, p<0.001) (Fig. 3.2B"). In addition, in the MPO and LSV there is a surprising significant effect of FEMALE (p<0.03 in both nuclei) with virgins showing globally (pooling females exposed to both stimuli) higher cFos immunoreactive (cFos-ir) cell density than LP females.

By contrast, the paraventricular hypothalamic nucleus (anterior part, PaA), whose oxytocinergic cells have been involved in some aspects of maternal behaviour (e.g. anxiety suppression; Knobloch et al., 2012), showed no significant differences in the density of cFos-ir cells between stimuli (two-way ANOVA, $F_{1,26}$=2.749, p=0.11; see Fig. 3.2A") or females (p=0.588). Given the preeminent role attributed to oxytocin (OT) in behavioural changes associated with motherhood (e.g. Numan & Young, 2016; Tsuneoka et al., 2013), we specifically analysed an area of the preoptic region that is enriched in OT-immunoreactive cells, the so-called anterior commissure nucleus/anterodorsal preoptic area (AC/ADP; Otero-García et al., 2016). Here, there is not just increased activation by pups as compared with buttons ($F_{1,26}$=37.623, p<0.001) but also a significant FEMALExSTIMULUS interaction ($F_{3,26}$=4.789, p=0.038) that indicates sharper differences between stimuli in LP as compared with virgin females (Fig. 3.1C"). Similar findings are observed in the MePD (significant FEMALExSTIMULUS interaction; $F_{3,26}$=15.277, p=0.001; see Fig. 3.1A"), a nucleus that is usually considered the chemosensory interface of the SBN. In both nuclei, AC/ADP and MePD, analysis of this interaction using post-hoc tests revealed that the response to pups is similar in LP and virgin females (p=0.57 for AC/ADP, Fig. 3.1C"; although there is a trend in MePD, p=0.055, Fig. 3.1A"), but there is a significant decrease in the response to buttons in LP females as compared

with virgins (p=0.018 for the AC/ADP; p=0.002 for MePD); this suggests that LP females may partially ignore non-social salient stimuli, such as buttons.

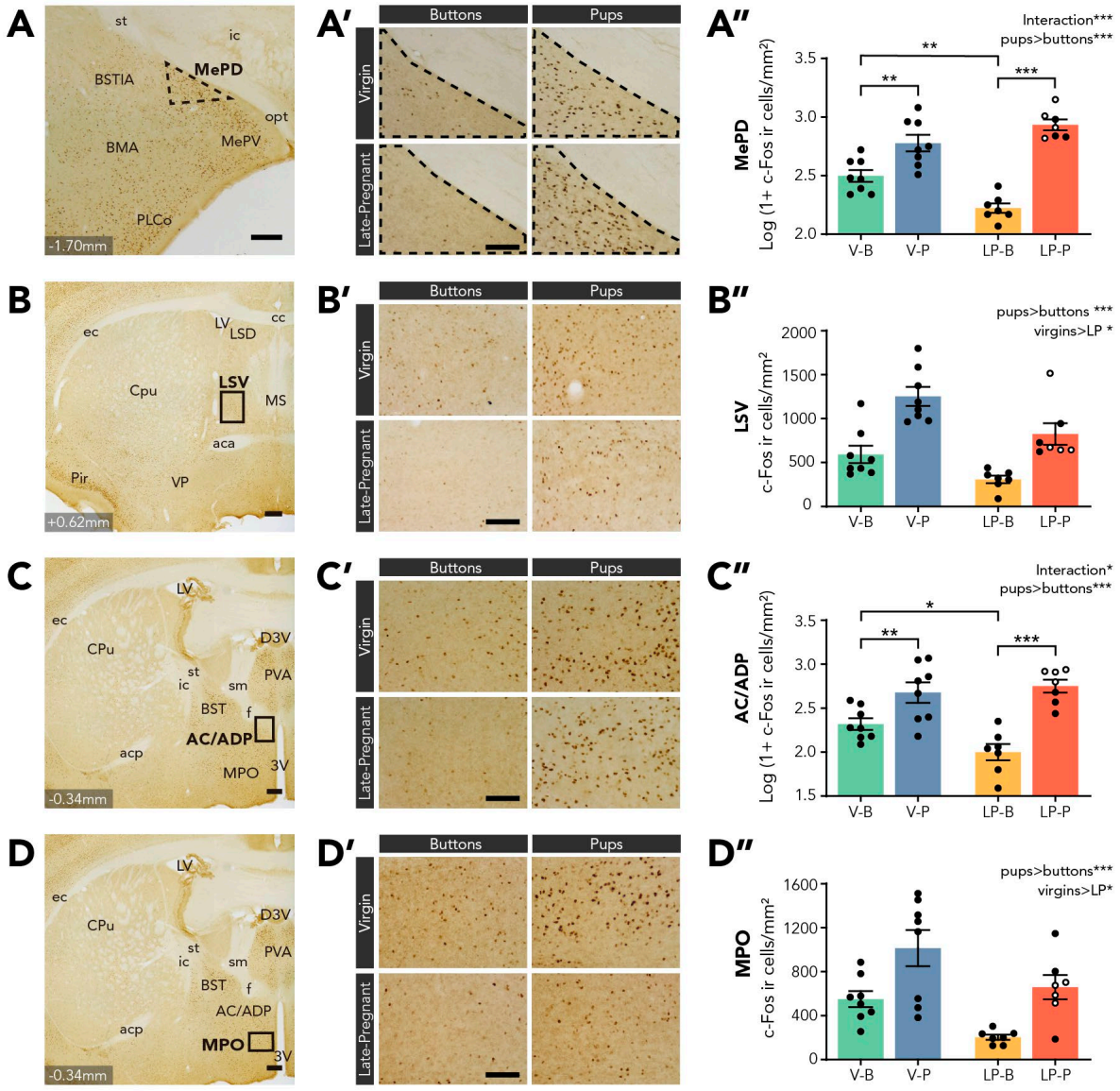


Figure 3.1. Expression of cFos (immunohistochemistry) in some nuclei of the socio-sexual brain network (SBN) of late-pregnant (LP) and virgin (V) females exposed to pups (P) or buttons (B). **A-D**. The column at left shows low-power pictures of the sections with indication of their approximate antero-posterior level relative to bregma (according to Paxinos and Franklin, 2004) and frames or dotted lines (regions-of-interest, ROI) delineating the location of the nuclei analysed. The nuclei sampled are the posterodorsal medial amygdala (MePD; A), the ventrolateral septum (LSV; B), the region of the nucleus of the anterior commissure/ anterodorsal preoptic nucleus (AC/ADP; C), and the medial preoptic area (MPO; D). **A'-D'**. The central column shows examples of microphotographs of the brain regions analysed for each experimental group: virgins exposed to buttons (V-B), virgins exposed to pups (V-P), late-pregnant exposed to buttons (LP-B), and late-pregnant exposed to pups (LP-P). Scale bars correspond to 250 mm for the first column and 100 mm for the second one. **A''-D''**. The column at right shows bar histograms illustrating the density of cFos positive cells (mean GSEM; log-transformed data for those nuclei not showing a normal distribution of data) of each nucleus in each group of females (V-B, green; V-P blue; LP-B yellow; LP-P orange). Individual data of each animal are also represented (circles; open circles correspond to females showing pup-directed attacks). When significant, the main effects revealed by the ANOVA are indicated on top of each histogram. Where there is a significant FEMALExSTIMULUS interaction, significant post-hoc comparisons (with Bonferroni corrections) are also illustrated on the histogram (***p<0.001, **p<0.01, and *p<0.05). Other abbreviations: 3V, third ventricle; aca, anterior commissure, anterior part; acp, anterior commissure, posterior;

BMA, basomedial amygdaloid nucleus, anterior part; BST, bed nucleus of the stria terminalis; BSTIA, intra-amygdaloid division of bed nucleus of the stria terminalis; cc, corpus callosum; CPu, caudate putamen; D3V, dorsal third ventricle; ec, external capsule; f, fornix; ic, internal capsule; LSD, lateral septal nucleus, dorsal part; LV, lateral ventricle; MePV, medial amygdaloid nucleus, posteroventral part; MS, medial septum; opt, optic tract; Pir, piriform cortex; PLCo, posterolateral cortical amygdaloid nucleus; PVA, paraventricular thalamic nucleus, anterior part; sm, stria medullaris of the thalamus; st, stria terminalis; VP, ventral pallidum.

Finally, we also analysed the cFos-ir cells in the lateral column of the periaqueductal gray (PAG) (Fig. 3.2C"), a brain region modulating social and motivated behavioural responses and known to be involved in intermale (Haller et al., 2006) and maternal aggression (Gammie & Nelson, 2001). Our results show an increased activation by pups as compared with buttons ($F_{1,26}$=22.870, p=0.016) but also a significant FEMALExSTIMULUS interaction ($F_{3,26}$=13.170, p=0.003). The post hoc analysis reveals that pups induce higher response in LP compared with virgin females (p=0.016), whereas buttons induce higher response in virgins than in LP females (p=0.047). Moreover, pups raise higher response than buttons in LP females (p<0.001), but no differences between stimuli are observed in virgin females (p=0.6). In other words, pups activate the PAG more than buttons only during pregnancy.

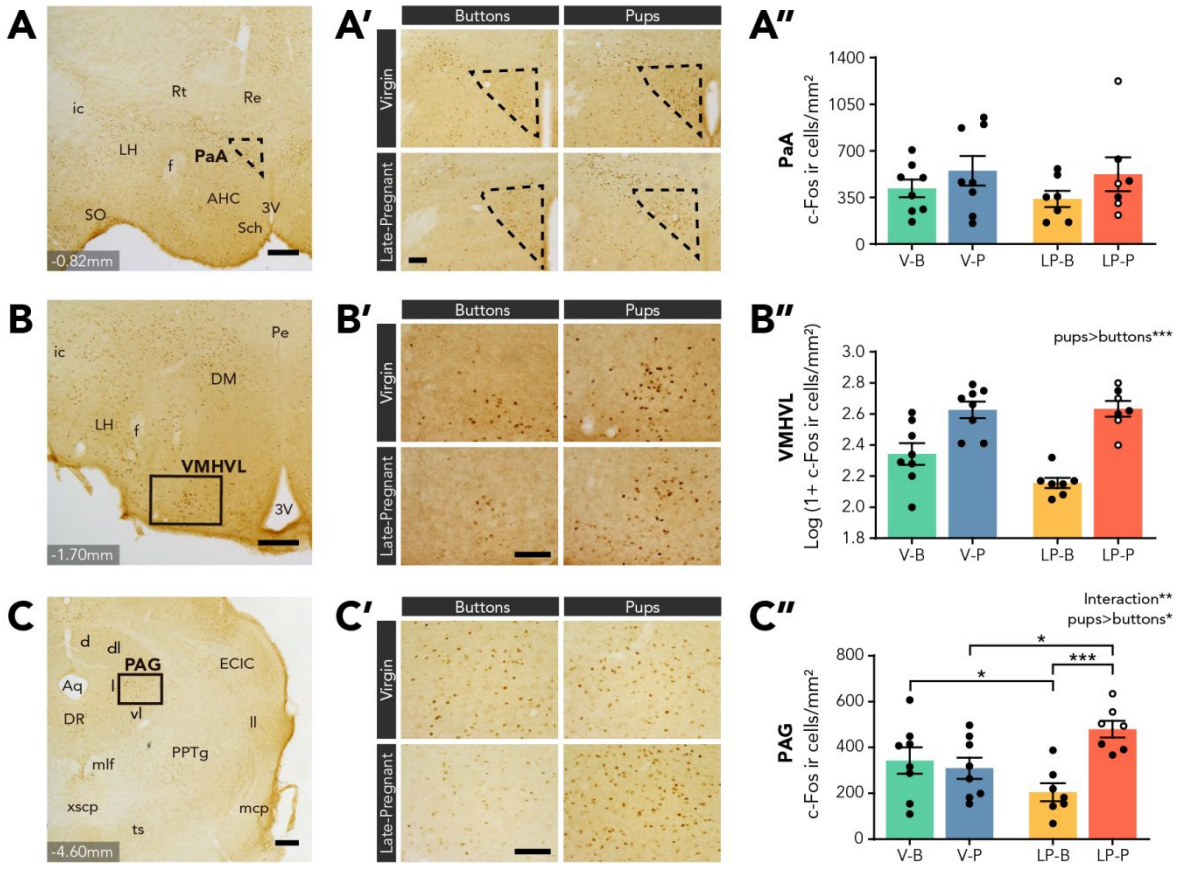


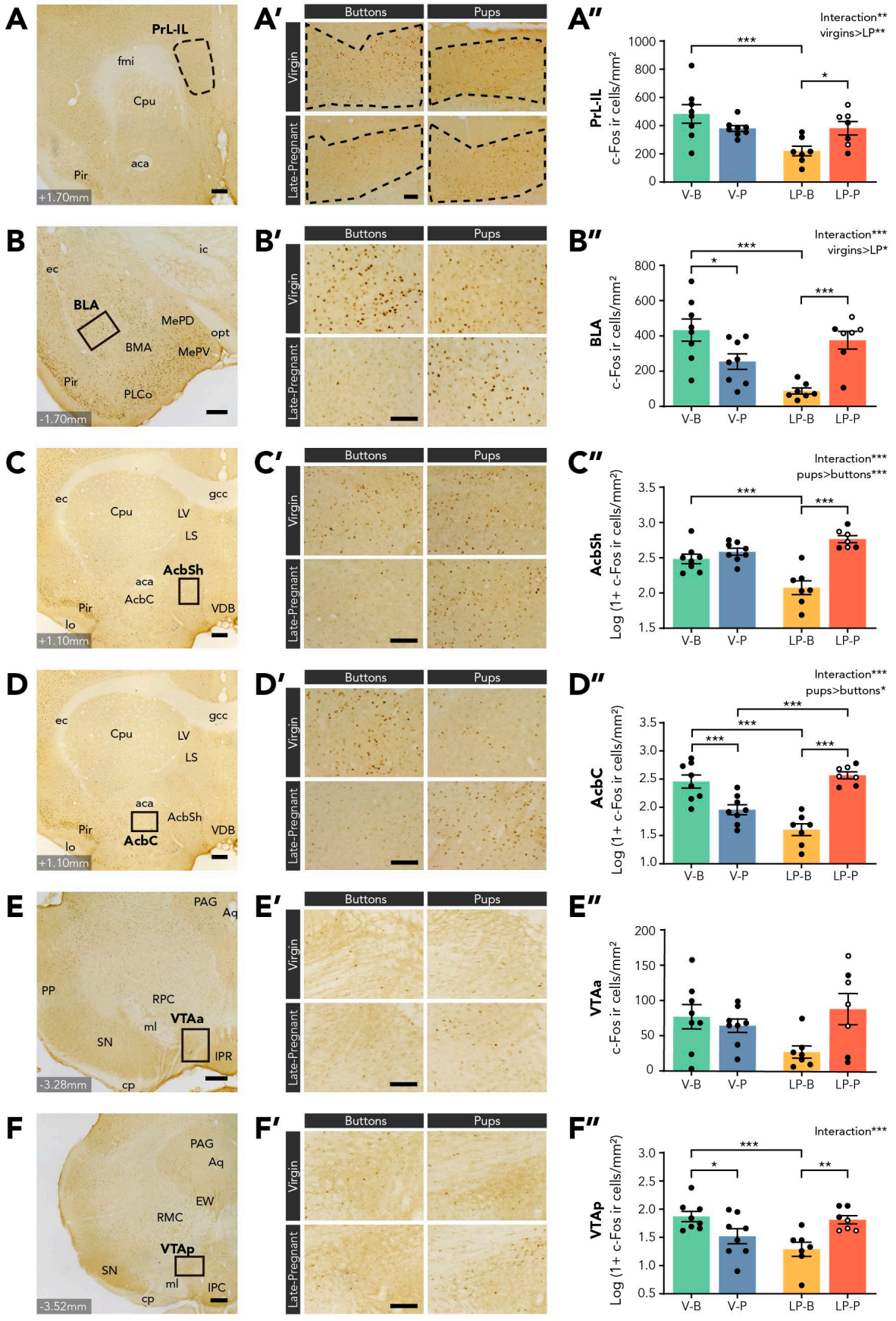
Figure 3.2. Expression of cFos (immunohistochemistry) in some nuclei of the socio-sexual brain network (SBN) of late-pregnant (LP) and virgin (V) females exposed to pups (P) or buttons (B). **A-C**. The column at left shows low-power pictures of the sections with indication of their approximate antero-posterior level relative to bregma (according to Paxinos and Franklin, 2004) and frames or dotted lines (regions-of-interest, ROI) delineating the location of the nuclei analysed. The nuclei sampled are the anterior portion of the paraventricular nucleus (PaA; A),

the ventrolateral portion of the ventromedial hypothalamic nucleus (VMHVL; B), and the lateral column of the periaqueductal gray (PAG; C). **A'-C'**. The central column shows examples of microphotographs of the brain regions analysed for each experimental group: virgins exposed to buttons (V-B), virgins exposed to pups (V-P), late-pregnant exposed to buttons (LP-B), and late-pregnant exposed to pups (LP-P). Scale bars correspond to 250 mm for the first column and 100 mm for the second one. **A''-C''**. The column at right shows bar histograms illustrating the density of cFos positive cells (mean GSEM; log-transformed data for those nuclei not showing a normal distribution of data) of each nucleus in each group of females (V-B, green; V-P, blue; LP-B, yellow; LP-P, orange). Individual data of each animal are also represented (circles; open circles correspond to females showing pup-directed attacks). When significant, the main effects revealed by the ANOVA are indicated on top of each histogram. Where there is a significant FEMALExSTIMULUS interaction, significant post-hoc comparisons (with Bonferroni corrections) are also illustrated on the histogram (***p<0.001, **p<0.01, and *p<0.05). Other abbreviations: 3V, third ventricle; AHC, anterior hypothalamic area, central part; DM, dorsomedial hypothalamic nucleus; f, fornix; ic, internal capsule; LH, lateral hypothalamic area; Pe, periventricular hypothalamic nucleus; Re, reuniens thalamic nucleus; Rt, reticular thalamic nucleus; SCh, suprachiasmatic nucleus; SO, supraoptic nucleus.

3.3.2. The motivational circuitry changes its response to pups/buttons during pregnancy

We also analysed cFos-ir cell density in the main nuclei of the motivational brain circuitry in female mice: ventral tegmental area (anterior, VTAa; and posterior division, VTAp), nucleus accumbens (core, AcbC; and shell AcbSh), anterior basolateral amygdaloid nucleus (BLA), and prelimbic/infralimbic areas of the prefrontal cortex (PrL/IL) (Fig. 3.3).

Two-way ANOVAs revealed a highly significant FEMALExSTIMULUS interaction in almost all the regions analysed: PrL-IL ($F_{1,26}$=8.341, p=0.008; see Fig. 3.3A"); BLA ($F_{1,26}$=23.660, p<0.001; Fig. 3.3B"); AcbSh ($F_{1,26}$=18.882, p<0.001; Fig. 3.3C"); AcbC ($F_{1,26}$=56.418, p<0.001; Fig. 3.3D"); VTAp ($F_{1,26}$=15.897, p<0.001; Fig. 3.3F"). The only exception was the VTAa where data did not allow performing a parametric ANOVA analysis (Fig. 3.3E"), but nonparametric test failed to show significant differences between females or stimuli. Further analysis of the significant interactions using post-hoc comparisons with Bonferroni corrections reveals that, in all cases, there is a significantly higher activation by pups as compared with buttons in LP females (p=0.023 in the PrL/IL, Fig. 3.3A"; p<0.001 in BLA, AcbSh and AcbC, Fig. 3.3B", C" and D" respectively; p=0.003 in VTAp, Fig. 3.3F"). This contrasts with a similar activation of both stimuli in virgin females (AcbSh and PreL/IL) or even higher activation by buttons than pups in virgin females (p=0.011 in the BLA, Fig. 3.3B"; p=0.001 in the AcbC, Fig. 3.3D"; p=0.026 in the VTAp, Fig. 3.3F"). In addition, in all these cases, buttons elicit higher activation in virgin than in LP females (p<0.001 in all cases, Fig. 3.3A"-F"), thus reinforcing the view that, during late pregnancy, females show a loss of interest on buttons, which become less salient for LP females. Interestingly, pups induce similar activation (cFos-ir cell density) in the brain of LP and virgin females, except for the AcbC where pup-induced activation is significantly higher in LP than in virgin females (p<0.001, Fig. 3.3D").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Interaction**
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Late-Pregnant
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Interaction***
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LP-B
LP-P

Figure 3.3. Expression of cFos (immunohistochemistry) in nuclei of motivation brain circuitry of late-pregnant (LP) and virgin (V) females exposed to pups (P) or buttons (B**). A-F**. The column at left shows low-power pictures of the sections with indication of their approximate antero-posterior level relative to bregma (according to Paxinos and Franklin, 2004) and frames or dotted lines (regions-of-interest, ROI) delineating the location of the nuclei analysed. The nuclei sampled are prelimbic and infralimbic cortical areas (PrL-IL; A), the anterior part of the basolateral amygdaloid nucleus (BLA; B), the nucleus accumbens shell and core (AcbSh and AcbC respectively; C and D), and the anterior and posterior parts of the ventral tegmental area (VTAa and VTAp; E and F). **A'-F'**. The central column shows examples of microphotographs of the brain regions analysed for each experimental group: virgins exposed to buttons (V-B), virgins exposed to pups (V-P), late-pregnant exposed to buttons (LP-B), and late-pregnant exposed to pups (LP-P). Scale bars correspond to 250 mm for the first column and 100 mm for the second one. **A"-F"**. The column at right shows bar histograms illustrating the density of cFos positive cells (mean G SEM; log-transformed data for those nuclei not showing a normal distribution of data) of each nucleus in each group of females (V-B, green; V-P, blue; LP-B, yellow; LP-P, orange). Individual data of each animal are also represented (circles; open circles correspond to females showing pup-directed attacks). When significant, the main effects revealed by the ANOVA are indicated on top of each histogram. Where a significant FEMALExSTIMULUS interaction was present, significant post-hoc comparisons (with Bonferroni corrections) are also illustrated on the histogram (***$p<0.001$, **$p<0.01$, and *$p<0.05$). Abbreviations: aca, anterior commissure, anterior part; Aq, aqueduct (Sylvius); BMA, basomedial amygdaloid nucleus, anterior part; cp, cerebral peduncle, basal part; CPu, caudate putamen; ec, external capsule; EW, Edinger-Westphal nucleus; fmi, forceps minor of the corpus callosum; gcc, genu of the corpus callosum; ic, internal capsule; IPC, interpeduncular nucleus, caudal subnucleus; IPR, interpeduncular nucleus, rostral subnucleus; lo, lateral olfactory tract; LS, lateral septal nucleus; LV, lateral ventricle; MePD, medial amygdaloid nucleus, posterodorsal part; MePV,medial amygdaloid nucleus, posteroventral part; ml, medial lemniscus; opt, optic tract; PAG, periaqueductal gray; Pir, piriform cortex; PLCo, posterolateral cortical amygdaloid nucleus; PP, peripeduncular nucleus; RMC, red nucleus, magnocellular part; RPC, red nucleus, parvicellular part; SN, substantia nigra; VDB, nucleus of the vertical limb of the diagonal band.

These data reveal that pregnancy changes the response of the motivation circuitry of the brain of females, by reducing activation induced by a novel non-social stimulus, whereas increasing activation by pups specifically in the AcbC.

3.3.3. Pregnancy reduces salience of non-social objects

As indicated earlier, in several nuclei of the SBN and motivation brain circuit, pregnancy seems to reduce the activity induced by non-social control objects, e.g. buttons; this can be due to a pregnancy-induced insensitivity to button-derived stimuli, a decreased salience of those objects resulting in reduced exploration, or both. To study this, we performed an additional experiment already described in Chapter 2 (see section 2.2.2.B), in which we analysed the behaviour of two groups of females identical to those of the cFos experiment (virgins and LP; pairs of same-condition females) for 5 min after introducing eight buttons in their home cage.

The results (Chapter 2 section 2.3.2, Fig. 2.3) indicate that virgins explore buttons significantly more than LP females (more episodes of button sniffing; more time engaged in sniffing buttons); this is not a general effect of pregnancy on mobility or on interest on exploring objects in their environment, as inter-female interactions (sniffing to the other female in the same cage) do not differ between virgin and LP females. Hence, it seems that pregnancy reduces specifically interest in non-social objects such as buttons; this might have resulted in

reduced cFos activity in many nuclei of the brain in LP females exposed to buttons (as compared with button-exposed virgin females).

3.3.4. Correlation between brain activity and maternal behaviours

Using the same strategy as described in the previous Chapter (see section 2.3.5), here we explore anatomo-functional relationships within the SBN and motivation circuits of the brain based on analysis of correlation between cFos-ir cell density and behaviour in pup-exposed females. There are significant correlations between brain activity and a few behaviours, but those differ between LP and virgin females.

First, cFos-ir cell density and pup aggression score showed a highly significant, strong, and negative correlation in the VTAp (Rho=-0.925; p=0.003; see Table 3.1), e.g. the higher the activation of this brain region, the lower the likelihood that the female attacks pups. By contrast, the cFos-ir cell density in the PAG shows a positive correlation with pup aggression (Rho=-0.964; p<0.001; see Table 3.2). Virgin females do not show pup attacks and, therefore, there is no correlation of this behaviour with brain activity in virgins.

Another behaviour displayed by females in the presence of pups is ‘‘approach-and-retreat,’’ a reaction similar to risk assessment: the female approaches a pup, sniffs at it for a while, and then retreats without retrieving the pup, e.g. the female takes the decision of not to pick up a pup after exploring it. Although virgin and LP females do not differ in the expression of these approach-and-retreat responses (see Chapter 2, Table Fig.2.2), in LP females this behaviour is significantly and negatively correlated with cFos-ir cell density of two nuclei of the motivation brain circuitry, the BLA (Rho=-0.852; p=0.015) and the AcbC (Rho=-0.778; p=0.039) (Table 3.1). Although causal relationships cannot be established through correlational analyses, the activation of these two connected centres of the motivation circuitry (Novejarque et al., 2011) is associated with LP females not avoiding pups after detecting and sniffing them. By contrast, in virgin females the more approach-and-retreat responses to pups, the higher the activity in the AcbC (Rho=0.801; p=0.017). This finding further reveals important changes in the activity of the motivation circuitry of the brain in response to pups during pregnancy. A similar situation is found in the MePD and the PAG, where approach-and-retreat responses to pups are positively correlated with cFos-ir cell density in virgins (Rho=0.801; p=0.017 for the MePD and Rho=0.726; p=0.041 for the PAG) but not in LP females (Table 3.2).

Table 3.1: Spearman's correlations between cFos expression in motivational network nuclei and behavioural events. Abbreviations: PrL-IL, the infralimbic cortical areas; BLA, the anterior part of the basolateral amygdaloid nucleus; AcbSh and AcbC, the nucleus accumbens shell and core; VTAa and VTAp, the anterior and posterior parts of the ventral tegmental area. Statistically significant correlations are indicated by bold values in group-specific coloured cell background (blue for virgins, orange for late-pregnant females) and asterisks *p<0.05; **p<0.01.

			PrL-IL	BLA	AcbSh	AcbC	VTAa	VTAp
Off Nest	V-P	Correl. Coef.	0,184	0,098	-0,221	-0,233	0,395	-0,303
		Sig. (bilateral)	0,662	0,817	0,599	0,578	0,333	0,466
		N	8	8	8	8	8	8
	LP-P	Correl. Coef.	0,487	0,094	0,293	0,487	0,430	-0,391
		Sig. (bilateral)	0,268	0,842	0,524	0,268	0,335	0,386
		N	7	7	7	7	7	7
Approach and Retreat	V-P	Correl. Coef.	0,701	0,250	0,175	**0,801***	-0,239	0,466
		Sig. (bilateral)	0,053	0,550	0,678	**0,017**	0,568	0,245
		N	8	8	8	**8**	8	8
	LP-P	Correl. Coef.	0,185	**-0,852***	-0,729	**-0,778***	-0,630	0,094
		Sig. (bilateral)	0,691	**0,015**	0,063	**0,039**	0,129	0,841
		N	7	**7**	7	**7**	7	7
On Nest	V-P	Correl. Coef.	-0,381	0,381	0,095	-0,357	0,060	0,407
		Sig. (bilateral)	0,352	0,352	0,823	0,385	0,888	0,317
		N	8	8	8	8	8	8
	LP-P	Correl. Coef.	-0,408	0,371	0,206	0,037	0,185	-0,132
		Sig. (bilateral)	0,364	0,413	0,658	0,937	0,691	0,778
		N	7	7	7	7	7	7
Nest Building	V-P	Correl. Coef.	0,460	0,498	0,077	0,038	0,167	-0,026
		Sig. (bilateral)	0,252	0,209	0,857	0,928	0,693	0,952
		N	8	8	8	8	8	8
	LP-P	Correl. Coef.	-0,094	0,699	0,448	0,586	**0,756***	-0,048
		Sig. (bilateral)	0,840	0,080	0,313	0,167	**0,049**	0,918
		N	7	7	7	7	**7**	7
Retrieval	V-P	Correl. Coef.	-0,013	-0,026	-0,089	0,153	-0,019	-0,507
		Sig. (bilateral)	0,976	0,952	0,833	0,717	0,964	0,199
		N	8	8	8	8	8	8
	LP-P	Correl. Coef.	0,459	0,073	-0,037	0,569	0,624	0,402
		Sig. (bilateral)	0,300	0,876	0,937	0,182	0,134	0,371
		N	7	7	7	7	7	7
In Nest	V-P	Correl. Coef.	0,192	-0,707	-0,144	0,275	-0,211	-0,355
		Sig. (bilateral)	0,649	0,050	0,734	0,509	0,616	0,388
		N	8	8	8	8	8	8
	LP-P	Correl. Coef.	-0,037	-0,037	0,019	-0,094	-0,356	0,114
		Sig. (bilateral)	0,937	0,937	0,968	0,842	0,434	0,807
		N	7	7	7	7	7	7
Maternal Score	V-P	Correl. Coef.	0,012	-0,623	-0,168	0,144	-0,084	-0,380
		Sig. (bilateral)	0,978	0,099	0,691	0,734	0,843	0,354
		N	8	8	8	8	8	8
	LP-P	Correl. Coef.	0,107	0,357	0,306	0,607	0,429	0,327
		Sig. (bilateral)	0,819	0,432	0,504	0,148	0,337	0,474
		N	7	7	7	7	7	7
Pup Aggression Score	V-P	Correl. Coef.						
		Sig. (bilateral)						
		N	8	8	8	8	8	8
	LP-P	Correl. Coef.	0,259	0,519	0,280	0,111	0,259	**-0,925****
		Sig. (bilateral)	0,574	0,233	0,542	0,812	0,574	**0,003**
		N	7	7	7	7	7	**7**
Female Interaction	V-P	Correl. Coef.	-0,085	0,606	0,218	0,473	-0,268	0,439
		Sig. (bilateral)	0,842	0,111	0,604	0,237	0,521	0,276
		N	8	8	8	8	8	8
	LP-P	Correl. Coef.	-0,094	0,430	-0,123	-0,112	0,075	0,048
		Sig. (bilateral)	0,842	0,335	0,793	0,811	0,873	0,919
		N	7	7	7	7	7	7

Table 3.2: Spearman's correlations between cFos expression in socio-sexual network nuclei and behavioural events. Abbreviations: LSV, the ventrolateral septum; MePD, posterodorsal medial amygdala; MPO, the medial preoptic area; VMHVL, the ventrolateral portion of the ventromedial hypothalamic nucleus; AC/ADP, the region of the nucleus of anterior commissure/anterodorsal preoptic; PaA, the anterior portion of the paraventricular nucleus; PAG, the lateral column of the periaqueductal grey. Statistically significant correlations are indicated by bold values in group-specific coloured cell background (blue for virgins, orange for late-pregnant females) and asterisks *p<0.05; **p<0.01.

			LSV	MePD	MPO	VMHVL	AC/ADP	PaA	PAG
Off Nest	V-P	Correl. Coef.	-0,221	0,061	-0,516	-0,528	-0,295	-0,368	0,160
		Sig. (bilateral)	0,599	0,885	0,191	0,179	0,479	0,369	0,706
		N	8	8	8	8	8	8	8
	LP-P	Correl. Coef.	0,529	0,393	0,150	0,711	0,299	-0,674	0,374
		Sig. (bilateral)	0,222	0,383	0,749	0,073	0,514	0,097	0,408
		N	7	7	7	7	7	7	7
Approach and Retreat	V-P	Correl. Coef.	0,150	**0,801***	-0,250	0,125	0,125	0,401	**0,726***
		Sig. (bilateral)	0,723	**0,017**	0,550	0,768	0,768	0,325	**0,041**
		N	8	**8**	8	8	8	8	**8**
	LP-P	Correl. Coef.	0,299	0,148	0,074	0,185	-0,259	0,185	-0,408
		Sig. (bilateral)	0,515	0,751	0,875	0,691	0,574	0,691	0,364
		N	7	7	7	7	7	7	7
On Nest	V-P	Correl. Coef.	0,476	-0,143	0,357	-0,405	0,429	-0,024	0,071
		Sig. (bilateral)	0,233	0,736	0,385	0,320	0,289	0,955	0,867
		N	8	8	8	8	8	8	8
	LP-P	Correl. Coef.	0,019	-0,630	0,222	-0,630	00,000	0,296	0,148
		Sig. (bilateral)	0,968	0,129	0,632	0,129	10,000	0,518	0,751
		N	7	7	7	7	7	7	7
Nest Building	V-P	Correl. Coef.	0,434	0,089	-0,562	-0,166	0,128	-0,217	0,358
		Sig. (bilateral)	0,282	0,833	0,147	0,694	0,763	0,606	0,385
		N	8	8	8	8	8	8	8
	LP-P	Correl. Coef.	0,486	-0,567	0,057	00,000	0,094	-0,397	0,472
		Sig. (bilateral)	0,269	0,184	0,904	10,000	0,840	0,378	0,284
		N	7	7	7	7	7	7	7
Retrieval	V-P	Correl. Coef.	-0,549	0,294	-0,077	0,013	-0,192	0,166	-0,064
		Sig. (bilateral)	0,159	0,480	0,857	0,976	0,650	0,694	0,881
		N	8	8	8	8	8	8	8
	LP-P	Correl. Coef.	-0,167	-0,441	0,441	00,000	0,312	**-0,826***	-0,312
		Sig. (bilateral)	0,721	0,323	0,323	10,000	0,496	**0,022**	0,496
		N	7	7	7	7	7	**7**	7
In Nest	V-P	Correl. Coef.	-0,467	-0,252	-0,036	0,683	-0,287	0,012	-0,479
		Sig. (bilateral)	0,243	0,548	0,933	0,062	0,490	0,978	0,230
		N	8	8	8	8	8	8	8
	LP-P	Correl. Coef.	-0,548	0,636	-0,543	0,150	-0,056	0,262	-0,037
		Sig. (bilateral)	0,203	0,125	0,208	0,749	0,905	0,570	0,937
		N	7	7	7	7	7	7	7
Maternal Score	V-P	Correl. Coef.	-0,419	-0,240	0,024	0,563	-0,395	0,012	-0,455
		Sig. (bilateral)	0,301	0,568	0,955	0,146	0,333	0,978	0,257
		N	8	8	8	8	8	8	8
	LP-P	Correl. Coef.	-0,396	00,000	-0,036	0,107	0,143	-0,429	-0,071
		Sig. (bilateral)	0,379	10,000	0,939	0,819	0,760	0,337	0,879
		N	7	7	7	7	7	7	7
Pup Aggression Score	V-P	Correl. Coef.							
		Sig. (bilateral)							
		N	8	8	8	8	8	8	8
	LP-P	Correl. Coef.	0,056	0,259	-0,445	-0,259	0,667	0,074	**0,964****
		Sig. (bilateral)	0,905	0,574	0,317	0,574	0,102	0,875	**0,000**
		N	7	7	7	7	7	7	**7**
Female Interaction	V-P	Correl. Coef.	0,679	0,436	0,509	0,267	0,546	0,655	0,303
		Sig. (bilateral)	0,064	0,280	0,197	0,523	0,162	0,078	0,466
		N	8	8	8	8	8	8	8
	LP-P	Correl. Coef.	-0,736	-0,412	-0,505	**-0,954****	0,337	0,356	0,206
		Sig. (bilateral)	0,059	0,359	0,247	**0,001**	0,460	0,434	0,658
		N	7	7	7	**7**	7	7	7

Finally, nest building and retrieval are quite common behaviours in virgin and LP females exposed to pups (see Chapter 2, Table Fig.2.2). Our data indicate that occurrence of nest building behaviour is significantly and positively correlated with activation of the VTAa only in LP females (Rho=0.756; p=0.049; see Table 3.1), whereas pup retrieval is negatively correlated with activation of the PaA in LP females (Rho=-0.826; p=0.022; see Table 3.2). As couples of same-condition females were tested together, we also analysed inter-female interaction (affiliative behaviours), which negatively correlates with the activity of the VMHVL in LP females (Rho=-0.954, p=0.001) (see Table 3.2).

3.3.5. Principal component analysis supports the SBN/motivation circuitry identity

In the previous sections, we have analysed independently the data of cFos expression of each nucleus and their possible correlation with pup-induced behaviours. To further understand how the whole brain network (SBN and motivation brain circuits) responds to pups and buttons in the brain of female mice and how these responses change during late pregnancy, we also carried out a principal component analysis (PCA).

We performed a PCA after standardizing each variable to have mean equal to zero and SD (standard deviation) equal to 1. From the 13 principal components obtained, we focus on the two main principal components that together explain almost a 65% of the variance (Fig. 3.4A). The contribution of the remaining principal components (PCs) to data variability is much lower (<9%).

For the first principal component, the loading factor vector φ_1= (φ_{11}, …,φ_{p1}; see Material and Methods) only shows positive factors (see first coordinate of vectors in Fig. 3.4B), so that PC1 is a weighted average of the degree of activity of the whole system. As all the variables have been standardized (transformed to mean equal to zero and SD equal to 1), positive and negative PC1-scores are obtained. PC1 scores are high for females exposed to pups (either virgins or LP), thus indicating a strong brain activation induced by pups in both kinds of females. However, buttons activate brain centres of virgins (high values of PC1), but not those of LP females, thus fitting the results of group comparison analysis (standard statistics).

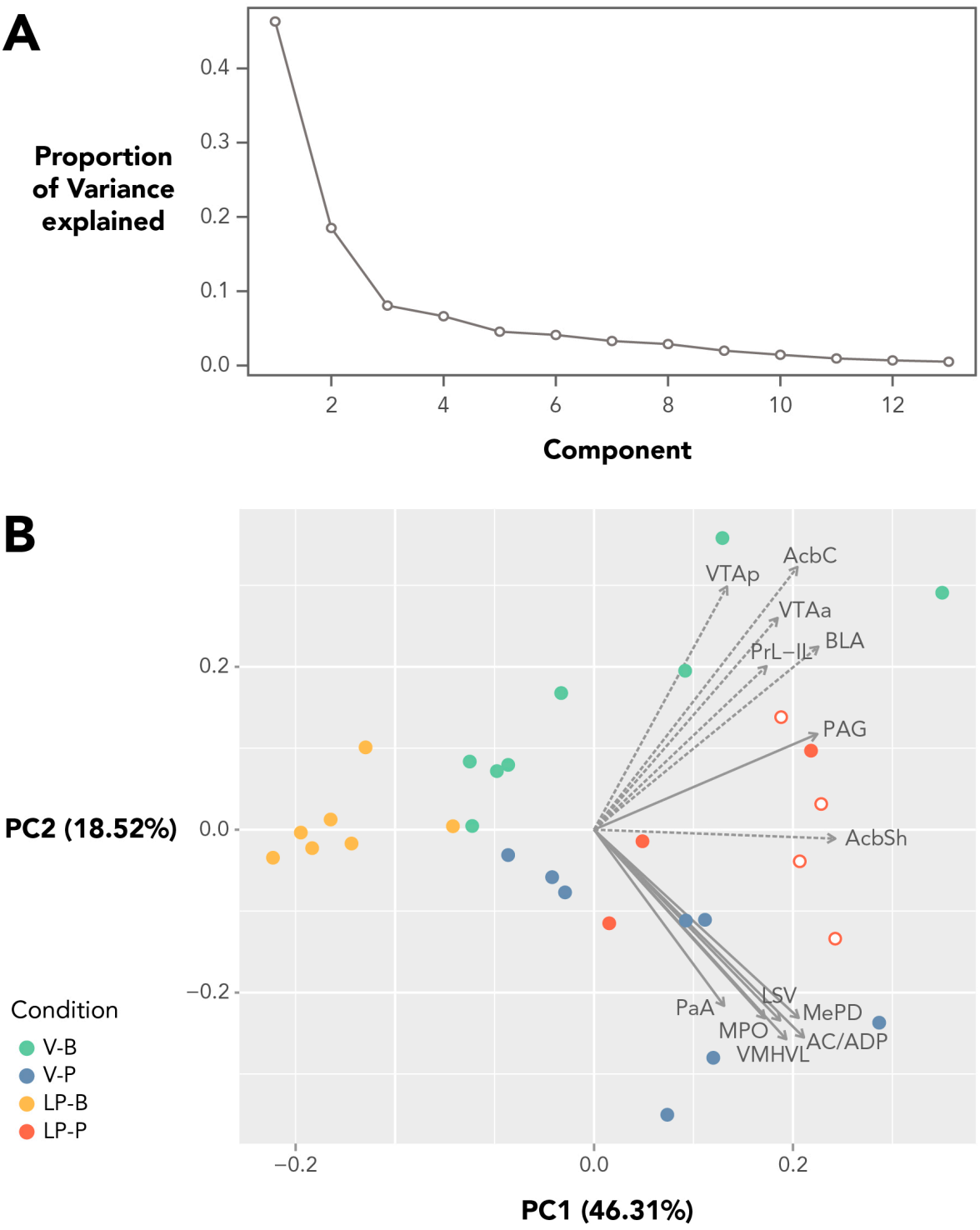

Figure 3.4. Principal components analysis (PCA) of the data of cFos-ir cell density in the socio-sexual and motivational circuits. A. Diagram showing the proportion of variance explained by each one of the 13 principal components obtained using the PCA. **B.** Diagram showing a biplot of the two main principal components (PC1 and PC2) for each animal (dots, the colour code for the different groups of females: virgins exposed to buttons (V-B in green), virgins exposed to pups (V-P in blue), late pregnant exposed to buttons (LP-B in yellow), and late-pregnant exposed to pups (LP-P in orange), combined with a graphic representation of the loading factors (41 and 42) applied to the density of cFos-ir cells for each brain centre analysed (vectors). Open circles represent those animals having attacked pups (only LP females did so, there is only orange open circles). Arrows in the graphic represent the loading factors of each nucleus in the two principal components. Abbreviations: AC/ADP, the region of the nucleus of anterior commissure/anterodorsal preoptic; AcbSh and AcbC, the nucleus accumbens shell and core; BLA, the anterior part of the basolateral amygdaloid nucleus; LSV, the ventrolateral septum; MePD, posterodorsal medial

amygdala; MPO, the medial preoptic area; PaA, the anterior portion of the paraventricular nucleus; PAG, lateral column of the periaqueductal gray; PrL-IL, the infralimbic cortical areas; VMHVL, the ventrolateral portion of the ventromedial hypothalamic nucleus; VTAa and VTAp, the anterior and posterior parts of the ventral tegmental area.

By contrast, PC2 has positive and negative loading factors for the different nuclei. Positive loading factors correspond to the nuclei belonging to the motivation brain circuitry (with the only exception of AcbSh, whose loading factor is nearly null; 0.00928) and the PAG, whereas the remaining nuclei of the SBN show negative loading factors (see the second coordinate of the vectors in Fig. 3.4B); this indicates that the studied nuclei actually belong to two different functional systems that respond differently to pups and buttons in the two groups of females. The PAG is an exception to that rule, as it belongs to the SBN but presents a positive loading factor, similar to most motivational nuclei; this is probably reflecting a role of the PAG not just in the expression of social behaviour (as part of the SBN) but also in motivation (e.g., expression of motivated attacks to pups, see Discussion), as suggested by direct neuroanatomical connections between the PAG and VTA and other functional and neurochemical data (Ntamati et al., 2018; Vázquez-León et al., 2021).

The vectors representing the loading factors of PC1 and PC2 within a given functional system are all of them oriented similarly as the angles they form with the axes are similar, thus indicating that the activities of these nuclei are positively correlated; this is true for both the SBN and the motivation circuitry (with the exceptions of the AcbSh and PAG). By contrast, the vectors corresponding to the loading factors of the SBN centres are nearly perpendicular to those of the motivation circuitry, revealing a lack of correlation between the activities of both systems.

Notably, PC2 gives a quantitative measure of the balance between both systems under the different situations, e.g. female status and stimulus presented. High, positive PC2 scores indicate a large activation of the motivational circuit with minor activation of the SBN. As shown in Fig. 3.4B, this is the case of virgin females exposed to buttons. On the other hand, virgins exposed to pups showed negative scores for PC2, fitting a higher activation of the SBN than the motivation circuit.

In addition, PC2 scores for LP females exposed to buttons are virtually zero in most cases, probably reflecting a low, similar activation of socio-sexual and motivational nuclei in these conditions. Finally, LP females exposed to pups display more variability in PC2 scores than LP-B females, meaning a variable, high activation of the SBN and the motivational system. But

altogether, LP-P females showed more neutral scores of their PC2 scores than both virgin groups.

3.3.6. Discriminant analysis: Activity pattern of the SBN/motivational circuitry predicts the condition of the female and stimulus

Finally, we decided to study whether the pattern of activity of the brain of the females of each condition (group/stimulus) was able to predict the animal condition. There are different classification techniques, such as multinomial regression or linear discriminant analysis, which can be used in this context. We have carried out a linear discriminant analysis (LDA) to predict a response variable, Y (with 4 categories: Class 1=LP-P; Class 2=LP-B; Class 3=V-P; Class 4=V-B) from:

a) The cFos-ir cell density values of the individuals in the p nuclei analysed (p=13 in our case).

b) In addition, as the medial preoptic area is claimed to be the main nucleus controlling expression of maternal behaviours (see Kohl and Dulac, 2018), we also performed a linear discriminant analysis using as predictor just the data of the MPO, in order to clarify whether its activity can explain by itself the response of the females to the stimuli.

A. Linear discriminant analysis using the original data (cFos-ir cell density)

The coefficients of the three linear discriminant functions (LD) built from the cFos-ir cell density in the 13 nuclei analysed are shown in Table 3.3, along with the proportions of trace explained by of each LD function. According to the results, the two first LD functions explain more than 86% of the between-class variance (Table 3.3).

A 3-D representation of the individual scores of each experimental subject for the three linear discriminant functions (LD1–LD3) is shown in Figure 3.5A, along with 2-dimension graphs with two-by-two projections on the three LDA functions (Figure 3.5C-E). As the figure shows, the first discriminant function (LD1) gives positive scores for females exposed to pups and negative values for females exposed to buttons, allowing to distinguish between both groups (Fig. 3.5C and D). The scores of the individuals in the second discriminant function (LD2) show negative values for LP-P and positive values for most V-P, allowing to distinguish between both groups, although the distinction is not so clear between LP-B and V-B (Fig. 3.5C and E). Last,

the third discriminant function (LD3) gives positive scores to LP-B and negative scores for V-B, whereas in females exposed to pups the majority of scores are closer to zero (Fig. 3.5D and E).

Table 3.3. Coefficients of the three linear discriminant functions and proportion of trace corresponding to each discriminant function. Abbreviations: AC/ADP, the region of the nucleus of anterior commissure/anterodorsal preoptic; AcbSh and AcbC, the nucleus accumbens shell and core; BLA, the anterior part of the basolateral amygdaloid nucleus; LSV, the ventrolateral septum; MePD, posterodorsal medial amygdala; MPO, the medial preoptic area; PaA, the anterior portion of the paraventricular nucleus; PAG, lateral column of the periaqueductal gray; PrL-IL, the infralimbic cortical areas; VMHVL, the ventrolateral portion of the ventromedial hypothalamic nucleus; VTAa and VTAp, the anterior and posterior parts of the ventral tegmental area.

		LD1	LD2	LD3
Coefficients	MePD	0.0029278591	-0.003644660	0.0005810748
	LSV	0.0020256285	0.002325294	-0.0001076949
	AC/ADP	0.0020912811	-0.000335981	0.0022131538
	MPO	-0.0002219098	0.000631026	-0.0017167591
	PaA	0.0010603764	0.001539820	-0.0001493084
	VMHVL	0.0021069854	0.001708304	-0.0038422846
	PAG	-0.0042428780	-0.001455463	-0.0001967822
	PrL-IL	-0.0004298770	0.002267811	-0.0025844942
	BLA	-0.0074353863	0.002221791	-0.0077058302
	AcbSh	0.0053308183	-0.003947955	0.0028311393
	AcbC	-0.0084052367	-0.005666441	0.0003816735
	VTAa	0.0313508976	0.005201454	0.0043151862
	VTAp	0.0129003910	0.001268623	-0.0051621317
Proportion of Trace		63.31%	22.93%	13.76%

Classification methods are applied with these data based on the iterative leave-one-out cross-validation procedure. The results indicate that, when applied to cFos-ir cell density in the 13 nuclei of interest (see Fig. 3.5), classification based on linear discriminant analysis assigns correctly all the females to their actual group with the exception of only two individuals, i.e. a V-B is classified as an LP-B, and a V-P is classified as an LP-P. No mistakes relative to the stimulus occur, therefore. This classification renders an accuracy of 93.33% with an associated p-value of 2.028×10^{-14}, and the sensitivity is 1.000 for LP-B and LP-P and 0.8750 for V-B and V-P (Fig. 3.5B).

Overall, statistical analysis reveals that data of brain activity (cFos-ir cell density) in the 13 nuclei of the SBN and motivation brain centres of our experimental females are sufficient to predict with high accuracy the status of a female (either virgin or LP) and whether the animal was exposed to a social stimulus (pups) or a non-social one (buttons).

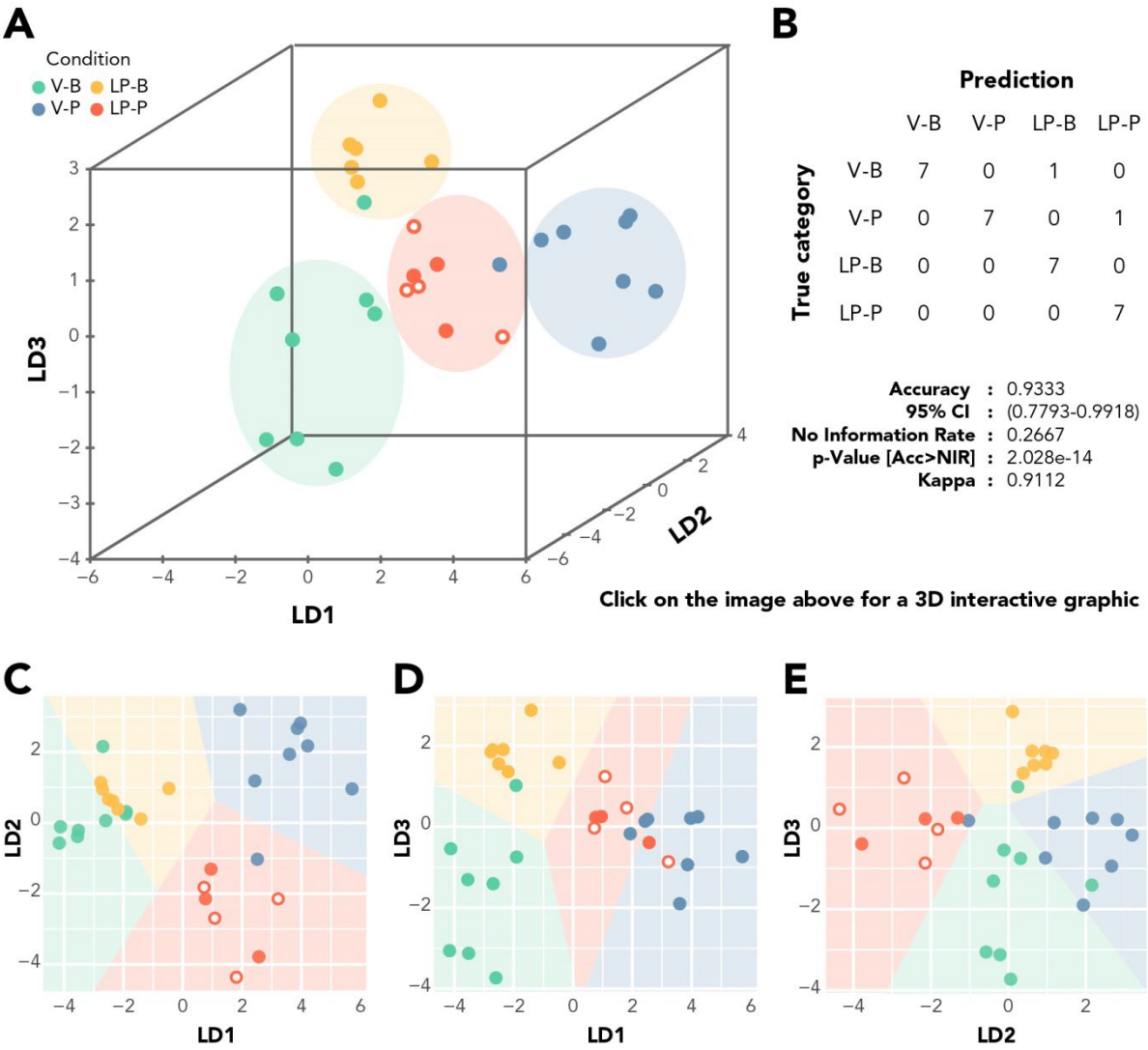

Figure 3.5. Linear discriminant analysis using the original data of cFos-ir cell density in the socio-sexual and motivational circuits. The colour code represents the groups of females: virgins exposed to buttons (V-B in green), virgins exposed (V-P in blue), late-pregnant exposed to buttons (LP-B in yellow), and late-pregnant exposed to pups (LP-P in orange). Open dots correspond to females having attacked pups. **A.** 3D representation of the individual scores (dots) of each subject, for the three linear discriminant functions (LD1–LD3) obtained using the full set of data (cFos cell density in all the 12 nuclei analysed). In order to help visualization, coloured regions are drawn to approximately representing the term of the partition obtained after the LDA to which the individuals of each category belong (LP-P, LP-B, V-P, V-B). By clicking on the graph, the reader can get access to an accurate interactive 3D graph that plots the actual limits of the LDA partition and the classification of individuals according to that partition. **B.** Confusion Matrix table indicating the number of animals of each group (True group) assigned to each group by means of the prediction procedure (Prediction). The results of the statistical analysis are summarized below. **C-E.** 2D graphs with two-by-two projections on the three LDA functions.

B. Linear discriminant analysis using only cFos-ir cell density in the MPO

Because previous literature suggests that the MPO is the key centre for the expression of maternal behaviours, which would change by the end of pregnancy, we next checked whether data on the MPO (density of cFos-ir cells in the MPO) might allow appropriately classifying the animals in their experimental group. When linear discrimination analysis was applied to the

MPO, dataset rendered a single linear discrimination function (LD1) with a coefficient <0.001 (Fig. 3.6A). Once again, we checked the performance of the discriminant functions by leave-one-out cross-validation, and the comparison between the real and the predicted classes is shown in Fig. 3.6B. In this case, there are multiple mistakes in the V-B, V-P, and LP-P groups. This classification results in an accuracy of 66.67%, with an associated p-value of 5.374×10^{-6}, with a sensitivity of 1.000 for LP-B, but zero for LP-P and 0.6250 for V-B and V-P.

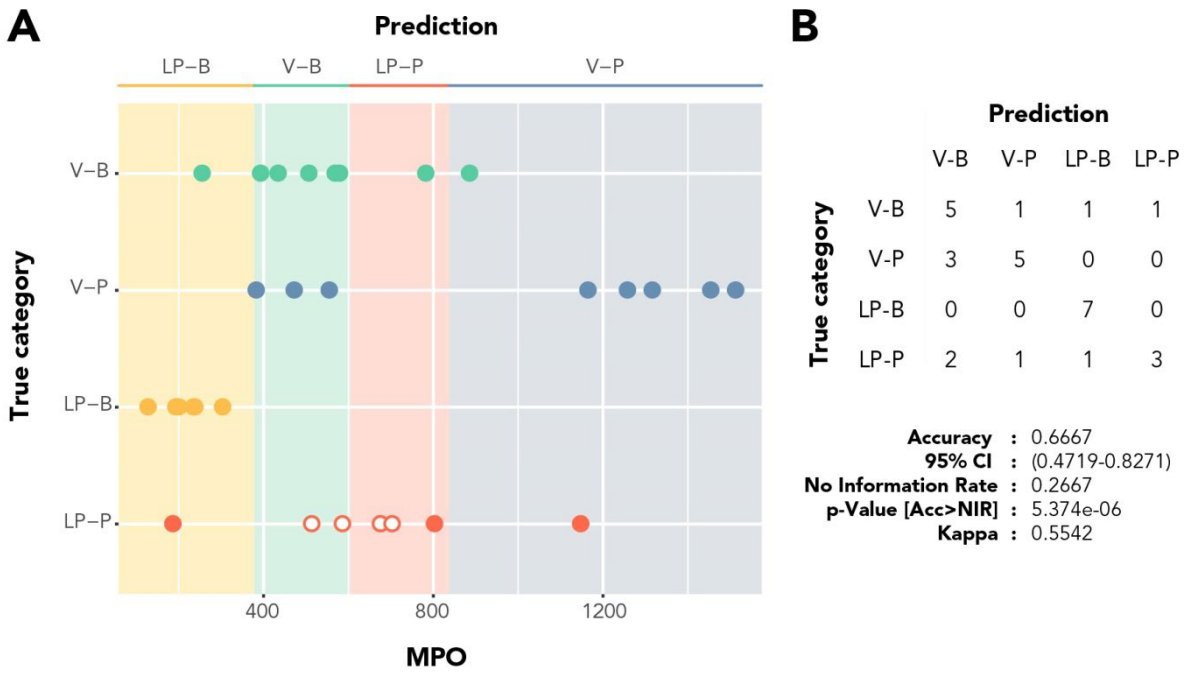

Figure 3.6. Linear discriminant analysis using only cFos-ir cell density in medial preoptic area. **A.** Distribution of the scores of the individuals of the different groups in the linear discriminant function LD1, using only the data of cFos-ir cell density of the medial preoptic area (MPO). The colour code of the groups: virgins exposed to buttons (V-B) in green, virgins exposed to pups (V-P) in blue, late-pregnant exposed to buttons (LP-B) in yellow, and late pregnant exposed to pups (LP-P) in orange. Open circles represent those females that attacked pups. **B.** Confusion Matrix table indicating the number of animals of each group (True group) assigned to each group by means of the prediction procedure (Prediction).

As a conclusion, using the data of the MPO alone renders a very low performance in the classification as compared with using data from the whole set of nuclei.

3.4. DISCUSSION

Using cFos expression assessment, we have shown that pups (a source of relevant novel social stimuli) and buttons (novel non-social objects) activate differently the brains of female mice, and these responses change by the end of pregnancy. We have focused on relevant nuclei known to participate in the expression of different social behaviours (the SBN) and in motivational aspects of behaviour (the motivation circuitry of the brain). Neither late-pregnant

nor virgin females had previous experience with pups during their adult life, so that novelty cannot contribute to the variability of the results and does not explain differences between groups and stimuli.

3.4.1. Pup care and maternal motivation: Changes induced by pregnancy

Our results confirm, firstly, that the SBN is more activated by a social stimulus (pups) than by a non-social one (buttons), thus validating our experimental design, as we are comparing two relevant stimuli that differ in their capacity to activate the social brain. The second conclusion is that this activation of the SBN seems largely independent of the status of the female, e.g. virgin and LP females show not differential response to the stimuli, no significant FEMALExSTIMULUS interaction. The exceptions to this are the AC/ADP, the MePD, and the PAG. In all three cases, FEMALExSTIMULUS interaction is mainly due to a decrease in the activity elicited by buttons in LP females, a fact that fits the decreased investigation of buttons observed in pregnant females (see Chapter 2, Fig. 2.3).

By contrast, in the reward brain circuitry, there are clear differences in the activity induced by social (pups) and non-social stimuli in both groups of females. Although the conditions of our experiment are not appropriate to reveal changes in motivation toward pups (e.g. interaction with the pups requires low effort; see discussion in Abellán-Álvaro et al., 2021; Salais-López et al., 2021), changes of this somewhat hidden aspect of behaviour can be inferred from the expression of cFos in the brain motivation circuit. In fact, among virgin females, buttons activate the motivation circuitry of the brain as much as pups or even more (as in the AcbC, BLA, and VTAp); this contrasts with the situation in LP females, in which pups are a more powerful, activating stimulus of the motivation brain circuit than buttons. In other words, pregnancy changes the salience of non-social stimuli, leading to less exploration to buttons (see Chapter 2, Fig. 2.3) and decreased activation of the reward brain circuitry. In addition, during pregnancy there is an enhanced activation by pups of some nuclei, such as the AcbC where the activity in LP females exposed to pups is significantly higher than in virgins.

Our results on this point partially agree with those obtained by Matsushita et al. (2015) that, using a cFos approach, also observed that pup exposure is a powerful stimulus to activate the motivational brain regions in lactating females. However, in that study, lactating females of 3–5 days postpartum were used as experimental animals, so that pups constituted novel stimuli only for virgins. Moreover, they did not expose control females to a non-social stimulus but

use just non-exposed females as controls. Therefore, our results are not completely comparable, although both studies suggest an impact of motherhood on the activation of the motivational circuitry by pups that, according to our results, starts before parturition, namely due to the action of pregnancy hormones on the brain.

The PCA analysis gives interesting information on the differential response of the SBN and brain reward circuitry during pregnancy. First, the second principal component, PC2, shows positive loading factors for the centres of the reward brain circuitry (with the exception of the AcbSh) and negative loading factors for the nodes of the SBN (with the exception of the PAG, as discussed later); this indicates neat differences in the activity of both brain circuits between animals.

The fact that loading factors' vectors of nuclei of a functional system (SBN or motivation circuitry) form a small angle between them reinforces the view that these nuclei act cooperatively (showing intra-system correlation). In addition, the fact that vectors of one system form large angles with those of the other system (see Fig. 3.4B) reveals that both systems are largely independent (their activity is not correlated), thus demonstrating that motivation is a somewhat independent component of different behaviours (e.g., caring for pups or sniffing/gnawing buttons).

The distribution of individuals in the PCA biplot (PC1 vs PC2; Fig. 3.4B) elegantly summarizes the differences between groups. Because PC1 somewhat reflects the general activation of the considered brain centres, the results of the PCA fit those of the standard statistics in showing that LP females exposed to buttons show less activation than the rest of the females (yellow dots are grouped at left), reflecting the curiosity of virgins toward buttons and the power of pups as stimulus for both types of females. Regarding PC2, the inverse sign of its loading factors for the SBN and the motivation brain circuit allow considering those individuals with positive scores as ''more motivated than social'' and those with negative scores as ''more social than motivated.'' Accordingly, virgins exposed to buttons are ''more motivated than social,'' although virgins exposed to pups are ''more social than motivated.'' Late-pregnant females show both positive or negative PC2 scores, but globally the scores are closer to zero, thus suggesting that LP are both ''social and motivated'' while interacting with pups. Considering that both groups exposed to pups (LP-P and V-P) show similar maternal behaviour, these results fit previous work of the group describing how motherhood- associated hormones specifically enhance the motivational aspects of maternal behaviour (Salais-López et al., 2021).

3.4.2. Pregnancy hormones indirectly influence maternal motivation

Because the only difference between virgin and LP females is pregnancy, our data reveal a strong effect of pregnancy hormones onto the brain of females, in the activity of the motivation circuitry. LP females seem to lose interest in non-social stimuli (there is a reduction of button-induced activity as compared with virgin females due, at least in part, to a reduced investigation of buttons) and focus on pups, thus resulting in a pup-elicited higher activation of, at least, the AcbC. It is interesting to note that, apparently, this higher activation of the AcbC is not due to increased interaction with pups in LP females, as there are not significant behavioural differences on that point (see Fig. 2.2), but probably due to enhanced motivational valence of pup-derived stimuli, paralleled by a decrease in motivational valence of buttons during pregnancy.

These findings raise the question of which are the mechanisms that allow pregnancy hormones to modify the functioning of the motivation circuitry of the brain of females. There is strong evidence indicating that sexual steroids (late-pregnancy progesterone withdrawal and oestradiol surge) plus placental lactogens (late pregnancy) and/or hypophysial prolactin (postpartum) (see Bridges, 2020) are needed to induce fully motivated maternal behaviours. Recent work has shown that during pregnancy and postpartum, lactogens are able to influence the SBN nodes (Salais-López et al. 2017, 2021) that also concentrate oestradiol-sensitive cells (Mitra et al., 2003; Simerly et al., 1990), whereas the nuclei composing the motivational brain circuitry do not express prolactin receptors (Kokay et al., 2018; Salais-López et al., 2018) or few oestrogen receptors (Mitra et al., 2003; Simerly et al., 1990). Therefore, the influence of pregnancy hormones on motivation must be indirect, e.g. they likely act onto neurons projecting to the motivation circuitry rather than on the motivation circuitry itself. The only exception to this rule is the VTA and the reticular division of the substantia nigra, where according to the study of Mitra et al. (2003) in the mouse, the beta-receptor of oestrogens is expressed at high levels, thus suggesting a locus for the action of oestrogens on motivation during motherhood. In this respect, the medial amygdala may also play an important role. Previous studies with juvenile individuals as a social, rewarding stimulus, have shown that a medial amygdala-to-hypothalamus pathway is critical for social motivation (Hu et al., 2021). Thus, optogenetic stimulation of GABAergic MePD neurons projecting to the MPO (or simply with their axons terminating in the MPO) induces (a) dopamine release in the Acb; (b) place preference acquisition; and (c) auto-stimulation. By contrast, optogenetically blocking this pathway interferes with social, but not other natural rewards. In fact, using fiber photometry

of calcium signals, these authors report that non-social rewarding items (chocolate, sugar) seem not to activate MePD-to-MPO projecting neurons, whereas social stimuli do it, thus fitting our cFos-expression data (pups > buttons induced cFos expression in the MePD and the MPO).

These data also fit a previous study of our group in which, using a motivated pup retrieval test, a clear correlation was observed between prolactin signalling and maternal motivation in several nuclei of the SBN, including the MePD and the MPO (as well as the PaA), plus other nuclei not belonging to the SBN (e.g. central amygdala and posterior intralaminar thalamus) (Salais-López et al., 2021). Thus, lactogens, acting onto several nuclei of the SBN (e.g. MePD and MPO), may change specifically the response to pup stimuli of some cell populations, enhancing the rewarding valence of pups by the end of pregnancy. In our current experiment, this is revealed by a differential activation by pups/buttons in the nuclei of the reward/motivation system of the brain during late pregnancy, together with a similar effect in the MePD (and the PAG, see below).

The lack of a similar differential response to pups/buttons in the MPO between LP and virgin females suggests that concurrent changes must occur in other targets of the MePD also involved in reward/motivation of social stimuli. In this respect, direct amygdalo-striatal pathways (Novejarque et al., 2011) have been involved in some forms of social reward, e.g. female attraction for male pheromones (Agustín-Pavón et al., 2014; Dibenedictis et al., 2015). The possibility exists, therefore, that amygdalo-striatal projections sensitive to sexual steroids and lactogens, such as pathways from the medial amygdala to the ventral striatum— either direct (Pardo-Bellver et al., 2012) or indirect—using intra-amygdaloid connections (basolateral/ basomedial amygdala, cortical amygdala [Novejarque et al., 2011; Pardo-Bellver et al., 2012]), might also contribute to changes in maternal motivation during late pregnancy. In fact, Numan et al. (2010) demonstrated the importance of basolateral/basomedial amygdala, projecting to the Acb (Novejarque et al., 2011), in pup-directed maternal responses in postpartum rats (inhibited by muscimol injected in the BLA/basomedial amygdala), thus fitting our data on cFos expression in the BLA and the AcbC induced by pups and buttons in LP females (see Fig. 3.3B" and D" respectively). The lack of effect of muscimol inhibition of the medial amygdala on maternal behaviour by Numan et al. (2010) suggests that the MePD-BLA-Acb pathway may have an important role on maternal motivation, whereas the MePD-MPO projection might be involved in other forms of social reward (Hu et al., 2021).

3.4.3. Females possess specific circuitry for decision-making of whether to care, ignore/avoid, or attack pups

In females exposed to pups, we monitored the behaviour during the first 8min of exposure and, with a single exception, LP and V females displayed no significant differences; this might seem surprising because previous work of ours (Martín-Sánchez et al., 2015b) and other groups (Alsina-Llanes et al., 2015; Stolzenberg & Rissman, 2011) indicate that lactating dams exhibit more and quicker maternal care when pups are (re) introduced in their cages than age-matched virgin females. However, in those previous studies dams differed from virgins not only in the influence of motherhood-associated hormones (during pregnancy, parturition, and postpartum) but also in their previous intensive experience with pups (from parturition to the day of the experiment), which virgins lacked. Our experiment suggests, therefore, that experience with pups has a pivotal role in the increase in maternal care observed in postpartum dams, and the lack of such experience in LP females minimizes differences with pup-naïve virgin females.

The only pup-directed behaviour that significantly differs between LP and virgins is pup-directed attacks (see Figure 2.2), displayed only by some LP females but not virgins. Although maternal behaviour is usually equated to pup care (maternal care), in some conditions attacking and eating pups is quite frequent and should be considered an adaptive maternal behaviour (Hrdy, 2000). In other words, contrary to what is usually thought, a truly adaptive maternal behaviour does not consist of taking care of every possible pup in every possible moment but choosing when it is convenient (given the conditions) to do so, or to eat the pups instead, postponing motherhood until a better occasion. When confronted to alien pups, LP females apparently have the two drives, caring and eating. A control of when to attack and eat pups is therefore important for maternal behaviour to be fully adaptive.

Our previous study on this subject (see Chapter 2) revealed a specific pathway within the vomeronasal system, apparently related to pup attack, as cFos expression in the accessory olfactory bulb (AOB) and posteromedial part of the medial bed nucleus of the stria terminalis (BSTMPM) is positively correlated with pup-aggression score. Here we have found that in LP females there is a highly significant, strong and negative correlation between pup-aggression score and activity in the VTAp (Table 3.1) and a strong positive correlation in the PAG (Table 3.2). These data lead us to hypothesize that during pregnancy, pup-induced activity of the mesolimbic dopaminergic system is part of the decision-making mechanisms for

attacking/caring pups. Because the increased activity of the VTAp (mainly containing dopamine neurons) triggers a higher activity of the mesolimbic dopaminergic pathway (see Fields et al., 2007; Ikemoto, 2010), it seems that elevated activity of this pathway may be associated with pup caring, whereas reduced activity would be associated with pup aggression. The lack of pup attacks in virgin females, in which the VTAp shows relatively low cFos-ir cell density after exposure to pups, indicates that high VTAp activity in LP refrains a drive to attack pups that might depend on another brain region (in males, a region of the posteromedial BST seems involved; Tsuneoka et al., 2015; in our females BSTMPM activity is correlated with pup attacks; see Table 2.1). This brain region is likely not activated by pups in virgins. Therefore, only in LP females an attack-or-care decision is necessary, and the VTAp seems to be related to this decision-making system.

In addition, also in LP females the activity (cFos-ir cell density) of the PAG shows a positive and significant correlation with pup aggression score, suggesting a role of this brain region in pup-directed aggression. Different studies (summarized by Canteras, 2012) relate the PAG with the medial hypothalamic column involved in aggressive/defensive reactions to predators (dorsolateral column) and to conspecifics (lateral column), e.g. agonistic behaviours. In this context, our findings suggest that pup-directed attack is an additional reaction to conspecifics (a social behaviour) that occurs in virgin males but also in late-pregnant or postpartum females under specific conditions (cold-induced stress, Zafar et al., 2018; caloric restriction during pregnancy, Bronson & Marsteller, 1985). Pup attack seems to involve part of the social behaviour network (e.g. portions of the bed nucleus of the stria terminalis; see Table 2.1; Tsuneoka et al., 2015) and, according to our results, also the PAG. Therefore, in LP females, pup-directed attacks are under the control of two neural systems acting antagonistically: activity in the VTAp is negatively correlated with pup attacks, whereas activation of the BSTMPM/PAG correlates positively with this behaviour.

Another behaviour displayed by females in our context is what we call ‘‘approach-and-retreat,’’ a reaction similar to risk assessment: the female approaches a pup, sniffs at it for a while, and then retreats without retrieving it, what can be interpreted as a ‘‘not-to-retrieve/care’’ decision. This behaviour is displayed similarly by virgin and LP females (see Chapter 2, Figure 2.2) but only in LP females it is negatively correlated with cFos-ir cell density in the BLA and the AcbC (Table 3.1). Although causal relationships cannot be established with this experiment, activation of these two interconnected (Novejarque et al., 2011) centres of the reward circuitry is associated with LP females not retreating after detecting and sniffing pups. By contrast, in virgin females approach-and-retreat responses to pups positively

correlate with activation of the MePD, the AcbC, and the PAG (Tables 3.1 and 3.2). This finding further reveals important changes in the activity of the motivation- socio-sexual circuitry of the brain in response to pups during pregnancy, leading to not avoiding or ignoring pups, but caring or attacking them instead.

Concerning virgin females, although in mice they approach pups with a short delay (see Alsina-Llanes et al., 2015; Martín-Sánchez et al., 2015b; Stolzenberg & Rissman, 2011), it seems that pup-sniffing related activation of the AcbC and the MePD facilitates or is facilitated by not retrieving the pups. This recalls the situation in the rat, where lesions of the MePD in virgin females dramatically shorten their sensitization period (Sheehan et al., 2001). By contrast, the strong activation of the BLA and the AcbC related to pup sniffing in LP females seems to be associated with not avoiding pups after detecting and sniffing at them, fitting the results of lesion/inactivation experiments of the BLA obtained in rats (Lee et al., 2000; Michael Numan et al., 2010). In fact, although inhibition with 100 ng of muscimol infused in the BLA of female rats did not alter pup sniffing, it significantly delayed pup retrieval.

Other social and maternal behaviours are also correlated with activation of specific nuclei. Thus, pup retrieval shows a strong, negative correlation with activation of the PaA in LP females (but not virgins; see Table 3.2). We tentatively interpret this as related to the parental style of the females. According to Bosch (2011), the basal level of anxiety is reduced during motherhood, with the paraventricular hypothalamus being involved in the regulation of this basal anxiety. Nonetheless, high-anxiety mothers display a more protective maternal style than low-anxiety ones: they retrieve pups more quickly and display more frequently arched-back posture and pup licking/grooming. Our results suggest that PaA activity has an inhibitory role of anxiety, may be mediated by somato-dendritic or axonal oxytocin release (Neumann, 2007), so that the higher the activity, the lower the anxiety and, consequently, the less frequent is pup retrieval. The anxiolytic activity of the PaA would already be present in LP females.

Moreover, nest building is a quite common behaviour in virgin or LP females exposed to pups, but our data indicate that occurrence of this behaviour is significantly and positively correlated with activation of the VTAa only in LP females (see Table 3.1). Similarly, affiliative behaviours between adult females (inter- female interactions), which are similarly expressed by virgin and LP females (see Chapter 2, Figure 2.2), show a negative, highly significant correlation with activity in the VMHVL in LP but not virgin females. Because LP females already show aggression to unfamiliar intruders (prepartum or pregnancy-induced aggression; Mann & Svare, 1982), and the VMHVL has been involved in intermale (Lin et al., 2011) and maternal aggression

(Hashikawa et al., 2017), our results can be interpreted as a tonic inhibition of aggression allowing affiliative contact with the familiar female, with which the experimental LP female is living in the same home cage.

3.4.4. A neural network view of social behaviours: SBN versus hierarchical labelled-line circuits

Most of the studies on the neural or neuroendocrine basis of social behaviours try to identify a key nucleus for the expression of a given social behaviour. For maternal behaviours, the classic studies by Michael Numan and his group (Numan et al., 1990; Numan & Numan, 1996) revealed that lesions restricted to, or isolating the medial preoptic area/ventral BST, resulted in a reduction of maternal care. Similar lesion experiments in mice led Tsuneoka et al. (2013) to identify a region in the MPO, what they called the central MPO, the lesion of which in females (using fiber-sparing neurotoxic drugs) resulted largely in pup killing instead of pup care.

This analysis was further detailed by Wu et al. (2014), who identified a specific galanin-expressing population of neurons in the MPO that project to the VTA that seems involved in motivational aspects of maternal behaviour. Ablation of these cells abolishes pup care in virgin and lactating females and promotes pup killing in virgin females and male mice. Otherwise, their activation promotes pup care in males and is correlated with parental behaviours in males, virgin females, and mothers.

However, the detailed analysis carried out by Kohl et al. (2018) has shown that the cell population of galanin- expressing cells in the MPO is interconnected with some 20 nuclei, and this network seems to encode together the complex interactions with pups during parenting and other aspects of non pup-directed parental behaviours. The analysis of the effects of optogenetic activation and inhibition of MPO-galaninergic cells suggests the involvement of some of the pathways arising from the MPO in motivation for pups (MPO to VTA), pup-directed grooming (MPO to PAG), and reduction of social interactions with adults (MPO to medial amygdala). However, the complex, reciprocal connections of the MPO with these centres (e.g. the medial amygdala) makes difficult to accept a hierarchical organization within the social brain. For instance, MPO galanin cells projecting to the medial amygdala are active during different components of parenting (sniffing, grooming, retrieving, and entering the empty nest), but inhibiting or activating these cells seems to have no clear effects on interaction with pups (see Kohl et al., 2018).

Overall, although not specifically focused on the galaninergic population of the MPO, our data suggest that the functioning of the whole brain network, instead of just the MPO, encodes maternal behaviours. First, although the MPO is activated by pups more than by buttons, this is also the case for every centre of the SBN. Second, late-pregnant and virgin females without previous experience with pups show similar activation by pups in the MPO, as it happens in most of the remaining nuclei of the SBN. In this respect, the discriminant analysis performed indicates that the data of activity of the MPO are not enough to predict the nature of the stimulus the animal has been exposed to (pups or buttons) and the physiological status of the female (virgin or late-pregnant). By contrast, considering for the discriminant analysis all 13 nuclei belonging to the SBN and motivation brain circuit allows a nearly perfect classification of the animals into their respective groups, with an accuracy of 93.33% and no mistakes on the stimulus the animals are exposed to (buttons or pups); this suggests that, although the MPO would be an important node of the network, the whole system composed of the SBN and the circuit for motivated behaviours encodes together the nature of the stimulus the animal is perceiving and the changes in the response to it associated with pregnancy.

This gives direct support to the view, already proposed by Sarah Winans Newman more than 20 years ago (Newman, 1999), that social behaviours, including maternal ones (pup care and non pup-directed behaviours), are encoded by the activity of a network of brain centres. The opposite view, namely that there are specific circuits and centres controlling the expression of different social behaviours (e.g. male and female sexual behaviours, aggressive/agonistic behaviours; affiliative relationships), considers a series of linear pathways (labelled line model) in which there is a hypothalamic nucleus responsible for the execution of a given social behaviour (male sexual behaviour; female sexual behaviour; agonistic/territorial behaviour, including aggression; affiliative interactions; pup care/attack) that would be triggered by a key social stimulus under a given hormonal status. This hypothalamic key nucleus (e.g. the hypothalamic aggression locus; Lin et al., 2011) would activate the behaviour through descending projections to the midbrain/brainstem (Falkner et al., 2020) but would work under a certain control of telencephalic centres (e.g. the lateral septum; Wong et al., 2016). In some cases, this scheme has been applied to the control of maternal behaviours, with the MPO as the hypothalamic key centre projecting to the ventrolateral periaqueductal gray and the medial amygdala as the telencephalic centre for control.

Our data indicate, instead, that the social brain network (SBN) encodes not only interactions between adult individuals, as proposed by Newman (1999), but also pup-directed behaviours, another special form of social behaviour. According to the hypothesis by Winans Newman, the

resulting behaviour would not depend on the activity of one of the nodes of the SBN, but on the pattern of activity on the whole network (see Fig. 3.7A). The whole SBN responds strongly to the presence of pups (as compared with buttons), with a different pattern in virgin and LP females, with higher activity in the MePD and the PAG, and low activity in the MPO and the LSV of LP females as compared with virgins. The results relative to the centres controlling motivational aspects of behaviour in our study reveal that they play an important role in the response of the females to social and non-social stimuli (Fig. 3.7B). Here, the status of the female (virgin or late-pregnant) determines their response to the stimuli, with pregnancy inducing a high activity in the whole system in response to pups, whereas virgins react strongly to buttons.

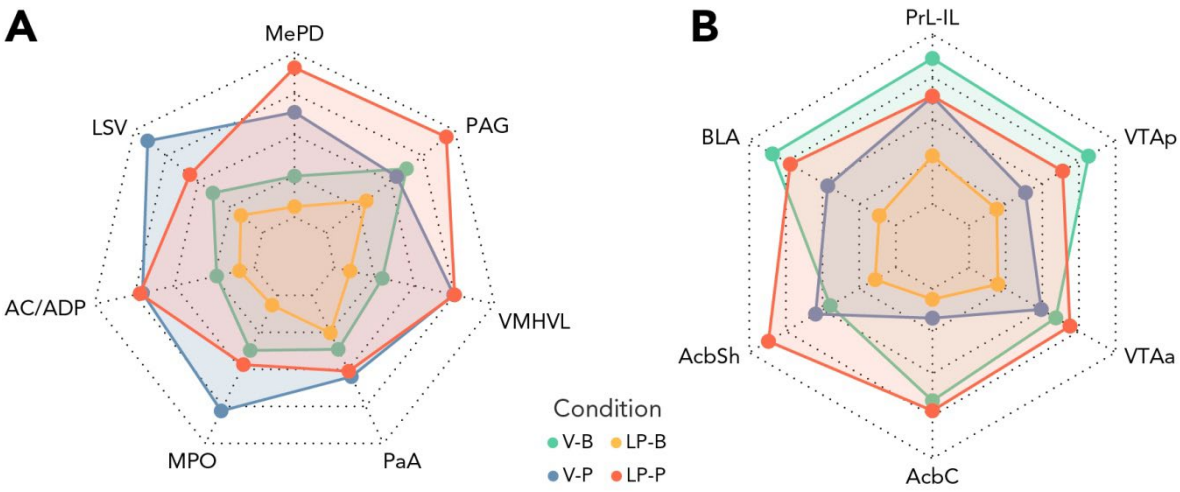

Figure 3.7. Pattern of activity of the socio-sexual and motivational circuits in females (virgin and late-pregnant) exposed to pups or buttons. Hexagons representing the relative activity (cFos-ir cell density) of the different nuclei belonging to the socio-sexual (A) and motivation circuitry of the brain (B) of the different female groups, as indicated by the colour code (green, virgins exposed to buttons, V-B; blue, virgins exposed to pups, V-P; yellow, late-pregnant exposed to buttons, LP-B; orange, late pregnant exposed to pups, LP-P). Each vertex of the hexagon represents a nucleus, as indicated. The area of the coloured hexagon is related to the degree of activation of the system, whereas its shape gives information on the pattern of activity within the system. Concerning the socio-sexual brain network (A), this system is clearly more activated by pups (irrespective of the kind of female) than by buttons, and pups elicit a different pattern of activity in LP and virgin females. On the other hand, the motivation brain circuitry (B) is more activated in LP-P and V-B, whereas buttons barely activate the motivation circuitry of LP females. By contrast, pups elicit a much higher activation of this circuitry in LP than virgin females, with a profile of activity that also differs between females. Abbreviations: AC/ADP, the region of the nucleus of anterior commissure/anterodorsal preoptic; AcbSh and AcbC, the nucleus accumbens shell and core; BLA, the anterior part of the basolateral amygdaloid nucleus; LSV, the ventrolateral septum; MePD, posterodorsal medial amygdala; MPO, the medial preoptic area; PaA, the anterior portion of the paraventricular nucleus; PAG, lateral column of the periaqueductal gray; PrL-IL, the infralimbic cortical areas; VMHVL, the ventrolateral portion of the ventromedial hypothalamic nucleus; VTAa and VTAp, the anterior and posterior parts of the ventral tegmental area.

As discussed earlier, the change in the response of both systems is with all likelihood due to the action of pregnancy hormones that, according to the distribution and action of oestrogens and prolactin, are concentrated in the SBN nodes; this would indirectly modulate the activity

of the motivation brain circuit, thus enhancing the incentive properties of pup-derived stimuli and reducing those of non-social stimuli. Altogether, these changes would result in motivated, pup-directed behaviours once the litter is born, ensuring their survival and well-being (Kohl et al., 2018; Salais-López et al., 2021).

3.4.5. Limitations of the study

A possible limitation of our study, as commented in Chapter 2, is that we have not recorded and measured the interactions with the control stimulus (buttons) in the same females in which cFos has been analysed. We considered buttons as a non-social novel object, appropriate as a simple control object (pups were also a novel object for the females). However, after the experiments we realized that many of the buttons had been intensely gnawed, thus indicating that they are not just novel but salient enough as to induce goal-directed behaviours (gnawing).

In fact, our results of cFos immunoreactivity indicate that button-induced activation is observed in most nuclei of the motivation circuit. There, virgins show generally more activation by buttons than by pups (opposite to what happens in LP females), and button-elicited cFos immunoreactivity is denser in virgin than LP females (see Fig. 3.3A”-F”). In a different set of females (LP and virgins), we measured button-induced behaviours, and this confirms that increased button-elicited brain activity in virgins relates to more prolonged interaction with buttons, enhanced rewarding properties of buttons, or both. Therefore, the pattern of activity in the SBN and the motivation circuitry and direct analysis of behaviour indicate a reduction of salience of buttons during late pregnancy. Because females were exposed to either pups or buttons, this is not due to a competing effect of the presence of pups, but it reflects an actual decrease in salience of this control, non-social stimulus during pregnancy.

Another limitation of our study (concerning both Chapter 2 and 3) is related to the use of cFos immunoreactivity to evaluate brain activity. A more dynamic procedure (calcium imaging, electrophysiological procedures) would allow analysing temporal aspects of brain activity, related to specific conducts, thus helping to clarify causal relationship. However, using these techniques, it is technically difficult to analyse simultaneously the activity of a network of 13 nuclei distributed in distant regions of the brain (plus the 7 nuclei and the VNO analysed in Chapter 2). Our data may help to focus future research on specific groups of these nuclei, to evaluate how their activity relates to maternal care in postpartum and virgin females. For

instance, the possible role of amygdalo-striatal pathway systems in maternal motivation can be further explored using these techniques.

GENERAL DISCUSSION

Our experiments tried to check the hypotheses that: a) mice use chemosignals for intra-species communication (chemosignals/pheromones) for the expression of maternal behaviours; and b) that the expression of social (including parental) behaviours and processing of these chemosignals change during late pregnancy in preparation to motherhood, thus mediating the expression of motivated pup care. The results of our experiments partially confirm these working hypotheses, and expand our knowledge on the neural basis of maternal behaviours.

D.1. Female mice detect and intensely chemoinvestigate conspecific chemosensory cues.

The results of preference tests carried out in Chapter 1 indicate that all females tend to spend more time exploring chemosignals contained in material soiled by a conspecific than clean material (Fig. 1.4A and 1.5A). Pup-naïve virgins, pup-experienced virgins and dams all preferred to sniff postpartum and prepartum nests rather than clean nest material. This is not surprising, since mice are social species and usually prefer interaction with a conspecific rather than with a non-social stimulus (Moy et al., 2004). Being a macrosmatic species, the drive of adult female mice to explore conspecific chemosensory cues was foreseeable. In fact, recently Contestabile et al. (2021) demonstrated in a very complete study about conspecific cues involved in social preferences that, although mice prefer to explore complex social stimuli rather than isolated cues, chemosensory cues are sufficient to elicit preference in approaching behaviour.

In our test, preference towards social chemosensory cues only appeared when analysing the time that females spent near the containing jars, not when analysing the time spent in whole compartments. This particularity of our results points to a role of low-volatility chemosensory signals, or at least signals that require proximity to the source of the stimulus to be detected. As discussed in the Introduction, non-volatile chemosignals are only detected by the vomeronasal organ. In addition, an active process of pumping during investigation close to the source is required to introduce chemicals into the VNO even to detect volatiles, due to the fact that it is a blind tube with no air stream running through it (Meredith, 1994), as it was demonstrated in behaving animals by Luo et al. (2003). Therefore, preference results in Chapter 1 suggest that the accessory olfactory system might have a relevant role in the detection of conspecifics chemosignals. Indeed, findings in Chapter 2, in which we exposed females to pups or non-social stimuli (buttons), are in line with these conjectures. In fact, we found that the VNO of females was activated by pups (Egr1 expression) more than by a control stimulus (buttons). Morever, the entire vomeronasal system responded more to pups than buttons regardless of the hormonal status of the female (Fig. 2.4-2.6), and this did not happen

in the main olfactory system in which only the anterior piriform cortex showed that trend (Fig. 2.7). Furthermore, this preferential response to pups (over buttons) of the PirAnt is due to a significantly higher activation by pups in late-pregnant groups, which is not observed in virgins, and a similar situation occurs in the PirPost. Hence, it is possible that VNO-detected chemosignals are indeed marking pups as "social-objects" to all females.

Supporting this view, the results of Chapter 2 indicate that pups activate similarly the VNO of virgin and LP females and other centres of the vomeronasal system. Differences between females only appear in olfactory-vomeronasal secondary centres, where olfactory and vomeronasal stimuli converge, e.g. the PMCo and piriform cortex (see Fig. 2.6 and 2.7 respectively) (Gutiérrez-Castellanos et al., 2014; Martínez-García et al., 2012). This strongly suggests that, although VNO-detected stimuli signals pups as social objects, pregnancy enhances the convergence with olfactory stimuli for the expression of pup-directed behaviours (maternal care or pup-aggression).

D.2. Infant and adult chemosensory cues seem similarly attractive for adult females

As discussed above, our results indicate that females are indeed detecting conspecific chemosensory cues and prefer them relative to control (clean material). However, they do not seem to prefer or even explore more P03-soiled samples over adult- or P21-soiled material (see Fig. 1.6 and 1.17 respectively). When presented with chemosensory cues, all females seem, at least, equally interested in both pup-derived (P03) and control conspecific stimuli, either prepartum collected nest (nE17; but see section 1.2.2) or juvenile collected stimuli (P21). In fact, sometimes females even explore more the P21 than the P03 stimuli (Fig. 1.17B and 1.18B and C).

In relation to that, in 1989 Londei et al. explored the role of mouse pup urine as a pheromonal signal by comparing the response of virgin females towards foetuses treated with pup or adult urine. In their research they found that pup urine was more effective in eliciting maternal behaviour than adult urine. Moreover, adult female urine was more effective than adult male urine. Hence, they proposed that adult females might retain some "infant factor" in their urine. This could be related to our results of females not showing preference for P03 soiled nest when tested against E17 soiled nest (Fig. 1.6), since maybe they are not able to distinguish between them. In this regard, in 2018 Isogai et al. found that both adults and pups produce chemical ligands for the same vomeronasal receptors (Vmn2r65 and Vmn2r88), although they

found this to be true only for salivary and lacrimal glands extracts, not urine. In their work, they proposed that chemosignals associated with pups are indeed from both pups and dams and identified two pheromones that elicit male aggression towards infants: the submandibular gland protein C and haemoglobins.

This fits with the results of our habituation-dishabituation tests, according to which females were not able to distinguish between P03 and E17 chemosignals, at least in the way we collected and presented them (Fig. 1.14). This is interesting because there are several studies in which preference for pup chemosensory cues has been established by using clean stimulus or even empty chambers as control stimuli (Smotherman et al., 1974; Wang & Storm, 2011; Wansaw et al., 2008), and therefore the measured preference may be due to the mere presence of "conspecific" cues rather than specifically "pup" cues. In fact, some of the traditional studies in rats used lactating females nest material as "pup-related odours" opposed to clean nesting material, what, in the light of our results in mice, might be inadequate for rodents (Fleming et al., 1989). Therefore, one of the main highlights of this thesis is the importance of employing adequate controls when analysing the preference response towards social stimuli, taking into account the possible limitations of the experimental design.

D.3. Motherhood does not alter attraction for collected pup- or adult-derived cues

In Chapter 1, we were interested in how pregnancy and lactation may be affecting the preference for conspecific chemosensory stimuli, as we expected that motherhood would lean the scale towards pup-derived stimuli when opposed to other conspecific cues. Surprisingly, we did not find that dams explored P03 stimuli over that of other conspecifics, and their behaviour in preference and exposure tests was mostly equal to that of virgin females (see Fig. 1.4-1.8, 1.17 and 1.18). That was unexpected, because literature in rats exploring motivation for pups shows that early postpartum mothers spent more time in newborn-associated chambers than in juveniles-associated ones in a conditioned place preference paradigm. In postpartum females, preference develops during lactation for pups aged as their own offspring (Ferreño et al., 2018). Nonetheless, the vast majority of research works on that topic did not use conspecific-derived stimuli as controls in preference tests, or tested preference between pups and males, which has its particularities because of the powerful effect of male cues in females in different physiological status (Agrati et al., 2008).

Also, previous works about preference of dams towards pup-derived cues has been centred in comparing different sensory cues of pups, such as vocalizations, shapes, warmth, etc. (Cohen et al., 2011; Isogai et al., 2018; Okabe et al., 2013; Smotherman et al., 1974). In those studies, which have been discussed in Chapter 1, the described synergistic effect of different stimuli may explain why the mere exposure to chemosensory cues did not allow observing enhanced preference towards pups (over other social stimuli) in dams. However, recent work by our group (Goterris-cerisuelo et al., 2021) shows that dams and pup-experienced virgins (but not pup-naïve virgins) show preference towards anaesthetized hidden pups, meaning that pup chemosignals are indeed enough to elicit preference in female CD1 mice. However, in their experiments, Goterris-Cerisuelo et al (2021) preclude direct contact of females to pups, which were enclosed in a stainless-steel infuser. This suggests that preference for pups depends on volatiles, whereas access to non-volatiles (as in our experiments) may mask such a preference, especially when pup-derived chemosignals are confronted to other social stimuli.

D.4. Difficulties in collecting chemosignals from P03 pups: different procedures have different problems.

There are two possible explanations of the lack of preference for pup-derived chemosignals observed in Chapter 1 (Fig.1.6): the difficulties of collecting substrates enriched with enough amounts of pup chemosignals to present them to the females, and the problems derived from working with very young pups. In our work, we decided to use P03 pups because younger pups elicit more maternal behaviour than older ones (Hasen & Gammie, 2011; Svare, 1977) and this effect seems mainly dependant on chemosensory cues, since it happens even when the female is tested with non-vocalizing dead pups (Londei, 1983). Furthermore, previous works stablished that as pups mature the olfactory system is less involved in the maternal behaviour and other cues become more important (Gandelman, 1971a).

Therefore, in the preference tests, we used prepartum E17 soiled nest vs postpartum P03 soiled nest, which include peripartum chemosignals. As commented before, however, dam-derived chemosignals present in the nest material might mask the litter ones, because adult female and pups share relevant secretions (Isogai et al., 2018; Londei et al., 1989). In fact, when tested in habituation-dishabituation test 1 and 2 (Fig. 1.14A and B), virgin females did not seem able to distinguish the odour of E17 and P03 nest material, which points to a similar profile in chemosensory cues in both kinds of samples. Nonetheless, in those tests we presented the stimuli inside the hanging tea infuser that has its particularities, which have been discussed in Chapter 1, and may not be a proper way to present them.

When we tried to avoid adult female chemosignals, by using P03 pups-soiled cotton swabs or bedding, the results did not improve. In fact, in these conditions, females apparently did not distinguish P03 pup-derived from clean stimuli in habituation-dishabituation tests (Fig.1.14C-F). A possible explanation for these results is that P03 pups are so young that they secrete a very low amount of chemosignals, which might be difficult to collect to be detected by the females. In fact, previous literature usually employs older donor pups. For instance, Wang & Storm (2011) collected urine of P05-P06 pups by holding the animals by the scruff of the neck, which we could not replicate with P03 pups, because they do not urinate enough volume. Moreover, according to our results P03 secretions are exiguous not only in the ano-genital area, but also in the oro-facial area (the two areas that we rubbed with the cotton swabs). This was also surprising, because Ferrero et al. (2013) had described an age-specific peptide secreted by infant lacrimal glands (ABP27), which could be a candidate for neonatal pup pheromone.

The last attempt we made to try to collect stimuli was based on Okabe et al. (2013), who also used pups older than ours. They placed three 6-day-old pups in a glass beaker full of cotton for 3 hours under a warm light bulb. As our approach used younger and more vulnerable pups we shortened the collection time to 90 minutes to avoid separation stress, and those two factors (short age and short collection time), may have hindered the results of the habituation-dishabituation tests 4 (Fig. 1.14E and F). Nevertheless, Okabe et al. (2013) did not find preference for pup chemosignals alone even with their conditions of collection, and pup odours only elicit preference when accompanied with pup vocalizations.

In this respect, Nakahara et al. (2016) managed to successfully collect chemosensory stimuli from even younger pups (P0 to P2.5 pups), which elicited the same response as pups in the Olfr62 VNO neurons of male adults. However, we did not use their approach of collecting pup chemosignals ("pup wash"), since they did not observe VNO activation of Olfr62 cells in females (only in males), maybe because they avoided the face in their collection procedure.

Finally, as commented in Chapter 1, the high variability that might have hindered the results of habituation-dishabituation tests 1 to 3 (Fig.1.14A-D), may be attributed to the use of tea infusers or naked cotton swabs hanging on the wall, both being pretty distracting for the females. This induced females to play with them, consequently masking the effects of the actual exploration of the odorous stimuli. By contrast, presenting the stimulus in a laboratory well fixed to the wall seemed an adequate procedure, since pilot with male bedding showed significant dishabituation from clean bedding (results not shown). This rendered habituation

and dishabituation after presentation of P03- and P21-soiled bedding, but the results again suggest that P03 pups secrete tiny amounts of chemosignals to the bedding, barely detectable by the females (Fig. 1.14E and F).

Taken together, the results of Chapter 1 point to the need of alternative procedures and strategies to present pup chemosignals to females. Thus, our work with pup-derived chemosensory cues opened new research lines of our group that include the study of the response of females to specific compounds of neonatal pups volatolome (Lacalle-Bergeron et al., 2021), and the response to complete anaesthetised pups (which do not vocalise) enclosed in stainless-steel infusers (thus avoiding somatosensory and visual stimuli) (Goterris-Cerisuelo et al., 2021).

D.5. The use of control stimuli and pup-naive females allows revealing pregnancy-related changes in processing of pup cues

While our group continued with the search of the "pup pheromone" with the new research lines, we decided to simultaneously take a different approach towards understanding pup chemosignals and its role in maternal behaviour by analysing the response of the chemosensory systems of females during interaction with pups.

The experimental design in Chapter 2 and 3 (see section 2.2) has two particularities that are worth commenting. First, we used females exposed to pups and control females exposed to a novel, non-social stimulus, buttons. Comparison of pup- and button-exposed groups allowed us to attribute specifically the differential neural activation to pups, while neutralising the effects of novelty (Rinaldi et al., 2010). This contrasts with previous works in the literature that conclude that pups activate specific brain centres or neural populations by comparing pup-exposed with isolated females, not exposed to any control object (e.g. Matsushita et al., 2015; Tsuneoka et al., 2013, 2015).

In addition, the use of buttons as non-social control objects has revealed that: a) the vomeronasal and social brain systems respond specifically to pup cues (social stimuli, see Fig. 2.4E, 2.6A"-D", 3.1A"-D" and 3.2A"-C"); b) late pregnant females show a diminished motivation for non-social stimuli (Fig. 2.3), which might have resulted in reduced activation of chemosensory and motivational brain systems (see Fig. 2.7A"-C" and 3.3A"-F"). Both findings have further implications that will be discussed later.

Second, in our experiments we used late-pregnant females instead of pup-experienced dams and compared them with pup-naïve virgin females. This allows avoiding the effect of experience in the interaction with pups and in the processing of pup-derived stimuli. As discussed in Chapter 2, our results showed no differences in maternal care between pup-naïve virgins and late-pregnant (also pup-naïve) females exposed to pups (Fig. 2.2). These results differ from those of previous works in which researchers compared pup-naïve virgins with pup-experienced dams (Alsina-Llanes et al., 2015; Martín-Sánchez et al., 2015b; Matsushita et al., 2015; Stolzenberg & Rissman, 2011) meaning that, at least in mice, experience with pups plays a significant role in the behaviour of lactating dams. Therefore, differential responses to pups in those previous works may not be attributed solely to the central effects of pregnancy hormones. Hence, our approach improves previous works by revealing changes in brain activity induced by pregnancy, without the interference of previous experience with pups.

D.6. Pup-derived chemosignals activate the VNO of both virgin and late-pregnant females

In Chapter 2, we found that the vomeronasal organ showed a higher activation in the groups exposed to pups when compared with the groups exposed to buttons, and this effect was equal in virgins and late-pregnant females (Fig. 2.4E). The involvement of VNO in the detection of conspecific stimuli is not a surprise, since it has been traditionally considered the main organ involved in pheromonal communication (see Introduction, section I.5.3). In fact, a recent work by Isogai et al. (2018) studied the expression of Egr1 in the VNO of males and females exposed to pups. Their results demonstrate a clear, significant activation of the VNO of males by pup-derived chemosignals, especially when males attacked the pups. Conversely, the authors do not comment on the apparent activation of the VNO of females illustrated in their Fig. 2B. Our results confirm that the VNO of females is also able to detect pup-derived chemosignals.

As discussed in Chapter 2, literature shows solid evidence that VNO is crucial for the display of pup-directed behaviours in males (parental care or infanticidal behaviour; Isogai et al., 2018; Kimchi et al., 2007; Nakahara et al., 2016; Tachikawa et al., 2013). The use of trpc2$^{-/-}$ mutants (Hasen & Gammie, 2011; Nakahara et al., 2016; Tachikawa et al., 2013) or lesions of the VNO (Lepri et al., 1985) has shown that in female mice vomeronasal-detected stimuli are needed for the expression of normal maternal behaviour. However, the most relevant functions described in females for which the VNO seems indispensable are the detection of adult male chemosignals eliciting maternal aggression towards intruders (Mann & Svare, 1982; Martín-

Sánchez et al., 2015a) and attraction for male-derived chemosignals (Martínez-Ricós et al., 2008).

Our results from Chapter 2 demonstrate that females detect some pup-derived chemosignals that might be involved in the expression of maternal behaviour. However, as discussed above, apparently, virgin and LP females VNO respond similarly to pups, since there is no FEMALExSTIMULUS interaction, nor differences between females in the expression of Egr1 (Fig. 2.4E). These results suggest that pregnancy effects on vomeronasal signals processing may not reside in the sensory transduction of these cues (although see Dey et al., 2015; Oboti et al., 2015), but in information processing in higher association nuclei (e.g. PMCo and MePD, see Fig. 2.6B" and C"). In this regard, when Nakahara et al. (2016) explored the activation of VNO by adult, juvenile and pup odours they found that, in males, pup cues specifically activate an olfactory receptor (Olfr692) expressed in sensory cells of the VNO. Those receptors seem involved in the shift from male aggression towards paternal care. However, although dams seem to have a higher number of Olfr692-expressing cells than virgin females, the activation of those cells when exposed to juveniles was equal between virgin females and dams, similar to our results in Chapter 2.

D.7. Pregnancy entails coupling of the main and accessory olfactory systems to process pup chemosignals

One of the most interesting results in Chapter 2 is the positive correlations we found between main and accessory olfactory centres, particularly numerous in LP females exposed to pups (see Table 2.2). The coupling of both systems matches recent literature, already discussed in the Introduction, that challenged the traditional dual-olfactory hypothesis towards a more interconnected integrative model for chemosensory function (Cádiz-Moretti et al., 2013; Martínez-García et al., 2009; Matsuo et al., 2015; Pardo-Bellver et al., 2017). In this regard, our work not only provides more evidence about the functional interrelation between both chemosensory systems, but also stresses the enhancement of such an interrelation during pregnancy. Future studies will help to clarify the mechanistic role of pregnancy hormones in the functional coupling of both systems.

Moreover, this finding might be reflecting a facilitation, during pregnancy, of association between odour cues and vomeronasal stimuli that may allow identifying the offspring through its odour (olfactory stimuli), which is very advantageous to locate pups, e.g. during retrieval. As

far as we are concerned, this effect has not been studied in relation to pup odour, but some research suggest that preference for male urine airborne chemosignals in female mice depends on their previous association with non-volatile ones detected by the VNO (Moncho-Bogani et al., 2005; Ramm et al., 2008). Maybe a similar learning process occurs in the case of preference for pups, and it is possible that this association between pup-derived vomeronasal and volatile chemosignals (odorants), which might occur in all females, is somehow facilitated by pregnancy hormones. This learning process would also contribute to the differences found in the literature between pup-naïve and pup-experience females (see Alsina-Llanes et al., 2015; Martín-Sánchez et al., 2015b; Stolzenberg & Rissman, 2011), thus endorsing the adequacy of our experimental design, which uses only pup-naïve females to unravel the actual influence of pregnancy alone in the chemosensory processing of pup cues (see section D.5).

D.8. Pregnancy changes the response towards pups in chemosensory secondary cortical nuclei, but not in primary chemosensory nuclei

As explained in section D.7, one of the main outcomes of Chapter 2 is that pregnancy does not change sensory transduction of pup-derived chemosignals in the VNO, but alters processing of pup cues in higher centres of the chemosensory pathways. Pregnancy-induced changes in pup-odours processing were observed in the vomeronasal cortex (posteromedial cortical amygdala, PMCo), and the olfactory cortices, e.g. anterior and posterior portions of the piriform cortex (see Fig. 2.6B" and 2.7B" and C" respectively). All three cortical regions showed an increased activation by pups during pregnancy. There are two possible explanations for this finding. First, this could be related to the enhancement of the olfactory-vomeronasal associative learning described in section D.7. Second, cortical activation may be increasing the salience of pup chemosensory cues in LP females.

As discussed in Chapter 2, PMCo projects to the basolateral amygdaloid division (Gutiérrez-Castellanos et al., 2014), especially to the basomedial nucleus. In addition, the cortex also projects to the basolateral nucleus of the amygdala (Johnson et al., 2000). Both the basomedial and basolateral (BLA) nuclei of the basolateral division of the amygdala give rise to massive projections to the ventral striatum (Novejarque et al., 2011) and are therefore crucial nodes of the motivational brain network. In this respect, our work in Chapter 3 showed increased BLA activation in LP females exposed to pups as compared to buttons, whereas in virgins, buttons activate more than pups (see Fig. 3.3B"). This change in the response to pups and buttons during pregnancy in the BLA, might be related to changes observed in their afferents, e.g. the

olfactory and vomeronasal cortices (Pir and PMCo, respectively). In fact, literature has described some neurons of the piriform cortex to respond preferentially to rewarded odours (Choi et al., 2011; Schoenbaum & Eichenbaum, 1995), endorsing this relationship between pregnancy effects on the processing of chemosensory information and its effects on increasing salience of pup-derived cues. Furthermore, in Chapter 2 results both PMCo and Pir cortex activities are significantly correlated to maternal behaviours (see table 2.1), meaning that chemosensory function may be involved not only in the perception of chemosensory cues, but also in providing essential information to socio-sexual and motivational networks, in order to prepare them for motherhood.

D.9. Pup-naïve late-pregnant and virgin females show similar maternal behaviour, except for pup aggression in LP females

As discussed in section D.5, our results prove that when we abolish the effect of experience in the display of maternal behaviour, pup-naïve virgins and pup naïve late-pregnant females display similar maternal behaviour when exposed to pups (see Fig. 2.2A-C), except for pup-directed attacks that will be further discussed in the next section.

Nonetheless, we have observed that in some neural centres, activation differs between females exposed to pups. Since the behavioural output is essentially the same for both kinds of females, this suggests that pregnancy influences the processing of pup-derived stimuli rather than the behavioural output of basic (motivation-independent) maternal care.

Nonetheless, our experimental design aimed to evaluate the processing of pup stimuli by females in neutral effortless presentations, meaning we needed all females to easily access, contact and interact with pups in a similar way. Unfortunately, these requirements could not fit with testing motivational aspects of maternal behaviour, which, according to previous literature (Salais-López et al., 2021) and our own results from Chapter 3, are the specific aspects that might be modified by pregnancy hormones. So the lack of differences in the maternal behaviour between groups may be due to the non-challenging experimental design. In this regard, further research is needed to evaluate how pregnancy changes the processing of pup-derived information while displaying goal-directed behaviours with pups.

But there was a behavioural difference between virgin and late-pregnant females that surprised us; the display of aggression towards pups by half of the late-pregnant females (see Fig.2.2D). Nevertheless, according to literature this might not be so unexpected. As discussed

in Chapter 3, in some occasions an adequate maternal behaviour entails the sacrifice of offspring in order to postpone breeding to a better occasion (Bronson & Marsteller, 1985; Hrdy, 1979, 2000; Zafar et al., 2018). Under this light, it is not surprising that pregnant females have a drive of eating alien pups, since some of the main allostatic mechanisms of pregnancy, described in section I.3, are metabolic adjustments that alter satiety regulation in order to gain weight (Ladyman et al., 2012). Hence, it is possible that the pup aggression we found in late-pregnant females is indeed an adaptive maternal behaviour, responding to allostatic pressures and postponing maternal care until their own offspring arrives.

D.10. Caring or attacking pups: a system for decision-making in the brain of females

The study of the correlations of nuclei activity with pup-directed aggression in the LP females revealed a positive correlation with cFos expression in AOB, BSTMPM (see Table 2.1) and PAG (Table 3.2), and a negative correlation with the VTAp (Table 3.1). Although the number of pup-aggressive females is quite small, behaviour-cFos correlation analysis lead us to hypothesize that in pregnant females, two different pathways may be involved in the decision to care or to attack the pups.

As discussed in Chapter 3, positive correlations with pup aggression score suggest an “attack” pathway somehow activated by vomeronasal signals, since it involves the AOB, like it has also been shown in males (Nakahara et al., 2016; Tachikawa et al., 2013; Trouillet et al., 2019), and the BSTMPM, a secondary vomeronasal centre (see section I.5.2). The correlation of the activity of this pathway with pup attacks is likely reflecting that the posteromedial BST, as part of the medial extended amygdala, also belongs to the social brain network (SBN). As part of the SBN the BSTMPM and PAG express receptors for sexual steroids (Mitra et al., 2003; Simerly et al., 1990) and are also influenced by prolactin and placental lactogens (Salais-López et al., 2017, 2018), and consequently they are very likely the target where pregnancy hormones facilitate pup-directed attacks.

Moreover, both BST and PAG have been previously linked to aggressive behaviours (Canteras, 2012; Fukui et al., 2019; Tsuneoka et al., 2015). So maybe pup-aggression is just another agonistic behaviour coded by a specific activity pattern in the chemosensory and the socio-sexual brain networks, that is particularly triggered in LP females since it may be adaptive in some circumstances (as discussed in section D.9).

However, pup-aggression score is also negatively correlated with the activity of the posterior part of the VTA (see Table 3.1), which happens to be one of the only motivational nuclei of our study that express high levels of beta-oestrogen receptors (Mitra et al., 2003). Maybe pregnancy hormones are directly influencing its activation to promote inhibition of the "attack" pathway (and/or activation of motivation for pup care). Furthermore, pup-odours may be also involved in this inhibition of attack, since the work of Hernandez-Gonzalez et al. (2005) showed that the response of VTA activity towards pup-derived chemical cues is increased in lactating rats. This inhibition might also involve the meso-limbic dopaminergic system which is triggered by VTA activation (Fields et al., 2007; Ikemoto, 2010). Hence, an elevated activity of the dopaminergic system would inhibit the pup-aggression behaviour and, as seen in previous studies with rats, promote pup care instead (see section I.6; Hansen, 1994; Hansen et al., 1991b, 1991a; Keer & Stern, 1999; Li & Fleming, 2003; Pereira & Morrell, 2011). Nonetheless, in our work we did not find correlations between the activity of nucleus accumbens with neither pup aggression score, nor with maternal score. Nevertheless, we did find that the activity of the AcbC is negatively correlated with the "approach and retreat" behaviour in LP females and positively correlated in virgin females (Table 3.1). This may suggest that, specifically in LP females, higher activity in the dopaminergic system diminishes non-caring interactions with pups, supporting a role in the pup-care pathway.

As a conclusion, there seems to be two different antagonistic brain systems promoting motivated pup care and pup attack respectively, both of which are activated during pregnancy as a consequence of the action of oestrogens and lactogens. A flip-flop balance between both systems would constitute the decision-making mechanism that would result in adaptive expression of one or the other behaviour, depending on the circumstances (Hrdy, 1979, 2000).

Further research is needed to explore the involvement of other nuclei in pup-aggression and pup care, the role of pup-derived chemical cues in these behaviours, and how pregnancy switches on and off the attack and care pathways.

D.10. Pregnancy entails changes in motivational but not social network

One of the major findings of our study is that, when females lack experience with pups, the differences between virgin and pregnant females are primarily in the way the motivational brain circuit process the different stimuli (except for the PAG, which also shows this trend). Consequently, results suggest that, although the expression of maternal care may be

dependent on the social brain network, pregnancy influences specifically the motivational brain circuit (Fig. 3.3A”-F”), although the influence might be indirect since its nuclei barely express prolactin and oestrogen receptors, except for the VTA (Mitra et al., 2003; Simerly et al., 1990). Brain nuclei activity results are consequent with the behavioural display of females, since virgin and late-pregnant females exposed to pups showed similar levels of pup care and also similar levels of social brain activation (see Fig. 2.2A-C, 3.1A”-D” and 3.2A”-C”). As we commented before, maybe with a more effort-demanding experimental design, we would have seen behavioural differences between groups that match the differences in motivational brain circuit.

As reflected in the section I.6 of the Introduction, the involvement of motivational brain network in maternal behaviour has been already analysed in rats (summarized by Numan & Young, 2016), and in this regard our results have been largely discussed in Chapter 3. But the particularity of our results reside in the fact that, although AcbC activation suggested an increase in the salience of pups in LP-females when compared to virgins (Fig. 3.3D”), the major pregnancy-related change we found was the decrease in the salience of non-social stimuli, an effect that occurred in almost the whole motivational brain network (Fig. 3.3A”-F”). To our knowledge, the particular pregnancy-related decrease of salience of non-social stimuli has not been described before, and opens new lines of research that may give new perspectives in the study of motivational changes occurring during pregnancy and motherhood.

In this regard, a supplemental experiment showed that LP-females interact less with buttons than virgin females do (Fig. 2.3), thus demonstrating a decrease in salience of non-social stimuli during pregnancy, which diminishes motivated interaction with buttons. Very likely, LP females exposed to buttons in the cFos experiment investigated less the buttons than virgins did, secondarily to a decreased motivation for non-social objects. This would have resulted in less exposure to button-derived odorants, thus explaining the reduced activity (as compared to virgins) of the nuclei of the main olfactory system observed in LP females exposed to buttons.

D.11. Assembling de puzzle: Network vs linear pathways

The complexity of interpreting all brain nuclei activity results from Chapter 3 lead us to perform a principal component analysis to evaluate globally the activity of the SBN and motivation brain circuit (Fig. 3.4). This, together with linear discriminant analysis (Fig. 3.5), resulted in the major take-home message of this thesis: the hormonal state of the female

(pregnant or not pregnant) and the stimuli they are exposed to (pups vs non-social) are encoded by the pattern of activity of the whole socio-sexual/motivational brain network.

Many current studies on the neural basis of maternal and other social behaviours are adopting a reductionist perspective, by focusing on specific cell populations of specific brain nuclei that are considered critical for triggering specific behaviours. For instance, the group of Catherine Dulac has identified galaninergic cells in the medial preoptic hypothalamus (MPO) of mice that activate, through different pathways, several components of maternal behaviours (Kohl et al., 2018). Similarly, Susana Lima and her colleagues have performed high-tech experiments showing that progesterone receptor-expressing cells in the ventrolateral portion of the ventromedial hypothalamus play a crucial role in the control of sexual behaviour in females, which allows adapting their response to males during different stages of the oestrous cycle (Dias et al., 2021). These approaches are indisputably essential for the understanding of the neurobiological basis of social behaviours and constitute the key pieces we need for assembling the puzzle. Nonetheless, these studies sometimes result in a simplified view by considering independent hierarchical linear pathways as responsible for each social behaviour, missing the role of other brain areas in the expression of those behaviours.

Our experiments allowed us to check this view by analysing whether the MPO cells alone are encoding the maternal status and/or the interaction with pups (Fig. 3.6). Based on our results, although MPO is an important node of the network, its activity alone cannot successfully predict the hormonal state of the female, and more importantly, the stimulus the female is facing. So our work backs up the Sarah Winans Newman idea of the whole socio-sexual network nuclei encoding information all at once, with different patterns of activation. These findings also extend to the motivational network, ultimately considering that the whole socio-sexual/motivational brain encodes environmental and self-state information by different patterns of activation of its different sub-systems (Fig. 3.7). Furthermore, the PCA analysis revealed that the activity of the nuclei within each system (socio-sexual or motivational) correlates (Fig. 3.4B). Hence, the study of social behaviours must consider the assessment of the activity of both systems at once, since the relationship between them gives more information than studying each system separately. For instance, in our work, the key for understanding the variance of our groups was the relation between both systems, rather than the activity of a particular system (or nucleus). This approach allowed us to observe that what made LP females different from virgins is that their balance between social and motivational activity was more equilibrated, and when they interact with pups, both systems seemed to

“light up” together. On the contrary, virgins balance always leaned on one side; towards social network when exposed to pups or towards motivation network when exposed to buttons.

In this regard, one of the limitations of this study has been the lack of time to test the involvement of chemosensory systems along with motivational and socio-sexual networks in the systematic approach of Chapter 3. Nevertheless, when we compare Chapter 2 and Chapter 3 results, some interesting hypothesis emerge. For instance, the effect of motherhood in the processing of stimuli by the motivational system (and the PAG), somehow mimics the effect found in the MOS system (compare Fig. 3.3A”-F” with Fig. 2.7A”-C”), since in both systems pregnant females show more activation when exposed to pups than to buttons, while virgins show similar activation between stimuli or even the opposite effect. This suggests an interesting functional relation between chemosensory cues and motivational aspects of maternal behaviour directed to pups. Since the main olfactory system responds similar to the motivational network, we may hypothesize that odorants are involved in motivational aspects of maternal behaviour. This fits the interpretation of the results of Chapter 1 exposed in D.3, suggesting that contact with pup-derived cues during preference tests may be masking the differences between dams and virgin females, which might be more dependent on air-borne molecules.

On the other side, except for the PAG, SBN activation pattern in the four groups of females is in its turn similar to the pattern found in the vomeronasal network (compare Fig. 3.1A”-D” and 3.2A”-C” with Fig. 2.4E and 2.6A”-D”), e.g. both kinds of females present more activation in those systems when they are presented with pups than with buttons.

Taken together, these data suggest a differential effect of the diverse compounds of pup-derived odours based on the chemosensory system involved in their detection. Broadly, we hypothesise that chemosignals processed by the AOS would elicit basic non-motivated maternal care in all females, while chemosignals processed by the MOS, likely airborne molecules, would be related with the motivational aspects of maternal behaviour. Further research is needed to unravel the actual pathways and implications of chemosensory cues in different aspects of maternal behaviour.

CONCLUSIONS

1. Female mice detect chemosensory cues in conspecific-soiled nest, but apparently they do not discriminate between prepartum and postpartum-derived odours (the latter including neonatal cues).
2. In the tested conditions, pregnancy does not seem to change attraction towards chemosensory pup-derived cues present in postpartum nest.
3. To collect and isolate chemosensory signals from neonatal pups (postpartum day 3-4) present several methodological difficulties mainly due to their young age and their dependence on their mother, but also from trying to collect samples with non-invasive and non-stressful methods.
4. The assessment of brain activity with the cFos immunostaining method requires the use of adequate control stimuli (non-social stimuli).
5. The use of virgins and late-pregnant females, both pup-naïve, allows studying hormones-induced changes in the processing of pup-derived cues, without the masking effect of previous experience with pups (which is present in experienced postpartum dams).
6. The vomeronasal organ is activated in the presence of pups, this effect being not significantly different between virgins and late-pregnant females.
7. The cFos correlational data suggest that the main and accessory olfactory systems couple their activity during interaction with pups, especially in late-pregnant females.
8. Pregnancy changes the response to pups in the vomeronasal and olfactory cortices, but not in the primary olfactory centres (olfactory bulbs) or in the extended medial amygdala.
9. Maternal behaviour is similar between virgin and late-pregnant females, both pup-naïve, except for the emergence of pup-directed aggression in late-pregnant females.
10. At the end of pregnancy, females present two drives towards pups: to care or to attack. The decision to display one instead of the other seems to be governed by specific circuits of the brain that generate an unstable equilibrium.
11. Pups activate the socio-sexual brain nuclei more than non-social stimuli in all females, thus reinforcing the concept and identity of the socio-sexual brain network.
12. Pregnancy entails significant changes in the response of the motivational brain nuclei, but not in the response of most of the socio-sexual brain regions.
13. These motivational changes not only increase the salience of pups (as literature describes), but also decreases the salience of non-social stimuli.
14. Information about the hormonal state of the female and the stimulus an animal is exposed to is encoded by the global pattern of both motivation and social brain

networks, rather than the activity of a specific nucleus or a hierarchical linear pathway. Hence, the study of socio-sexual behaviours must include a holistic view that integrates the results obtained from studies using a reductionist approach.

Resumen en castellano

Objeto y objetivos de la tesis

La maternidad conlleva cambios reversibles en la conducta que incluyen un aumento de la motivación por las crías y una gran agresividad contra congéneres que se aproximen al nido (agresión maternal). Estos cambios son debidos en gran medida a la acción de las hormonas del embarazo en el cerebro. En el ratón, nuestro modelo animal, la comunicación intraespecífica ocurre principalmente vía quimioseñales, de manera que hipotetizamos que las hormonas del embarazo podrían estar aumentando el carácter reforzante de las quimioseñales de cría y modificando la manera en que las hembras las perciben y responden a ellas.

Por lo tanto, el objeto principal de la presente tesis es evaluar los cambios que sufren las hembras durante la gestación en relación a la percepción de las crías, haciendo hincapié en el procesamiento de las señalas químicas de las mismas.

Los objetivos específicos de la tesis serían los siguientes:

Objetivo 1. Analizar la respuesta conductual de las hembras a olores derivados de crías y evaluar si esta respuesta cambia durante la maternidad.

Objetivo 2. Evaluar cómo la gestación cambia la detección, percepción y el procesamiento de las señales químicas de crías.

Objetivo 3. Estudiar las diferencias en la respuesta de los sistemas socio-sexual y motivacional del cerebro entre hembras vírgenes y gestantes durante la interacción con crías.

Planteamiento y metodología utilizados

Los objetivos propuestos se plantean en 3 capítulos a lo largo del manuscrito. El primer capítulo **"La exploración de señales químicas de crías en las hembras de ratón"** corresponde al objetivo 1. En él, se muestran los resultados de diversos experimentos en los que se exponen hembras lactantes y vírgenes (con y sin experiencia con crías) a diferentes sustratos que contienen señales químicas derivadas de crías. Usando estas fuentes de olor de cría, se llevan a cabo 3 tipos de pruebas conductuales: un test de preferencia, un conjunto de experimentos de habituación-deshabituación y, por último, un test de exposición simple.

El primer experimento es un test de preferencia entre material de nido recolectado en el día 3 postparto (P03, contiene señales de cría) frente a material de nido recolectado en los días

previos al parto (E17, sólo contiene señales de hembra adulta). Como sujetos experimentales se utilizan 3 grupos de hembras: hembras lactantes, hembras vírgenes sin contacto previo con crías, y "comadres", es decir, hembras vírgenes que han convivido con una madre durante su gestación y posterior lactancia, de manera que han adquirido amplia experiencia con crías. En este test se evalúa el tiempo que pasan explorando cada uno de los estímulos, si existe preferencia por alguno y si esta preferencia difiere entre grupos.

El segundo experimento consta de 4 pruebas de habituación-deshabituación en las que hembras vírgenes son expuestas a diferentes fuentes de señales químicas de conespecíficos. Las pruebas difieren tanto en la forma de recolección como en la forma de exposición del estímulo. Se utilizan estímulos de cría en día P03, estímulos control de conespecíficos tales como aquellos derivados de hembras pre-parto (E17, día post-concepción 17), o individuos juveniles en la edad de destete (día 21 postparto; P21), y adicionalmente estímulo limpio u olor de naranja como controles no-sociales. La recolección se lleva a cabo en diferentes sustratos; nido de P03 y E17, lecho "ensuciado" por crías de P03 o P21 durante 90 minutos, o bastoncillos de algodón restregados por la zona anogenital de los individuos donantes de P03 o P21. Los diferentes formatos de presentación incluyen un infusor de acero colgado en un lateral de la caja, los bastoncillos de algodón desnudos también colgados en un lateral, y un dispositivo rígido adaptado a la pared, fabricado *ex profeso* para presentar el estímulo sin posibilidad de movimiento. En estos test se evalúa si las hembras son capaces de detectar y distinguir un estímulo de otro mediante el procedimiento de la habituación seguida de deshabituación.

Por último, el capítulo 1 se cierra con un test de exposición simple a lecho de P03 y de P21. En él, hembras lactantes y vírgenes tienen acceso total a lecho de P03 o de P21, pero no de forma simultánea, sino en sesiones diferentes. En este test se evalúa el tiempo que pasan explorando cada uno de los estímulos, y si esta conducta difiere entre grupos.

En resumen, utilizando esta metodología se intenta comprobar si las hembras prefieren quimioseñales de crías sobre las de otros conespecíficos, y si hay cambios en esta preferencia durante la maternidad. Sin embargo, los resultados negativos de los experimentos de preferencia nos obligaron a abordar la cuestión de si las hembras de ratón eran capaces de detectar los estímulos de crías neonatales y de discriminarlos de crías juveniles y de hembras adultas. Los resultados de estos últimos experimentos sugieren que los métodos de extracción de olores de crías y de presentación de estos extractos a las hembras adultas, necesitan se refinados para obtener resultados concluyentes.

El segundo capítulo, **"La gestación cambia la respuesta de los sistemas vomeronasal y olfativo frente a crías en ratón"** explora el efecto de la gestación sobre la respuesta de los sistemas de detección y procesamiento de señales químicas de crías. Para ello se expone a hembras vírgenes y gestantes (en los días pre-parto) a crías de 4 días de edad o a botones, que constituyen un estímulo control no-social de tamaño similar. Por un lado se analiza la conducta maternal y quimio-exploratoria de las hembras expuestas a crías para evaluar si existen diferencias en la interacción con crías entre grupos. Por otro lado, se perfunden todas las hembras, se extrae el órgano vomeronasal (VNO) y el encéfalo, y se procesan los tejidos (fijación y corte) para realizar una inmunohistoquímica de marcadores de activación (Egr1 para VNO y cFos para los núcleos del cerebro), con la finalidad de analizar la respuesta de activación neuronal de las hembras gestantes y vírgenes frente a los dos estímulos (vírgenes expuestas a botones V-B, vírgenes expuestas a crías V-P, gestantes expuestas a botones LP-B y gestantes expuestas a crías LP-P).

Una vez realizada la tinción inmunohistoquímica de los marcadores de actividad, se capturan imágenes del VNO, de núcleos del sistema vomeronasal (bulbo olfativo accesorio, AOB; núcleo amigdalino posteromedial cortical, PMCo; amígdala medial posterodorsal, MePD; y parte medial dela división posteromedial del lecho de la estría terminal, BSTMPM) y núcleos del sistema olfativo principal (bulbo olfativo principal, MOB; y las divisiones anterior y posterior de la corteza piriforme, PirAnt y PirPost respectivamente). Las imágenes de todas las áreas cerebrales se capturan a niveles antero-posterior, medio-lateral y dorso-ventral específicos, lo que permite la comparación de la densidad de células marcadas (relacionada con la densidad de células activadas) entre los 4 grupos en cada uno de los centros estudiados. En el caso del VNO se lleva a cabo un registro manual (herramienta *Cell counter* de ImageJ) del número de células marcadas, por un observador ignorante del grupo al que pertenece cada preparación (impidiendo así sesgos). Para el resto de centros nerviosos, la imagen se procesa y analiza con el software ImageJ usando un umbral de segmentación predeterminado a partir de la moda del nivel de gris de la imagen que se aplica sistemáticamente a las muestras de todos los animales, para obtener la densidad de células en la región de interés (cels/mm^2), o en su defecto el porcentaje de área marcado (AOB y MOB), de forma libre de sesgo. Obtenidos estos datos se evalúan las diferencias en activación de cada una de las áreas medidas aplicando un ANOVA de 2 vías, definiendo como factores "TIPO DE HEMBRA" Y "ESTÍMULO". Posteriormente, en los grupos de hembras expuestas a crías (V-P y LP-P) se exploran las correlaciones entre conducta (maternal y quimioexploratoria) y activación neuronal, de

manera independiente en cada grupo. A su vez, en estos dos grupos se exploran las correlaciones entre la activación de todos los núcleos medidos.

Los resultados de estos experimentos indican que las crías activan de forma significativa el VNO de las hembras adultas, sin que se haya observado alteraciones significativas de la transducción sensorial durante la gestación. Esto no obstante, las hembras gestantes atacan a las crías en una alta proporción mientras las vírgenes no lo hacen, aunque el resto de conductas maternales y quimioexploratorias son similares en los dos grupos expuestos a crías. En cuanto al procesamiento central de los estímulos de crías, en los centros vomeronasales la respuesta es mayor frente a crías que frente a botones, tanto en hembras vírgenes como en gestantes. En el AOB y el BSTMPM, aunque no hay respuesta diferencial ente hembras, en gestantes (no así en vírgenes que no atacan a las crías) la actividad se correlaciona de forma positiva con la conducta infanticida. Por el contrario, en la amígdala cortical (PMCo) y medial (MePD) hay una respuesta diferencial ente hembras, con menor respuesta a botones en hembras gestantes, y mayor activación inducida por crías en PMCo. Los análisis de correlación indican además una relación directa en hembras gestantes (no tanto en vírgenes) entre la actividad en ambos núcleos y algunos aspectos de la conducta maternal como la recogida de crías (presente también en hembras vírgenes) y una variable derivada de todas las conductas pro-maternales (*maternal score*; PMCo), y una variable derivada del olfateo de las crías (*olfactory score*; MePD). Todo ello revela un cambio durante la gestación en el procesamiento de señales vomeronasales de crías relacionado con la expresión de conducta maternal/infanticida, y un papel relevante del PMCo en la conducta maternal, hasta ahora ignorado.

En cuanto al sistema olfativo también hay cambios importantes en el procesamiento de los estímulos de crías, así como de botones. Así, en los bulbos olfatorios principales, el córtex piriforme anterior y posterior, los botones activan la expresión de cFos en las vírgenes en mayor medida que en las hembras gestantes. Por el contrario, en la corteza piriforme anterior y especialmente posterior, las hembras preñadas muestran más activación inducida por crías que por botones. La actividad reducida en los centros olfativos en respuesta a botones parece tener relación con una menor interacción con estos objetos en hembras gestantes, relacionada probablemente con cambios en la motivación durante la gestación, que estudiamos también en el tercer capítulo de esta tesis.

Por último, los estudios de correlación indican un acoplamiento funcional (correlación positiva) entre los sistemas olfativo y vomeronasal en respuesta a la interacción con crías, que se acentúa durante la gestación.

El tercer capítulo, **"La gestación cambia la actividad de los sistemas social y motivacional del cerebro en ratón"**, evalúa las diferencias en ambos sistemas entre los grupos de hembras del experimento anterior (capítulo 2; V-B, V-P, LP-B y LP-P).

Esta vez se toman imágenes de niveles concretos bien caracterizados de varios núcleos del sistema social (porción ventral del septum lateral, LSV; amígdala medial posterodorsal, MePD; núcleo preóptico medial, MPO; núcleo hipotalámico ventromedial; VMHVL, parte anterior del núcleo hipotalámico paraventricular, PaA; área entre el núcleo de la comisura anterior y el área preóptica anterodorsal, AC/ADP; y columna ventrolateral del área gris periacueductal, PAG) y motivacional (área ventral tegmental anterior y posterior, VTAa y VTAp respectivamente; corteza y zona central del núcleo accumbens, AcbSh y AcbC respectivamente; amígdala basolateral, BLA; y corteza medial prefrontal incluyendo el área prelímbica y límbica, PreL-IL). Obtenidas estas imágenes, se evalúa la densidad de células inmuno-positivas para cFos en cada una de los núcleos escogidos de la misma forma que en capítulo anterior, usando un procedimiento automático no sesgado de análisis de imágenes con ImageJ. Con estos datos analizamos las diferencias en activación en cada núcleo en las hembras mediante un ANOVA de 2 vías, definiendo como factores "TIPO DE HEMBRA" Y "ESTÍMULO". También en este caso se exploran las correlaciones entre conducta maternal y activación neuronal en cada uno de los grupos de hembras expuestas a crías (V-P y LP-P). Los resultados indican que los estímulos sociales (crías) activan los centros pertenecientes al cerebro socio-sexual significativamente más que objetos no sociales (botones) en hembras vírgenes y gestantes, confirmando así la naturaleza e identidad del cerebro socio-sexual. El análisis de correlación entre conducta y actividad del cerebro socio-sexual en hembras expuestas a crías nos permite completar la identidad del sistema de toma de decisiones cuidar/atacar que actúa en hembras gestantes cuando se enfrentan a crías ajenas. Así, la actividad del polo posterior de la VTA (VTAp) correlaciona significativa y negativamente con el infanticidio, mientras que la actividad del PAG correlaciona de forma muy significativa y positivamente con el ataque a crías. Esto sugiriere un equilibrio inestable entre dos estados en el que los estímulos vomeronasales que actúan sobre el AOB y BSTMPM facilitarían el infanticidio a través de la actividad del PAG, mientras que la activación del VTAp frenaría el ataque incrementando probablemente la actividad del sistema motivacional hacia el cuidado de las crías. En hembras vírgenes, por el contrario, la actividad del PAG y del sistema MePD-

AcbC se correlaciona con una respuesta de evitación de las crías, una conducta que hemos denominado *sniff and retreat* (olisquear y retirarse), similar a la evaluación de riesgos frente a estímulos nuevos (*risk-assessment behaviour*).

Por otra parte, los centros relacionados con la motivación muestran un perfil de actividad que revela mayor motivación por botones en hembras vírgenes que en gestantes. Contrariamente, en las hembras gestantes las crías activan los centros cerebrales de la motivación más que los botones. Estos resultados sugieren que durante la gestación se produce un aumento en la motivación frente a estímulos sociales (crías) y un decremento de la motivación por objetos salientes no sociales (botones), como consecuencia de la acción de hormonas (esteroides y lactógenos placentarios) sobre el cerebro.

En este capítulo se aborda además un estudio global de las relaciones entre los núcleos de ambos sistemas y su aportación a la varianza muestral mediante un análisis de componentes principales (PCA) con los datos de densidad de células activadas (cFos) en todos los núcleos analizados de todas las hembras experimentales. Finalmente, se evalúa si el patrón de activación de ambos sistemas es capaz de predecir la condición de un animal (tipo de hembra y estímulo al que se le expone) mediante un análisis discriminante lineal (LDA) con la información de todos los núcleos estudiados. Los resultados de este análisis se comparan con los de otro análisis discriminante realizado únicamente con los resultados del MPO, tradicionalmente considerado el núcleo responsable de la conducta maternal. Los resultados apoyan los conceptos e identidad de los sistemas cerebrales socio-sexual y motivacional, e indican que los datos del MPO por sí mismos son insuficientes para predecir el tipo de hembra y estímulo, mientras que el patrón global del sistema socio-sexual/motivacional es un buen predictor para ello. Esto sugiere que, tal como propuso Sarah Winnans Newman, la conducta social no depende de vías neurales lineales jerarquizadas, sino de circuitos en red, en los que el patrón global (y no la actividad de núcleos concretos) codifica la conducta resultante.

Aportaciones originales

Las principales aportaciones del presente manuscrito pueden resumirse en dos: la importancia del uso de grupos y controles adecuados cuando se trata de evaluar conductas sociales, y la necesidad de completar los estudios de neurobiología de la conducta con aproximaciones más globales.

Respecto al primer punto, parte de la literatura sobre preferencia por estímulos olfativos de cría descrita hasta la fecha utilizaba estímulo limpio como control, o incluso cámaras vacías sin

estímulo. Estos estudios concluían que existe una preferencia en las hembras lactantes por los estímulos de cría. Sin embargo, a la luz de nuestros resultados, es posible que esa preferencia documentada no responda específicamente a estímulos de cría, sino a que las hembras muestran preferencia por cualquier estímulo de conespecífico si se enfrenta a un estímulo socialmente neutro o no saliente. También en esta línea cabe destacar que la mayoría de estudios se han realizado en hembras lactantes frente a hembras vírgenes, sin tener en cuenta que las primeras tienen experiencia con crías mientras que las segundas no. De nuevo, nuestro diseño experimental consigue salvar esta limitación mediante el uso de madres gestantes y vírgenes, ambas sin ninguna experiencia previa con crías. De hecho, nuestros resultados ponen de manifiesto que cuando no existe experiencia con crías, gestantes y vírgenes muestran una conducta maternal similar (con la excepción del infanticidio presente en gestantes), al menos en nuestra cepa de ratón. Esta novedosa aproximación del uso de gestantes en vez de madres lactantes permite atribuir las diferencias encontradas exclusivamente al factor hormonal y los consecuentes efectos alostáticos de la gestación.

Por último, el uso de los métodos estadísticos dirigidos a evaluar globalmente la respuesta de activación cerebral en los sistemas motivacional y socio-sexual es pionero en nuestro campo. Si bien el análisis de cada núcleo por separado ya sugiere que la percepción de las crías y de los estímulos control es diferente entre gestantes y vírgenes, el abordaje global nos ha permitido comprender mejor cómo se codifica esta información en el cerebro. Mediante el análisis de componentes principales y el análisis discriminante hemos podido comprobar que las diferencias no residen en núcleos concretos y aislados, sino que los estímulos sociales (crías) y no sociales (botones) generan patrones de activación diferenciales en hembras vírgenes y gestantes en ambos sistemas (socio-sexual y motivacional), que determinarían la conducta resultante en cada caso. Estos resultados sitúan esta nueva aproximación holística como el complemento perfecto de los estudios centrados en núcleos o áreas concretas, ofreciendo un enfoque global en el que ir encajando todas las piezas que conforman la base neurobiológica de la conducta.

Conclusiones obtenidas y futuras líneas de investigación

Las principales conclusiones obtenidas son las siguientes:

1. Las hembras de ratón detectan las señales de conespecíficos en el material de nido, pero aparentemente no discriminan entre señales pre-parto (de hembra gestante) y post-parto (que incluye señales de cría).

2. En las condiciones testadas, la gestación no parece alterar la atracción hacia señales químicas de crías presentes en el nido post-parto.
3. Recoger y aislar las señales de crías neonatales (día post-parto 3 y 4) entraña muchas dificultades metodológicas, principalmente debido a la juventud de las crías y su dependencia materna, pero también de la necesidad de emplear métodos no invasivos y no estresantes.
4. La evaluación de la actividad cerebral mediante inmunohistoquímica de cFos requiere del uso de estímulos control adecuados (en nuestro caso estímulos no sociales, botones).
5. El uso de hembras vírgenes y gestantes, ambos grupos sin ninguna experiencia previa con crías, permite estudiar los cambios que se deben específicamente al efecto de las hormonas de la gestación, sin interferencias debidas a la experiencia previa.
6. El órgano vomeronasal responde a la presencia de crías, aunque este efecto no parece diferir entre hembras gestantes y vírgenes.
7. Los datos de correlación entre núcleos del sistema olfativo principal y vomeronasal sugieren que existe un acoplamiento entre ambos sistemas, especialmente facilitado en hembras gestantes.
8. La gestación cambia la respuesta frente a crías en las cortezas olfativa y vomeronasal, pero no en los centros olfativos primarios (bulbos olfativos) o en la amígdala medial (MePD).
9. La conducta maternal es similar entre vírgenes y gestantes en sus últimos días preparto, ambas sin experiencia previa, salvo por la aparición de la agresión dirigida a crías en las hembras gestantes.
10. Al final de la gestación, las hembras presentan dos tendencias: cuidar o atacar. La decisión de mostrar una conducta u otra parece estar regida por circuitos específicos del cerebro que generan una especie de equilibrio inestable.
11. Las crías activan el sistema socio-sexual más que los estímulos no-sociales en ambos grupos de hembras, lo que refuerza el concepto y la identidad del sistema.
12. La gestación conlleva cambios significativos en la respuesta de los núcleos del sistema motivacional, pero no ocurre así en la mayoría de núcleos del sistema socio-sexual.
13. Los cambios en el sistema motivacional no sólo aumentan el carácter reforzador de las crías (tal y como describe la literatura), sino que además disminuyen el interés por los estímulos no sociales.
14. La información sobre el estado hormonal y el estímulo al que se encuentra expuesta una hembra se codifica mejor mediante el patrón global de activación de ambos

sistemas, motivacional y socio-sexual, que mediante la activación de un solo núcleo o una vía jerárquica lineal. Por lo tanto, el estudio de las conductas socio-sexuales debería incluir una visión holística que integrase los resultados obtenidos en los trabajos con una aproximación más reduccionista.

Las futuras líneas de investigación abarcan desde el estudio en profundidad de las señales químicas de crías hasta la aplicación de esta nueva visión holística a otras relaciones intraespecíficas que ahonden en la base neurobiológica de las conductas sociales.

Respecto a la búsqueda de una posible feromona de cría, el equipo ya ha hecho avances mediante el análisis cromatográfico de las emisiones de cría (volatoloma), cuyos primeros resultados han sido publicados y forman parte de otra tesis del grupo. También se explorará la respuesta de las hembras frente a crías anestesiadas, que permiten un mayor abanico de diseños experimentales en los que se facilita la exposición única y específica a estímulos olfativos (volátiles y/o no volátiles).

Por otro lado, la novedosa aproximación en el diseño experimental y el tratamiento de los datos de esta tesis se podrá aplicar fácilmente a los estudios de otro tipo de relaciones intraespecíficas. Por ejemplo, ya hemos comenzado el análisis de la respuesta de hembras en diferentes estadios vitales (prepúberes, vírgenes postpúberes y lactantes) a lecho ensuciado por machos adultos, en relación con los cambios en la respuesta de las hembras a machos durante la infancia (aversión), la edad adulta (atracción y respuesta sexual) y la maternidad (agresión maternal).

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Cartoons

CHAPTER 1: Pyle, N. W. (2019). *Strange planet*. Harper Collins USA.

CHAPTER 2: Watterson, B. (2001). *Calvin and Hobbes: Sunday pages 1985-1995.* Andrews McMeel Publishing

CHAPTER 3: Gauld, T. (2021). *Tom Gauld's cultural cartoons*. The Guardian.

APPENDIX

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
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Pregnancy Changes the Response of the Vomeronasal and Olfactory Systems to Pups in Mice

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Motherhood entails changes in behavior with increased motivation for pups, induced in part by pregnancy hormones acting upon the brain. This work explores whether this alters sensory processing of pup-derived chemosignals. To do so, we analyse the expression of immediate early genes (IEGs) in the vomeronasal organ (VNO; Egr1) and centers of the olfactory and vomeronasal brain pathways (cFos) in virgin and late-pregnant females exposed to pups, as compared to buttons (socially neutral control). In pup-exposed females, we quantified diverse behaviors including pup retrieval, sniffing, pup-directed attack, nest building and time in nest or on nest, as well as time off nest. Pups induce Egr1 expression in the VNO of females, irrespective of their physiological condition, thus suggesting the existence of VNO-detected pup chemosignals. A similar situation is found in the accessory olfactory bulb (AOB) and posteromedial part of the medial bed nucleus of the stria terminalis (BSTMPM). By contrast, in the medial amygdala and posteromedial cortical amygdala (PMCo), responses to pups-vs-buttons are different in virgin and late-pregnant females, thus suggesting altered sensory processing during late pregnancy. The olfactory system also shows changes in sensory processing with pregnancy. In the main olfactory bulbs, as well as the anterior and posterior piriform cortex, buttons activate cFos expression in virgins more than in pregnant females. By contrast, in the anterior and especially posterior piriform cortex, pregnant females show more activation by pups than buttons. Correlation between IEGs expression and behavior suggests the existence of two vomeronasal subsystems: one associated to pup care (with PMCo as its main center) and another related to pup-directed aggression observed in some pregnant females (with the BSTMPM as the main nucleus). Our data also suggest a coactivation of the olfactory and vomeronasal systems during interaction with pups in pregnant females.

Keywords: pregnancy, pup chemosignals, vomeronasal system, olfactory system, mice, IEGs

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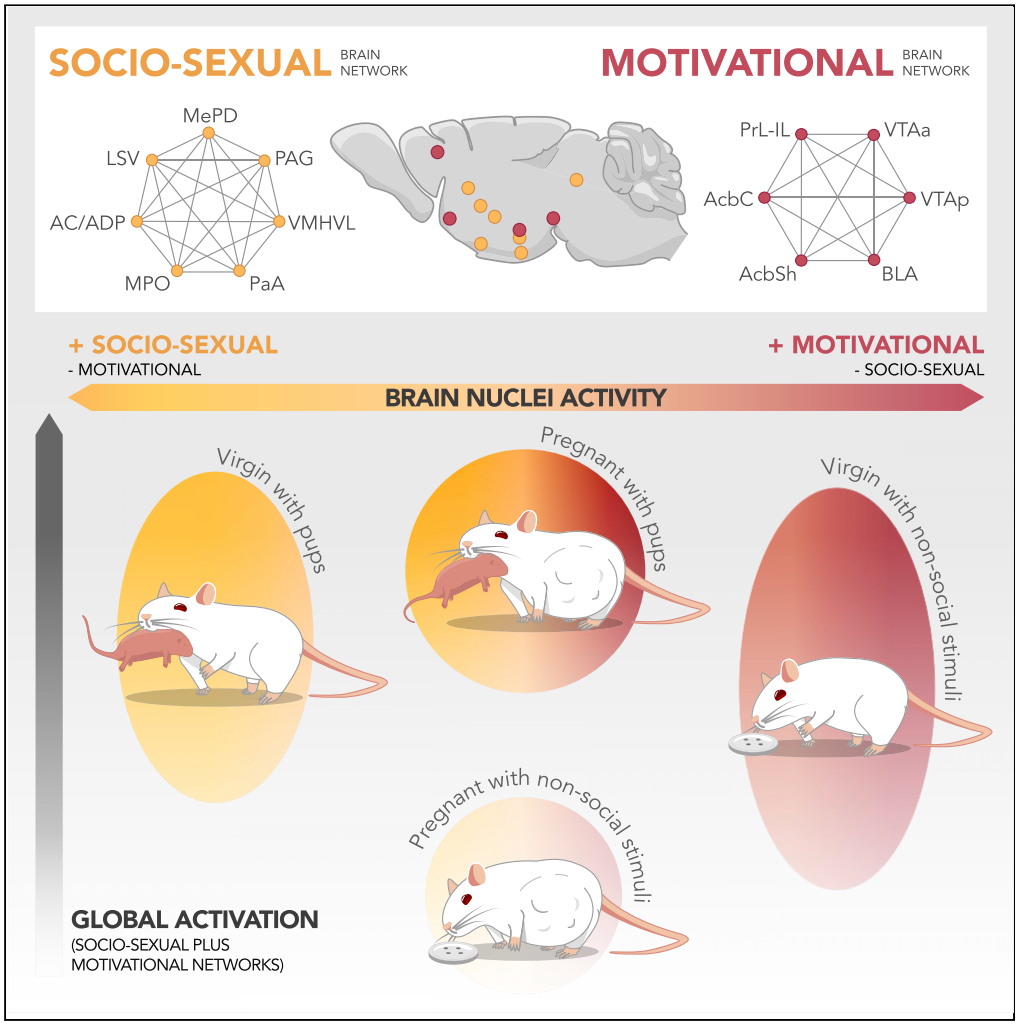
Becoming a mother shifts the activity of the social and motivation brain networks in mice



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Highlights

Pups activate the sociosexual brain network of females more than nonsocial objects

Pregnancy boosts motivation for pups and reduces incentive salience of buttons

During pregnancy, specific circuits govern decision of caring or attacking pups

The socio-motivational brain works as a network rather than a labelled-line circuit

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