The role of hormone-mediated neural remodelling in parental behaviour

Francesco Monaca

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Declaration

I, **Francesco Monaca**, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. Importantly, findings reported in this thesis have been published in the following article where I hold co-first authorship*:

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Francesco Monaca

London, United Kingdom

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Abstract

Understanding how physiological states shape information processing in neural circuits is a fundamental question in neuroscience. Parenting is an instinctive behaviour the onset of which is linked to pregnancy-related hormonal fluctuations. While dramatic progress has recently been made in dissecting the neural circuitry underlying parental behaviour, very little is known about the modulation of these circuits by the females reproductive state. This thesis proposes that pregnancy hormones remodel specific neurons in the hypothalamus to trigger changes in parental behaviour in mice. To examine how pregnancy-associated hormonal signals alter circuit function to promote parenting, parental behaviour was characterised during pregnancy. It was found that aspects of parental behaviour are changed during pregnancy and that these changes are most pronounced in late pregnancy. Most behavioural adaptations persisted until at least a month after pregnancy, indicating that they might result from long-lasting remodelling of the brain by pregnancy hormones. Ablation of receptors for ovarian hormones estradiol and progesterone from galanin-expressing MPOA (MPOA^{Gal}) neurons, a population critical for parenting, impaired parenting, showing that hormone action is required for pregnancy-induced behavioural changes. Electrophysiological recordings from these neurons in brain slices from virgin and pregnant females showed that, while estradiol silences MPOA^{Gal} neurons and increases their excitability, progesterone permanently rewires this circuit node by promoting dendritic spine formation and recruitment of excitatory synaptic inputs. Finally, *in vivo* endoscopic calcium imaging of MPOA^{Gal} population revealed that MPOA^{Gal}-

specific neural remodelling sparsens population activity, resulting in stronger and more selective responses to pup stimuli. This work therefore demonstrates that the ovarian hormones estradiol and progesterone therefore act on MPOA^{Gal} neurons during pregnancy, thereby remodeling parenting circuits in preparation for motherhood.

Impact Statement

Our knowledge of the neural underpinnings of instinctive behaviours, such as aggression, parenting, feeding and mating, has been progressing in discovery stages. In the 1980s and 1990s, classical anatomical methods guided a first discovery stage into the basic structures underlying instincts in rodents — *i.e.* brain regions and gross connections amongst them. Recently, a second discovery stage has harnessed modern techniques — chemogenetics, optogenetics, circuit tracing and neural activity imaging — permitting a more granular understanding of the different neuronal populations constellating neural circuits responsible for instinctive behaviours. We currently sit at the transition between this discovery stage and the next one, where new discoveries are bringing contextual information to the anatomical and functional descriptions of their neural substrate. This work brings an important contribution to neuroscience and its next discovery stage by revealing how physiological states affect neural circuits underlying instinctive behaviours.

Instinctive behaviours are indeed not merely implemented by hardwired neural circuits, but they are flexibly shaped by the animal's internal state and environment. These factors modulate behaviours by remodelling neural circuits at several levels, from neuronal gene expression to neural ensemble activity. Understanding the mechanisms governing such plasticity from genes to behaviour is particularly challenging. Parenting, a key instinctive behaviour ensuring offspring's survival in many species, provides an ideal framework to study the neural and molecular bases of state-dependent behavioural flexibility. Parental behaviour is indeed

highly sex- and reproductive state-dependent. While sexually-naive female rodents typically ignore infants, lactating females engage in caregiving behaviours. It has been proposed that the hormonal milieu of pregnancy is responsible for these changes in parental behaviour, but its mechanisms of action have not been described yet. In this work, we discover that estradiol and progesterone, two important ovarian hormones whose levels rise during gestation, remodel a distinct population of hypothalamic neurons, thus mediating the onset of parental behaviour in female mice prior to parturition. We characterise hormone-mediated behavioural, biophysical and functional changes underpinning the onset of parenting in female mice, thereby showing that pregnancy opens a window of plasticity in the adult brain during which neural remodelling orchestrates behavioural adaptations for the future challenges of parenthood.

Findings of this work constitute the first demonstration of hormones reshaping a neural circuit to anticipate a future behavioural need. Connecting steroid signalling to neuronal activity and behavioural flexibility provides conceptual advances for other neural circuits and instinctive behaviours, as well as for species in which pregnancy hormones have documented effects in the brain, including humans.

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This work would have not been possible without the unwavering support and supervision of Jonny Kohl. From guiding me through my first ever surgery, to the countless *What's next?*, I thank you for always being present over the past 4 and half years. As you would keep reminding me, most things are challenging, but doable.

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Mamma e papà, questi ultimi 5 anni, così come i precedenti 3, non sono stati tra i più facili, soprattutto per voi. Non ho idea di cosa si possa provare ad essere lontani 2775 km dal vostro unico figlio. Sebbene non ve lo dica spesso, siete voi la ragione di tutti i miei successi, e spesso la motivazione per risollevarmi dai miei fallimenti. Dovete essere fieri di quello che avete fatto fin'ora insieme, dell'esempio di amore ma soprattutto di forza e testardaggine che siete sempre stati, perché nonostante i chilometri di distanza e le diverse realtà in cui oggi viviamo, ho sempre cercato di rendervi fieri, di onorare tutti i sacrifici che avete fatto affinché io possa essere qui. In questa famiglia, non posso che non includere Asia, la mia sorella a quattro zampe. Asiuccia, meriti un paragrafo a parte, così come meriti i miei stessi riconoscimenti accademici dopo essere stata promossa ogni anno di scuola media e liceo scientifico, ascoltando ore e ore di lezioni di biologia, filosofia, letteratura, matematica. Te ne sei andata elegante e composta (e acculturata) così come hai vissuto la tua lunga e, spero, piacevole vita, addolcendo anche i più amari dei giorni con la tua silenziosa e fedele presenza.

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Ad meliora et maiora semper.

Contents

Table of Acronyms

Notation	Description
AAV	Adeno-associated virus.
ADP	Anterodorsal preoptic nucleus.
AHN	Anterior hypothalamic nucleus.
AHI	Amygdalohippocampal transition area.
AOB	Accessory olfactory bulb.
ARH	Arcuate hypothalamic nucleus.
AVP	Anteroventral preoptic nucleus.
AVPV	Anteroventral periventricular nucleus.
BNST	Bed nucleus of the stria terminalis.
BNSTp	Posterior bed nucleus of the stria terminalis.
BNSTpr	Principal nucleus of the bed nucleus of the stria terminalis.
BNSTrh	Rhomboid bed nucleus of the stria terminalis.
BNSTv	Ventral bed nucleus of the stria terminalis.
Calcr	Calcitonin receptor.
CoApm	Posteromedial cortical amygdala.
DA	Dopaminergic.
DMH	Dorsomedial nucleus of the hypothalamus.
Esr1	Estrogen receptor 1.
E2	Estradiol.

Notation	Description
FOV	Field of view.
GABA	Gamma-aminobutyric acid.
Gal	Galanin.
GRIN	Gradient refractive index.
HY	Hypothalamus.
KO	Knock-out.
LPO	Lateral preoptic area.
LS	Lateral septum.
MeA	Medial amygdala.
MeApd	Posterodorsal medial amygdala.
MEPO	Median preoptic nucleus.
MMme	Medial mammillary nucleus.
MOB	Main olfactory bulb.
MOE	Main olfactory epithelium.
MPN	Medial preoptic nucleus.
MPOA	Medial preoptic area.
NAc	Nucleus accumbens.
OSN	Olfactory sensory neurons.
OVX	Ovariectomised.
Oxt	Oxytocin.
Oxtr	Oxytocin receptor.
PAG	Periaqueductal grey.
PD	Posterodorsal preoptic nucleus.
PeF	Perifornical nucleus.
PeFA	Perifornical area.
PH	Posterior hypothalamic nucleus.
PMd	Dorsal premammillary nucleus.

Notation	Description
PMv	Ventral premammillary nucleus.
PR	Progesterone receptor.
Prl	Prolactin.
Prlr	Prolactin receptor.
PS	Parastrial nucleus.
PVa	Periventricular hypothalamic nucleus - anterior part.
PVi	Periventricular hypothalamic nucleus - intermediate part.
PVp	Periventricular hypothalamic nucleus - posterior part.
PVpo	Periventricular hypothalamic nucleus - preoptic part.
PVH	Paraventricular hypothalamic nucleus.
P4	Progesterone.
RCH	Retrochiasmatic area.
SBPV	Subparaventricular zone.
SO	Supraoptic nucleus.
SUM	Supramammillary nucleus.
TH	Tyrosine hydroxylase.
TMd	Tuberomammillary nucleus - dorsal part.
TMv	Tuberomammillary nucleus - ventral part.
TU	Tuberal nucleus.
Ucn3	Urocortin 3.
VLPO	Ventrolateral preoptic nucleus.
VMH	Ventromedial hypothalamic nucleus.
VNO	Vomeronasal organ.

Notation	Description
VSN	Vomeronasal sensory neuron.
VTA	Ventral tegmental area.

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Chapter 1

General Introduction

1.1 Introduction

Instinctive behaviours rely on neural circuits that are assumed to be largely genetically hard-wired. However, even though such behaviours require little or no prior learning, they can be modified by experience and physiological state. Hormones and neuromodulators can reconfigure network output by altering the activity of individual neurons within circuit nodes. Parenting provides a highly relevant paradigm for state-dependent circuit function: virgin female mice generally ignore or avoid their infants (pups), whereas mothers display high parental care. Because the neural circuits controlling parenting are anatomically largely indistinguishable between virgins and mothers, it can be hypothesised that pregnancy functionally alters neural circuits to instruct the switch to parental behaviour. The mechanisms and dynamics underlying such modulation are yet to be uncovered. While considerable progress has been made in dissecting the neural circuit underpinning instinctive behaviours, such mating, parenting and aggression (Falkner et al., 2014; Kohl, 2020; Lenschow and Lima, 2020), how internal states shape information processing within these circuits is still poorly understood. This work aims to elucidate the neural mechanisms by which a profound physiological state change — pregnancy — affects the brain to instruct parental responsiveness.

1.2 Instinctive social behaviours

Across species, animals can execute sets of behaviours without prior learning or experience. These behaviours, often referred to as "innate" or "instinctive", have developed through countless iterations of natural selection, and ensure the survival of the species by standardising the spectrum of behavioural responses to a situation or stimulus within a given environmental context. From escaping a rapidly approaching predator to mating with a conspecific and foraging for food, innate behaviours are thought to be stereotyped — i.e. consisting of structured

and robust action sequences (Tierney, 1986).

Since Konrad Lorenz and Nikolaas Tinbergen's foundational work (Lorenz, 1950; Tinbergen, 1951), instinctive social behaviours have been extensively described and traditionally divided into appetitive and consummatory components. In rodents, during the initial appetitive phase, the animal detects the social stimulus through species-specific sensory cues. Based on its internal readiness to engage with the conspecific, the animal subsequently approaches the social stimulus or, alternatively, avoids or simply ignores it. Approach towards a social target normally leads to further investigation. Exploration of the social stimulus permits to gather sensory and social information which, together with its internal readiness, determines whether the animal engages with the target by releasing goal-directed, and species-specific, consummatory sequences endemic to innate behaviours (Fig. 1.1).

Importantly, at the detection-to-investigation and investigation-to-consummation transition stages, the animals motivation — or social interest — dictates the likelihood of its behavioural commitment. Motivation is modulated by a variety of factors:

- Internal factors, such as sex, reproductive and circadian states, energy levels, age.
- Environmental factors, such as nutrition, space availability, and population density.
- Experiential factors.

By modulating the animal's internal readiness to approach and engage with a social target at those two critical transition points, experience, environment and internal state make stereotyped and robust instinctive behaviours highly malleable. The flexibility of these behaviours is supported by plasticity changes in the underlying neural circuits which, despite being developmentally-shaped and hard-wired, are amenable to modulation (reviewed in D. Wei et al., 2021).

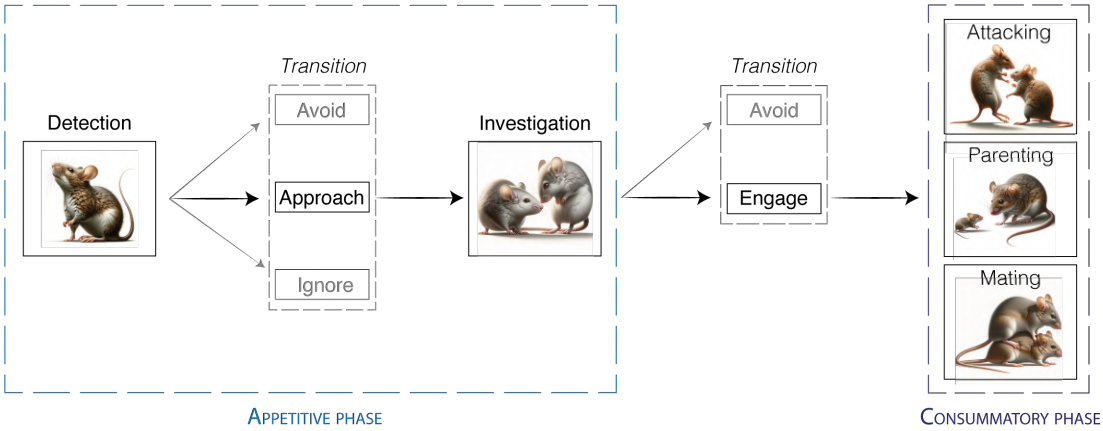

Figure 1.1: **Phases of instinctive social behaviours**. The four main stages — *detection*, *approach*, *investigation*, *consummation* — constitute the appetitve and consummatory phases of social behaviours. The transitions from detection to approach, and from investigation to consummation, are subject to the animal's internal, environmental and experiential factors. Adapted from D. Wei et al., 2021

Amongst social behaviours, parental behaviour is an ideal paradigm to explore the relationship between neural plasticity and behavioural flexibility (Mei, Osakada, and Lin, 2023).

1.3 Parental behaviour

One of the most fascinating forms of instinctive social behaviour is parenting. Parenting involves intricate social interactions which are evolutionary tuned to maximise the survival and well-being of the caregiver's offspring, without any immediate benefit to the caregiver itself (Numan, 2020). As previously discussed, similar to other social innate behaviours, parenting is a multifaceted naturalistic behaviour involving the detection of a social target (infant cues), the integration of these cues with the adults physiological state, and execution of stereotypical behavioural programmes (Kohl, 2020; Kohl et al., 2017; Mei, Osakada, and Lin, 2023). In rodents, parental care involves retrieval of pups to the nest, grooming, nest building, and crouching over them for protection and nurturing (Numan, 2003, 2015). With some quantitative differences — and with the exception of nursing — parental care can be performed by both male and female rodents

(Lonstein and De Vries, 2000), although the occurrence and the quality of the behaviour are reproductive state-dependent in both sexes (Gandelman et al., 1970; Lonstein et al., 1999). Pregnancy is indeed a period in which drastic hormonal fluctuations are accompanied by changes in parental behaviour (Numan and Woodside, 2010; Numan and Young, 2016). As I will review in the sections below, the neural circuitry underlying parental behaviour is affected by hormonal changes associated with pregnancy. How this happens had been unclear at the time of starting this research project. To explore the neural mechanisms underpinning the hormonal modulation of parental behaviour, I will first describe its neural circuitry.

1.3.1 Circuit basis of parental behaviour

In the following section, I will briefly review current knowledge on the neural circuitry underlying parental behaviour in rodents, from brain structures supporting the detection of pup sensory cues to those modulating behavioural execution (Fig. 1.2).

Sensory detection. As macrosmatic animals, rodents use their sense of smell as a prominent sensory modality to investigate their environment. Detection of conspecific cues relies on two main chemosensory pathways underpinned by structures in the nasal cavity: the main olfactory epithelium (MOE) composed of primary olfactory sensory neurons (OSNs) that project to the main olfactory bulb (MOB) and the vomeronasal epithelium (VNE) of the vomeronasal organ (VNO) made up of vomeronasal sensory neurons (VSNs) projecting to the accessory olfactory bulb (AOB) (Spehr et al., 2006). The MOE detects volatile odours, while the VNO detects soluble compounds with low volatility (Spehr et al., 2006). In mice, initiation and coordination of parental behaviour relies on both structures. Following olfactory bulbectomy, anosmic mothers and virgin females display severely disrupted nest building and maternal care, with the majority of females eating their pups (Gandelman et al., 1971; Vandenbergh, 1973). While MOB ablation

by nasal irrigation of zinc sulphate induces pup cannibalism (Vandenbergh, 1973), removal of the VNO does not interfere with the expression of maternal behaviour (Lepri et al., 1985), as females show intact pup retrieval. Similarly, females carrying a mutant *Trpc2* gene, which encodes an ion channel of the transient receptor potential family that is expressed almost exclusively in VNO neurons, display intact pup retrieval behaviour (Fraser and Shah, 2014; Liman et al., 1999; Stowers et al., 2002). Additionally, mutation of the G proteins Gαi2 and Gαo, which are expressed in the apical and basal layers of the vomeronasal sensory epithelium respectively, does not impair pup retrieval (Chamero et al., 2011; Fraser and Shah, 2014; Norlin et al., 2003; Trouillet et al., 2019). Conversely, targeted mutation of elements essential for MOE signal transduction disrupts maternal behaviour. Specifically, genetic deletion of *Scn9a*, which encodes the voltage-gated sodium channel Nav1.7, as well as deletion of genes *Adcy3* and *Cnga2*, encoding adenylyl cyclase-3 and cyclic nucleotide-gated channel sub-unit alpha 2 respectively, result in deficient pup retrieval and nest building, and reduced maternal aggression (Fraser and Shah, 2014; Z. Wang et al., 2011; Weiss et al., 2011). The VNO seems to have a more prominent role in mediating maternal aggression. Mice mutant for *V1r* and *V2r*, which are selectively expressed in VNO sensory neurons, display substantially altered maternal aggression (Del Punta et al., 2002). *Trpc2* mutant females also show impaired maternal aggression (Fraser and Shah, 2014; Kimchi et al., 2007; Leypold et al., 2002). However, the VNO is not entirely futile to pup retrieval. Mothers with both MOE-specific $Cnga2^{-/-}$ and VNO-specific $Trpc2^{-/-}$ mutations showed significantly diminished pup retrieval compared to $Cnga2^{-/-}$ females even though $Trpc2^{-/-}$ females were comparable to wild-type (WT) mothers (Fraser and Shah, 2014). While in females lesioned VNO results in intact parenting and reduced maternal aggression, in males surgical or genetic ablation of the VNO abolishes pup-directed aggression and evokes parental behaviour (Mennella and Moltz, 1988; Tachikawa et al., 2013; Trouillet et al., 2019; Wu et al., 2014). Despite these sex differences, the vomeronasal pathway appears

to regulate maternal aggressive behaviours.

Overall, these lesion and genetic ablation studies highlight how maternal care is differentially regulated by MOE and VNO pathways: pup retrieval requires the MOE, whereas maternal aggression requires both sensory structures to be functional, with the VNO necessary for pup-directed aggressive behaviours. These findings support the view of an approach-avoidance model for the onset of maternal behaviour, first proposed by Rosenblatt and Mayer (Rosenblatt and Mayer, 1995). According to this model, the onset of maternal behaviour relies on the combination of increased attraction to, and reduced aversion of, novel infant stimuli (Numan, 2006, 2007; Numan and Sheehan, 1997; Rosenblatt and Mayer, 1995). In this framework, infanticidal and pro-parental circuit patterns might be loosely juxtaposed with the vomeronasal and main olfactory pathways, respectively. Neural substrates enabling a bidirectional control of parenting extend beyond the sensory periphery into downstream areas (Mei, Yan, et al., 2023), which I will further explore in the next section.

Amygdalar integration. Deeper in the brain, the medial nucleus of the amygdala (MeA) is a key area relaying olfactory information to downstream hypothalamic nuclei (Simerly and Swanson, 1986). The posterodorsal medial amygdaloid nucleus (MeApd) is the main recipient of direct projections from mitral and tufted (M/T) cells of the AOB, which convey vomeronasal information in the form of pheromones and kairomones (Ben-Shaul et al., 2010; Dulac and Wagner, 2006; MohedanoMoriano et al., 2007; Papes et al., 2010; ProSistiaga et al., 2007; Scalia and Winans, 1975; Von Campenhausen and Mori, 2000). Volatile odour information from the MOB also reaches the MeApd via higher-order projections from the piriform cortex (PirCtx) as well as from the anterior and posterolateral cortical amygdaloid nuclei (CoAa and CoApl); however, minor direct connections from the MOB to the rostral parts of the MeA have also been documented (Bergan et al., 2014; Cádiz-Moretti et al., 2016; Kang et al., 2009; ProSistiaga et al., 2007; Sosulski et al., 2011). Seminal studies have implicated the MeA in the regulation

of parental behaviour. Large lesions of the MeA result in increased maternal behaviour (Fleming et al., 1980; Numan et al., 1993; Sheehan et al., 2001), and increased immediate early gene expression — a proxy for neuronal activity — has been observed in the MeA of non-maternal rodents during pup exposure (Sheehan et al., 2000). Together with the VNO and downstream hypothalamic nuclei — e.g. ventral lateral septum (LS), ventromedial hypothalamus (VMH), anterior hypothalamic nucleus (AH), among others —, the MeA has been traditionally regarded as a component of the "central aversive network" negatively controlling pup-directed behaviours. Contradicting this model, one recent study has uncovered a new level of granularity. *Chen et al.* reported that stimulation of GABAergic neurons in the MeApd — which constitutes >70% of the MeA (Y. Li et al., 2017) — promotes parental behaviour in females (Chen et al., 2019). In males, the MeA is active during exposure to pup sensory cues (Kohl et al., 2017; Y. Li et al., 2017; Tachikawa et al., 2013), but its role in paternal behaviour is still elusive. The same study reported that in males optogenetic activation of GABAergic MeApd neurons also leads to parenting, but high stimulation results in infanticide, suggesting scalability in response (Chen et al., 2019). The apparent discrepancy with the long-standing view of the MeA as suppressing parenting might be explained by experimental differences in the activation of neuronal subpopulations with distinct roles. Powered by finer cell-type specific manipulations, new studies indeed paint a more complex picture of the MeA. Standing at the intersection between the main olfactory and vomeronasal pathways and outputs associated to both the pro-social and aggression networks (Hu et al., 2021; Raam and Hong, 2021; Unger et al., 2015), recent studies have highlighted the diversity of cell types and associated projections in the MeA (Hong et al., 2014), especially within its GABAergic population (Bupesh et al., 2011; Demir et al., 2020; Lischinsky et al., 2017; Padilla et al., 2016), This has led to the view that the MeA is involved in both infant-directed affiliative and aggressive behaviours, modulating transitions between the two modalities rather than playing an exclusive role

in suppressing parenting (Bailey and Isogai, 2022).

Alongside the MeA, the posterior part of the bed nucleus of the stria terminalis (BNSTp), considered to be part of the extended amygdala, and the posteromedial cortical amygdala (CoApm) also receive inputs from the AOB. Not only is the BNST densely interconnected with the MeA, but it has also been extensively implicated in both parental and infanticidal behaviours. Neurons from ventral BNST (BNSTv) are activated during parental behaviour. Moreover, heightened *c-fos* activity was observed in rhomboid BNST (BNSTrh) following infanticide (McHenry et al., 2015; Tsuneoka et al., 2015). Plasticity changes in the BNST underlies the transition from parenting to infanticide in males (Amano et al., 2017), as well from ignoring to parental females (Mei, Osakada, and Lin, 2023; Mei, Yan, et al., 2023). Sensory inputs from the VNO converge onto the AOB, which innervates both the BNST and the MeA. BNST and MeA, in turn, project to the MPOA. Conversely, sensory neurons from the MOE innervate the MOB which sends efferents to the piriform cortex (PirCtx), and from there to the nucleus accumbens (NAc) and the MPOA.

A picture has emerged where sensory information originating from the VNO and relayed to the hypothalamus via the MeA and BNST negatively affects parental behaviour, whereas the downstream hypothalamic medial preoptic area (MPOA) positively regulates pup-directed behaviours by inhibiting this "central aversion network". Despite the heterogeneous composition of neuronal populations and their differential contribution to parental behaviour, recent studies have consolidated the view of the MPOA as a hub gathering sensory inputs from pups and regulating parental behaviour by inhibiting areas controlling pup aversive behaviours and disinhibiting areas controlling pup affiliative behaviours.

Hypothalamic hub.

Vomeronasal and main olfactory pathways converge onto individual cells in the MeA (Keshavarzi et al., 2014). Downstream of the MeA, the MPOA has long been known as a central node for the positive regulation of parenting. Lesion-

ing this area abolishes parental behaviours, whereas direct hormonal stimulation of the MPOA elicits parental responses (Numan, 2003, 2015). The MPOA and its connected areas will be discussed below in the following section. Briefly, the MPOA projects to, or forms reciprocal connections with, several areas involved in distinct aspects of parental behaviour, such as execution of parenting actions, hormonal modulation and release of pro-social neuromodulators, and parental motivation. For instance, activation of MPOA neurons projecting to the ventral tegmental area (VTA) increases maternal motivation by disinhibiting dopamine release in the nucleus accumbens (NAc) (Fang et al., 2018). MPOA projections to the VTA also regulate approach to pup and retrieval (Fang et al., 2018), whereas periaqueductal grey (PAG)-projecting MPOA neurons have been implicated in pup grooming (Kohl et al., 2018). GABAergic neurons in the PAG are mainly contacted by GABAergic MPOA neurons, suggesting that this projection regulates pup-directed behaviours by disinhibiting PAG activity (Zhang et al., 2021). Conversely, activation of PAG by glutamatergic MPOA neurons has been shown to induce anxiety-like states, thereby suppressing parenting (Zhang et al., 2021). In the context of pup-aversive neural circuitry, activation of urocortin 3-expressing neurons in the perifornical area (PeFA^{Ucn3}) has been reported to promote pup-directed neglect (Autry et al., 2021). Inhibition of PeFA^{Ucn3} neurons also induces pup retrieval (Abdelmesih et al., 2023). Since half of the inputs onto PeFA^{Ucn3} neurons are constituted by GABAergic MPOA neurons (Autry et al., 2021), the MPOA likely activates parental behaviour by inhibiting PeFA. In addition, extensive connectivity has been observed between the MPOA and other hypothalamic nuclei. The MPOA projects to the adjacent anteroventral periventricular nucleus (AVPV), whose neurons in turn project to oxytocinergic paraventricular hypothalamic (PVH) neurons, thus modulating release of a neuropeptide involved in infant-parent bonding, parturition and lactation (Okabe et al., 2017; Young Iii et al., 1996). Curiously, oxytocinergic PVH neurons densely innervate the pup-neglect promoting PeFA^{Ucn3} neurons, indicating that

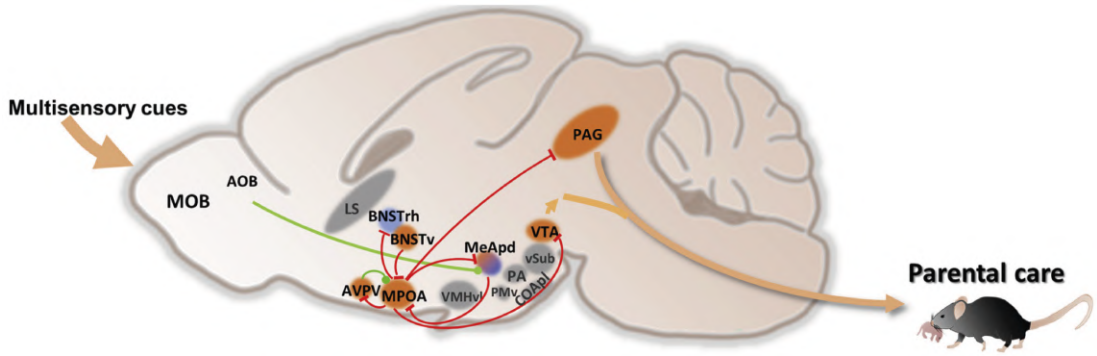

Figure 1.2: **Neural circuitry underlying parental behaviour in mice**. Areas regulating the expression of parental behaviour. Blue regions suppress parental behavior while orange regions promote the behaviors. Red lines indicate inhibitory connections, while green line denotes excitatory connections. Not all known connections and areas are shown. AOB: accessory olfactory bulb; AVPV: anteroventral periventricular nucleus; BNSTp: posterior part of the bed nucleus of the stria terminalis; BNSTrh: rhomboid nucleus of the bed nucleus of stria terminalis; BNSTv: ventral part of the bed nucleus of stria terminalis; COApl: posterolateral cortical amygdala; LS: lateral septum; MeA: medial amygdala; MPOA: medial preoptic area; PA: posterior amygdala; PAG: periaqueductal grey; PMv: ventral premammillary nucleus; VMHvl: ventrolateral part of the ventromedial hypothalamus; VMHvll: lateral subdivision of the VMHvl; vSUB: ventral subiculum; VTA: ventral tegmental area. Adapted from Wei et al., 2021.

the MPOA might also indirectly promote parental behaviour via multisynaptic circuit pathways.

In summary, in this section I have described — although not comprehensively — some of the most critical component areas of the neural circuits underlying parental behaviour, and their connectivity. Amongst them, the MPOA, which I will discuss next, is one of most well-studied areas of this network, for its neuronal populations have been implicated in all aspects of the behaviour, and its position in the circuit makes it the ideal target for state-dependent modulation.

1.3.2 The role of the medial preoptic area (MPOA) in parental behaviour

The MPOA is a heterogeneous hypothalamic area, receiving inputs from, and sending projections to, many brain regions (Simerly and Swanson, 1986, 1988; Simerly et al., 1986). It contains cells expressing several fast-acting neurotrans-

mitters — e.g. glutamate, GABA, dopamine — and neuropeptides (Tsuneoka et al., 2013, 2017), and it is involved in many instinctive behaviours and physiological functions, including thermoregulation (Szymusiak and Satinoff, 1982), sleep (Chung et al., 2017), sexual behaviour (McHenry et al., 2017) and parenting (Numan, 2003).

The pioneering work of Michael Numan and others established the role of the MPOA in parental behaviours in rodents. Pharmacological and surgical lesions of the MPOA result in abolished maternal and paternal behaviour (Miceli et al., 1983; Numan, 2006; Numan and Stolzenberg, 2009; Numan et al., 1988, 1977; Rosenblatt et al., 1996). Immediate early gene expression analyses confirmed that the MPOA is functionally associated with the onset of parental behaviour (Fleming et al., 1994; Kalinichev et al., 2000; Kirkpatrick et al., 1994). Moreover, electrical stimulation of the MPOA facilitates parental behaviour and preference for pup-associated cues (Morgan et al., 1999).

Since then, and over the past decades, an astonishing 677 studies have explored in depth the role of the MPOA in parenting (Carollo et al., 2021). Seminal studies have attempted a finer parcellation of the MPOA. For instance, *Lonstein et al.* first reported that many MPOA neurons showing increased *c-fos* activity after maternal behaviour are GABAergic, hinting at the presence of an inhibitory circuit regulating parenting (Lonstein and De Vries, 2000). Others documented an increase in local excitatory activity within the MPOA of parturient females (Komisaruk et al., 2000). In addition, *Tsuneoka et al.* reported that, among the gross sub-regions of the MPOA, its central part is indispensable for parenting in mice, for its lesion leads to infanticide, with GABAergic and/or peptidergic — galanin, neurotensin, and/or tachykinin2 — neurons in the area being transcriptionally activated during maternal behaviour (Tsuneoka et al., 2015, 2013). It was also found that GABAergic and glutamatergic MPOA neurons antagonistically regulate parental behaviour, and that reciprocal inhibition exists between the two populations (Zhang et al., 2021). In particular, in contrast to glutamater-

gic MPOA neurons, GABAergic neurons — which account for nearly half of the MPOA — positively influence parental behaviour in females (Zhang et al., 2021).

Only recently, however, have advances in circuit-level neuroscience and cell-type specific investigations permitted a closer inspection of parenting-relevant neuronal populations. *Wu et al.* first uncovered a subset of galanin-expressing neurons in the MPOA that are specifically activated during parenting. MPOA^{Gal} neurons comprise roughly 20% of the MPOA, and their function is critical for parental behaviour: cell-type specific ablation of MPOA^{Gal} neurons significantly impairs all components of parental care — e.g. nest building, pup grooming and sniffing — in both fathers and mothers, hinting at the existence of a core, non-sexually dimorphic parenting circuit (Wu et al., 2014).

Inspired by research establishing the effect of estrogen on the MPOA of female rats (Siegel and Rosenblatt, 1975), other studies explored the functional contribution of MPOA neurons expressing estrogen receptor α (MPOA^{Esr1} neurons) in parenting (Fang et al., 2018; Mei, Yan, et al., 2023; Y.-C. Wei et al., 2018). Esr1 is an intracellular receptor and transcription factor activated by the steroid estradiol, and is highly expressed in the MPOA (Mitra et al., 2003). In a recent study, MPOA^{Esr1} neurons were shown to have a higher excitability in mothers compared to females. Conversely, Esr1-expressing neurons in the BNST, to which MPOA^{Esr1} neurons send reciprocal inhibitory connections, are less excitable in mothers than those in virgin females (Mei, Yan, et al., 2023). It was therefore proposed that opposing changes in neuronal properties, and the antagonistic relationship between the two areas, regulate parental behaviour. Moreover, chemogenetic and optogenetic inhibition of MPOA^{Esr1} neurons in females abolishes pup retrieval to the nest, whereas their activation induces pup retrievals (Fang et al., 2018; Y.-C. Wei et al., 2018). Whilst other pup-directed behaviours were investigated, the effects of MPOA^{Esr1}-specific manipulations are confined to pup retrieval, with pup grooming and crouching remaining unchanged (Fang et al., 2018). The same behavioural phenotype is also observed when all MPOA

neurons are optogenetically activated (Y.-C. Wei et al., 2018). Together with the observation that >50% of MPOA neurons are Esr1-positive (X.-Y. Li et al., 2019), these manipulation studies suggest that Esr1 is not a highly specific genetic marker in the MPOA. In addition, MPOA^{Esr1} neurons account for approximately 50% of MPOA neurons expressing vesicular GABA transporter (Vgat) — i.e. GABAergic neurons (X.-Y. Li et al., 2019). Activation of MPOA Vgat+ neurons also elicits pup retrieval, alongside nest building, further highlighting the high level of overlap between the identified MPOA populations (X.-Y. Li et al., 2019).

Since brain regions participate in many behaviours and physiological functions, manipulating a marker-defined albeit very large population of neurons within an area, as is the case for MPOA^{Esr1} but also GABAergic MeApd neurons, expectedly results in context-dependent phenotypes. Both pup retrieval and mounting can be elicited by MPOA-wide stimulation in males and females in a context-dependent manner, and MPOA^{Esr1} photometric recordings confirmed that ramping neural activity strongly correlates with either behaviour (Y.-C. Wei et al., 2018).

By combining single-cell RNA sequencing and multiplexed fluorescence *in situ* hybridisation, *Moffitt et al.* observed that both Esr1- and galanin-expressing neurons overlap, and that each population comprises many transcriptome types (Moffitt et al., 2018). In addition, they identified calcitonin receptor-expressing MPOA (MPOA^{Calcr}) neurons as another molecularly defined population important for parental behaviour. Approximately 70% of MPOA^{Calcr} and MPOA^{Esr1} overlap. In females, the activity of MPOA^{Calcr} neurons is upregulated during parenting and silencing them impairs caregiving behaviours. Conversely, pharmacological activation of MPOA^{Calcr} neurons abolishes infanticide in virgin males (Yoshihara et al., 2021). Collectively, these results strongly suggest that MPOA neurons are highly heterogeneous in their molecular makeup and functional roles.

Although the relationship between molecular identity and functional special-

isation of MPOA clusters is still uncertain, the identification of genetically defined MPOA neuronal sub-populations has accelerated our understanding of the circuit logic underlying parenting. Starting from MPOA^{Gal} neurons, *Kohl et al.* uncovered non-overlapping projection-defined neuronal pools in charge of distinct aspects of parenting (Kohl et al., 2018). MPOA^{Gal} neurons are embedded in a reciprocally connected, brain-wide network of more than 20 brain regions. While the MPOA^{Gal} population is active during all active episodes of pup-directed behaviours in both sexes, each projection-defined MPOA^{Gal} subset is tuned to defined motor, motivational and hormonal aspects of the behaviour. Combined with tracing, optogenetic manipulations revealed that MeA-projecting MPOA^{Gal} neurons are active throughout most parental episodes, suggesting that this projection does not directly regulate parenting but rather inhibits competing social interactions with adult conspecifics. Its inhibition hampers male-male aggression and chemoinvestigation of male intruders in females, but does not affect pup-directed behaviours. In addition, the MPOA^{Gal} $\rightarrow$ ventral tegmental area (VTA) projection mediates the motivation to interact with pups, as evidenced by females' increased frequency of barrier crossings towards a pup upon VTA-projecting MPOA^{Gal} neuronal stimulation (Kohl et al., 2018). Interestingly, activation of VTA-projecting MPOA^{Esr1} neurons results in pup retrieval (Fang et al., 2018). Since stimulation of all MPOA^{Esr1} neurons also leads to retrieval of rubber pups (Y.-C. Wei et al., 2018), VTA-mediated pup retrieval might be the consequence of increased maternal motivation and social reinforcement. Consistent with this view, dopaminergic VTA (VTA^{DA}) neurons are transiently active during pup retrieval, and have been shown to encode social reward signals, thus enabling females to efficiently learn and perform retrieval (Miyamichi, 2024; Xie et al., 2023). Given that MPOA^{Esr1} neurons mainly project to non-dopaminergic, presumably GABAergic, VTA neurons, the MPOA^{Esr1} $\rightarrow$ VTA^{DA} projection might positively regulate maternal behaviour via disinhibition of VTA^{DA} neurons (Fang et al., 2018; Miyamichi, 2024). Finally, the MPOA^{Gal} neurons contact

arginin vasopressin (AVP)-, oxytocin (Oxt)- and corticotropin-releasing hormone (CRH)-expressing neurons in the hypothalamic paraventricular nucleus (PVH), thus also regulating the neuromodulatory component of parenting (Kohl et al., 2018). This connection seems to be sexually dimorphic, as MPOA^{Gal} neurons more densely innervate Oxt-expressing PVH neurons in females, whereas more MPOA^{Gal} neurons contact AVP- and CRH-expressing PVH neurons in males (Kohl et al., 2018). Oxytocinergic PVH neurons, in turn, receive monosynaptic inputs from tyrosine hydroxylase (TH)-expressing neurons in the anteroventral periventricular (AVPV), and involvement of this projection in parenting is sexually dimorphic (Scott et al., 2015). Since MPOA^{Gal} neurons directly contact TH-expressing AVPV neurons, it is possible that MPOA^{Gal} population affects oxytocin release, thus influencing parental behaviour, via the circuit MPOA^{Gal} $\rightarrow$ TH-expressing AVPV $\rightarrow$ Oxt-expressing PVH, and that this MPOA^{Gal}-mediated oxytocin release occurs in females. Consistent with this, more TH-expressing neurons are found in the AVPV of females compared to males (C. F. Yang et al., 2013). More recently, it was found that the expression of oxytocin receptors in the AVPV is exclusive to females and that it is estradiol-dependent (Sharma et al., 2019). As I will later discuss, circulating estradiol rises during pregnancy. It is therefore plausible that a positive feedback loop exists between oxytocin receptor-expressing and TH-expressing neurons in the AVPV, driving parental behaviour in lactating females.

Notably, MPOA^{Gal} neurons integrate inputs from many brain areas, mainly from within the MPOA itself (20% of total presynaptic inputs) and other hypothalamic nuclei (60% of total presynaptic inputs), including VHM, BNST and PVH, as well as from the mesolimbic reward system — e.g. nucleus accumbens (NAc), VTA —, and from areas involved in sensory processing — e.g. MeA — and other homeostatic functions — e.g. arcuate (Arc) nucleus. Interestingly, some of these areas, such as TH-negative AVPV neurons, more densely project to MPOA^{Gal} neurons in mothers than virgin females, although the functional

consequence of this has not been explored yet (Kohl et al., 2018).

I have discussed that the MPOA emerges as a crucial integrative area within the neural circuit underlying parental behaviour. Several studies have revealed the connectivity of molecularly-defined MPOA neuronal pools, further showing that this structure is anatomically well-placed to integrate sensory and internal state information and modulate behavioural execution accordingly. In females, a large body of knowledge, which I will review in the next section, indicates that hormones and neuromodulators play an important role in the transition to motherhood by acting on the MPOA.

1.4 Hormonal modulation of parental behaviour

In absence of hormonal stimulation, experiential factors, such as exposure to pups, can increase parental responsiveness in sexually naive mice (Stolzenberg and Mayer, 2019). A high degree of parental responsiveness can be observed in virgin female laboratory mice which, unlike their wild-type counterparts and female rats, may display affiliative infant-directed behaviours even during their first encounter with alien pups (Chalfin et al., 2014; Kuroda et al., 2011; Scott et al., 2015). Nonetheless, although wild-type female mice and rats are generally aggressive towards pups on their initial exposure, they are capable of developing caregiving behaviours when co-housed with them over several days, through a process termed "sensitisation" (Rosenblatt, 1967). These studies suggest that the hormonal events associated with pregnancy are dispensable for parental behaviours of female rodents. However, when pups are placed in a novel T-maze extension of the home cage, lactating rats perform better than pup sensitised females in retrieval behaviours, thereby showing higher maternal motivation and/or a decrease in risk aversion (Stern and Mackinnon, 1976). Similarly, although some virgin female laboratory mice display immediate parental behaviour upon pup presentation in their home cage, they do not readily retrieve pups placed in a T-maze cage extension, whereas lactating females do so. These sexually-naïve mice

require a few days of pup sensitisation in the home cage to retrieve pups in a novel environment (Stolzenberg and Rissman, 2011; Stolzenberg and Mayer, 2019). In addition, in an operant conditioning paradigm, lactating mice were observed to press a lever to receive a pup as a reward more frequently than virgin females, in spite of showing comparable parental responsiveness in the home cage, indicating a higher motivational state towards pups in post-partum females (Hauser and Gandelman, 1985). Parental care of virgin females is therefore less robust than that of postpartum females (Lonstein et al., 2015). Consistent with this view, maternal aggression, an important component of the parental behaviour repertoire, is usually absent in sexually naive mice (Ferreira et al., 2002; Martín-Sánchez et al., 2015). Seminal studies confirmed that pregnancy hormones underlie the rapid onset of parental behaviour. Transfusion of blood plasma from a lactating female can induce parental behaviour in virgin females (Terkel and Rosenblatt, 1968). A later study reported that transfusion of blood from a pregnant female also elicit parenting in inexperienced virgins (Terkel and Rosenblatt, 1972), wheres using blood from a pup sensitised female did not have the same results (Terkel and Rosenblatt, 1971). Altogether, these studies demonstrate that, even in parental laboratory mice, and especially in rats and wild mice, the hormonal events of pregnancy are needed to elicit the full expression of parenting.

1.4.1 Pregnancy hormones

Pregnancy is a period of drastic change in physiological state. The gestation period is associated with pronounced fluctuations in the levels of several hormones (Fig. 1.2). The ovary is the main secretory source of the steroids 17β-estradiol (E2) and progesterone (P4). Both hormones are necessary to support pregnancy (Rubinstein and Forbes, 1963). In mice, plasma E2 levels transiently rise on day 4 of gestation, playing a critical role for embryo implantation (McCormack and Greenwald, 1974), and then continue to rise from mid-pregnancy, declining rapidly before parturition (Barkley et al., 1977). Plasma P4 levels reach

a peak around day 16 of gestation, decreasing before parturition (Murr et al., 1974). Along with these ovarian hormones, plasma levels of the peptide hormones prolactin (Prl) and oxytocin (Oxt) also change during and after gestation. Prl exhibits mating-induced twice-daily surges in the initial stages of pregnancy, followed by a steady increase up until parturition (Phillipps et al., 2020). Oxt, in contrast, first peaks at parturition, and shows pulsatile release supported by suckling stimulation in the post-partum period (UvnäsMoberg et al., 2020). Therefore, during the second half of pregnancy, the mouse brain is exposed to high levels of E2, which sharply declines before parturition, and steadily increasing levels of prolactin on a background of progesterone withdrawal, while oxytocin first peaks at parturition.

Evidence of the central role of ovarian hormones on parental behaviour originates from early studies on the administration of pregnancy-like E2/P4 priming regime to non-pregnant and ovariectomised rats, which normally ignore pups. When exposed to a pregnancy-like hormonal stimulation consisting of rising E2 and P4 withdrawal, treated female rats readily display full parental behaviour (Bridges, 1984; A. D. Mayer et al., 1990; Moltz et al., 1970). Other early studies have reported that pregnant rats undergoing hysterectomy, and subsequent pregnancy termination via caesarean section, during mid- or late gestation show shorter latencies of parental responses when compared to virgin females (Bridges, 1977; Rosenblatt, 1969) or even intact pregnant females (Rosenblatt and Siegel, 1975), suggesting that endocrine events occurring in the second half of gestation, and not parturition, are sufficient to elicit parental behaviours. In further support of these findings, rats ovariectomised at the time of hysterectomy and caesarean section in mid-pregnancy fail to display immediate parenting, yet they still show shorter-latency parental responses than virgins (Moltz and Wiener, 1966; Rosenblatt and Siegel, 1975). When these hysterectomised-ovariectomised females were systemically injected with high doses of E2 after the hysterectomy-ovariectomy procedure, however, immediate onset of parenting was restored (Siegel and Rosen-

blatt, 1975). In these experiments, surgical removal of ovaries and termination of pregnancy were performed in mid-pregnancy, when E2 starts to rise and P4 levels are already high. Following hysterectomy-ovariectomy, both E2 and P4 decline, but the action of E2 (and prolactin) until that point, combined with the surgery-induced drop of P4, might explain why these females display shorter parenting onset than virgins. By ensuring elevated circulating levels of E2 and low P4 levels, further systemic administration of E2 after the procedure would better simulate peri-partum endocrine events, thereby inducing a rapid onset of parental behaviors (Numan, 2015). It has been proposed that P4 primes the brain of females to become more sensitised to pup cues and control the timing of its sensitivity (Bridges, 2015). This cumulative priming effect becomes apparent when high circulating P4 levels drop, either before parturition (Slotnick et al., 1973) or in pregnant females subjected to ovariectomy in the late stages of gestation (Bridges et al., 1978). In ovariectomised females treated with E2 and P4, if P4 is not withdrawn prior to behavioural testing, an immediate onset of parental responsitivity is not observed (Bridges and Russell, 1981). Additionally, administration of an anti-progesterone monoclonal antibody in pregnant females impairs post-partum maternal behaviour, and these behavioural effects are not due to deficits in lactation but presumably to impaired pre-partum priming of the brain (M. Wang, Crombie, Mais, et al., 1995). These studies implicate the hormonal events associated with pregnancy in the onset of parental behaviour.

The MPOA has been established as a site of ovarian hormone modulation. Intracellular receptors for E2 (Esr1) and P4 (PR) are expressed in the MPOA (Giordano et al., 1989; Quadros and Wagner, 2008; Shughrue et al., 1997; Simerly et al., 1990), and their expression is affected by pregnancy (Francis et al., 2002; Giordano et al., 1989). Interestingly, upregulated expression of Esr1 during pregnancy is correlated with the latency of parental behaviour (Giordano et al., 1990, 1989). Implants of E2 directly into the MPOA shorten the onset of parental behaviour in both ovariectomised (Fahrbach and Pfaff, 1986) and pregnancy-

terminated rats (Numan et al., 1977). Notably, MPOA stimulation with E2 recapitulates the behavioural phenotype observed upon systemic E2 administration in hysterectomised-ovariectomised female rats, whereas infusion of E2 into other hypothalamic areas, such as the ventromedial hypothalamus, or of cholesterol into the MPOA, did not elicit immediate parental responses in pregnancy-terminated females (Numan et al., 1977). Moreover, at the end of pregnancy, P4 withdrawal combined with increasing E2 levels activates MPOA neurons, whereas P4 administration alone does not induce parenting nor result in increased Fos expression in the MPOA (Sheehan and Numan, 2002). More recently, short hairpin interference of Esr1 mRNA in the MPOA was reported to abolish the onset of parental behaviour (Ribeiro et al., 2012).

Together with the ovarian steroid hormones, the neuropeptide hormones Prl and Oxt are involved in parenting, as their administration induces a rapid onset of full parental behaviour in ovariectomised and steroid-primed female rats (Moltz et al., 1970; Pedersen et al., 1982; Riddle et al., 1935). In presence of ovarian hormones, the facilitatory role of prolactin in parental behaviour is in part mediated via the MPOA. Central — but not systemic — infusion of prolactin stimulates the onset of parenting only in steroid-primed ovariectomised females, and MPOA administration in these females further enhances parental responses (Anderson et al., 2006; Bridges et al., 1990). Prolactin is normally released into the MPOA of post-partum females during suckling (Torner et al., 2004). Treatment with prolactin increases prolactin receptor (Prlr) expression in the MPOA (Anderson et al., 2006). Moreover, deletion of Prlr in MPOA neurons of post-partum females results in parental deficits (Brown et al., 2017). These females do not spend time with pups, which starve to death. However, other parental behaviours, such as retrieval, are not impacted by Prlr ablation. This indicates that, while Prl is clearly involved in the regulation of parental behaviour, its behavioural effects might not be as prominent as those induced by ovarian hormones. In addition, a pregnancy-like steroid regime has been reported

to alter Prlr expression in the MPOA, suggesting that Prl action is modulated by steroid hormonal changes during gestation (Bridges and Hays, 2005). Similar to Prl, Oxt infusions in steroid-primed females also promote parental behaviour, while administration of an Oxt antagonist negatively affects parenting (Pedersen and Prange, 1979; Pedersen et al., 1994; Van Leengoed et al., 1987). In addition to the MPOA, whose neurons express Oxtr, Oxt acts on several other brain areas, where it can modulate other social behaviours as well as experience-dependent (and pregnancy hormone-independent) parental behaviour in virgin mice (Scott et al., 2015; Sharma et al., 2019, and reviewed in Fineberg and Ross, 2017; Froemke and Carcea, 2017). The mechanisms and sites of action of Oxt, both in lactating and sexually-naïve animals, have not been fully described yet (Numan and Woodside, 2010; Numan and Young, 2016; Yukinaga et al., 2022). An example of Oxt action is provided in Chapter 6 in the context of alloparental care (see Conclusions and future directions).

Collectively, I have provided evidence which implicates pregnancy-associated hormonal signalling in the upregulation of parental responsiveness, and establishes the MPOA as a site of hormonal modulation. However, how these hormones affect MPOA neurons to induce parental behaviour is an open question.

1.5 Hypotheses

It had been known that the hormonal milieu of pregnancy is important for the induction of parental behaviour. In addition, several studies had demonstrated that the endocrine events of late gestation, rather than parturition or lactation, are sufficient to drive behavioural changes in parenting. There had also been evidence that stimulation of the MPOA with ovarian hormones results in the full expression of parental behaviours. Concurrently, MPOA neurons and specifically MPOA^{Gal} neurons have been implicated in the regulation of parental behaviour. However, despite the wealth of studies implicating hormones in the onset of parental behaviour via MPOA action, how these hormones act on MPOA neurons to induce

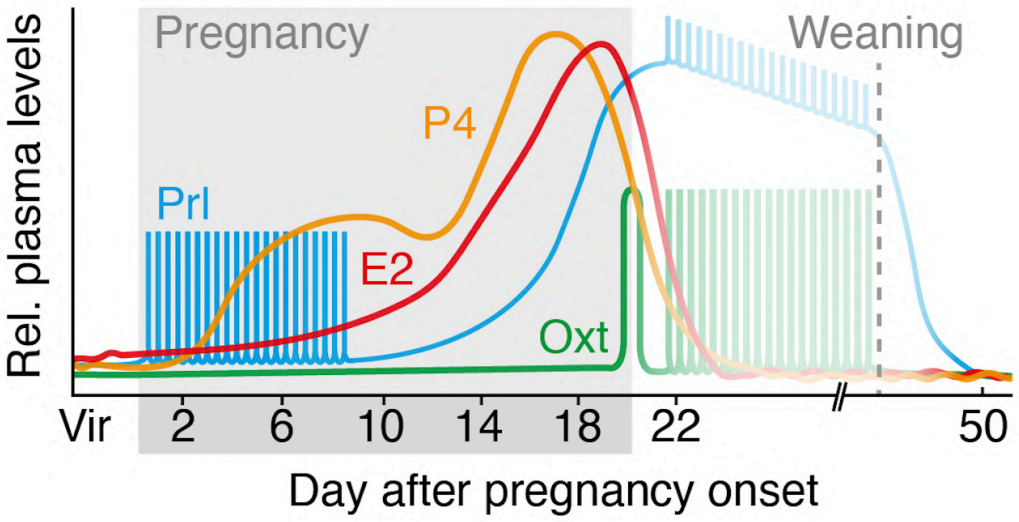

Figure 1.3: **Hormonal fluctuations during pregnancy**. Relative plasma levels of canonical pregnancy hormones in female mice. Adapted from Ammari and Monaca et al., 2023.

parental behaviour had been unclear. This led to four main hypotheses:

- Parental behaviour changes already during pregnancy.
- Steroid hormones mediate changes to pup-directed behaviour by acting on $MPOA^{Gal}$ neurons.
- The biophysical properties of $MPOA^{Gal}$ neurons are affected by steroid hormone action during pregnancy.
- Pregnancy affects the population activity of $MPOA^{Gal}$ neurons to support better maternal performance.

1.6 Thesis overview

To test these hypotheses, I first investigate whether pregnancy affects parental behaviour in Chapter 3. I also explore which behavioural parameters change, and the time course of such behavioural adaptations. In Chapter 4, after confirming that receptors for ovarian pregnancy hormones are enriched in the MPOA, I perform receptor ablation experiments to demonstrate the necessity of ovarian hormone action on MPOA and MPOA^{Gal} neurons for pregnancy-induced behavioural changes. I also examine how E2 and P4 affect the biophysical and morphological properties of these neurons during pregnancy. Finally, in Chapter 5, I proceed with cellular-resolution *in vivo* calcium imaging from the MPOA^{Gal} population, and probe single neuron- and population-level activity changes during pregnancy.

Chapter 2

Methods

2.1 Animals

Mice were from a C57BL/6J background. They were housed in individual cages on a 12 h:12 h light-dark cycle (light on: 22:00-10:00). Food and water were available *ad libitum*. Timed pregnancies were set up during mid-dark phase (16:00) and females were checked for vaginal plugs early in the mornings on subsequent days. Gestation length averages 19.3 days in the C57BL/6J strain. Pregnancy Day (D) 1 was defined by when a vaginal plug was detected. Postpartum time points (e.g. D22, D50) were counted from vaginal plug detection. For behavioural testing, C57BL/6J mice were between 8-14 weeks old, and provided by the Francis Crick Biological Research Facility. For electrophysiology experiments, Gal-Cre BAC mouse line (Stock: Tg(Gal-cre) KI87Gsat/Mmucd, 031060-UCD) was crossed to Cre-dependent Rosa26 Tomato (Ai9, JAX #007909).

CRISPR/Cas9-mediated knock-in of a P2A-FlpO cassette 3' of the STOP codon (Exon 6) of the galanin gene was employed to generate the Gal-Flp transgenic line (Biocytogen). The Esr1^{loxP} line was imported from the European Mouse Mutant Archive (EM:11179), and the Francis Crick Genetic Modification Services (GeMS) generated the PR^{loxP} line was generated by the Francis Crick Genetic Modification Services (GeMS). For hormone receptor KO experiments, the Gal-Flp line was crossed to Esr1^{loxP} (estrogen receptor 1 conditional knockout) or PR^{loxP} (progesterone receptor conditional knockout). All mouse lines were back-crossed to C57BL/6J.

2.2 Behavioural profiling

2.2.1 Experimental groups

In the pregnant repeated exposure (*Preg*) group, virgin females were tested a week before being paired with a sexually-experienced (stud) male. Interactions with pups were assessed every four days during pregnancy (see Pup-directed behavior assay). After parturition, females were allowed to stay with their own litters to

avoid any negative impact on social behavior that could result from separating them (Fabricius et al., 2008). Females' own pups were, however, removed before behavioural testing. In the ovariectomised (*OVX*) group, animals were given a week to recover before behavioural testing. They were then paired with stud males for 3 days, and behaviourally assessed every 4 days. The pairing with stud males did not result in pregnancy in this group, but controlled for exposure to a male conspecific. Behavioural testing for this group started after males were separated. In the dual exposure group (*Dual*), animals were only tested twice — once at the virgin (Vir) state and then on the equivalent day 18 (D18) of pregnancy. In the single exposure group (*1x*), females were only tested when pregnant on day 18 of gestation. In the long-term exposure group (*Lex*), females were tested at virgin (Vir) and, once pregnant, at D18, D50, D90, D180 and D270.

2.2.2 Pup-directed behaviour assay

Animals were single-housed for a week before behavioural testing. Tests were preceded by a 10-minute habituation period. Experiments were conducted during the first half of the dark phase in the animals home cage. Two foster pups, aged 1-3 days (randomly picked from litters of 5-8), were placed in opposite corners of the cage, away from the nest. Interactions with pups were recorded for 15 minutes using a camera positioned above the cage (Basler Ace GigE, acA1300-60gmNIR), recording at 30 Hz. Behaviours were scored manually using EthoVision XT 13 (Noldus), and segmented behaviours were then analysed with custom Python scripts. Duration of each behavior was expressed as a percentage of the total assay time, while the latency of behaviours was measured from introduction of the first pup.

The following pup-directed behaviours were quantified: Contact latency referred to the time it took for the animal to make its first nose contact with a pup. Pup grooming was defined as close contact with the pups, including licking

and pup displacing. Pup sniffing involved close interaction without direct contact (chemoinvestigation). Retrieval latency was defined by the animal picking up the pup and successfully carrying it to the nest. The fraction of mice retrieving or crouching over at least one pup was recorded. Time in nest was defined as the time animals stayed in the nest with at least one pup. Crouching was defined as the animal positioned over the pups whilst stationary. Pup-directed aggression was defined as rough handling or violent grooming of at least one pup by the female. When females exhibited pup-directed aggression, the pups were immediately removed, and the experiments stopped. Nest building included activities like gathering nesting material or shaping it into a nest. Nest quality was assessed at the beginning of each testing session and scored during the habituation phase, with scores defined as follows: 0 no nest or scattered material; 1 flat nest; 2 plate-shaped nest with low walls; 3 plate-shaped nest with higher walls; and 4 fully closed, dome-shaped nest (as previously reported in Topilko et al., 2022).

To calculate behavior transition probabilities, scored behavioural events from virgin females (n = 10) and pregnant females on day 18 (n = 10) from the *Preg* group were compiled. Both lists of behaviours were split into pairs (i.e. behaviour 1, behaviour 2). To calculate the probability for each given pair of behaviours, the number of instances for the pair was divided by the total number of pairs featuring behaviour 1. U tests were conducted for each behaviour pair between Vir and D18, and differences highlighted if $P < 0.05$.

2.3 Surgery and viral injections

The day before surgery, animals were provided with 0.15 ml carprofen was in 200 ml drinking water as analgesia. Animals received isoflurane for anaesthesia (4% for induction, 1.5% for maintenance) in oxygen-enriched air and were head-fixed on a stereotaxic frame (Model 940, Kopf Instruments). Meloxicam (10 mg / kg body weight) and Buprenorphine (0.1 mg / kg body weight) were given subcutaneously before the surgical procedure. Surgery sites were closed using

Vicryl sutures (Ethicon) or Vetbond (3M). During the surgery, eyes were protected with ophthalmic ointment (Viscotears, Alcon). Rectal body temperature was maintained at 37 °C during surgeries using a heating pad (Harvard Apparatus). For pain management, the anti-inflammatory Carprofen was then provided post-operatively for 48 hours diluted in drinking water.

2.3.1 Receptor knockout experiments

For MPOA-wide receptor KO experiments, 250 nl of AAV2/5-CMV-EGFP-Cre (Addgene 105545) or AAV2/5-CMV-EGFP (Addgene 105530) at a titre of 2 × 10^{13} GC/ml were bilaterally injected into the MPOA of Esr1^{loxP} or PR^{loxP} animals. For MPOA^{Gal}-specific receptor ablation and slice physiology experiments, the MPOA of Gal-Flp;Esr1^{loxP} or Gal-Flp;PR^{loxP} mice was injected with 250 nl of AAV2/1-syn-fDIO-EGFP-2A-iCre (packaged by Vectorbuilder) or AAV2/1-syn-fDIO-EGFP (packaged by Vectorbuilder) at a titre of 4 × 10^{13} GC/m and 2 × 10^{13} GC/m, respectively. MPOA injection coordinates: AP 0.0 mm (from bregma), ML ± 0.5 mm (from midline), DV 5.05 mm (from pia). Two weeks after the surgical procedure, animals were injected at virgin state and D18. The efficiency of ablation was separately assessed in another cohort of mice which received unilateral injections (see Image acquisition and analysis).

2.3.2 Deep brain imaging

For calcium imaging experiments, 100-200 nl of AAV2/1-syn-FLEx-jGCaMP7s-WPRE (Addgene 104491), or AAV2/1-syn-GCaMP7s (packaged by the Francis Crick Vector Core) was unilaterally injected into the MPOA of Gal-Cre or C57BL/6J mice, respectively, with a Nanoject II or Nanoject III injector (Drummond Scientific) at a titre of 2 × 10^{13} GC/ml. Weak GCaMP expression was observed in contralateral soma, suggesting potential retrograde transport of AAV, which may have contributed to labeling in these regions. MPOA injection coordinates: AP 0.0 mm (from bregma), ML ± 0.5 mm (from midline), DV 5.05

mm (from pia). After injection, virus would be allowed to diffuse for at least 8 minutes. Injection needle was then slowly retracted and a gradient-index lens (0.6 × 7.3 mm, Inscopix) was implanted at a depth of 4.95 mm (from brain surface). The implant was cemented in place using UV lightcurable dental cement (RelyX Unicem, 3M) and Superbond dental cement (Prestige Dental). Imaging sessions started 6-8 weeks after surgery.

2.4 Histology

Animals were sacrificed and perfused with PBS and 4% paraformaldehyde (PFA) in PBS. Brains were dissected, fixed in 4% PFA overnight, and washed in PBS. 60 μm coronal sections were cut using a vibratome (Leica) and mounted on Superfrost Plus slides (VWR, 48311-25 703) with DAPI-containing Vectashield mounting medium. For immunostaining, PBS-T (0.3% Triton X-100 in PBS) was used for 30 minutes to permeabilise sections, which were then fixed with 4% PFA in PBS for 10 minutes, and washed in PBS-T (3 × 20 min). Blocking buffer (0.3% Triton X-100, 1% BSA, 2% normal donkey serum in PBS) was then applied to sections for 2 hours. Incubation with primary antibody (rabbit anti-Esr1, Millipore 06-935, 1:5,000) was carried out for 24 hours at 4°C. Sections were washed in PBS-T (5 × 60 min) and secondary antibody (Alexa Fluor-488 anti-rabbit, Thermo Fisher A-11008, 1:1,500) was added for 48 hours at 4°C. After PBS-T washes (5 × 60 min), NeuroTrace 530/615 Red Fluorescent Nissl Stain (Thermo Fisher N21482, 1:100) was added for 30 minutes at room temperature. Sections were again washed in PBS-T (3 × 20 min) and finally mounted.

2.5 Hormone receptor expression

2.5.1 Hybridisation and immunostaining

Animals were sacrificed and perfused with ice-cold PBS, their brain removed, embedded in OCT and frozen with dry ice (Tissue-Tek, 4583). 18 μm cryo-sections were collected on Superfrost Plus slides (VWR, 48311-703) in three series. Subsequently, one of the three series was stained and imaged. Slides were fixed in 10% Neutral Buffered Formalin (NBF), and a series of dehydration steps in ethanol (5 min each of 50%, 70%, 100%, 100% v/v EtOH) followed. Slides were pretreated with RNAscope Protease III reagent. Single-molecule fluorescent *in situ* hybridization (smFISH) and immunohistochemistry (IHC) against NeuN were performed on slides using the RNAscope LS Multiplex Reagent Kit (Advanced Cell Diagnostics) and LS 4-Plex Ancillary Kit and Multiplex Reagent Kit on a robotic staining system (Leica BOND-III). RNAscope probes: Esr1 (ACD, Cat. No. 475248), Pgr-C2 (Cat. No. 318928-C2, which detects both the PR-A and PR-B isoforms), Gal (Cat. No. 400961, 400961-C2), FlpO (Cat. No. 520791-C3), Prlr-C3 (Cat. No. 430798-C3), Oxtr-C4 (Cat. No. 412178-C4). NeuN antibody (Millipore MAB377) was used at 1:500.ă

2.5.2 Image acquisition and analysis

Vectra Polaris Automated Quantitative Pathology Imaging System from Akoya Biosciences was used at 20× magnification to acquire images. Phenochart software and inForm Tissue Analysis software from Akoya Biosciences were used to select regions of interest (ROIs) and spectrally unmix image tiles. QuPath custom routines were used to stitch spectrally unmixed image tiles and for neuron and subcellular spot detection. NeuN and DAPI were employed to detect neuronal cell bodies.

Subcellular detection of receptor transcripts was carried out on these identified neurons. The distance between sections was 56 μm because every third

section, each of 18 μm, was processed. The Fiji plugin ABBA was used to align coronal brain sections with the Allen Brain Atlas (https://biop.github.io/ijp-imagetoatlas/). For a given brain, X and Y rotations were adjusted for all sections, and two rounds of affine registration were done using Elastix. Non-rigid registration was performed using the BigWarp tool (with DAPI as the sample channel and Nissl as the atlas channel). From QuPath, the transformed cell detections were then exported to a custom Python app for visualisation (https://github.com/nickdelgrosso/ABBA-QuPath-RegistrationAnalysis), and further analysed with custom Python routines.

Immunostaining against Esr1 was performed on 18 μm MPOA sections to determine a threshold for categorising neurons as Esr1-positive. NeuTrace (a neuronal counterstain) was used to quantify the percentage of Esr1-positive neurons in the MPO and MPN, two MPOA subregions which featured in all sections. The percentage of Esr1-positive neurons in these regions was calculated to be 40.4% $\pm$ 5.9% (mean $\pm$ SEM, 28,543 neurons, 3 animals), corresponding to $\geq$3 transcripts per cell. For PR-positive neurons, published data on the number of PR-positive neurons in the hypothalamic ARH and AVPV was used due to a lack of commercially available PR antibodies. Based on Moore et al., 2015, a threshold of $\geq$5 transcripts per cell was chosen.

To assess the efficiency of receptor ablation, AAV2/5-CMV-EGFP-Cre or AAV2/5-CMV-EGFP was unilaterally injected into the MPOA of $Esr1^{loxP}$ or PR^{loxP} mice. Two weeks after the injection, immunostaining against Esr1 or in situ hybridisation for Esr1 and PR were employed to quantify Esr1 or PR-expressing neurons in injected and non-injected hemispheres. For each section, the ratio of receptor-expressing neurons (immunostaining) or staining intensity (*in situ* hybridisation) was calculated between injected and non-injected sides of the MPOA using QuPath.

2.6 Electrophysiological recordings

2.6.1 Whole-cell patch clamp

For targeted $MPOA^{Gal}$ recordings, the *Gal-cre* and floxed tdTomato line (Ai9) were crossed, and their progeny used. For recordings from mice with $MPOA^{Gal}$-specific receptor ablation, animals were obtained as described above in "Receptor knockout experiments". Animals were anesthetized with 3% isoflurane in oxygen and sacrificed by decapitation. The brain then placed in ice-cold slicing solution containing (in mM): sucrose (213), KCl (2.5), NaH_2PO_4 (1.3), $NaHCO_3$ (26), $MgSO_4$ (2), $CaCl_2$ (2), and D-glucose (10), equilibrated with carbogen (95% O_2 / 5% CO_2). 250 μm-thick coronal brain slices containing the MPOA were cut on a vibratome (Leica VT1200S) in ice-cold slicing solution and then transferred to an incubation chamber filled with artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (127), KCl (2), NaH_2PO_4 (1.2), $NaHCO_3$ (26), $MgSO_4$ (1.3), $CaCl_2$ (2.4) and 10 D-glucose (10), which was continuously oxygenated with carbogen. After a recovery period of at least 1 hour at 37°C, slices were moved to a submersion chamber under an upright microscope equipped with infrared Nomarski differential interference contrast optics (Slicescope, Scientifica). During the experiments, the slices were submerged and continuously perfused (2-3 ml/min) with aCSF at a near-physiological temperature (33°C), while being bubbled with carbogen. Glass micropipettes with a resistance of 3-6 M were made from borosilicate capillaries (World Precision Instruments, Aston, UK) using a P-97 Flaming/Brown micropipette puller (Sutter, Novato, CA) and filled with an internal solution containing (in mM): K-Gluconate (140), KCl (10), KOH (1), EGTA (1), Na_2ATP (2), HEPES (10), pH 7.3, 280290 mOsm. To record spontaneous excitatory postsynaptic currents (sEPSCs) and inhibitory postsynaptic currents (sIPSCs), voltage clamp recordings were performed at the reversal potential of chloride (-70 mV) or the reversal potential for fast excitatory neurotransmission (0 mV), respectively, using an internal solution of Cesium methane

sulfonate (140), CsCl (10), HEPES (10), 120 EGTA (10), and Na_2ATP (2) (pH 7.3 adjusted with KOH).

For neuronal morphology reconstruction (see below), Neurobiotin (SP-1120, Vector Laboratories) was added at a concentration of 0.2% (w/v). Access resistance was monitored throughout the experiment, and neurons with a series resistance above 25 MΩ or changes of ≥20% were excluded from the analysis. The liquid junction potential was 16.4 mV and was not compensated. Intrinsic electrophysiological properties of the cells were characterised using a standard current-clamp protocol, which included I/V curves, ramps, and current injections. To measure excitability, depolarising current ramps (10 pA/s) ranging from +25 to +165 pA were injected.

Recordings were performed using an Axon Instruments Multiclamp 700B amplifier (Molecular Devices), low-pass filtered at 10 kHz, and digitised with a Digidata 1322A digitizer (Molecular Devices). Slow and fast capacitive components were semi-automatically compensated. Data analysis was conducted off-line using Clampfit 10 software (Molecular Devices), WinEDR v4, and WinWCP v5 (http://spider.science.strath.ac.uk/sipbs/software_ses.htm), along with custom Python routines (Anaconda distribution).

2.6.2 Morphological reconstruction

After slice physiology recordings, slices were post-fixed overnight at 4°C in 4% PFA in PBS containing 200 mM sucrose and 0.1 M HEPES. Slices were then rinsed in PBS and washed in PBS-T for 1 hour. They were incubated with Streptavidin-647 (Thermo Fisher S32357, 1:2,000) at 4°C for 24 hours. Following another 1-hour wash in PBS-T, slices were rinsed in PBS and mounted on slides using Vectashield mounting medium with DAPI (Vector Laboratories, H-1200). Imaging was carried out using a Zeiss LSM 710 confocal microscope equipped with a 63X (NA = 1.4) oil immersion objective and a Z step size of 0.5 μm. Tile scans were performed to capture the processes of the recorded neurons within the slice.

Morphological reconstruction of the soma and neurites of each Neurobiotin-filled neuron was done using Neurolucida software (MBF Bioscience), while spine reconstruction was performed using the Filament Tracer module (auto-path method, spine diameter 0.25 μm) in Imaris 9.0 (Bitplane). Soma volumes from non-recorded MPOA^{Gal} neurons in the same brain slices were measured using the Surfaces module in Imaris.

2.7 Endoscopic calcium imaging

Mice were connected to a miniature microscope (nVista, Inscopix). Calcium imaging data was acquired using nVista software (Inscopix) at a frame rate of 20 Hz with 475 nm LED power of 0.1-0.2 mW/mm^2, gain of 5-8, and a resolution of 800 $\times$ 1,280 pixels. Miniscope acquisition was synchronised with behavioural video recording using a custom workflow on Bonsai software (Neurogears). After a 20-min habituation session, 15-min pup-directed behavioural assays were performed. As previously described in "Pup-directed behaviour assay", 2 pups were dropped at the cage corners opposite the nesting area. Females were allowed to freely interact with pups for 15 minutes, after which a set of social and non-social stimuli was presented following a randomised order. For pre-processing purposes, 1-min baseline was acquired before pup introduction for each ROI in the field of view (see Pre-processing).

2.7.1 Pre-processing

Imaging frames were spatially down-sampled to 400 $\times$ 540 pixels. To correct for drift in the baseline signal over time, a spatial bandpass filter was applied with lower cut-off spatial frequency of 0.005 oscillations per pixel and upper cut-off frequency of 0.5 oscillations per pixel. Motion artifacts were corrected and the relative fluorescence change ΔF for each pixel compared to baseline was calculated

as follows:

$$\frac{\Delta F}{F0} = \frac{F - F0}{F0}$$

(where F0 is the mean fluorescence value of each pixel during the baseline period). PCA/ICA-based cell detection was performed using a mean ROI radius of 79 pixels in Inscopix Data Processing Software (IDPS). All automatically identified cells were reviewed to exclude false-positives. For each verified cell, the relative change in calcium signal $\Delta F \div F0$ was normalised as follows:

$$\frac{\Delta F}{F0} = \frac{\frac{\Delta F}{F0} - min(\frac{\Delta F}{F0})}{max(\frac{\Delta F}{F0}) - min(\frac{\Delta F}{F0})}$$

2.7.2 Evoked activity analysis

Z scores were calculated based on the period of -2 s to 0 s before event detection (baseline period) and 0 to 2 s from event detection (activity period) as $z = \frac{\chi - \mu}{\sigma}$, where χ is ΔF/F of the current timestamp, μ is the mean ΔF/F of the baseline period and σ is the standard deviation of the baseline period. Responses were defined as significant when the z-scored ΔF/F of the baseline and activity periods were significantly different. ROIs were then categorised as exhibiting increased, decreased (or unchanged) evoked activity based on the sign of the t statistic. A neuron exhibiting increased evoked activity during a parental action or detection of stimulus number was therefore categorised as activated. Conversely, a neuron exhibiting decreased evoked activity during a parental action or detection of stimulus number was categorised as inhibited. The single neuron tuning index was derived from unpaired t tests between the activity and baseline periods, prior to Z-scoring. Tuning indices were normalised based on their absolute value using a MinMaxScaler estimator from the sklearn preprocessing package while keeping their original sign.

2.7.3 Selectivity index

To assess selectivity, stimuli were presented sequentially in a randomised order. The choice probability for each neuron was calculated to obtain the selectivity of individual neurons for pups versus other social and non-social stimuli. This process involved using the ΔF/F values of individual neurons during pairs of chemo-investigation behaviours (e.g. investigation of pups versus intruders) to estimate how well two behaviours can be distinguished based on their respective ΔF/F distributions. The first sniffing episode towards each stimulus was used to achieve better separation between activity transients evoked by different stimuli in sniffing sequences. This was particularly important given the gradual desensitisation with repeated sniffing of the same stimulus. To calculate the selectivity index, ΔF/F distributions were plotted as histogram pairs. Histograms were then plotted against each other to generate a ROC curve, and the selectivity index was calculated as: 1 — (*area under the ROC curve*). A selectivity index of 1 denotes neurons exclusively active during pup sniffing, a selectivity index of 0 denotes neurons exclusively active during sniffing of another stimulus, and a selectivity index of 0.5 denotes non-selective neurons.

2.7.4 Linear discriminant analysis

To assess how different social and non-social stimuli can be discriminated by $MPOA^{Gal}$ neuronal ensemble, linear discriminant analysis was performed, using timestamps as features (Y. Li et al., 2017). LDA is a supervised dimensionality reduction technique that projects input data into a subspace, where the axes are designed to maximise the separation between different classes. For each imaging session, LDA was computed using two inputs: i) a 3D array, x, representing the population activity of all n neurons recorded for 3 seconds following the onset of k behaviors (including the first sniff of a pup, male and female intruder sniff, screw sniff, dummy pup sniff, and food pellet sniff), forming $k = 4$ classes.With a sampling of 20 Hz over 3 s, this results in a matrix with a dimension of $n \times k \times 60$;

ii) vector y, assigning behaviour labels to each of k activity episodes in x, giving a k-dimensional vector. Given these two inputs, LDA maximizes the posterior log likelihood of input y belonging to class i out of k classes computed as ($\log P(y = i|x) = \omega_i^k x + \omega_{\text{i0}} + C_{\text{st}}$ where $\omega_i = \Sigma^{-1}\mu_i$ and $\omega_{\text{i0}} = -\frac{1}{2}\mu_i^k\Sigma^{-1}\mu_i + \log P(y = i)$. This can be achieved by minimising the term $(x - \mu_i)^k\Sigma^{-1}(x - \mu_i)$. This will minimise both the variance within each class and the distances between x and each of the k classes. Sklearn linear discriminant classifier was used for this analysis with an eigen solver and a 0.001 shrinkage parameter for regularisation. For better visualisation, ellipses comprising the 95% confidence intervals were fitted to LDA clusters. To quantify separability of LDA clusters, all episodes in x were again fitted to the derived LDA space with their class assigned by the fitted LDA model. The Rand index (RI) was then calculated based on this predicted class and ground truth class y as follows:

$$RI = \frac{No.of agreements(TP + TN)}{No.agreements(TP + TN) + No.disagreements(FP + FN)}$$

where TP = true positive, TN = true negative, FP = false positive, FN = false negative.

2.7.5 Quantification and statistical analyses

The SciPy stats module was used to calculate all test statistics and P values. Two-sided Mann Whitney U test was used to compare two independent groups. Wilcoxon signed-rank test or paired t test was used for paired data based on distribution and sample size. ANOVA was performed, followed by the Tukey HSD *post hoc* test, when >2 groups were compared. Dunnetts *post hoc* test was used instead when several groups were compared to a single reference group (e.g., Vir). Repeated-measures ANOVA was used for longitudinal analysis of behavioural data. Two-tailed Fishers exact test was used for categorical data, and Benjamini-Hochberg false discovery rate adjustment was performed for multiple comparisons

(FDR < 0.05). Kaplan-Meier (K-M) survival analysis was performed to evaluate behavioural latencies which contained saturated data (e.g. retrieval latency). The K-M estimator was computed using occurrence of retrieval (or contact) as event type 1; retrieval (or contact) failure over the course of the assay constituted a right censored event (event type 0). Pairwise P values were obtained by log-rank test on K-M curves and adjusted for multiple comparisons using the Benjamini-Hochberg FDR procedure. The KolmogorovSmirnov test was used to compare two distributions. In initial experiments, no statistical methods were used to predetermine sample sizes, but sample sizes were similar to those reported in similar studies. Details of statistical testing are provided in figure legends.

Chapter 3

Characterisation of pregnancy-induced parental behaviour

3.1 Introduction

Parental behaviour consists of motor actions which are stereotypically performed by sexually naive (virgin) and lactating females. These include infant-directed behaviours, such as retrieving pups to the nest, grooming and crouching over them, as well as behaviours aimed at protecting the offspring, such as aggression towards intruders and nest building (Lisk, 1971; Mann et al., 1984; Numan, 2020). Generally, while female rats and wild mice ignore or attacks pups, female laboratory mice may exhibit hormone-independent, spontaneous parental behaviour. In contrast, lactating females are robustly parental (Fleming and Rosenblatt, 1974; Rosenblatt et al., 1988). Several studies suggest that pregnancy-induced hormonal signalling triggers parenting onset (A. D. Mayer et al., 1990; Moltz et al., 1970; Rosenblatt, 1969), while others attribute increased maternal responsiveness to the hormonal events occurring around parturition (Brown et al., 2017; Rosenblatt et al., 1988). It is also known that exposure of virgin rats to pregnancy hormones evokes parental behaviour (A. D. Mayer et al., 1990; Moltz et al., 1970; Pedersen et al., 1982; Rosenblatt et al., 1998). However, despite the wealth of knowledge linking pregnancy hormones to the onset of parenting, it is unclear which aspects of parenting change during pregnancy, and when these changes occur.

To comprehensively characterise pregnancy-associated changes to parental behaviour, I employed a naturalistic behavioural assay. Females were monitored in their home cages for 15 minutes while presented with pups at regular 4-day intervals before, throughout and after pregnancy (Fig. 3.1, A). Subsequently, I quantified a set of behavioural parameters characteristic of parenting to address whether parental behaviour is changed by pregnancy, when such changes happen, and which behavioural parameters are affected. The complete list of behavioural parameters and their definition are presented above (see "Pup-directed behaviour assay" in Methods). I then asked whether ovarian hormones and/or exposure to pups are required for any observed pregnancy-induced behavioural adaptations

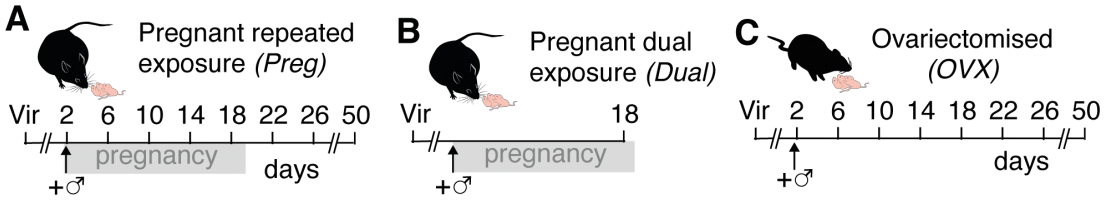

Figure 3.1: **Behavioural assays**. (**A**) Behavioural testing of pup-directed interactions in repeatedly pup-exposed pregnant females (*Preg*), (**B**) in pregnant females exposed to pups twice (*Dual*), and (**C**) in repeatedly pup-exposed ovariectomised females (*OVX*). Day of pregnancy shown in (A) and (B) or relative to mating with male shown in (C). Grey box, pregnancy.

by comparing parental performance in ovariectomised females (Fig. 3.1, C) and females minimally exposed to pups (Fig. 3.1, B). I finally examined the long-term behavioural effects of pregnancy in a separate cohort of females.

3.2 Effects of pregnancy and pup exposure on parental behaviour

3.2.1 Parental behaviour improves during pregnancy

I first characterised parental behaviour in female mice of the *Preg* cohort (Fig. 3.1, A). I exposed females to pups at regular intervals before, during and after gestation, and longitudinally assessed paternal behaviours, including both pup-directed and non-pup-directed behaviours. In *Preg* females, we found that all 12 screened behavioural parameters change across testing days when compared to baseline performance before mating and subsequent pregnancy (*Vir*)(Fig. 3.2). Excluding duration of pup grooming (*Grooming time*), 11 out of 12 parameters change during pregnancy — i.e. at any point between pregnancy day 2 (D2) and day 18 (D18; Fig. 3.2, M). Duration of pup grooming was the only aspect that significantly changed after parturition from pregnancy day 22 (D22; Fig. 3.2, L and M), whereas duration of nest building (*Nest build time*) was the only aspect approaching baseline levels, and thus not changed compared to *Vir* performance, in the post-partum period (Fig. 3.2, C and M). Whilst some aspects already

diverged from *Vir* baseline early in pregnancy (Fig. 3.2, A to E and M), the majority of behaviours was significantly different in mid-to-late pregnancy (Fig. 3.2, F to K). At D18 — i.e. late pregnancy —, I observed changes in 10 out of 12 assessed parameters, the highest fraction across all testing days (Fig. 3.2, M). At D18, all females retrieved pups with a very short latency (34.3 ± 9.2 seconds at D18; 477.9 ± 143.3 seconds at Vir; mean ± SEM; Fig. 3.2, D), crouched above them (17.3 ± 3.5% of assay; Fig. 3.2, E), and spent most of the time in the nest with them (81.2 ± 4.8% of assay; Fig. 3.2, G). During gestation, parental performance therefore increases, with behavioural changes occurring throughout pregnancy. Interestingly, some aspects of parental behaviours remain changed until one month after parturition (D50). Specifically, 90% of pregnant females from the *Preg* group retrieved pups to the nest with a very short latency of 11.5 ± 2.1 seconds (Fig. 3.2, E), and stayed with them in the nest for 75.7% ± 6.6% of the assay (Fig. 3.2, D, E, J).

To test whether pregnancy hormones play a role in the observed behavioural adaptations, I next characterised parental behaviours in a group of repeatedly pup-exposed, ovariectomised (*OVX*) females, which are unable to produce ovarian hormones (Fig. 3.1, C). I compared their parental performance with that of repeatedly pup-exposed pregnant (*Preg*) females (Fig. 3.2, A to L). Across many aspects of parenting, behaviours of *Preg* females diverged significantly from those of *OVX* females, which exhibited a very poor maternal performance (Fig. 3.2, A-L). In particular, the percentage of *OVX* females that retrieved pups remained low throughout the testing period, significantly diverging from that of *Preg* females at pregnancy day 6 (D6), and never rising above 30% (Fig. 3.2, J). While all *Preg* females retrieved at D18, only 10% of *OVX* females did so at the equivalent time point (Fig. 3.2, J). Concomitantly, no significant decrease in retrieval latencies was noticeable (Fig. 3.2, E). In addition, although the largest fraction of crouching *OVX* females was observed at D18, this value was significantly lower than in *Preg* females (*OVX*, 30%; *Preg*, 100%; Fig. 3.2, H). Even after repeated

pup exposure in our assay, *OVX* females therefore do not reliably retrieve pups, crouch over them or spend time with them in the nest.

These results indicate that the increased parental performance is due to hormonal changes occurring during pregnancy, and that repeated pup exposure alone is not sufficient to trigger the onset of parenting. However, repeatedly exposing females to pups over many testing sessions could still affect maternal responsiveness (Herrenkohl and Lisk, 1973; Rosenblatt, 1967). The next set of experiments was aimed at addressing this concern.

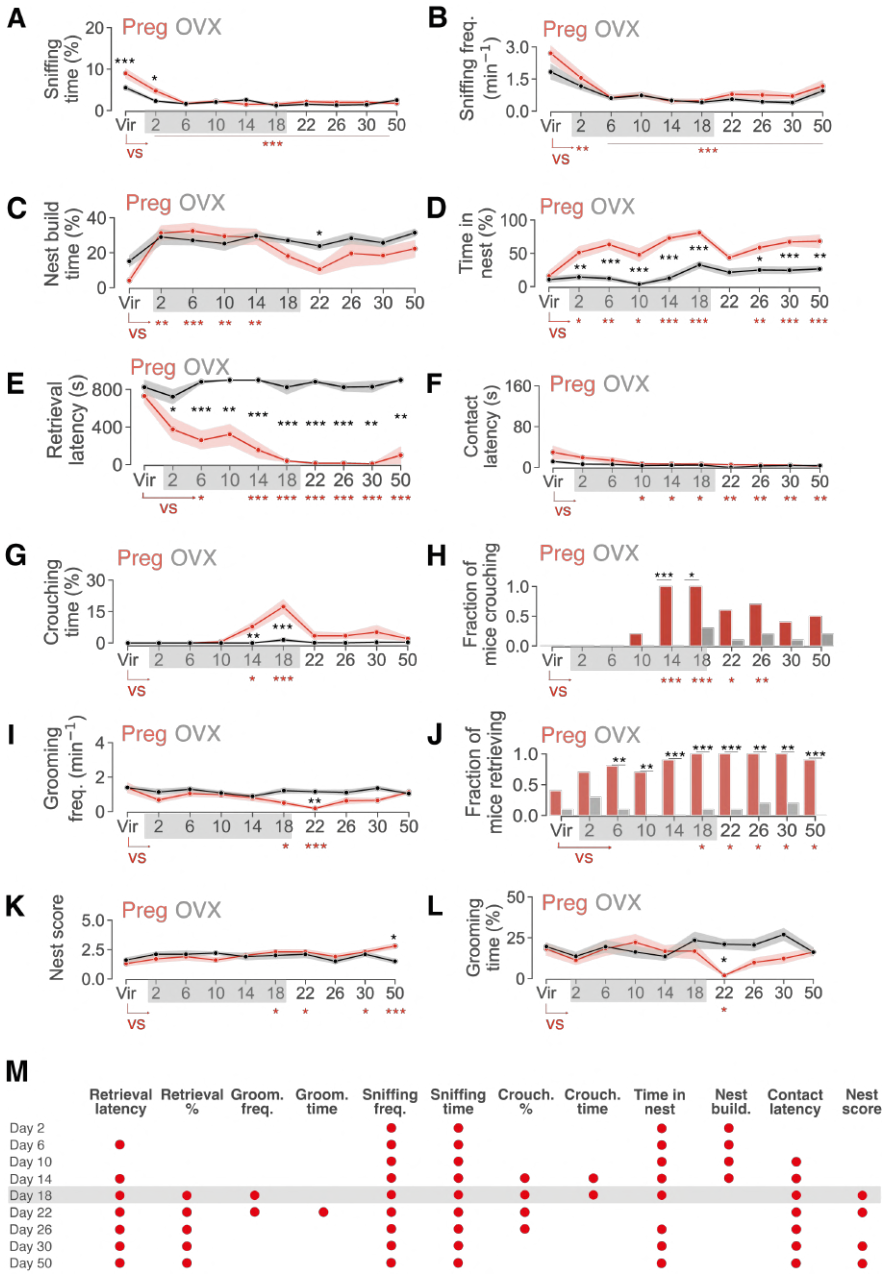

Figure 3.2: **Longitudinal quantification of parental behaviour in repeatedly pup-exposed females**. (**A** to **L**) Comparison of behavioural changes in repeatedly pup-exposed pregnant females (*Preg*, n = 10) and ovariectomised females (*OVX*, n = 10) across days. Grey box, pregnancy; solid lines in (A to F), (G), (I) and (K), mean; shaded area, SEM. Within-group [Preg, virgin (Vir) versus each subsequent timepoint; red asterisk] and between-group [(Preg versus OVX; black asterisks)] comparisons are shown. (**M**) Matrix of behavioural parameters and changes screened across days in *Preg* cohort. Red dot, significantly different from Vir timepoint; grey box, day with most number of behavioural changes. Statistics by repeated-measures ANOVA with Dunnett's *post hoc* test for within-group comparisons and one-way ANOVA with Tukey *post hoc* test for between-group comparisons in (A to D), (G), (I) and (K to L); by Kaplan-Meier survival analysis with log rank test in (E) and (F); by Fisher's exact test with Benjamini-Hochberg adjustment for multiple comparisons in (H) and (J). ***P $<$ 0.001; **P $<$ 0.01; *P $<$ 0.05.

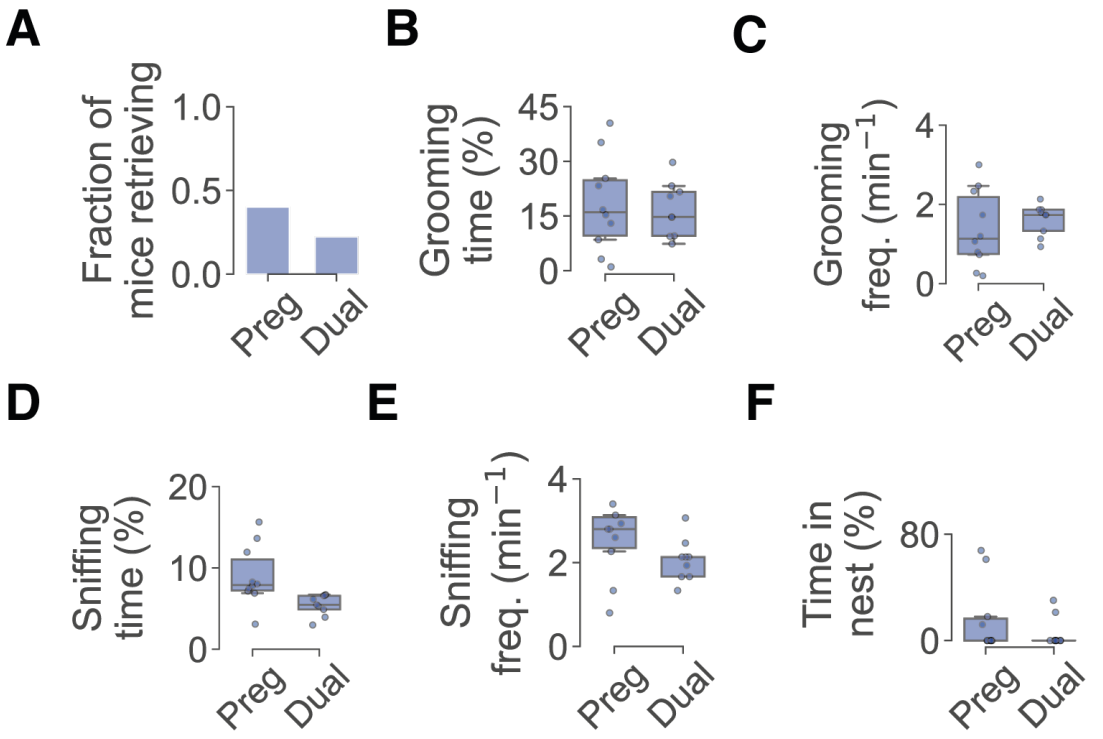

Figure 3.3: **Baseline parental behaviours in virgins**. (**A** to **F**) Comparison of a set of maternal behaviours at virgin (Vir) timepoint in *Preg* (n = 10) and *Dual* (n = 9) groups. Statistics by t-test in (B to F); by Fisher's exact test in (A). Error bars represent SEM.

3.2.2 Hormones are responsible for the onset of parenting

I next asked whether sensitisation due to repeated exposure to pups played a role in pregnancy-associated maternal behavioural changes. Sensitisation is indeed a well-documented phenomenon in which frequent exposure to pups facilitates parental responsiveness in adult rodents (Herrenkohl and Lisk, 1973; Rosenblatt, 1967). The lack of behavioural changes in the repeatedly pup-exposed *OVX* cohort already indicates that this is not the case. However, the neural mechanisms underpinning sensitisation remain largely unknown. Sensitisation to pups was previously reported in ovariectomised females (Stolzenberg and Rissman, 2011), but the frequency and length of pup exposure was substantially different from that employed here. To conclusively exclude potential effects of pup exposure-mediated changes from our observations, I further quantified maternal behaviours in a group of intact females exposed to pups only twice, at Vir and D18 (*Dual*; Fig. 3.1, B). I then compared behaviours at D18 in *Preg* and *Dual* groups to

assess whether, despite the different pup exposure frequency, both cohorts could reach comparable levels of parental performance.

In order to isolate the effects of pup exposure on D18 behaviours, I first compared a set of baseline behaviours at the virgin (Vir) timepoint in both cohorts to rule out batch effects (Fig. 3.3). Figure 3.3 shows the percentage of time virgin females spent grooming and sniffing pups and in the nest with them, as well as frequency of these behaviours and the fraction of animals that retrieved pups to the nest. I did not find any significant behavioural differences between *Preg* and *Dual* groups across any of these behaviours (Fig. 3.3). Importantly, not only did the absence of baseline differences allow us to proceed with between-group comparisons at D18, but also allowed me to pool virgin behaviours from both groups (Vir, Fig. 3.4).

I focussed on Vir and D18 timepoints in order to compare behavioural performance at baseline (Vir) and at the pregnancy stage in which parenting is maximally changed (D18; Fig. 3.2, M). I found that all parameters but contact latency (Fig. 3.4, F) and duration of pup grooming (Fig. 3.4, L) change significantly between Vir and at least one D18 timepoint, either from *Preg* or *Dual* group (Fig. 3.4). Among these, while some aspects of maternal behaviour were predominantly driven by frequent pup exposure, others were mainly affected by pregnancy-associated hormonal changes. Specifically, D18 females from all three groups spent significantly less time sniffing pups than virgins (Fig. 3.4, A), and they did so less frequently (Fig. 3.4, B). This suggests that sniffing behaviour, both in duration and frequency, is equally affected by both pregnancy hormones and pup exposure, since no significant difference was observed between pregnant females — regardless of their pup exposure frequency — and OVX females (Fig. 3.4, A, B). By contrast, time in nest (Fig. 3.4, D), retrieval (Fig. 3.4, E, J) and crouching (Fig. 3.4, G, H) show a similar pattern of changes. These three parameters significantly change between Vir and D18 of both *Preg* and *Dual* groups, regardless of pup exposure. Concurrently, for these parameters, *OVX* females

display a virgin-like behaviour at D18, despite being repeatedly exposed to pups. These findings indicate that retrieval, crouching and time spent in the nest are aspects of maternal behaviour mainly affected by pregnancy hormones, but not frequent pup exposure.

Therefore, out of the 12 behavioural aspects initially screened, I selected 5 which were significantly and selectively affected by pregnancy: time spent in nest with pup(s), pup retrieval (latency and fraction of animals) and crouching (duration and fraction of animals). I focused on these behavioural parameters in the subsequent experiments outlined in this thesis.

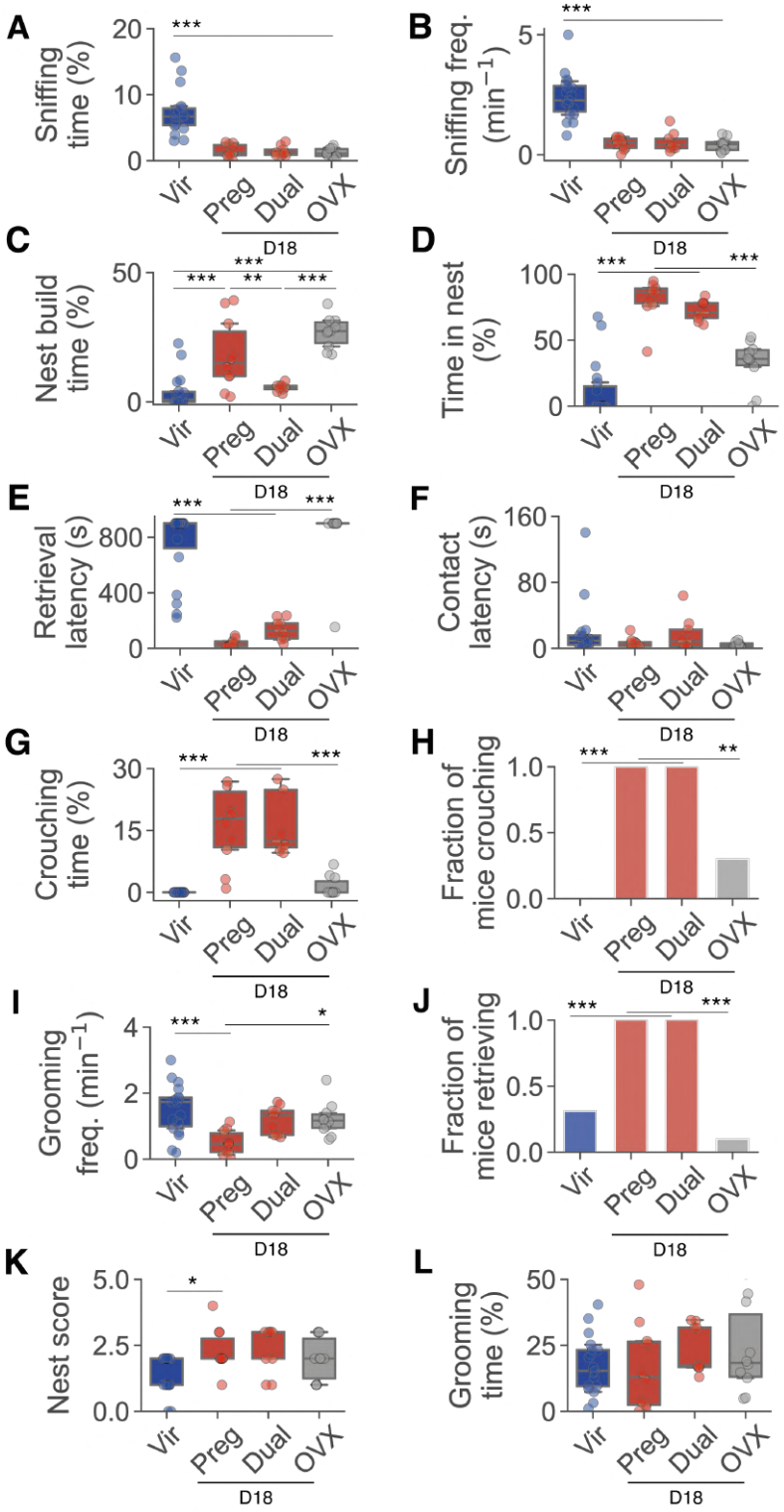

Figure 3.4: **Effects of pup exposure on pregnancy-associated maternal behaviours**. (**A** to **L**) Comparison of maternal behaviours at Vir and D18 in *Preg* (n = 10), *Dual* (n = 9) and *OVX* (n = 10) groups. Behaviour of virgins (Vir) was pooled from *Preg* and *Dual* groups, n = 19). Statistics by one-way ANOVA with Tukey *post hoc* test in (A to D), (G), (I), (K) and (L); by Kaplan-Meier survival analysis with log rank test in (E) and (F); by Fisher's exact test with Benjamini-Hochberg adjustment for multiple comparisons in (H) and (J). Error bars in (A to G), (I), (K) and (L) represent SEM. ***P $<$ 0.001; **P $<$ 0.01; *P $<$ 0.05.

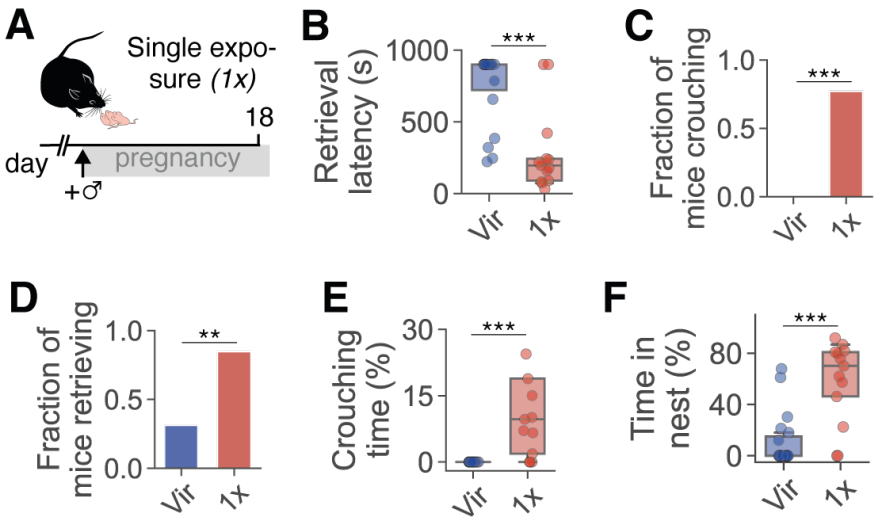

Figure 3.5: **Pregnancy-associated parental behaviours in females exposed to pups once**. (**A**) Timeline of behavioural assay for females exposed to pups once at D18 (*1x*). (**B** to **F**) Comparison of parental behaviours between virgins (Vir) and pregnant females exposed to females once at D18 (*1x*). Behaviour of virgins pooled from *Dual* and *Preg* groups (n = 19) and *1x* females (n = 13). Statistics by Kaplan-Meier survival analysis with log rank test in (B); by Fisher's exact test in (C) and (D); by t-test in (E) and (F). Error bars in (B), (E) and (F) represent SEM. ***P $<$ 0.001; **P $<$ 0.01; *P $<$ 0.05.

Finally, in order to exclude any potential effects of prior pup exposure, I assessed a group of pregnant females exposed to pups only once, at D18 (*1x*; Fig. 3.5, A). *1x* females displayed changes across the full set of pregnancy-induced behavioural parameters (Fig. 3.5, B-F). These results demonstrate that pregnancy induces parental behaviour in mice.

3.2.3 Pregnancy-induced changes to behavioural sequences

In the previous sections, I demonstrated that individual aspects of parental behaviour are affected by pregnancy, and that these behavioural adaptations, most of which last until after pup weaning, cannot be induced by repeated or prior pup exposure.

In addition to quantifying individual behavioural parameters, I assessed sequences of behaviours. I did this by computing behavioural transition matrices for virgins (Vir) and D18 females from the *Preg* group (Fig. 3.6). By representing the likelihood of a future behaviour given the animal's current action, transition matrices enable to compare sequences of motor actions between groups of animals, thus providing a complementary level of behavioural characterisation.

I found that virgin females spend most of their time performing pup grooming-sniffing-nest entering sequences, and were more likely than D18 females to transition between these three behaviours. By contrast, D18 females engaged in novel behavioural sequences comprised of pup grooming, sniffing, retrieval, nest building and crouching (Fig. 3.6). One of the most interesting insights is the transition from sniffing to retrieval, which becomes more likely in pregnant females compared to virgins. I previously showed that pregnant females spent significantly less time sniffing pups than virgins, and they did so less frequently (Fig. 3.4, A, B). This indicates that D18 females might need to chemoinvestigate less and proceed to retrieve pups faster than virgins.

In conclusion, behavioural state transition diagrams enable for deeper understanding

In summary, I have demonstrated that:

1. specific aspects of maternal behaviour change during pregnancy;
2. these parental behaviours are maximally changed at D18;
3. the hormonal milieu of pregnancy, and not previous pup exposure, contributes to the onset of parenting;

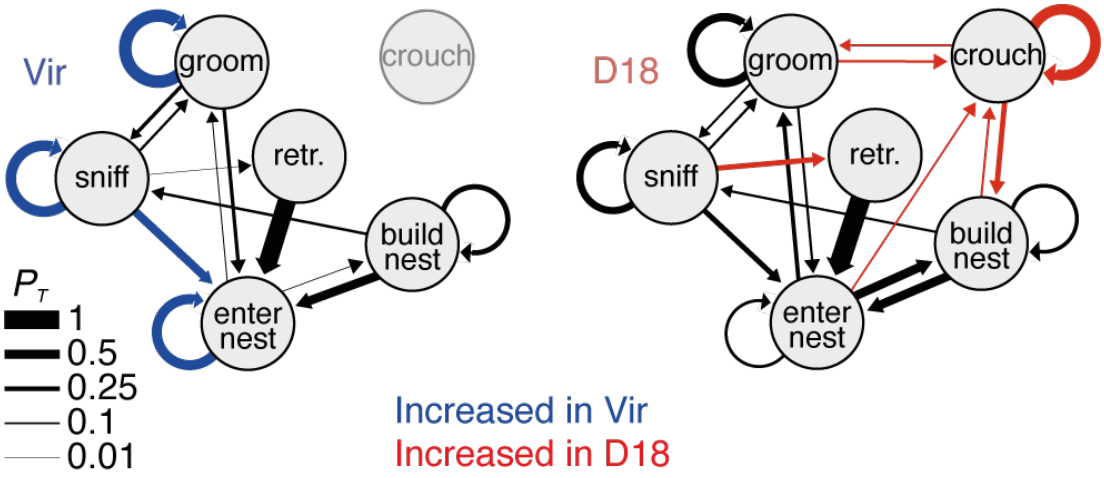

Figure 3.6: **Late pregnant females engage in different behavioural sequences**. Behavioural state transition diagrams for virgin (Vir) and late pregnant (D18) females from *Preg* cohort (n = 10). P_T, average transition probabilities. Highlighted are differences between Vir and D18 if P < 0.05 (U test). retr., retrieval; groom, grooming; sniff, sniffing; crouch, crouching; build nest, nest building; enter nest, entering nest.

4. pregnancy-induced behavioural changes persist at least one month after parturition;

5. entire behavioural sequences are affected by pregnancy.

3.3 Long-term behavioural adaptations induced by pregnancy

My characterisation of parental behaviours suggested that some aspects of parenting remain for at least one month after parturition, at D50.

I next investigated how long pregnancy-induced behavioural adaptations persist in a group of females (Long-term exposure, *Lex*; Fig. 3.7, A). I quantified time in nest with pups, retrieval latencies and fraction of retrievers at D18, D50, and subsequently every 3 months until pregnancy day 270 (D270), which corresponds to 8 months after parturition. Given a median lifespan of laboratory C57BL/6 mice of 27 months, this allows for a robust assessment of long-term behavioural effects.

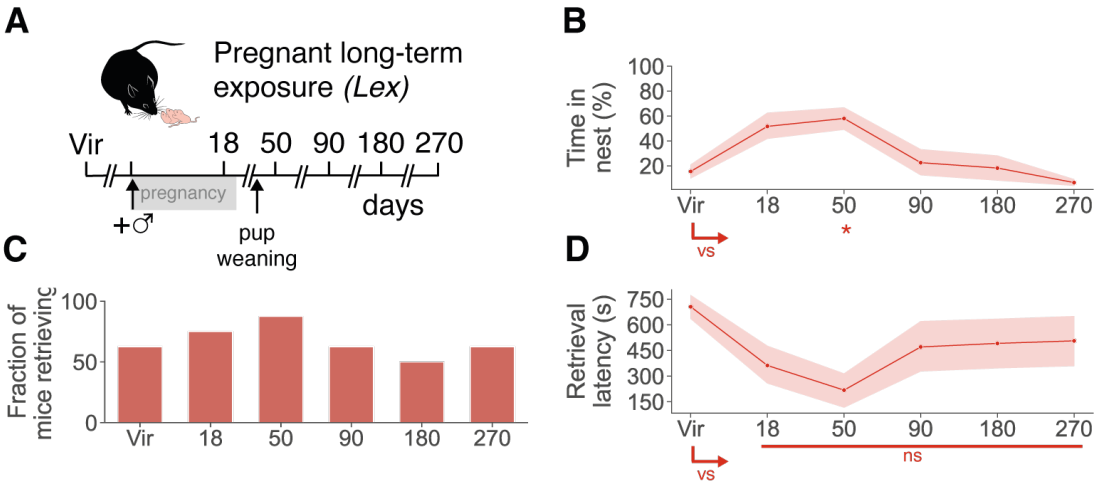

Figure 3.7: **Long-term effects of pregnancy in repeatedly pup-exposed females**. (**A** to **D**) Quantification of behavioural parameters in repeatedly pup-exposed pregnant females (*Lex*, n = 8) across days. (**A**) Timeline of behavioural assay for females exposed to pups until day 270. Grey box, pregnancy; solid lines in (B and D), mean; shaded area, SEM. Within-group [Preg, virgin (Vir) versus each subsequent timepoint; red asterisk] comparisons are shown. Statistics by repeated-measures ANOVA with Dunnett's *post hoc* test for within-group comparisons in (B); by Kaplan-Meier survival analysis with log rank test in (D); by Fisher's exact test with Benjamini-Hochberg adjustment for multiple comparisons in (C). ***P $<$ 0.001; **P $<$ 0.01; *P $<$ 0.05.

Surprisingly, despite an increase between Vir and D50 timepoints, the fraction of retrievers was not find significantly changed (Fig. 3.7, C). As expected, females spent more time in the nest with pups at D18 and D50 (Fig. 3.7, B), and retrieved with shorter latencies (Fig. 3.7, D). Subsequently, however, parental performance returned to baseline (Vir) levels. Time spent in the nest with pups progressively declined (Fig. 3.7, B). Similarly, retrieval latencies gradually increased after D50 (Fig. 3.7, D). Because of an uncharacteristically low parental performance in late pregnancy in this cohort of mice, I cannot draw firm conclusions regarding the long-term effects of pregnancy on maternal behaviour. Nevertheless, I observed a trend of improving parental performance until D50, in accordance with my previous findings. I also report a trend of decreasing maternal levels later in the post-partum period.

3.4 Discussion

While behavioural changes associated with motherhood have been extensively investigated in female rats, it had been unclear when pregnancy-associated maternal adaptations arise in female mice, how long they persist and which behavioural aspects are affected.

The aim of this chapter was to characterise parenting in laboratory female mice to determine which aspects of maternal behaviour are primarily driven by pregnancy. I confirmed that the onset of maternal behaviour occurs already during pregnancy in mice. By late pregnancy, several pup-directed behaviours maximally diverge from their virgin levels. Pregnant females retrieve pups to the nest faster and spend more time with them. Using minimal pup exposure, I determined that pregnancy, but not repeated pup exposure, indeed underlies parenting onset. By assessing pup-directed behaviours in ovariectomised animals, I further confirmed that frequent pup exposure alone is not sufficient to drive behavioural adaptations, and that ovarian hormones are necessary for the onset of parental behaviour. Additionally, I found that entire behavioural sequences are affected by the hormonal milieu of pregnancy. Finally, although pregnancy-induced behavioural changes persisted till late post-partum, I did not find statistically significant effects in our long-term assessment of parental behaviour. Given the prolonged single-housing of this cohort, the low parental performance months after parturition could be due to increased stress (Hatch et al., 1963). Moreover, the uncharacteristically high parental responsiveness at baseline timepoint might have masked any statistical significance for behavioural changes during pregnancy.

Unexpectedly, I did not observe pup exposure-induced parental changes in the ovariectomised cohort. Sensitisation is well-documented behavioural phenomenon which results from repeatedly exposing female rodents to pups. The paradigm used to facilitate parental behaviour via sensitisation generally involves longer and more frequent pup exposures than the assays used here. While females were

here exposed to pups for 15 minutes every 4 days, sensitisation typically requires daily 2-h exposure for 4 days (Stolzenberg and Rissman, 2011). Similar to my findings, a previous study found that a 20-min pup exposure session with 4-day intervals was not enough to to trigger statistically shorter latencies (Okabe et al., 2017).

I found that 40% of virgin females retrieve pups (*Preg* group). As discussed in sections 1.3 and 3.1, in contrast to female rats which typically ignore or avoid pups, virgin female mice exhibit hormone-independent spontaneous alloparental care during their first exposure to pups; this is especially true for laboratory animals (Chalfin et al., 2014; Kuroda et al., 2011; Numan, 2020). Therefore, it would be expected to observe a higher fraction of parental virgin females. However, other recent studies also reported average retrieval rates of 30% for virgin females during their first pup exposure (Kohl et al., 2018; Marlin et al., 2015), and rates of <40% were observed by others (Wu et al., 2014), in line with our findings. Interestingly, one study reported that none of virgins exhibited pup retrieval at first exposure to pups, effectively observing low parental performance (Carcea et al., 2021). In general, a certain degree of variability between experimental cohorts is expected. This held true for our behavioural assays. Only 10% of ovariectomised virgins retrieved pups to the nest, the lowest rate amongst all the cohorts assessed in this study. In contrast, virgins from the *Lex* cohort were more parental, as 50% of them retrieved. The reasons behind the inter-group variability in spontaneous maternal behaviour are still unclear, but subtle initial differences in levels of parental care that animals received as infants or differences in animal husbandry might contribute. Housing conditions of animals prior to the first testing day might be particularly important. High retrieval rates have indeed been observed for animals that were group-housed until the first pup exposure during the behaviour assay (Stolzenberg and Rissman, 2011), whereas animals that were single-housed for one week before testing exhibited retrieval rates comparable to those in this study — i.e. ca. 30-40% — (Kohl et al., 2018;

Wu et al., 2014) or even lower (Carcea et al., 2021). Because housing protocols in this study were standardised across groups, we also explored the possibility that the variability in spontaneous behaviour could be due to stress. This might play a crucial role in mice undergoing long recovery surgeries prior to behavioural testing, such as craniotomies and intracranial viral injections. Findings of stress phenotype will be presented in the following chapter, where comparisons with animals receiving viral vector-mediated hormone receptor deletion will be drawn. No stress phenotype was detected in groups of mice undergoing different types of surgery, and displaying different initial retrieval rates at baseline, suggesting that inter-group variability in this study might stem from subtle differences in animal husbandry or parental care during development.

While none of our experimental groups displayed retrieval rates of $\geq$50%, this was not the case for the *Lex* cohort, where 62.5% of females retrieved at least one pup to the nest. Unfortunately, this high parental baseline resulted in the absence of significant differences in the longitudinal assessment of pup-directed behaviours. Even at D50, when all but one animal retrieved with very short latency, no statistical difference was observed. In line with my previous findings, a trend of improving maternal performance was observed until D50. By contrast, parental behaviour started to decrease after D90. However, because parental behaviour was not maximally changed in late pregnancy, and no statistical difference was detected longitudinally due to high baseline maternal responsiveness, it is currently challenging to draw strong conclusions. Additional behavioural testings with an experimental cohort displaying typical virgin-like behaviours will be needed to characterise the long-term effects of pregnancy on parenting.

In summary, in this chapter I established that the hormonal milieu of pregnancy leads to changes in specific aspects of parental behaviour, which maximally diverge from virgin-like pup-directed behaviour in late pregnancy. Because most behavioural changes persisted until D50, when when pregnancy- and parturition-associated hormonal fluctuations have subsided, I reason that such behavioural

adaptations are the result of long-lasting remodelling of parenting circuits by hormones.

In the next chapter, I examine the necessity of the ovarian steroid hormones estradiol and progesterone for the onset of parenting during pregnancy, and investigate how these hormones remodel MPOA neurons.

3.5 Author contributions

Francesco Monaca and Johannes Kohl designed the experiments and the analysis. Francesco Monaca performed behavioural experiments. Francesco Monaca and Rachida Ammari performed manual behavioural annotation. Francesco Monaca and Johannes Kohl wrote custom Python code for behavioural analysis. Francesco Monaca performed behavioural analysis.

Chapter 4

Pregnancy hormone action on MPOA neurons

4.1 Introduction

In the previous chapter, I addressed which aspects of parental behaviour are affected by pregnancy, and established late pregnancy as a critical time period for such changes. As already mentioned, the MPOA is an ideal candidate area to examine the neural underpinnings of pregnancy-induced behavioural changes. In addition to being a crucial hypothalamic hub in the regulation of parental behaviour (Numan, 2006, 2003), the MPOA is also is a key hormonal target. Several studies have indeed demonstrated that MPOA stimulation with the steroid hormones estradiol (E2) and progesterone (P4), as well as with prolactin (Prl) and oxytocin (Oxt), elicits parental behaviour in virgin rodents (Bridges and Freemark, 1995; Fahrbach and Pfaff, 1986; Numan et al., 1977). However, how these hormones mediate pregnancy-induced parental responses is still unclear. Additionally, the effect of hormonal action on MPOA neurons had been unknown. In this chapter, I, together with Rachida Ammari — who performed electrophysiological recordings — and Johannes Kohl — who analysed hormone receptor expression —, investigated these outstanding questions. I first probed the expression of pregnancy hormone receptors in the MPOA. Mouse lines carrying floxed estrogen and progesterone receptor genes were generated. I therefore assessed pup-directed behaviour after hormone receptor ablation from the MPOA. I also examined whether the hormone sensitivity of MPOA^{Gal} neurons, a subpopulation critical for parenting (Kohl et al., 2018; Wu et al., 2014), is necessary for pregnancy-mediated parental adaptations. Finally, to explore how hormones affect this neuronal population, slice electrophysiology in intact and receptor-deleted MPOA^{Gal} neurons was performed to assess biophysical and morphological changes induced by pregnancy.

4.2 Expression of pregnancy hormone receptors in the MPOA

Since parental behaviour can be induced by administration of E2, P4, Prl and Oxt (Bridges and Ronsheim, 1990; A. D. Mayer et al., 1990; Moltz et al., 1970; Pedersen et al., 1982; Riddle et al., 1935), I hypothesised that the underlying neural substrate must also be sensitive to these hormones. E2 and P4 act on their intracellular cognate receptors, Esr1 and PR, respectively (Brinton et al., 2008; Ogawa et al., 1998). E2 also binds to another intracellular receptor, Esr2. Esr1, however, and not Esr2, has been extensively implicated in the regulation of several social behaviours, including parenting (Ogawa et al., 1998). Oxt and Prl exerts their function through their respective receptors, Oxtr and Prlr. Both have also been implicated in parental behaviour (Brown et al., 2017; Takayanagi et al., 2005). I therefore quantified the expression of receptors for the four canonical pregnancy hormones — Esr1, PR, Prlr and Oxtr.

Single-molecule fluorescent *in situ* hybridisation (smFISH) is a particularly powerful technique to detect RNA with sub-cellular resolution. This approach allows to image multiplexed gene expression, revealing abundance and spatial distribution of transcripts within individual cells. By performing smFISH on brain slices which were then registered to the Allen Brain Atlas, I measured expression and distribution of four receptor transcripts — Esr1, PR, Prlr, Oxtr — in hypothalamic areas (Fig. 4.1, A and B). NeuN and DAPI co-staining was used to detect nuclei and cell bodies, respectively. A threshold of $\geq$3 Esr1 transcripts per cell was employed to label a neuron Esr1-positive, while a threshold of $\geq$5 transcripts per cell was chosen to detect PR-, Prlr- or Oxtr-positive neurons. Criteria to establish thresholds were based on ground-truth data obtained by immunostaining against Esr1 in MPOA sections (Fig. 4.1, D) or on published data when receptor antibodies were commercially unavailable (see Methods).

I found that, amongst hypothalamic areas, MPOA sub-regions are highly en-

riched in neurons expressing Esr1, PR and Prlr. According to the Allen Mouse Brain Common Coordinate Framework, the medial preoptic area (MPO), parastrial nucleus, (PS), anterodorsal preoptic nucleus (ADP), PD and MPN are subregions of the MPOA (Q. Wang et al., 2020). Esr1 and PR are most enriched in two MPOA sub-regions: 72.44% ± 8.51% of neurons in the PD and 52.89% ± 4.01% of neurons in MPN express Esr1 (mean ± SEM; Esr1, Fig. 4.1, E). Esr1-expressing neurons were also present in other MPOA sub-regions (MPO, 28.29% ± 3.69%; PS, 20.09% ± 2.94%; ADP, 17.26% ± 5.31%; Esr1, Fig. 4.1, E).

Similarly, PR is highly expressed in the PD and MPN (PD, 74.56% ± 7.23%; MPN, 63.94% ± 1.19%), as well as in the MPO (52.57% ± 3.54%), ADP (50.92% ± 8.19%) and PS (46.95% ± 8.01%; PR, Fig. 4.1, E). Prlr is also highly expressed by MPOA sub-regions (Prlr, Fig. 4.1, E).

By contrast, very few neurons in the MPOA express Oxtr. Only 7.69% ± 2.86% of neurons in the PD and 7.49% ± 2.75% of neurons in the MPN expressed Oxtr, while even fewer Oxtr-expressing neurons were found in other MPOA sub-regions (Oxtr, Fig. 4.1, E).

Finally, while neurons expressing both Esr1 and PR occur in most hypothalamic areas, MPOA sub-regions are most enriched in Esr1/PR co-expressing neurons: 55% ± 11.35% of neurons in the PD and 37.64% ± 3.45% of neurons in the MPN co-express Esr1 and PR, and 17.34% ± 3.53% neurons in the MPO did. Lower expression levels are found in the PS and ADP (PS, 8.67% ± 2.58; ADP, 8.19% ± 3.78%; Fig. 4.1, C).

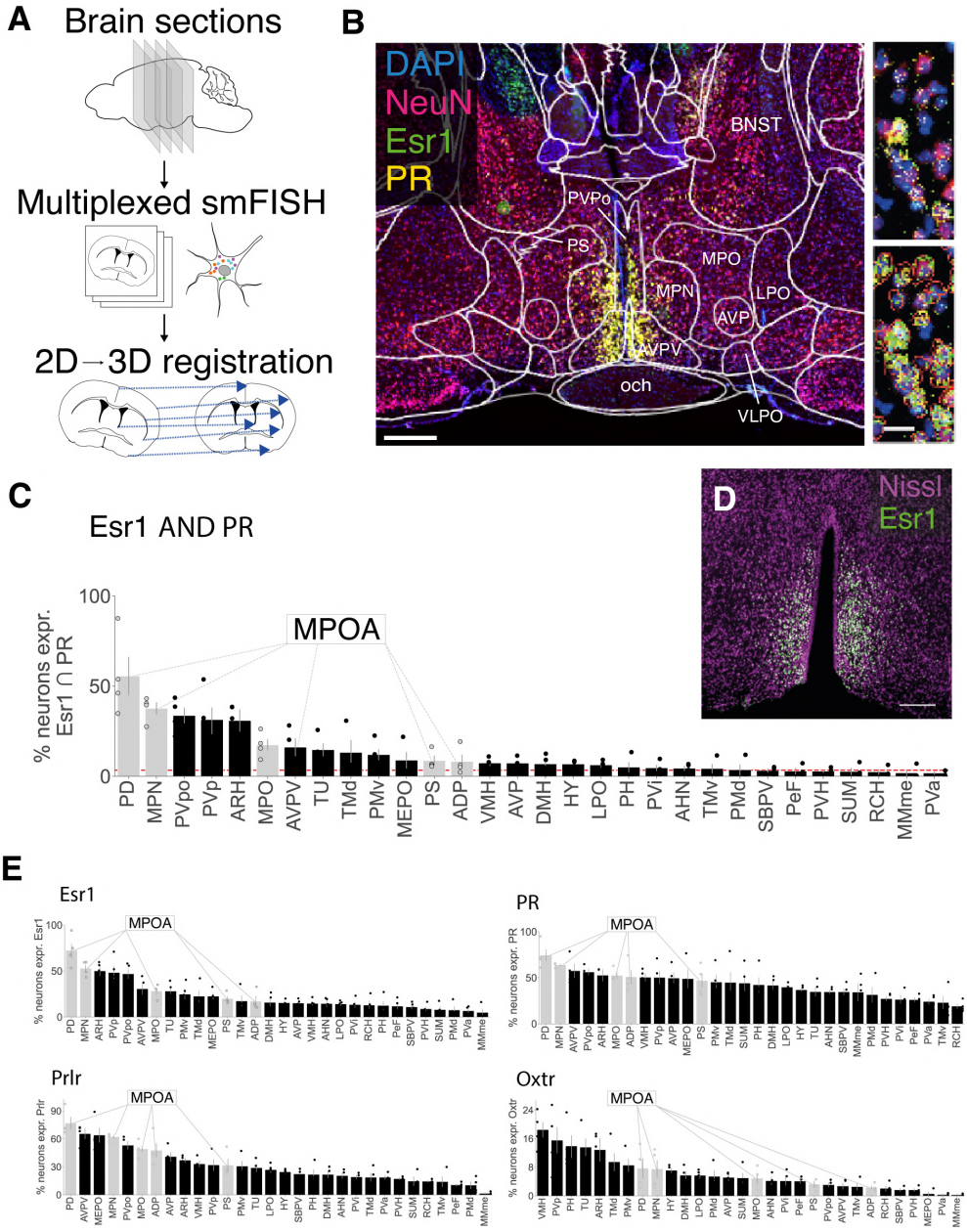

Figure 4.1: **Expression of pregnancy hormone receptors in the hypothalamus**. (**A**) Experimental strategy for multiplexed smFISH detection of mRNA hormone receptors, and subsequent brain section registration to brain atlas. (**B**) Representative brain section with Esr1 and PR transcripts, NeuN and DAPI staining. Lateral panels highlight sub-cellular transcript detection. Scale bars are 500 μm in main panel, 20 μm in lateral panels. (**C** to **E**) Quantification of neurons expressing the four canonical pregnancy hormones across different hypothalamic areas (n = 4 mice, 2 virgins, 2 D18). (**C**) Percentage of neurons co-expressing Esr1 and PR mRNA. Red dotted line indicates brain-wide average. (**D**). Immunohistochemistry brain section with Esr1 and NeuN staining. Scale bar is 200 μm. (**E**) Percentage of neurons expressing Esr1, PR, Prlr or Oxtr mRNA. Error bars indicate SEM. MPOA areas are indicated in grey. Abbreviations for brain regions are listed in "Table of Acronyms".

4.3 Ablation of pregnancy hormone receptors

I demonstrated that the MPOA is enriched in neurons expressing Esr1, PR and Prlr, as well as in those co-expressing Esr1 and PR, but not Oxtr. Next, I examined whether MPOA hormonal sensitivity is required for the onset of pregnancy-induced parental behaviours. Given that the co-administration of estradiol and progesterone is most effective in eliciting parenting in virgin females (Rosenblatt et al., 1988) and that the MPOA is highly enriched in Esr1/PR co-expressing neurons, I focussed on Esr1 and PR and tested whether deletion of these two receptors from the MPOA disrupts pregnancy-induced maternal behaviours.

4.3.1 Validation of experimental approaches

I first tested the efficacy of viral-genetic hormone receptor deletion in the MPOA. I injected either adeno-associated viruses (AAVs)-expressing either Cre recombinase or a green fluorescent protein (GFP) unilaterally into the MPOA of mice carrying floxed Esr1 or PR alleles (Fig. 4.2, A). By performing immunostaining against Esr1, I found that the number of Esr1-positive neurons in Cre-injected animals (KO) is significantly lower than in GFP-injected controls (*fraction of Esr1-positive neurons in injected/non-injected hemispheres*; KO, 0.11 ± 0.02; ctrl, 0.96 ± 0.26; mean $\pm$ SEM; Fig. 4.2, B-D). Because a good PR antibody was not available at the time of the experiment, I also tested whether this ablation approach disrupts receptor transcript expression. By performing smFISH on Cre- and GFP-injected MPOA of Esr1-floxed and PR-floxed animals, I observed a significant reduction of both Esr1- and PR-positive neurons in KO brain slices compared to controls (Fig. 4.2, E-H). This approach therefore effectively ablates receptors from MPOA neurons.

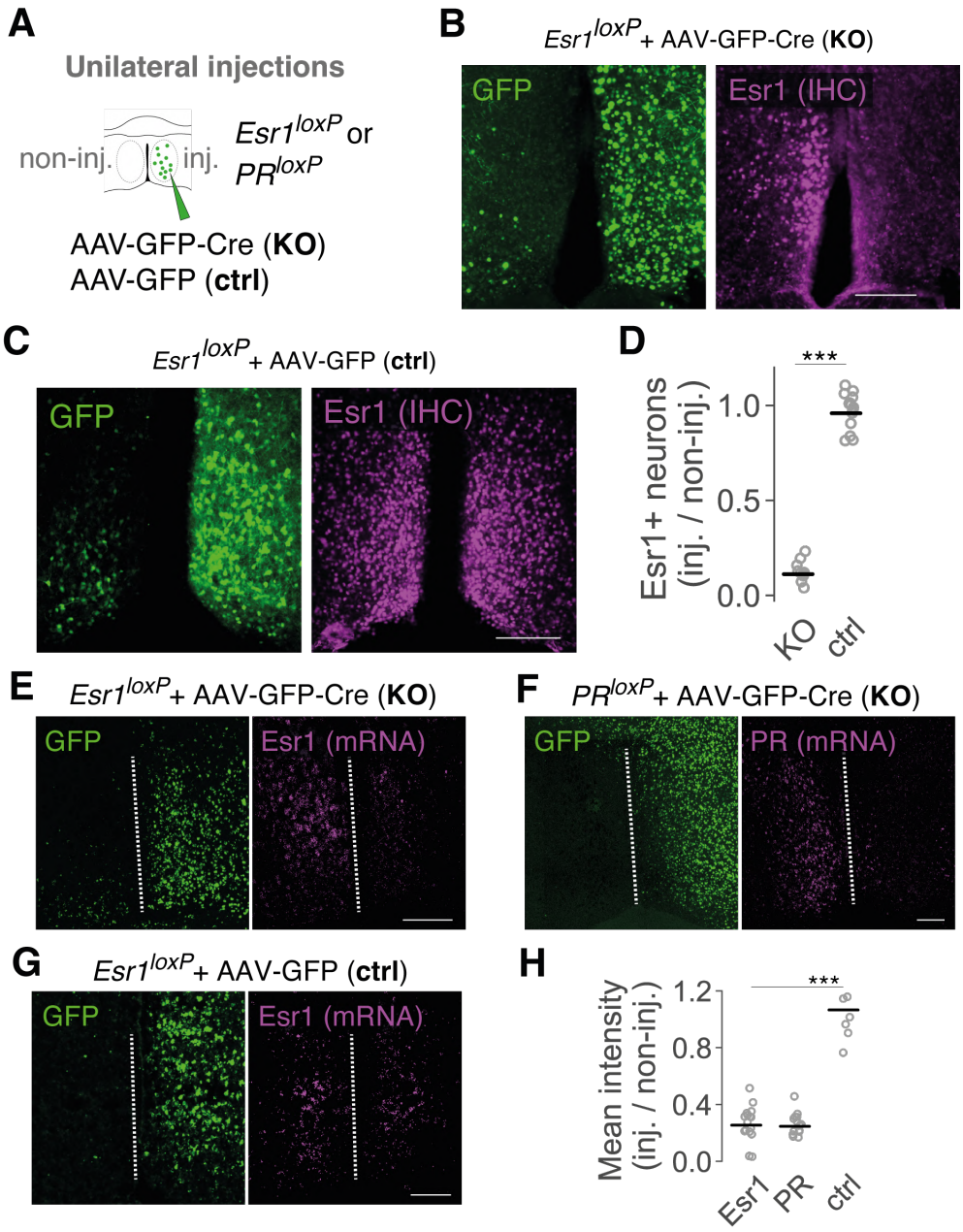

Figure 4.2: **Validation of AAV-mediated receptor deletion**. (**A**) Experimental strategy for unilateral ablation of hormone receptors from the MPOA (KO) and control (ctrl). (**B** and **C**) Representative MPOA sections of injected (right) and non-injected (left) hemisphere in Esr1-deleted (KO, B) and negative control (ctrl, C) animals with Esr1 immuno-staining. Scale bars are 200 μm. (**D**) Quantification of successful Esr1 deletion in KO and ctrl animals (n = 3 animals per group, 14 sections each). (**E** to **G**) Representative MPOA sections of injected (right) and non-injected (left) hemisphere in Esr1-deleted (KO, E), PR-deleted (KO, F) and negative control (ctrl, G) obtained by smFISH detection against hormone receptor transcripts and GFP. Scale bars are 200 μm. (**H**) Average staining intensity of injected vs. non-injected hemisphere across Esr1 and PR KO animals, and controls (n = 2 animals per group, 15 sections each). Statistics by Mann-Whitney U test in (D); by one-way ANOVA with Tukey *post-hoc* test in (H). ***P $<$ 0.001. All data obtained from D18 females.

4.3.2 Conditional deletion of hormone receptors across the MPOA

I first performed pan-neuronal AAV-mediated receptor ablation in the MPOA. Given the occurrence of viral expression in regions neighbouring the MPOA, such as AVPV, that I had observed for viral ablation validation (Fig. 4.2, B,C), only animals with AAV expression confined to the MPOA were considered for behavioural testing (Fig. 4.3, H). I performed my parental behaviour assay for virgin and D18 females and quantified pregnancy-modulated behavioural aspects. I observed a very low percentage of Esr1-deleted ($Esr1^{KO}$) and PR-deleted (PR^{KO}) virgins retrieving at least one pup to the nest ($Esr1^{KO}$ Vir, 14%; PR^{KO} Vir, 12%; Fig. 4.3, C). Similarly, none of the control virgins displayed retrieval behaviour (ctrl Vir; Fig. 4.3, C). Surprisingly, while all pregnant controls retrieved (ctrl D18; Fig. 4.3, C) with a latency of 195.2 ± 54.2 seconds (mean ± SEM; Fig. 4.3, B), only 14% of $Esr1^{KO}$ and 22% of PR^{KO} mice did at D18 (Fig. 4.3, C). Similarly, crouching behaviour was abolished in both receptor-deleted pregnant groups (Fig. 4.3, D and E). No difference was found in the time spent in the nest with pups between virgins and D18 females of both receptor-deleted groups (Fig. 4.3, F). By contrast, pregnant controls spent significantly more time in the nest compared to their virgin counterparts (ctrl D18, 55.1% ± 10.7%; ctrl Vir, 1.7% ± 1.6% of assay duration; Fig. 4.3, F). Interestingly, in some cases, I also observed the presence of pup-directed aggressive behaviour (see Methods). While this was not observed in control and $Esr1^{KO}$ virgins, several pregnant $Esr1^{KO}$ and PR^{KO} bit or aggressively groomed pups ($Esr1^{KO}$ D18, 2/7; PR^{KO} D18, 2/9; Fig. 4.3, G), while the remaining animals avoided pups or displayed affiliative pup-directed behaviours (Fig. 4.3, G). Finally, a small percentage of PR^{KO} virgins also showed pup-directed aggression (PR^{KO} Vir, 1/9; Fig. 4.3, G).

Ablation of Esr1 or PR in the MPOA thus abolishes the pregnancy-induced upregulation of parental performance in the late stages of gestation.

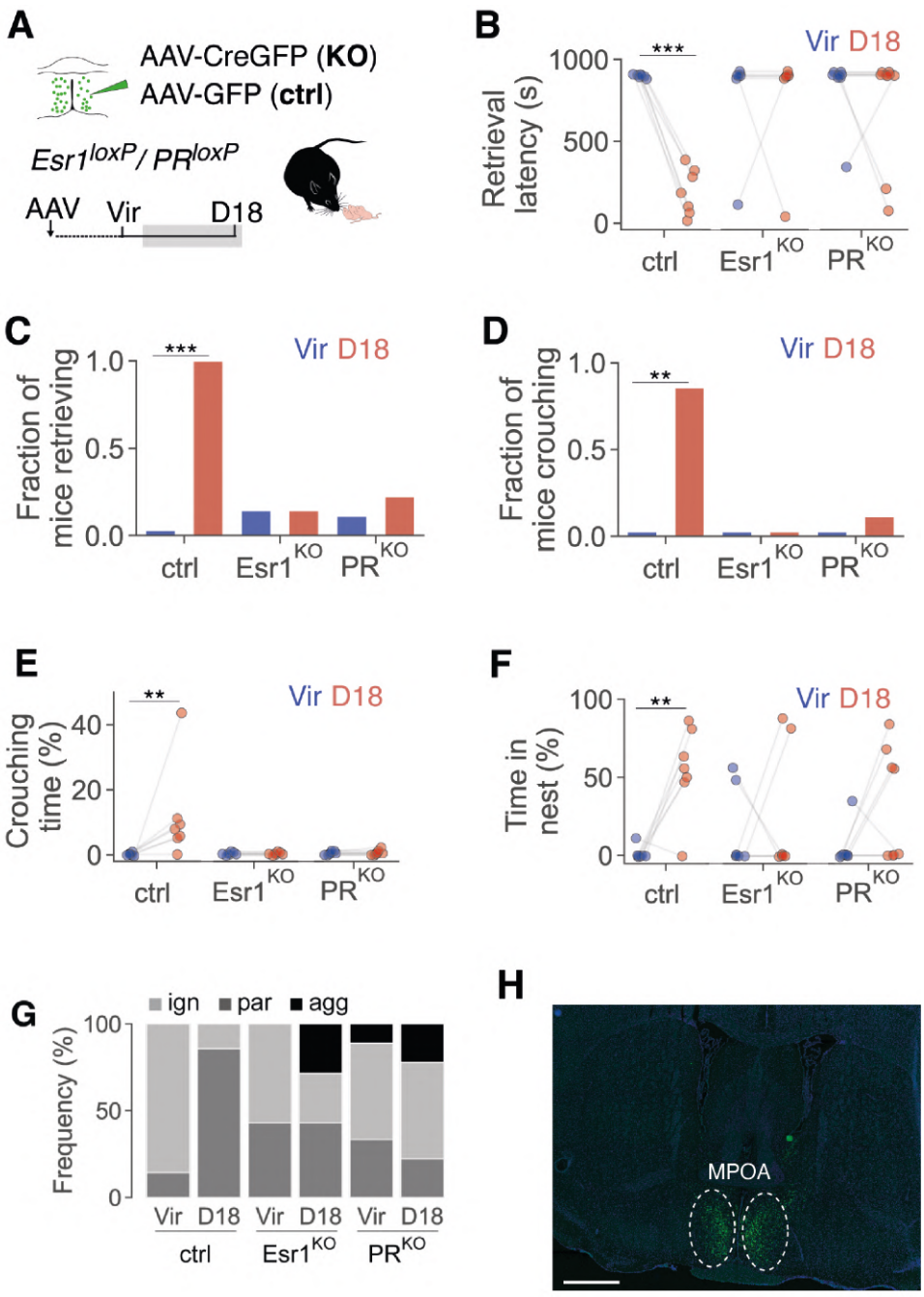

Figure 4.3: **Behavioural effects upon ablation of pregnancy hormone receptors from MPOA neurons**. (**A**) Timeline of behavioural assay for females with pan-MPOA AAV-mediated Esr1 (Esr1^{KO}) or PR (PR^{KO}) deletion (n = 7, 9), and controls (n = 7). Grey box, pregnancy. (**B** to **F**) Quantification of pup-directed parameters in Esr1^{KO} and PR^{KO} virgin (Vir) and pregnant (D18) females, and controls. (**G**) Proportion of ignoring (ign), parental (par) or aggressive (agg) females across reproductive states and groups. (**H**) Example histology following pan-MPOA injection with CreGFP-expressing AAV. Scale bar is 1 mm. Statistics by Kaplan-Meier survival analysis with log rank test in (B); by Fisher's exact test with Benjamini-Hochberg FDR correction for multiple comparisons in (C, D, G); by one-way ANOVA with Tukey *post-hoc* test in (E) and (F). ***P $<$ 0.001; **P $<$ 0.01; *P $<$ 0.05.

4.3.3 Deletion of hormone receptors from MPOA^{Gal} neurons

Within the MPOA, many partially overlapping sub-populations of neurons have been implicated in the regulation of parental behaviour (see General Introduction). Among them, galanin-expressing neurons, constituting approximately 20% of the MPOA (Wu et al., 2014), are especially important, as their ablation impairs parental behaviour in both females and males (Kohl et al., 2018; Wu et al., 2014). I therefore tested whether MPOA^{Gal} neurons are sensitive to pregnancy hormones by assessing Esr1 and PR expression in this sub-population (Fig. 4.4, B). I found Esr1- and PR-expressing neurons in the MPOA^{Gal} population. Interestingly, no significant difference in receptor expression was detected between virgin and D18 females (*fraction of neurons expressing*: Esr1 Vir, 72.02% ± 3.44%; PR Vir, 53.78% ± 9.73%; Esr1 D18, 80.09% ± 1.95%; PR D18, 70.8% ± 4.45%; mean ± SEM; Fig. 4.4, C).

To determine whether MPOA^{Gal} sensitivity to E2 and P4 is necessary for pregnancy-induced behavioural changes, I developed a viral-genetic strategy to ablate Esr1 or PR specifically from these neurons. A knock-in mouse line expressing FlpO recombinase in galanin neurons was generated and crossed with mice carrying floxed Esr1 (*Gal-Flp*; *Esr1*^{loxP}) or PR alleles (*Gal-Flp*; *PR*^{loxP}). Nearly all Gal-positive neurons expressed FlpO recombinase (98.5%), and vice-versa (99.3%)(Fig. 4.4, A; n = 2 *Gal-Flp*; *Esr1*^{loxP} virgin females). Two weeks before behavioural testing, I injected AAVs expressing either a Flp-dependent Cre recombinase or Flp-dependent GFP into the MPOA (Fig. 4.5, A). The DIO (Double-flanked Inverted Open reading frame) system was used to express Cre in a Flp recombinase-dependent manner in galanin neurons of females with either Esr1 or PR floxed genes (see Methods).

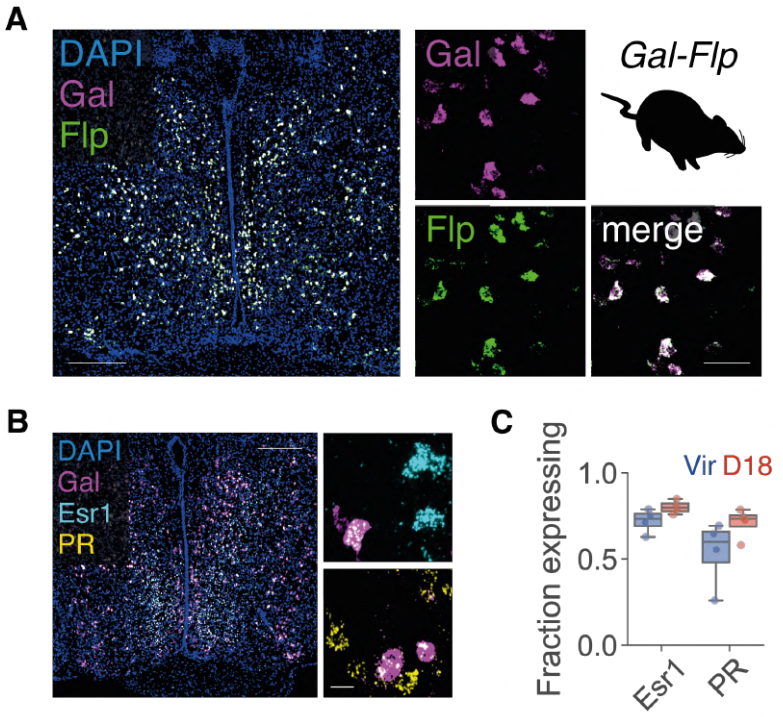

Figure 4.4: **Hormone receptor expression in MPOA^{Gal} neurons**. (**A**) Example MPOA section obtained by smFISH against Gal and FlpO mRNA from a *Gal-Flp* female. (**B**) Example MPOA section obtained by smFISH against Esr1, PR and Gal transcripts. (**C**) MPOA^{Gal} neurons expressing Esr1 or PR at virgin (Vir) and late pregnant (D18) stages (n = 4, 4). Scale bars are 250 μm (A), 50 μm (B), 20 μm (lateral panels in A and B). Error bars indicate SEM. Statistics by Mann-Whitney U test in (C).

I then performed our pup-directed behavioural assay for virgin and pregnant females. In line with my findings from MPOA-wide receptor KO, I observed that MPOA^{Gal}-specific Esr1 and PR deletion impaired retrieval and crouching behaviours in pregnant animals. While only 20% of Esr1^{KO} and 15% of PR^{KO} D18 animals retrieved (Fig. 4.5, C), a significantly higher fraction of control mice did at D18 (ctrl D18, 100%, Fig. 4.5, C) with a latency of 198.9 ± 61.9 seconds (mean ± SEM; Fig. 4.5, B). In addition, all pregnant controls displayed crouching (ctrl D18; Fig. 4.5, D, E). In contrast, none of Esr1^{KO} and only 7.7% of PR^{KO} pregnant animals crouched (Esr1^{KO} D18, PR^{KO} D18; Fig. 4.5, D). Similar to pan-MPOA receptor-deleted females, Esr1^{KO} and PR^{KO} pregnant females did not spend significantly more time in the nest with pups compared to their virgin counterparts, unlike control animals (ctrl D18, 58.9% ± 8.5%; ctrl Vir, 15.3% ±

8%; Fig. 4.5, F). Interestingly, while all pregnancy-modulated behavioural aspects were affected by MPOA^{Gal}-specific receptor KO, non pregnancy-modulated parameters were not affected, such as the latency to contact pups (Fig. 4.5, G).

In addition, I noted pup-directed aggressive behaviours in nearly half of the Esr1^{KO} and PR^{KO} pregnant females (Esr1^{KO} D18, 50%; PR^{KO} D18, 54%; Fig. 4.5, H). Pup-directed aggression was also observed across virgin groups but, unlike Esr1^{KO} and PR^{KO} pregnant animals, it was completely abolished in control pregnant females, which were parental (Esr1^{KO} Vir, 33%; PR^{KO} Vir, 7%; ctrl D18, 0%; Fig. 4.5, H).

Overall, MPOA^{Gal}-specific ablation of either Esr1 or PR fully recapitulated the effects observed following MPOA-wide receptor ablation. Therefore, direct action of estradiol and progesterone on MPOA^{Gal} neurons via their intracellular receptors is necessary for pregnancy-induced parental behaviour.

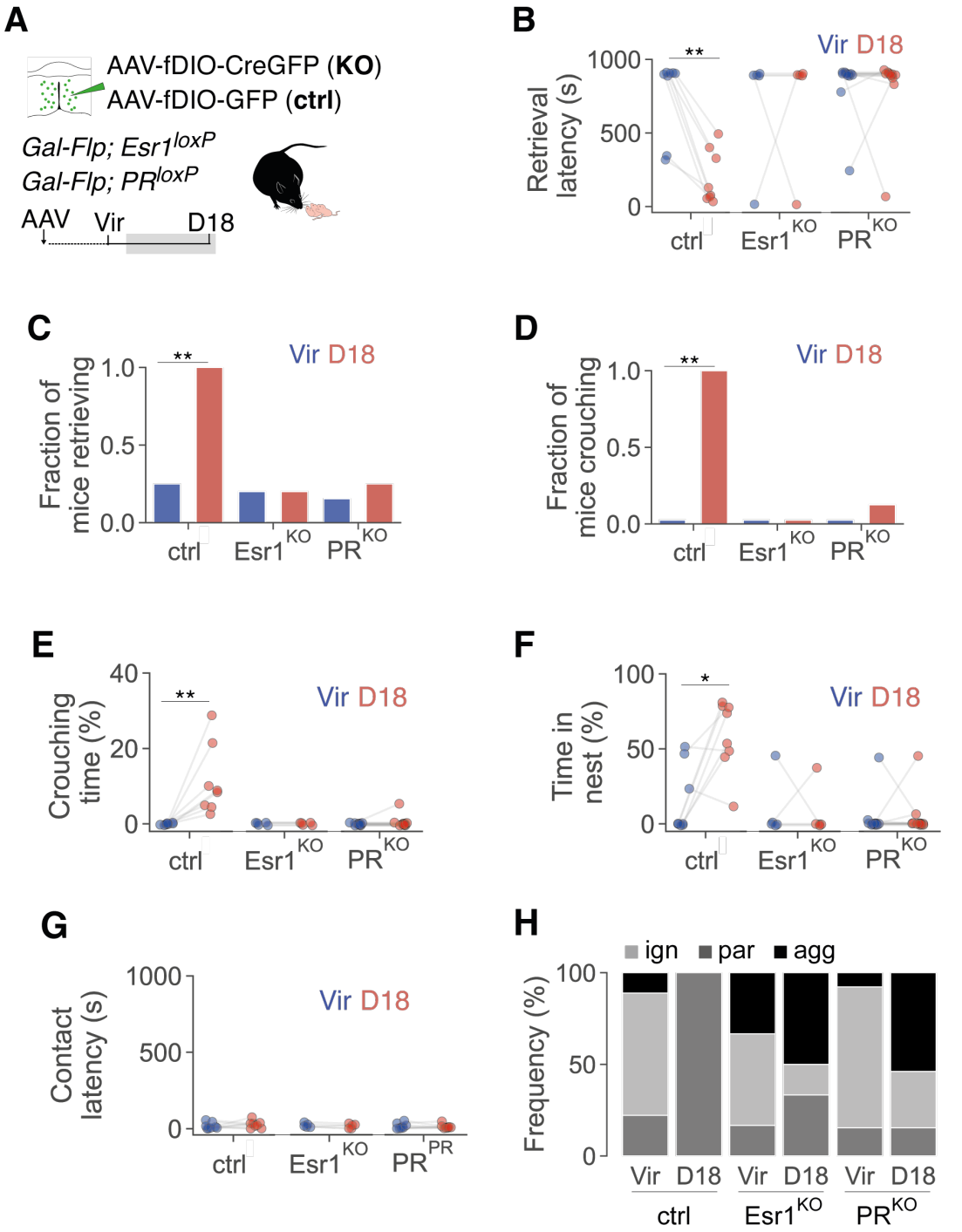

Figure 4.5: **Behavioural effects upon ablation of pregnancy hormone receptors from MPOA^{Gal} neurons**. (**A**) Timeline of behavioural assay for females with MPOA^{Gal} AAV-mediated Esr1 (Esr1^{KO}) or PR (PR^{KO}) deletion (n = 5,13), and controls (n = 8). The viral DIO (Double-Inverted Open reading frame) system was employed to express CreGFP or GFP in a Flp-dependent manner in galanin neurons. Grey box, pregnancy. (**B** to **G**) Quantification of pup-directed parameters in Esr1^{KO} and PR^{KO} virgin (Vir) and pregnant (D18) females, and controls. (**H**) Proportion of ignoring (ign), parental (par) or aggressive (agg) females across reproductive states and groups. Statistics by Kaplan-Meier survival analysis with log rank test in (B); by Fisher's exact test with Benjamini-Hochberg FDR correction for multiple comparisons in (C, D, H); by one-way ANOVA with Tukey *post-hoc* test in (E to G). ***P $<$ 0.001; **P $<$ 0.01; *P $<$ 0.05.

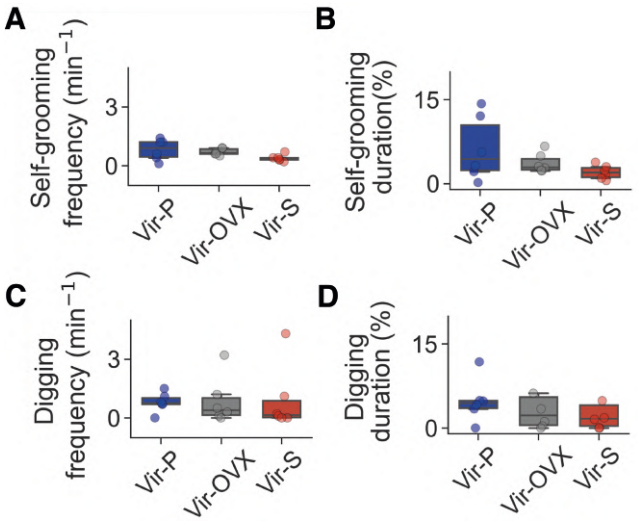

Figure 4.6: **Stress-related behaviours**. (**A** to **D**) Stress-related behavioural parameters of virgins after PR ablation from MPOA^{Gal} neurons (Vir-S, n = 8), virgins from *OVX* group (Vir-OVX, n = 10), and virgins from *Preg* group (Vir-P, n = 10). Statistics by one-way ANOVA with Tukey *post-hoc* test.

Given that animals were subjected to different surgical procedures, I tested whether behavioural deficits could be due to post-operative stress or pain (Roughan and Flecknell, 2004; Roughan and Flecknell, 2003). To exclude any stress-related effect from these behavioural observations, I assessed stress-associated behaviours, such as self-grooming and digging in the cage substrate (Kalueff et al., 2016; Kedia and Chattarji, 2014), during the 10-minute habituation prior to testing in virgin females that underwent craniotomy surgeries as part of the receptor ablation experiments (Vir undergoing surgery, Vir-S; Fig. 4.6). I compared their behaviours with those of intact females (Vir from *Preg* group, Vir-P) and of animals that underwent a different surgical procedure, ovariectomy (Vir-OVX; Fig. 4.6). No significant behavioural differences were observed between virgins subjected to either craniotomy or ovariectomy and intact females (Fig. 4.6). Since these groups showed different retrieval rates and levels of spontaneous maternal behaviour (Figs. 3.2, J and 4.5, C), this suggests that the absence of pregnancy-induced behavioural adaptations is not due to stress. While not quantitatively assessed, I observed no clear signs of pain, such as pilo-erection, hunched posture, or changes in locomotor activity (Burkholder et al., 2012).

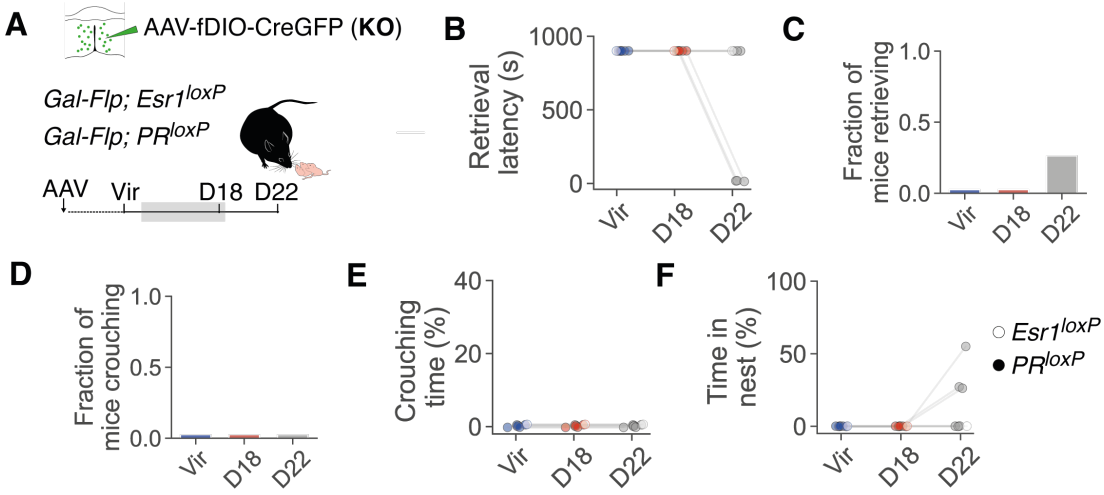

Figure 4.7: **Post-partum maternal behaviours upon MPOA^{Gal}-specific ablation of pregnancy hormone receptors**. (**A**) Timeline of behavioural assay for females with MPOA^{Gal} AAV-mediated Esr1 ($Esr1^{loxP}$) or PR (PR^{loxP}) deletion. (**B** to **F**) Quantification of pup-directed behaviours at virgin, late pregnancy and early post-partum (D22, n = 7; 1 $Esr1^{loxP}$, 6 PR^{loxP}). Statistics by Kaplan-Meier survival analysis with log rank test in (B); by Fishers exact with Benjamini-Hochberg FDR correction for multiple comparisons in (C) and (D); by one-way ANOVA with Tukey *post-hoc* test in (E) and (F).

While levels of E2 and P4 drop rapidly at the end of gestation, Oxt surges at parturition and Prl release is further stimulated by the suckling stimulation during interactions with pups (see General Introduction). It was therefore plausible that peri-partum hormonal events could rescue parental behaviour. I examined pup-directed parental parameters in early post-partum in receptor-ablated females that littered down and nursed their own pups (Fig. 4.7, A). I did not find any difference between early post-partum (D22) and either Vir or D18 timepoints across the set of pregnancy-modulated pup-directed behaviours (Fig. 4.7, B to F), suggesting that the hormonal events of parturition do not compensate for pre-partum behavioural deficits.

In summary, I demonstrated that E2 and P4 action on MPOA neurons and, specifically, MPOA^{Gal} neurons is necessary for pregnancy-induced behavioural changes, and that the parental behaviour of receptor-ablated females remains impaired after parturition.

4.4 Hormonal remodelling of MPOA^{Gal} neurons

Next, I explored how pregnancy hormones affect MPOA^{Gal} neurons. I closely collaborated with Rachida Ammari, who performed the electrophysiological recordings described in the following section. Whole-cell patch-clamp recordings were performed from MPOA^{Gal} neurons of wild-type females at different reproductive stages — virgin, mid-pregnancy (D10) or late pregnancy (D18; Fig. 4.8).

We observed a lower baseline firing in late pregnancy compared to virgins (Vir, 1.87 ± 0.30 Hz; D18, 1.20 ± 0.31 Hz; mean ± SEM; Fig. 4.8, B, C). Baseline firing was already reduced in mid-pregnancy (D10; 1.44 ± 0.52 Hz; Fig. 4.8, C). Similarly, membrane resting potential was significantly reduced in mid- and late-pregnancy (Vir, -42.93 ± 0.92 mV; D10, -48.23 ± 1.61 mV; D18, -46.67 ± 1.08 mV; Fig. 4.8, E). Concurrently, we observed a larger fraction of silent neurons in mid- and late-pregnant females (Vir, 13.2%; D10, 35.8%; D18, 33.3%; Fig. 4.8, F). Interestingly, application of tertian-Q prevented this increase, suggesting that GIRK channels might be involved in the silencing effect (Tert-Q; Fig. 4.8, F). Following positive current injections, a smaller fraction of neurons exhibited depolarisation block in late pregnancy (Vir, 73.5%; D18, 35.5%; Fig. 4.8, H, I), hinting at a higher neuronal excitability during pregnancy. Increased spontaneous post-synaptic currents (sPSCs) were also observed in late pregnancy (Vir, 1.17 ± 0.31 Hz; D18, 2.07 ± 0.28 Hz; Fig. 4.8, K). However, the above-mentioned silencing effect was not due to an increase in inhibitory inputs. We observed an increased frequency of excitatory, but not inhibitory, sPSCs (*EPSCs*; Vir, 0.80 ± 0.20 Hz; D18, 0.93 ± 0.12 Hz; Fig. 4.8, M). Increased sPSC frequency mainly occurred in active (non-silenced) neurons (Fig. 4.8, L). To assess morphological changes, recorded neurons were filled with Neurobiotin and reconstructed. While no overall changes to neurite morphology or complexity were found (data not shown), we observed higher dendritic spine density in late pregnancy compared to virgin state (Vir, 0.72 ± 0.12 spines/10 μm; D18, 1.73 ± 0.37 spines/10 μm; Fig. 4.8, O). As excitatory inputs are presumably made on spines in these neurons,

increased spine density mirrors the increase in sEPSC frequency (Fig. 4.8, P).

Pregnancy hormones increase the intrinsic excitability of MPOA neurons, while promoting the recruitment of excitatory synaptic inputs and reducing baseline neuronal activity. We next explored whether these neuronal effects are driven by hormones.

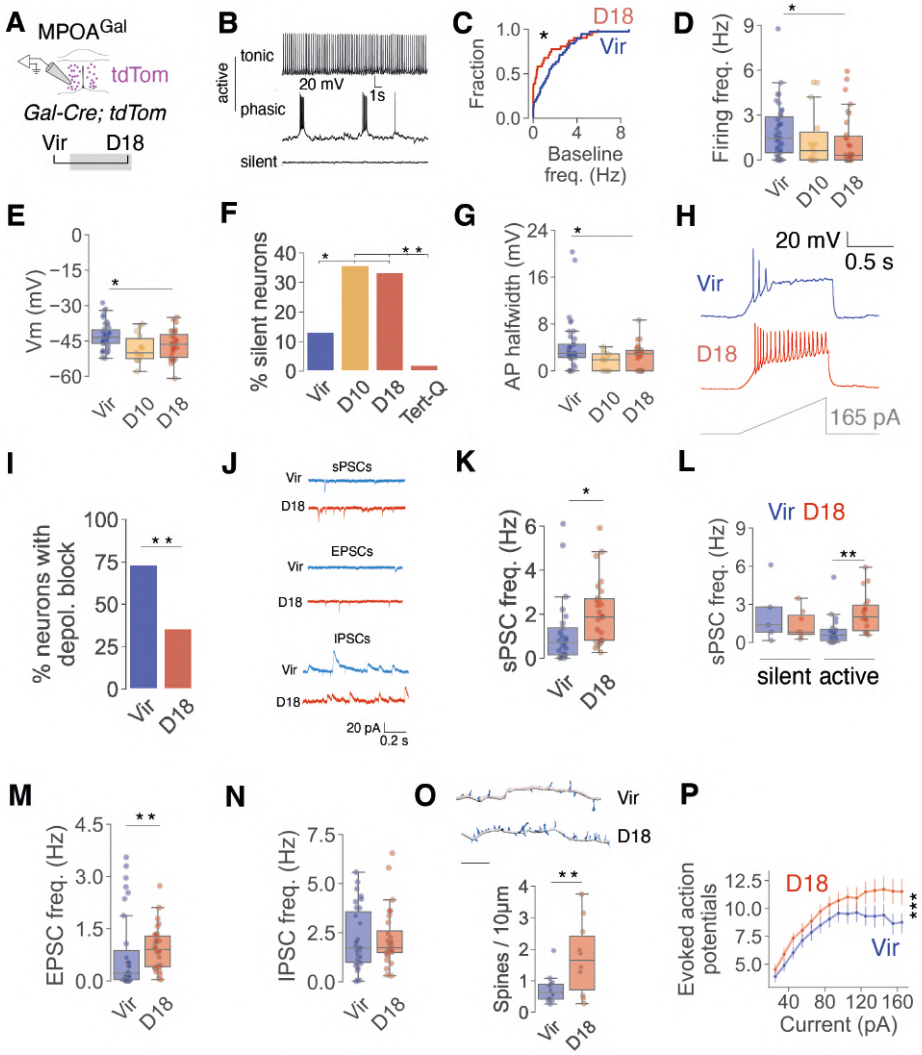

Figure 4.8: **Pregnancy-induced changes in biophysical and morphological properties of MPOA^{Gal} neurons**. (**A**) Slice electrophysiology from MPOA^{Gal} neurons. (**B**) Example traces of different MPOA^{Gal} activity patterns at resting membrane potential. (**C**) Distribution of baseline firing frequency (Vir and D18; 33 and 21 cells from $n = 15$ and 7 mice). (**D**) and (**E**) Spontaneous firing frequency and resting membrane potential (38, 14, and 31 cells from $n = 15$, 4, and 8 mice). (**F**) Fraction of silent neurons at different stages, and with Tertiapin-Q (38, 14, 30, 9 from $n = 15$, 4, 9, 3 mice). (**G**) Action potential half-width. (**H**) Representative current clamp traces of neurons at Vir and D18. (**I**) Fraction of neurons with depolarisation block (34, 30 cells from $n = 15$, 8 mice). (**J**) Representative voltage clamp traces with sPSCs, EPSCs, IPSCs from MPOA^{Gal} neurons. (**K**) and (**L**) sPSC frequency in Vir and D18 (21, 23 cells from $n = 9$, 6 mice), or silent and active neurons (5, 10, 33, and 20 cells). (**M**) EPSC frequency (31, 30 cells from $n = 5$, 4 animals). (**N**) IPSC frequency (31, 28 cells from $n = 5$, 4 mice). (**O**) Reconstructed MPOA^{Gal} dendrites and density of spines (14, 10 cells from $n = 10$, 4 mice). Scale bar is 10 μm. (**P**) F-I curves showing evoked action potentials following somatic current injection at Vir and D18 (33, 30 cells from $n = 12$, 9 animals). Statistics by Fisher's exact test with BH adjustment for multiple comparisons in (D, F, I); by one-way ANOVA with Dunnett's *post hoc* test in (C, E, G, K, O); by K-S test in (L, M, N); by two-way ANOVA with Tukey *post hoc* test in (P). ***P < 0.001, **P < 0.01, *P < 0.05.

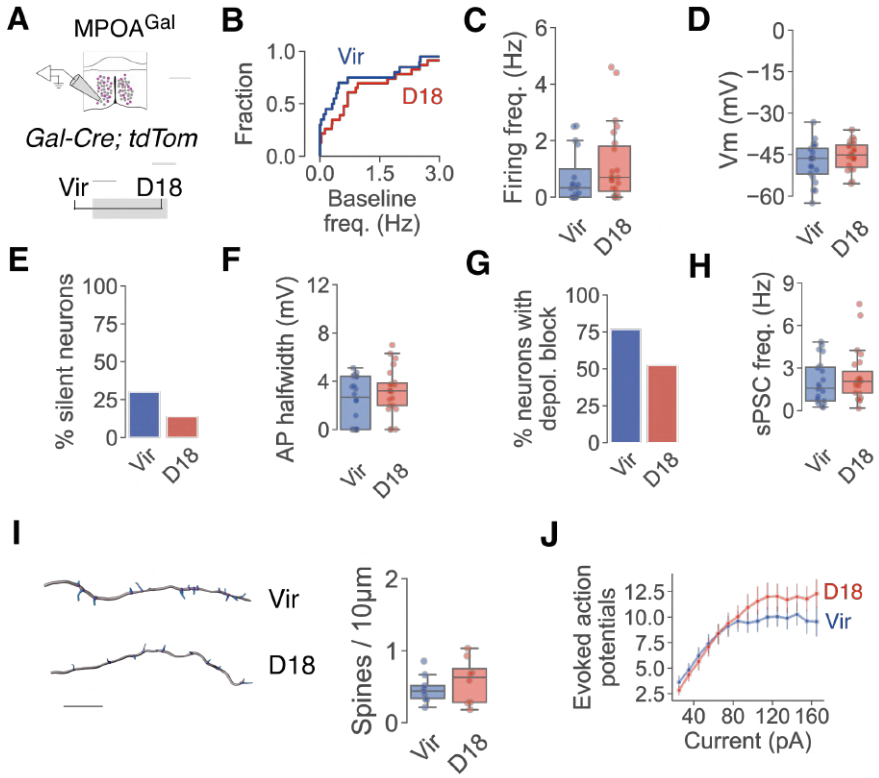

Figure 4.9: **Pregnancy-induced changes in biophysical and morphological properties of Gal-negative MPOA neurons**. (**A**) Slice electrophysiology recordings from wild-type galanin-negative MPOA neurons. (**B**) Distribution of baseline firing frequency. (**C**) Spontaneous firing frequency at different pregnancy stages. (D) Resting membrane potential at different pregnancy stages. (**E**) Fraction of silent neurons. (**F**) Action potential half-width. (**G**) Fraction of neurons exhibiting depolarisation block. (**H**) sPSC frequency (21, 23 cells from $n = 5$, 3 animals). (**I**) Reconstructed neuronal dendrites with spines and density of spines at different pregnancy stages (9 and 8 cells from $n = 4$ and 3 animals). Scale bar is 10 μm. (**J**) Evoked action potentials following somatic current injection (21, 23 cells from $n = 5$, 3 mice). Statistics by Fisher's exact test in (E, G); by U test in (B, C, D, F, I); by K-S test in (H); by two-way ANOVA in (J).

Importantly, the biophysical and morphological changes were specific to galanin-positive MPOA neurons. We did not observe equivalent pregnancy-associated changes to membrane, synaptic and morphological properties of galanin-negative neurons (Fig. 4.9).

To assess whether pregnancy-induced MPOA^{Gal}-specific neuronal plasticity is due to direct E2 and P4 action, recordings were performed from Esr1- and PR-deleted MPOA^{Gal} neurons at D18 (Fig. 4.10). We found that Esr1-deleted MPOA^{Gal} neurons of D18 females had a higher resting membrane potential than those of wild-type virgins (Vir, -42.93 ± 0.92; Esr1^{KO}, -40.46 ± 1.43 mV; Fig. 4.10, B). In contrast to Esr1 deletion, PR deletion did not prevent the pregnancy-induced lowering of membrane potential (PR^{KO}, -46.56±1.23 mV; Fig. 4.10, B). Similarly, the fraction of silent neurons in pregnant females approached virgin-like levels following Esr1, but not PR, deletion (Esr1^{KO}, 11.8%; PR^{KO}, 26.1%, Fig. 4.10, C). Notably, considerably more neurons exhibited depolarisation block in Esr1-deleted pregnant than wild-type pregnant females (D18, 35.5%; Esr1^{KO}, 93.8%, Fig. 4.10, D). This effect was not observed in MPOA^{Gal} neurons of PR-deleted pregnant females (PR^{KO}, 40%; Fig. 4.10, D). Esr1, but not PR, deletion therefore prevents pregnancy-induced reduction in resting membrane potential, increase in membrane excitability and silencing of MPOA^{Gal} neurons.

By contrast, while Esr1-deleted MPOA^{Gal} neurons exhibited levels of spontaneous post-synaptic currents at D18 comparable to those of D18 controls, PR deletion returned them to a virgin-like state (D18, 2.07 ± 0.28 Hz; Esr1^{KO}, 1.96 ± 0.40 Hz; Vir, 1.17 ± 0.31 Hz; PR^{KO}, 0.62 ± 0.16 Hz; Fig. 4.10, F). Virgin-like spine density was also observed on dendritic segments of PR-deleted, but not Esr1-deleted, MPOA^{Gal} neurons (Vir, 0.72 ± 0.12 spines/10 μm; PR^{KO}, 0.15 ± 0.06 spines/10 μm; Esr1^{KO}, 1.13 ± 0.16 spines/10 μm Fig. 4.10, G). Esr1 deletion therefore does not affect synaptic inputs and spine density.

No biophysical and morphological changes were detected in MPOA^{Gal} neurons from GFP-injected pregnant animals (ctrl cf. wt; Fig. 4.10, H-M).

In summary, Esr1 deletion prevents the reduction in resting membrane potential, the increase in membrane excitability and the silencing of MPOA^{Gal} neurons typically observed during pregnancy, but has no effect on synaptic inputs. PR deletion instead abolishes the upregulation of synaptic currents and the increase

in dendritic spine density, but does not alter membrane properties. These findings indicate that E2 and P4 exert distinct, non-overlapping effects on MPOA^{Gal} neurons during pregnancy: while E2 is necessary to the silencing and the increase in intrinsic excitability, P4 mediates the recruitment of excitatory synaptic inputs.

Finally, we explored how long-lasting MPOA^{Gal} neuronal remodelling is (Fig. 4.11). sPSCs remained elevated in late postpartum (D22, 2.83 ± 0.36 Hz; D50, 1.95 ± 0.20 Hz; Fig. 4.11, B). Receptor deletion-induced effect on synaptic inputs was still present at D22 (PR^{KO} D18, 0.625 ± 0.16 Hz; PR^{KO} D22, 1.24 ± 0.26 Hz; Fig. 4.11, I). Similarly, dendritic spine density remained increased until late post-partum (D50, 0.94 ± 0.08 spines/10 μm; Fig. 4.11, C) and its upregulation was abolished in post-partum PR-deleted neurons (PR^{KO} D18, 0.15 ± 0.06 spines/10 μm; PR^{KO} D22, 0.58 ± 0.11 spines/10 μm; Fig. 4.11, J). In contrast, biophysical changes reverted to virgin levels (Fig. 4.11, D-G). Resting membrane potential and baseline firing increased in the late post-partum period (*Vm*: Vir, -42.93±0.92 mV; D22, -45.61±1.29 mV; D50, -41.82±1.53 mV. *Firing freq.* Vir, 1.87±0.30 Hz; D22, 0.98±0.21 Hz; D50, 1.47±0.28 Hz; Fig. 4.11, D and E). The fraction of silent neurons and those exhibiting depolarisation block returned to virgin levels earlier at D22 (*% silent neurons*: Vir, 13.2%; D22, 20%; D50, 5%; *% neurons with depol. block*: Vir, 73.5%; D22, 79%; D50, 84.6%; Fig. 4.11 F,G). In summary, while synaptic changes persisted until at least one month after parturition, membrane properties returned to a virgin-like state in the post-partum period.

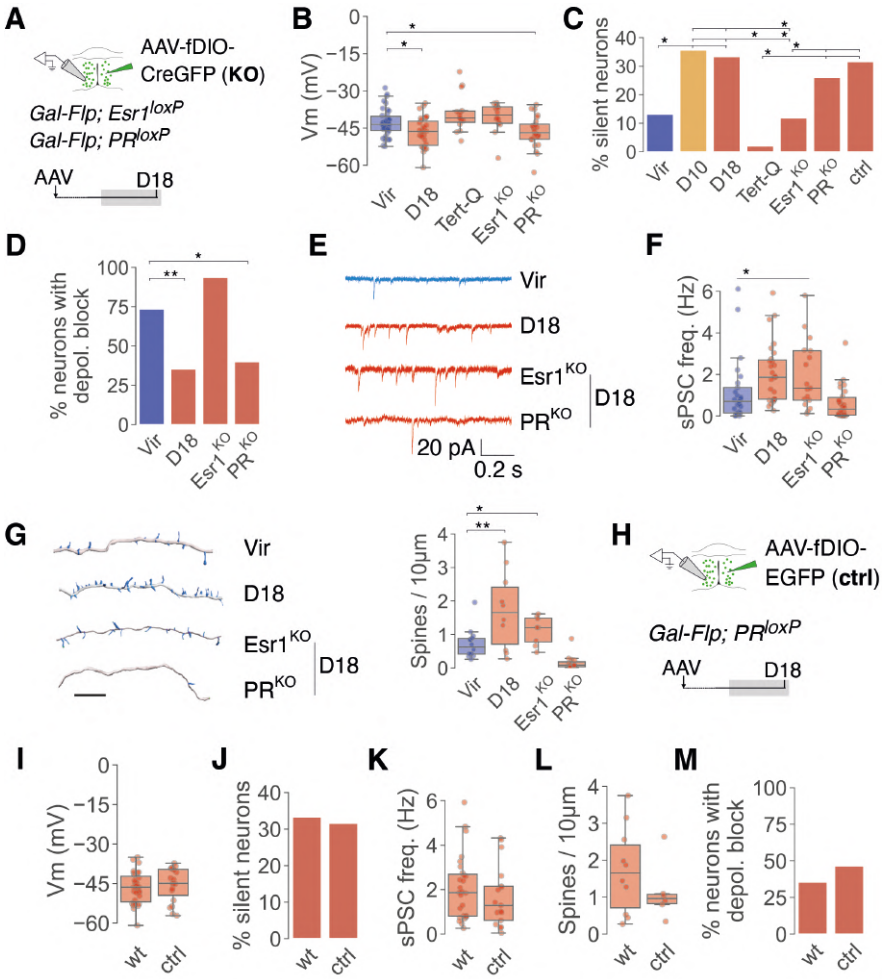

Figure 4.10: **Remodelling of MPOA^{Gal} neurons following hormone receptor ablation.** (**A**) Slice electrophysiology from receptor-deleted MPOA^{Gal} neurons. (**B**) Resting membrane potential from virgin and pregnant intact females (Vir and D18; 38 and 32 cells from $n = 15$ and 9 mice), receptor-deleted pregnant females (Esr1^{KO} and PR^{KO}; 18 and 26 cells from $n = 3$ and 5 mice), pregnant intact females with Tertiapin-Q (Tert-Q; 15 cells from $n = 3$ mice). (**C**) Fraction of silent neurons from virgin and pregnant females (Vir, D10, D18; 38, 14, 30 cells from $n = 15$, 4, 9 mice), pregnant intact females with Tertiapin-Q (Tert-Q; 9 cells from $n = 3$ mice), receptor-deleted pregnant females (Esr1^{KO}, PR^{KO}; 17, 23 cells from $n = 3$, 5 mice), and pregnant controls (19 cells from $n = 4$ animals). (**D**) Fraction of neurons exhibiting depolarisation block (34, 30, 18, 25 cells from $n = 15$, 8, 3, 5 mice) (**E**) Representative voltage clamp traces from intact and receptor-deleted females. (**F**) sPSC frequency (21, 23, 18, 26 cells from $n = 9$, 6, 3, 5 mice). (**G**) Example reconstructed MPOA^{Gal} dendrites with spines and density of spines (14, 10, 8, 15 cells from $n = 10$, 4, 3, 4 mice). Scale bar is 10μm. (**H**) Slice electrophysiology from GFP-expressing MPOA^{Gal} neurons. (**I**) to (**M**) Biophysical and morphological properties of MPOA^{Gal} neurons from control (ctrl) animals. I (38, 19 cells from $n = 15$, 4 mice); J (38, 19 cells from $n = 15$, 5 mice); K (Hz) (21, 19 cells from $n = 9$, 4 mice); L (14, 6 cells from $n = 10$, 2 mice); M (34, 30 cells from $n = 15$, 8 mice). Statistics by one-way ANOVA with Dunnett's *post hoc* test in (B, F, G, J, M); Fishers exact test with BH adjustment in (C, D); by U test in (I, K, L). ***P < 0.001, **P < 0.01, *P < 0.05.

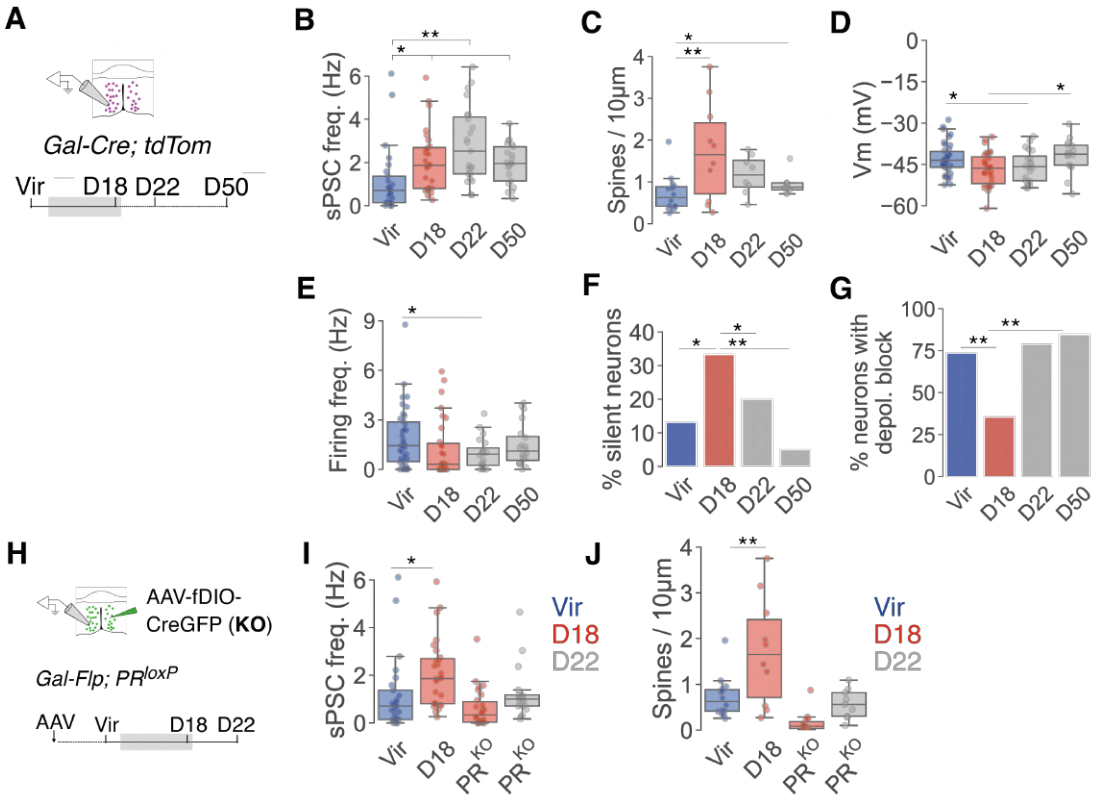

Figure 4.11: **Long-lasting hormone-mediated MPOA^{Gal} neuronal remodelling**. (**A**) Slice electrophysiology from wild-type MPOA^{Gal} neurons at different stages (virgin, Vir; pregnant, D18; post-partum, D22, D50). (**B**) sPSC frequency in intact virgin, pregnant and post-partum females (34, 30, 24, 14 cells from n = 15, 8, 5, 5 mice). (**C**) Density of dendritic spines (14, 10, 7, 9 cells from n = 10, 4, 4, 4 animals). (**D**) Resting membrane potential. (**E**) Baseline firing frequency. (**F**) Fraction of silent neurons. (**G**) Fraction of neurons with depolarisation block (34, 30, 24, 14 cells from n = 15, 8, 5, 5 animals). (**H**) Slice electrophysiology in PR-deleted MPOA^{Gal} neurons. (**I**) sPSC frequency in intact virgin and pregnant females (Vir and D18), receptor-deleted pregnant and post-partum females (D18 PR^{KO} and D22 PR^{KO}). (**J**) Density of spines in intact virgin and pregnant females (Vir and D18; 34 and 30 cells from n = 15 and 8 animals), PR-deleted pregnant and post-partum females (D18 PR^{KO} and D22 PR^{KO}; 15 and 5 cells from n = 4 and 3 mice). Statistics by.one-way ANOVA with Dunnett's *post hoc* test in (B, C, D, E, I, J); by Fisher's exact test with Benjamini-Hochberg adjustment for multiple comparisons in (F, G). ***P $<$ 0.001, **P $<$ 0.01, *P $<$ 0.05.

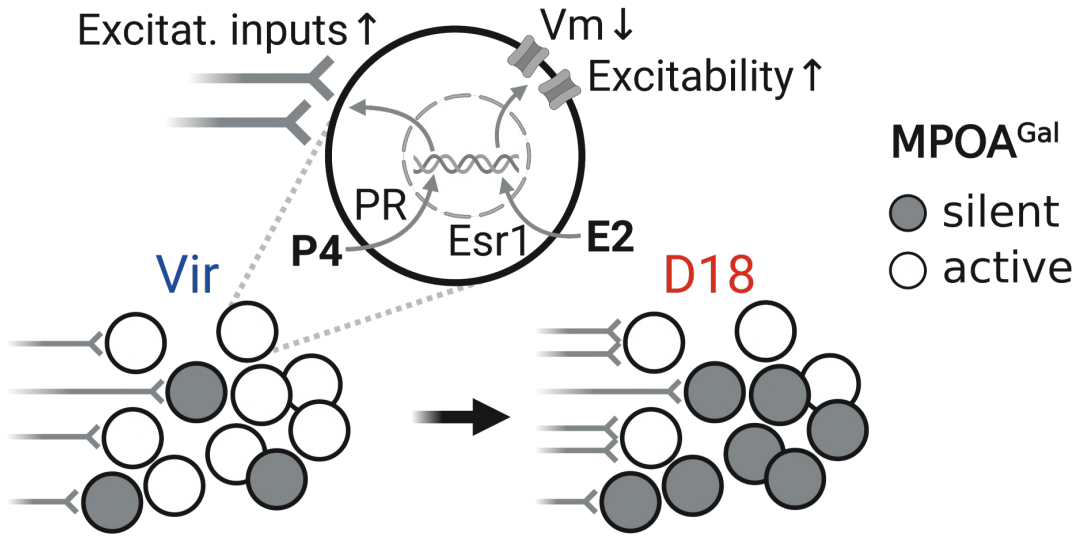

Figure 4.12: **Model of pregnancy hormone-mediated remodelling of MPOA^{Gal} neurons**. Cartoon summarising E2 and P4 action via intracellular receptors on MPOA^{Gal} neurons during pregnancy.

In conclusion, we propose a model for hormone-mediated MPOA^{Gal} neuronal remodelling (Fig. 4.12). During pregnancy, E2 and P4 govern distinct, non-overlapping aspects of MPOA^{Gal} -specific plasticity. E2, acting via its intracellular receptor Esr1, reduces neuronal baseline activity, and increases intrinsic excitability. At the same time, P4, also acting via its intracellular receptor PR, promotes recruitment of excitatory synaptic inputs onto spontaneously active MPOA^{Gal} neurons. Given that Esr1 and PR are also transcription factors, these effects are likely instructed by changes in gene transcription. Interestingly, while the PR-mediated remodelling of synaptic inputs and changes in spine density are long-lasting, Esr1-mediated plasticity is transient, reverting to a virgin-like state after parturition.

4.5 Discussion

In this chapter, I investigated the effect of pregnancy hormones on MPOA^{Gal} neurons. As already mentioned, several studies have implicated E2 and P4, as well as Oxt and Prl, in the induction and regulation of parental behaviour, and the MPOA has been established as a target for these hormones (A. D. Mayer et al., 1990; Moltz et al., 1970; Numan, 2003; Pedersen et al., 1982; Riddle et al., 1935). Although expression patterns of hormone receptors have been mapped in the mouse brain, these region-level maps lack cellular resolution and information on receptor co-expression (Brinton et al., 2008; Gould and Zingg, 2003; Kokay et al., 2018; N. Sugiyama et al., 2009). To confirm whether MPOA neurons are hormonal targets, I first probed and quantified the expression of receptors for E2, P4, Prl and Oxt in the broader hypothalamus. I found that Esr1, PR and Prlr are enriched in the MPOA compared to other hypothalamic regions. On the other hand, the MPOA did not show particularly high levels of Oxtr transcripts, which were instead more enriched in the ventromedial hypothalamus (VMH). This is in line with another study which, by leveraging an Oxtr-LacZ reporter mouse line, reported higher levels of Oxtr in both the ventrolateral VMH (VMHvl) and dorsomedial VHM (VMHdm) than the MPOA (Gould and Zingg, 2003). Interestingly, the MPOA had high levels of co-expression of Esr1 and PR. Notably, I did not observe a difference in Esr1 and PR expression in MPOA^{Gal} neurons between virgins and D18 females. This is surprising given that higher density of Esr1 was previously reported in the MPOA of lactating females (Byrnes et al., 2009). The lack of significant differences could be due to the small sample of virgins and D18 females in this experiment. Nonetheless, together with studies showing that co-administration of estradiol and progesterone is sufficient to elicit parenting (Rosenblatt et al., 1988), these findings hint at a particularly prominent role of Esr1 and PR in the MPOA in mediating the onset of parental behaviour.

I therefore asked whether hormonal sensitivity of MPOA neurons is indeed necessary for the pregnancy-induced onset of parental behaviour. We focussed

on intracellular receptors Esr1 and PR because of their well-documented role in maternal behaviour (Brinton et al., 2008; Ogawa et al., 1998; Ribeiro et al., 2012; Rosenblatt et al., 1988) and their high expression in the MPOA. Additionally, given the long-lasting nature of parental behavioural adaptations, intracellular receptors acting as transcription factors and directly affecting gene transcription are ideal candidates mediating long-lasting forms of neural plasticity.

By conditionally deleting Esr1 and PR in MPOA neurons, I determined that both receptors are required for the onset of maternal behaviour during pregnancy. These findings agree with earlier studies. It was previously reported that in female mice systemic knockout of Esr1 leads to dramatically impaired maternal behaviours, alongside disrupted sexual behaviour and increased aggression towards adult conspecifics and infants (Ogawa et al., 1998). Another study demonstrated that RNA interference-driven knock-down of Esr1 expression in the MPOA reduces levels of maternal and sexual behaviours (Ribeiro et al., 2012). I find that ablation of PR in the MPOA mirrors the behavioural effects observed in Esr1 knockout females. A previous study in mice showed that subcutaneous administration of a PR antagonist disrupts maternal behaviour (M. Wang, Crombie, Hayes, and Heap, 1995). In addition, a large body of literature in rats has provided evidence supporting the role of nuclear progesterone receptors in parenting and established their site of action in the MPOA (Numan et al., 1999). To our knowledge, however, the site of P4 action in regulating the onset of maternal behaviour has remained largely unexplored in mice. My work therefore establishes a role for P4 signaling in the MPOA for parental behaviour in mice.

Interestingly, I found that deletion of either receptor from the smaller MPOA^{Gal} neuronal subset recapitulates the behavioural phenotype of the pan-MPOA ablation. Not only are MPOA^{Gal} neurons crucial for regulating parenting, but their sensitivity to E2 or P4 is as important as that of the wider MPOA for the induction of parental behaviour. Given that MPOA^{Gal} neurons constitute only 20% of MPOA neurons (Kohl, 2020; Wu et al., 2014), such a drastic behavioural

effect might initially be a surprise. Several neuronal types are indeed found in the MPOA, where most neurons are (co-)sensitive to both steroid hormones. Alongside parenting, the MPOA regulates several other instinctive behaviours, such as mating (Láng et al., 2024). It is therefore likely that these hormones have downstream neuronal effects which are different across MPOA cell types. Correspondingly, it would be interesting to dissect which cellular characteristics underlie the behavioural specificity of E2 and P4-responsive MPOA^{Gal} neurons. Additional experiments involving non galanin-expressing MPOA neurons would provide important cues regarding the unique molecular makeup of pregnancy hormone-targeted neurons, as well as the identity and role of Esr1- and PR-mediated downstream cellular pathways. Recent efforts into determining the genomic mechanisms by which steroid hormone receptors act in neural circuits governing instinctive behaviours are promising (Gegenhuber et al., 2022). Another point to be explored is whether E2 or P4 action on MPOA^{Gal} neurons is sufficient to trigger parenting onset.

Despite the striking findings of our ablation experiments, I speculate that several neuronal types within the MPOA, and within the MPOA^{Gal} population, are differentially modulated by other pregnancy hormones, and that their action plays an important role in the regulation of parenting. Prl, for instance, whose receptors are enriched in the MPOA, might contribute to changes in pup-directed behaviours in the early stages of pregnancy (Ladyman et al., 2021), or in the post-partum period (Brown et al., 2017). It is also important to note that hormones might synergistically enhance parental behaviour, thus indirectly affecting non cognate receptor-mediated downstream pathways. For example, E2 fluctuations during pregnancy positively correlate with Oxtr expression in hypothalamic areas, including the MPOA (Caldwell et al., 1992; Coirini et al., 1989). It was recently reported that deletion of Esr1 drives a reduction of PR-responsive neurons in a hypothalamic area (Trouillet et al., 2022). Thus, although ablation of Esr1 or PR abolishes the onset of parental behaviour, other hormones are also expected to

influence parental behaviour by directly acting on MPOA and MPOA^{Gal} neurons, or by interacting with non-cognate hormone receptors via mechanisms which remain to be described. Nevertheless, effects triggered by other hormones, such as those of parturition, cannot compensate for the behavioural deficits induced by Esr1 or PR deletion.

I observed that receptor KO-induced behavioural disruptions persist until at least D22, indicating that endocrine events surrounding parturition are not sufficient to compensate for behavioural deficits induced by Esr1 or PR ablation. Anecdotally, while litter losses were common in KO animals, some pups survived for several days, suggesting that a minimal degree of parental behaviour was still performed by receptor-ablated mothers. This might result from compensatory mechanisms, either mediated by other hormones acting on other regions or, alternatively, by E2 and P4 action on areas connected to the MPOA. For instance, although exhibiting normal maternal behaviour, mothers with conditional deletion of Oxtr from both the paraventricular hypothalamus (PVH) and supraoptic nucleus (SO) failed to feed newly born pups, presumably because of impaired milk ejection (Inada, 2024). Although E2 and P4 actions on MPOA^{Gal} neurons is essential for the onset of parenting, this behaviour relies on the coordination of several motor actions. It is therefore plausible that the action of other hormones, either on the MPOA or other regions, might partially rescue parental behaviour, thus explaining the post-partum survival of pups observed in my ablation experiments. Additionally, I reported increased pup-directed aggression in receptor-ablated females. This is consistent with a recent study demonstrating reciprocal inhibition between Esr1-expressing neurons in the principal nucleus of the bed nucleus of stria terminal (BNSTpr) and MPOA^{Esr1} neurons. Making MPOA neurons insensitive to E2 and P4 might thus release their inhibiting effect, thereby leading to an up-regulation of the infanticide-promoting BNSTpr neural circuit (Mei, Yan, et al., 2023). Nonetheless, given the lack of quantitative data on pup survival, and in light of the robust behavioural deficits exhibited by

receptor-ablated females, our conditional receptor ablation experiments indicate that estrogen and progesterone act on MPOA^{Gal} neurons to mediate the onset of parenting during pregnancy.

Next, we asked how pregnancy hormones affect MPOA^{Gal} neurons. MPOA^{Gal} neurons exhibit different patterns of spontaneous activity, and we observed that P4 mediates the recruitment of synaptic inputs on spontaneously active, rather than silent, neurons. It is also known that the MPOA^{Gal} population consists of several molecularly distinct sub-populations, with galanin being expressed in five different inhibitory and two excitatory cell types in the MPOA (Moffitt et al., 2018). Considering that MPOA^{Gal} neurons are organised in projection-defined pools in charge of distinct aspects of parenting (Kohl et al., 2018), future work will explore whether a correlation between their projection-defined role in parenting and hormonal susceptibility exists. It would be interesting to test whether new inputs are preferentially made onto functionally or genetically-defined MPOA^{Gal} neuronal subsets.

Notably, we found that E2 and P4 govern distinct, non-overlapping aspects of pregnancy-induced plasticity. While E2 transiently tunes neuronal intrinsic excitability and baseline membrane potential, P4 mediates the long-term recruitment of excitatory inputs. In an earlier study, it was reported that E2 modulates the intrinsic excitability of neurotensin-expressing MPOA neurons likely by enhancing the function of delayed rectifier K^+ channels (McHenry et al., 2017). While the cellular pathways modulated by E2 and P4 are largely unknown, we hypothesise that increased MPOA^{Gal} excitability is also driven by upregulation of delayed rectifier K^+ channels. This would potentially allow for high-frequency firing, as indicated by the reduction in action potential half-width and enhanced evoked action potentials observed during pregnancy. Given its specific disruption in Esr1-deleted females, we report that E2 additionally governs transient MPOA^{Gal} neuronal silencing, which might be mediated by GIRK channels (Jin et al., 1999). Importantly, and at first counter-intuitively, we found that this neu-

ronal silencing is further enhanced by a P4-mediated upregulation of excitatory, rather than inhibitory, inputs primarily targeting the MPOA^{Gal} non-silenced subpopulation. Accordingly, we observed a PR-dependent increase in dendritic spine density on MPOA^{Gal} neurons. However, it remains to be established from where these additional inputs originate, and what information they convey. Because the majority of MPOA neurons is GABAergic, and thus inhibitory, we hypothesise that the recruited excitatory synaptic inputs originate from long-range projections, rather than local afferents. These might carry information on pup sensory cues, thereby providing a cellular correlate for the increased neuronal selectivity for, and improved population-level discriminability of, pup stimuli observed during pregnancy (see Chapter 5). These MPOA^{Gal} functional adaptations will be discussed in detail in the following chapter. Finally, while the pregnancy-induced membrane changes return to baseline levels after parturition, spine density and synaptic inputs remain increased, suggesting that the long-term increase in parental behaviour might rely on MPOA^{Gal} altered circuit integration.

Collectively, the transient changes in neuronal silencing and intrinsic excitability, and the long-lasting recruitment of additional excitatory inputs, set up the MPOA^{Gal} population for efficient parental behaviour. We propose that the combined steroid action on MPOA^{Gal} neurons has important functional implications on both neuronal and neural activity, which we will next examine.

4.6 Author contributions

Francesco Monaca, Rachida Ammari and Johannes Kohl designed experiments and analysis. Francesco Monaca performed surgical procedures for all mice used for behavioural profiling and slice electrophysiology. Francesco Monaca and Rachida Ammari performed behavioural annotation. Francesco Monaca provided custom Python code for behavioural analysis and performed behavioural analysis. Rachida Ammari performed slice electrophysiology experiments and analysis. Patty Wai harvested brain tissue, and performed sectioning and image acquisition for hormone receptor mapping. The Francis Crick Institute's Experimental Histopathology (EHP) Science Technology Platform performed automated in-situ hybridisation for hormone receptor mapping. Johannes Kohl performed analysis for hormone receptor mapping. The Francis Crick Institute's Genetic Modification Service (GeMS) generated the PR^{loxP} transgenic mouse line, while the $Esr1^{loxP}$ line was imported from EMMA (European Mouse Mutant Archive). Patty Wai performed further crosses to generate $Esr1^{KO}$ and PR^{KO} lines.

Chapter 5

The effects of pregnancy on MPOA^{Gal} population activity

5.1 Introduction

Previously, I established that sensitivity of MPOA^{Gal} neurons to pregnancy hormones is necessary for the onset of maternal behaviour. We also determined that hormones remodel MPOA^{Gal} neurons, potentially altering their circuit integration. Next, we asked how pregnancy hormones shape the activity of the MPOA^{Gal} neurons *in vivo*.

Previous studies used bulk calcium activity recordings to interrogate MPOA neural activity *in vivo* during instinctive behaviours, such mating and parenting (Qian et al., 2022; Y.-C. Wei et al., 2018; Zhang et al., 2021). Others used cellular-resolution calcium imaging to probe how internal states or experience modulate the activity of individual neurons, and ensemble representations of social stimuli in hypothalamic areas (Karigo et al., 2021; Kennedy et al., 2020; Remedios et al., 2017; B. Yang et al., 2022). Recent studies investigated MPOA activity with varying degrees of neuronal specificity by recording molecularly distinct MPOA neuronal pools (Bayless et al., 2023; Fang et al., 2018; Kohl et al., 2018; McHenry et al., 2017; Mei, Yan, et al., 2023). However, how internal states, such as pregnancy, modulate the activity of individual neurons and ensemble activity in the MPOA had still been unknown.

In this chapter, I (1) assess the activity of MPOA^{Gal} neurons during pup-directed and other behaviours at cellular resolution, and (2) address how pregnancy affects these responses. Specifically, I performed cellular resolution Ca^{2+} imaging in females to probe pregnancy-induced changes in the MPOA^{Gal} neuronal and neural activity. Each female underwent an imaging session as virgin (Vir), during late pregnancy (D18) and one month after parturition (D50; Fig. 5.1, A). 6-8 weeks before the first imaging session (Vir), sexually naive females were injected with a Cre-dependent GCaMP7s-expressing AAV to target galanin-positive MPOA neurons. Gradient-index (GRIN) lenses were then implanted above the MPOA (Fig. 5.1, B). Importantly, behavioral performance was not affected by GRIN lens implantation: in line with behavioural experiments conducted on in-

tact females (Fig. 3.2), 100% of implanted and imaged PD18 females retrieved pups to the nest. This is noteworthy given that the lens trajectory passes through cortical regions involved in social behavior, such as the cingulate cortex (M. Sato et al., 2023; M. L. Smith et al., 2021), potentially causing damage.

During each imaging session, animals were tethered to a miniaturised microscope in their home cage and subjected to our parental behaviour assay. At the end of the assay, animals were presented with additional social and non-social stimuli, including female and male intruders, a dummy pup, foreign cage bedding material, food pellets, and a novel object (Fig. 5.1, C). After the last *in vivo* recording session, animals were sacrificed, and viral and implantation targeting verified histologically. Baseline signal drift and motion artifacts were removed from the recordings by image pre-processing steps, and region of interests (ROIs) around cells were automatically detected by a PCA-ICA algorithm. The resulting cell set was then manually curated to exclude remaining artifacts or false positives. The typical field-of-view (FOV) from a MPOA^{Gal}-specific imaging session yielded between 15 and 30 verified cells, with a maximum of 60 neurons (Fig. 5.1, D). Behavioural annotation was then performed to extract evoked Ca^{2+} activity around behavioural or stimulus detection events. Finally, MPOA^{Gal} single neuron-level activity was assessed by measuring neuronal evoked responses, tuning and selectivity properties, while population-level neural activity was probed by performing linear discriminant analysis (LDA).

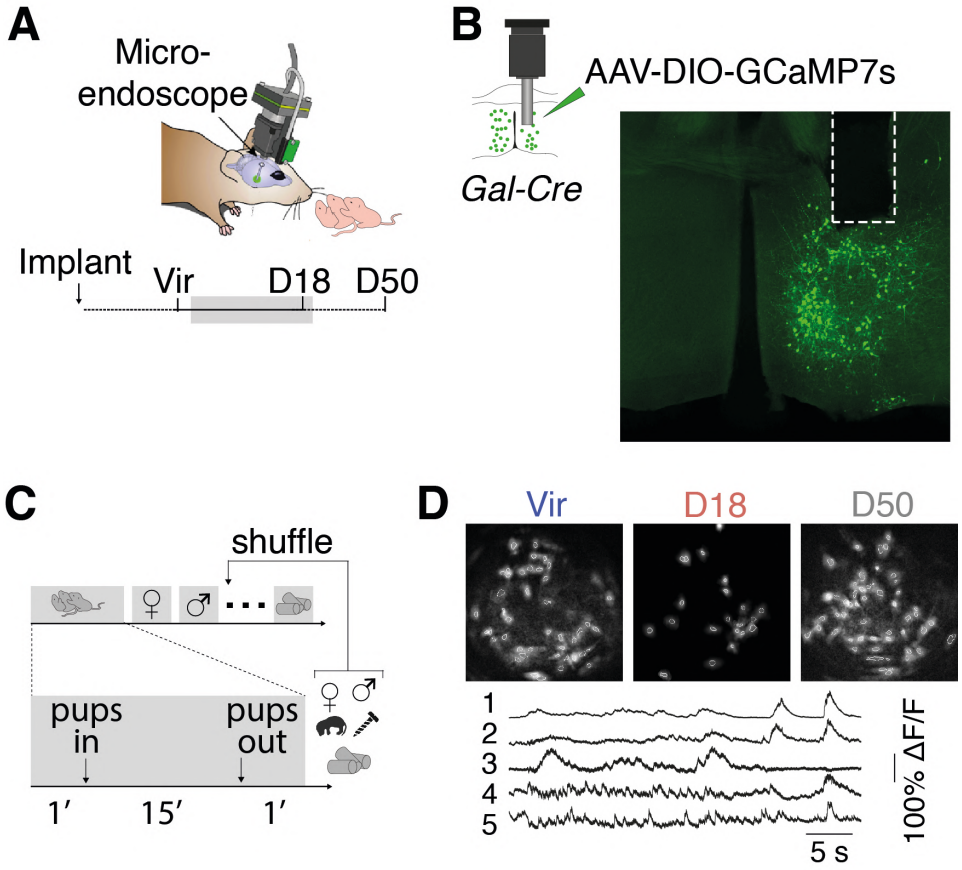

Figure 5.1: **Experimental pipeline for *in vivo* imaging**. (**A**) Experimental strategy for micro-endoscopic imaging before (Vir), during (D18) and after (D50) pregnancy. (**B**) Example histology of implantation site from a *Gal-Cre* female injected with a Cre-dependent GCaMP7s-expressing AAV in the MPOA. Scale bar is 500 μm. (**C**) Experimental design during an imaging session: 15-min maternal assay is followed by a shuffled presentation of intruders and objects. (**D**) Sample field-of-view (FOV) at different timepoints after pre-processing and cell detection, and representative neuronal activity traces from a virgin (Vir) animal. Scale bar is 200 μm.

5.2 Single-neuron analysis

In line with the *ex vivo* neuronal silencing, I observed a MPOA^{Gal}-specific reduction in spontaneously active — i.e. detected, or non-silent — neurons during pregnancy (Vir, 32 ± 7 neurons; D18, 15 ± 3 neurons; Fig. 5.2, G), hinting at a sparsening of MPOA^{Gal} spontaneous activity. To verify that MPOA^{Gal} activity sparsening was not due to a decline in GCaMP expression over time, I imaged MPOA^{Gal} neurons in virgin females at a time interval similar to gestation (21 days)(Fig. 5.2, F). Virgin females had a comparable number of active MPOA^{Gal}

neurons between imaging sessions (Vir-1, 29 ± 8 neurons; Vir-2, 29 ± 9 neurons; Fig. 5.2, H). The number of detected MPOA^{Gal} neurons reverted towards virgin levels in late post-partum, indicating that this change is reversible and does not constitute an experimental artifact (D50, 27 ± 8 neurons; Fig. 5.2, G). Imaging from non-specific MPOA further corroborated these findings. A small, but non-significant, decline in active MPOA neurons was observed at D18, suggesting that the MPOA activity sparsening is specific to MPOA^{Gal} neurons (Vir, 100 ± 11 neurons; D18, 95 ± 8 neurons; Fig. 5.2, A to C).

Neuronal activity was first assessed by quantifying MPOA^{Gal} evoked responses to pup cues or parental actions in females before, during and after pregnancy (Fig. 5.2, A, F). To determine whether an evoked response was significant, unpaired t tests were performed between the calcium activity during the 2 seconds preceding a behavioural event (baseline period) and the activity during the 2 seconds following event detection (activity period) for each neuron. Based on the sign of the t-statistic, each neuron was then categorised as activated or inhibited during a specific behaviour or investigation of a specific stimulus (see Methods).

During pregnancy, the fraction of activated neurons exhibiting pup retrieval-associated evoked responses decreased significantly (Vir, 0.48 ± 0.09; D18, 0.22 ± 0.08; mean ± SEM; Fig. 5.3, B). Conversely, no significant change was observed in the fraction of pup sniffing-activated neurons (Vir, 0.52 ± 0.09; D18, 0.52 ± 0.09; Fig. 5.3, G). Similarly, the fraction of pup retrieval-inhibited neurons and pup sniffing-inhibited neurons were unchanged during pregnancy (Fig. 5.3, D, I). Although the number of pup sniffing-activated neurons was not different between virgin and D18, responses occurred with significantly shorter latency at D18 (Fig. 5.3, C, H), suggesting that MPOA^{Gal} neurons are more excitable to pup stimuli during pregnancy.

To further investigate the specificity of MPOA^{Gal} neurons to sensory and behavioural cues, their tuning index was computed. Neuronal tuning measures how specific a neuronal response is to a given stimulus or a given behaviour, regard-

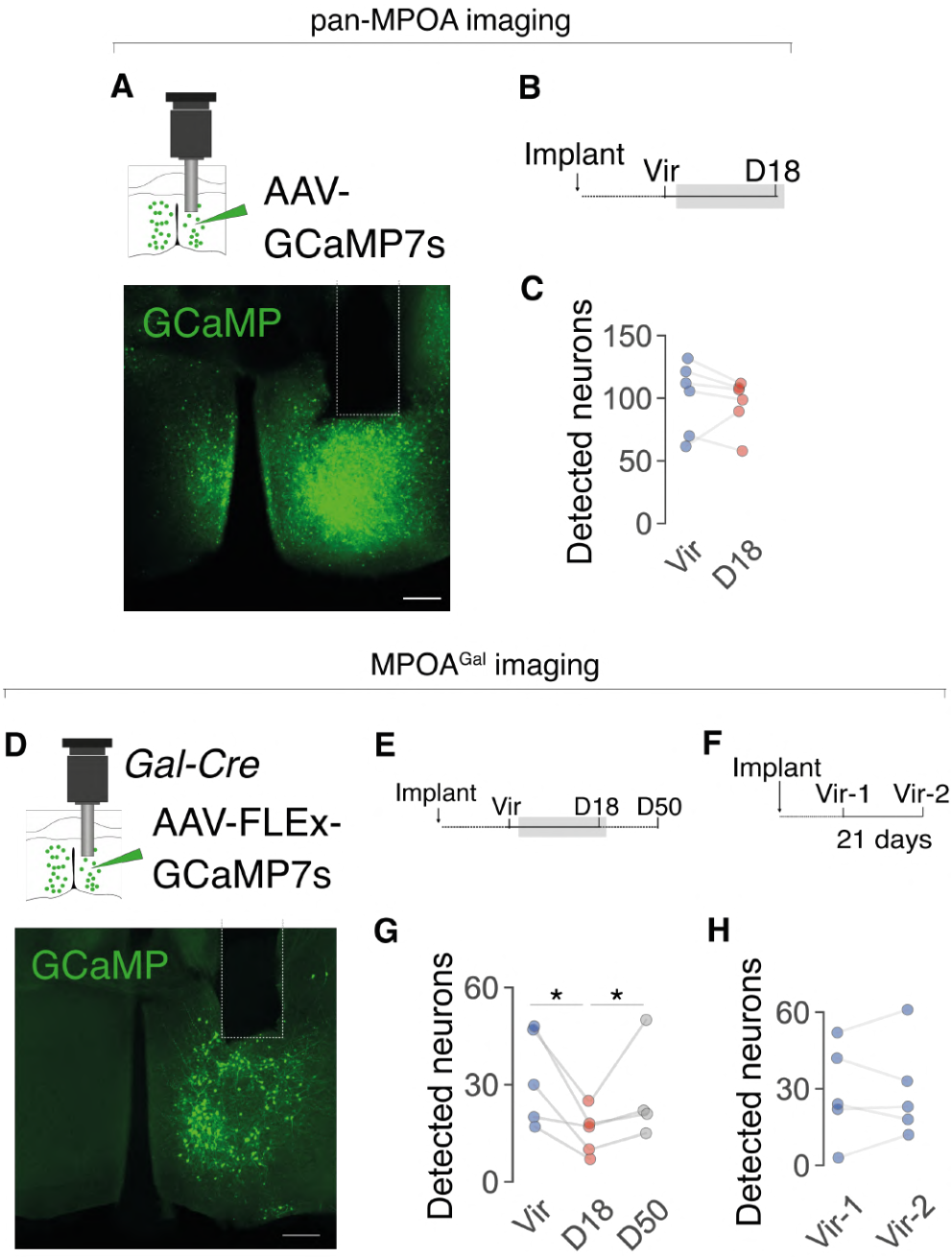

Figure 5.2: **Controls for MPOA^{Gal} population activity sparsening**. (**A** to **C**) MPOA-wide microendoscopic calcium imaging. (**D** to **H**) MPOA^{Gal}-specific microendoscopic imaging. (**A**) and (**B**) Experimental strategy for microendoscopic imaging before (Vir), during (D18) and after (D50) pregnancy, and sample histology of implantation site from a wild-type female injected with a non-conditional GCaMP7s-expressing AAV in the MPOA. Scale bar is 250μm. (**C**) Number of active neurons detected for each mouse (n = 6 animals). (**D**) MPOA^{Gal}-specific micro-endoscopic calcium imaging. Scale bar is 250μm. (**E** to **F**) Experimental strategy for micro-endoscopic imaging of females at Vir, D18 and D50 (E), or virgin females at a 21-day interval (F). (**G**) Number of active neurons detected per mouse at virgin, D18 and D50 (n = 5, 5, 4 animals). (**H**) Number of active neurons detected per mouse at first (Vir-1) and second (Vir-2) imaging sessions of virgin females (n = 5, 5 animals). Statistics by paired t test in (C, G, H). ***P $<$ 0.001; **P $<$ 0.01; *P $<$ 0.05.

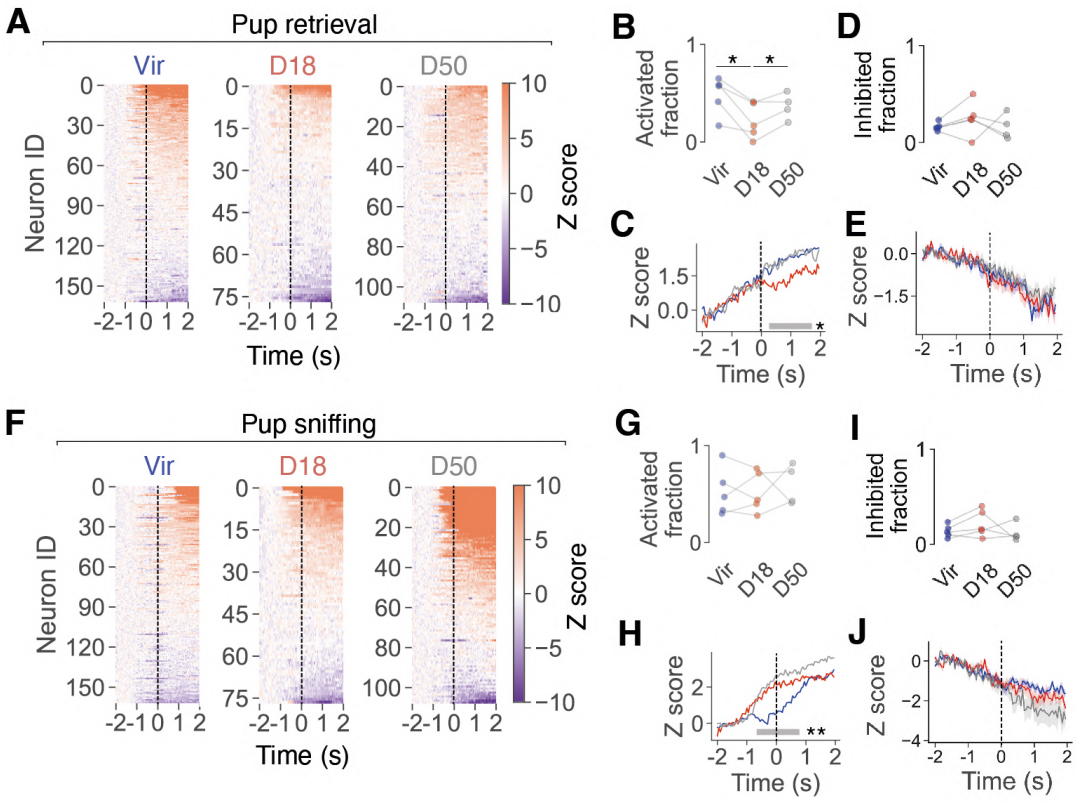

Figure 5.3: **MPOA^{Gal} evoked responses during pup interactions across reproductive stages**. (**A**) Z-scored calcium activity around a pup retrieval episode at virgin, D18 and D50 (n = 5, 5, 4 animals). (**B**) Fraction of neurons exhibiting positive evoked responses to pup retrieval at virgin, D18 and D50 (n = 5, 5, 4 animals). (**C**) Z-scored peri-stimulus time histograms of pup retrieval-activated neurons at virgin, D18 and D50 (n = 115, 41, 63 neurons from 5, 5 and 4 animals). (**D**) Fraction of neurons exhibiting negative evoked responses to pup retrieval at virgin, D18 and D50 (n = 5, 5, 4 animals). (**E**) Z-scored peri-stimulus time histograms of neurons inhibited during pup retrieval at virgin, D18 and D50 (n = 27, 20, 14 neurons from 5, 5, 4 animals). (**F**) Z-scored calcium activity around a pup sniffing episode at virgin, D18 and D50 (n = 5, 5, 4 animals). (**G**) Fraction of neurons exhibiting positive evoked responses to pup sniffing at virgin, D18 and D50 (n = 5, 5, 4 animals). (**H**) Z-scored peri-stimulus time histograms of pup sniffing-activated neurons at virgin, D18 and D50 (n = 112, 51, 86 neurons from 5, 5, 4 animals). (**I**) Fraction of neurons exhibiting negative evoked responses to pup sniffing at virgin, D18 and D50 (n = 5, 5, 4 animals) (**J**). Z-scored peri-stimulus time histograms of neurons inhibited during pup sniffing at virgin, D18 and D50 (n = 24, 16, 11 neurons from 5, 5 and 4 animals). Statistics by paired t test in (B, D, G, I); by unpaired t test in (C, E, H, J). Grey bars in (C, H) indicate significance between virgin and D18. ***P $<$ 0.001; **P $<$ 0.01; *P $<$ 0.05.

less of the direction of the response — i.e. increased or decreased (see Methods). Compared to virgins, MPOA^{Gal} neurons were less tuned to pup retrieval and grooming at D18, whereas they were similarly tuned to pup sniffing (Fig. 5.4, A to C). Together with the decreased number of retrieval-activated neurons, the reduced tuning to pup retrieval and grooming indicates that the overall MPOA^{Gal} population activity decreases during parental actions. This MPOA^{Gal} parenting-associated activity sparsening is reminiscent of the MPOA^{Gal} neuronal silencing effect reported in the slice electrophysiology experiments. I observed that the MPOA^{Gal} baseline activity was negatively correlated with neuronal tuning to pup sniffing, further linking the silencing of neuronal activity to stronger pup cue-evoked responses (Vir, $r^2 = 0.04$, $P < 0.005$; D18, $r^2 = 0.202$; Fig. 5.4, D, E).

By D50, while the number of spontaneously active and retrieval-activated neurons returned to virgin levels (Fig. 5.2, G; Fig. 5.3, B), neuronal tuning to parental actions remained low (Fig. 5.4, A and C), whereas the amplitude of the response evoked by, and tuning to, pup sensory cues were significantly higher than those at both virgin and D18 conditions (Fig. 5.3, H; Fig. 5.4, B). These findings indicate that, while the parenting-associated MPOA^{Gal} responses are temporarily sparsened during pregnancy, pregnancy-induced strengthening of pup-evoked responses persists at least until one month after parturition.

In summary, we observed a decline in spontaneously active, or non-silent, MPOA^{Gal} neurons during pregnancy. This decline hints at a sparsening of the MPOA^{Gal} activity, primarily occurring during the execution of parental actions, as suggested by the reduced fraction of neurons activated by pup retrieval, but not pup sniffing, and the decreased neuronal tuning to pup retrieval and grooming at D18. Interestingly, MPOA^{Gal} activity sparsening occurs during pregnancy and largely disappears by the late post-partum period. At the same time, pregnancy leads to a strengthening of pup-evoked responses, as indicated by the higher amplitude and shorter latency response to pup sniffing at D18. Pregnancy-induced

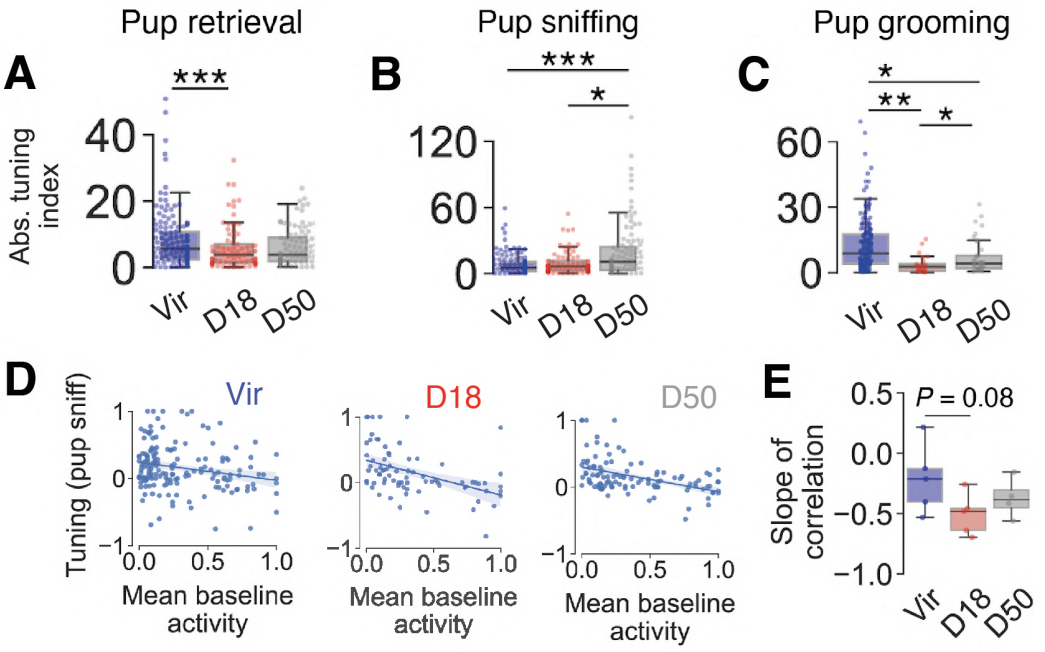

Figure 5.4: **Neuronal tuning to pup-directed behaviours**. (**A-C**) Single-neuron tuning index to pup retrieval (A), pup sniffing (B), pup grooming (C) at virgin, D18 and D50 (n = 5, 5, 4 animals). (**D**) Correlation between normalised neuronal tuning index to pup sniffing and normalised average baseline activity at virgin ($r^2 = 0.04$, $P < 0.005$), D18 ($r^2 = 0.202$, $P < 0.001$) and D50 ($r^2 = 0.194$, $P < 0.001$). (**E**) Slope of correlation between neuronal tuning index to pup sniffing and normalised baseline activity. Statistics by a mixed linear model in (A, B, C); by linear regression in (D); by paired t test in (E). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

strengthening of neuronal response evoked by, and increased neuronal tuning to, pup stimuli is still present at D50. The sparsening of spontaneous and parenting-associated MPOA^{Gal} activity *in vivo* seemed to mirror the silencing of MPOA^{Gal} neurons *ex vivo*.

Next, we examined neuronal responses evoked by chemoinvestigation of other social sensory stimuli and compared them to pup-evoked responses. Mirroring the effect observed for pup sniffing, detection of both male and female sensory cues did not result in significantly reduced fractions of activated neurons between virgins and D18 females (*male intruder contact*: Vir, 0.52 ± 0.12; D18, 0.21 ± 0.11; D50, 0.43 ± 0.08; *female intruder contact*: Vir, 0.44 ± 0.09; D18, 0.26 ± 0.06; D50, 0.42 ± 0.09; Fig. 5.5, A to D). By contrast, significantly stronger neuronal responses were evoked by pup stimuli at D18 and D50 (*D18*; *D50*; Fig.

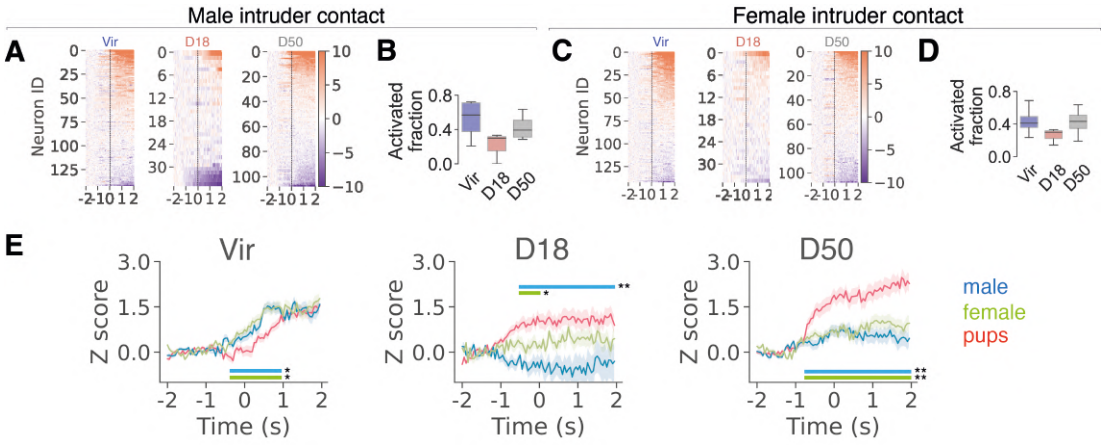

Figure 5.5: **MPOA^{Gal} evoked responses during interactions with intruders across reproductive stages**. (**A**) Averaged calcium activity around contact to a male conspecific at virgin, D18 and D50. (**B**) Fraction of neurons exhibiting positive evoked responses to male intruder contact at virgin, D18 and D50 (n = 5, 5, 4 animals). (**C**) Averaged calcium activity around contact to a female conspecific at virgin, D18 and D50. (**D**) Fraction of neurons exhibiting positive evoked responses to female intruder contact at virgin, D18 and D50 (n = 5, 5, 4 animals). (**E**) Z-scored peri-stimulus time histograms of neurons activated during detection of pups, male or female intruders at virgin, D18 and D50. Statistics by paired t test in (B, D); by unpaired t test in (E). Bars in (E) indicate periods of significance between pup- vs female-evoked activity (green) or pup- vs male-evoked activity (blue). ***P $<$ 0.001; **P $<$ 0.01; *P $<$ 0.05.

5.5, E). Interestingly, the opposite occurred in virgins, where male- and female-evoked responses had higher amplitude than pup-evoked ones (*Vir*; Fig. 5.5, E). In addition, at D18 and D50, pup-evoked responses had a shorter latency than those in virgins (Fig. 5.5, E). This further confirms stronger MPOA^{Gal} neuronal responses to pup sensory cues over other social targets during pregnancy.

To address whether pregnancy enhances the ability of MPOA^{Gal} neurons to discriminate between pups and other social or non-social stimuli, we calculated neuronal selectivity indices by determining the choice probability for each neuron (Fig. 5.6). For any given pair of stimuli, this measure estimates how well a stimulus can be distinguished from the other stimulus based on the distribution of their associated fluorescence activity change. Choice probability was computed between the first sniffing bout of pups and the first sniffing episode of another target from a battery of stimuli presented in a randomised order. A selectivity index of 1 indicates that a neuron was exclusively active during pup sniffing; a

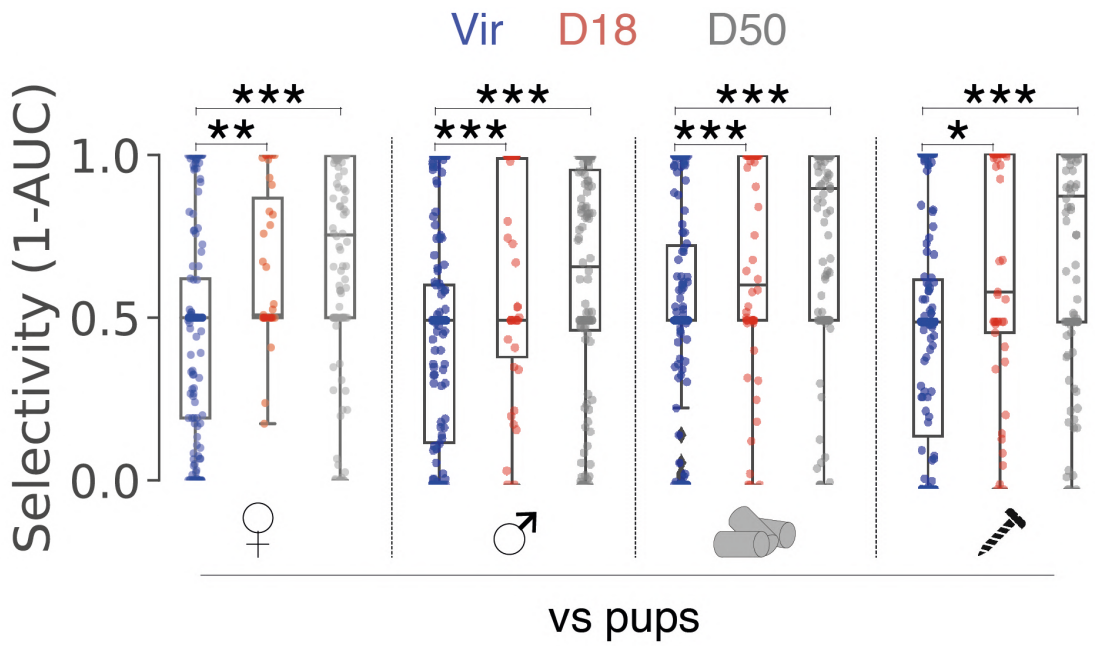

Figure 5.6: **MPOA^{Gal} neuronal selectivity to social and non-social stimuli**. Single-neuron selectivity index of responses evoked by chemoinvestigation of pups versus a female intruder, a male intruder, a food pellet and a novel object (screw) at virgin, D18 and D50 (n = 142, 35, 108 neurons from 4, 3, 4 animals). Statistics by mixed linear regression model. ***P $<$ 0.001; **P $<$ 0.01; *P $<$ 0.05.

neuron with an index of 0 was exclusively active during sniffing of a different social or non-social stimulus, while an index of 0.5 denotes non-selective neurons. Compared to the virgin state, we found that during pregnancy significantly more MPOA^{Gal} neurons were highly selective for pup cues versus all other social and non-social sensory stimuli (Vir vs. D18; Fig. 5.6). Interestingly, neurons retained their increased selectivity for pups in the post-partum period across all stimulus pairs (Vir vs. D50; Fig. 5.6).

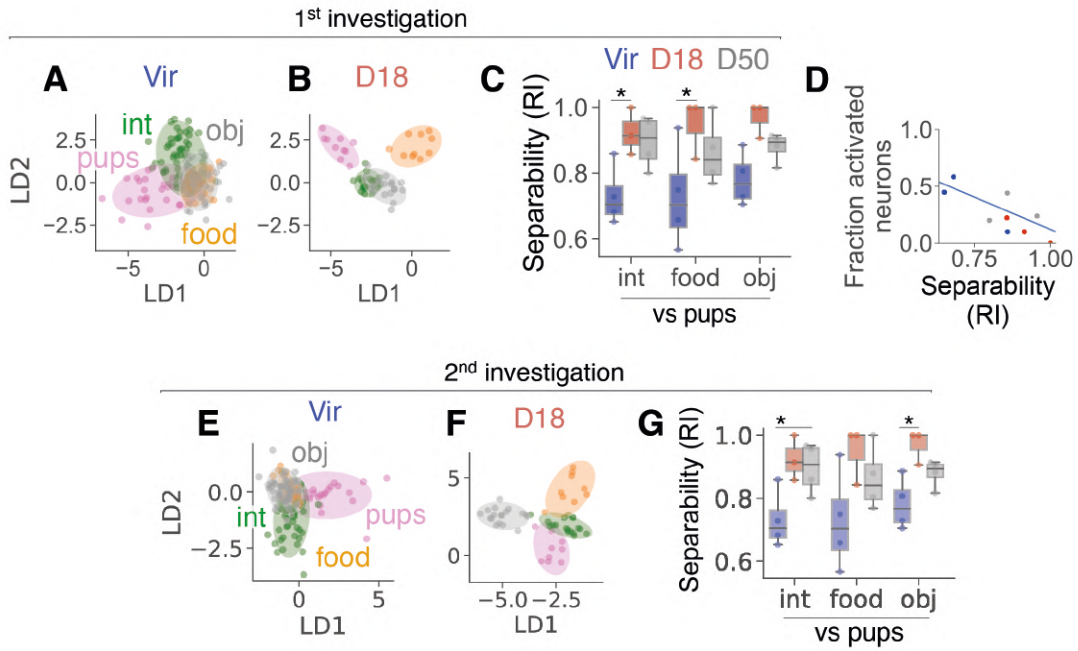

Figure 5.7: **MPOA^{Gal} population dynamics during chemo-investigation by LDA**. (**A** and **B**) Representative neural activity ensemble in LDA space from individual imaging sessions during the first chemo-investigation of pups, male and female intruders (int), food and a novel object (obj) at virgin (A) and D18 (B). (**C**) Separability of pairs of stimuli quantified by Rand Index RI (n = 4, 3, 4 animals). (**D**) Correlation between fraction of pup retrieval-activated neurons and separability of pups vs intruder by RI (r^2 = 0.56, P < 0.001). (**E** and **F**) Representative neural activity ensemble in LDA space from individual imaging sessions during the second chemo-investigation of pups, male and female intruders (int), food and a novel object (obj) at virgin (E) and D18 (F). (**G**) Separability of pairs of stimuli quantified by Rand Index RI (n = 4, 3, 4 animals). Statistics by unpaired t test in (C, G); by linear regression in (D). ***P < 0.001; **P < 0.01; *P < 0.05.

5.3 Analysis of MPOA^{Gal} population dynamics

The single-neuron analysis established that pregnancy affects MPOA^{Gal} neurons by strengthening neuronal responses to, and increasing selectivity for, pups over other stimuli. To address whether the neural representations of pups are affected by pregnancy, I performed a linear discriminant analysis (LDA), thereby classifying neural responses to different stimuli by the MPOA^{Gal} population. This approach projects high-dimensional neural ensemble activity into a lower-dimensional LDA space, where dimensions are chosen to maximise the separation between distinct classes of stimuli. In this instance, the four classes of stimuli

were sensory cues from pups, male and female intruders, a food pellet and a novel object (Fig. 5.7). LDA allowed me to visualise and quantify how well the four classes of stimuli can be separated according to their associated MPOA^{Gal} population activity.

I found that in virgins, neural ensemble activity during detection of all four classes of stimuli was highly overlapping, as visualised by principal LDA discriminants (Fig. 5.7, A). At D18, however, neural ensemble activity became significantly less overlapping (Fig. 5.7, B), suggesting that during pregnancy the separability of pup representations from that of other stimuli increases. The Rand index (RI) was then computed to quantify the degree of separability between the four clusters, and compare ensemble activities across virgin, D18 and D50 conditions. A high RI indicates that LDA separates neural activity well into clusters corresponding to their assigned stimulus categories, thus implying that LDA-obtained clusters are distinct and well-differentiated across conditions. Compared to virgin condition, we observed a significantly higher Rand index for pups versus the other three stimulus clusters at D18 and D50, suggesting that pregnancy preferentially enhances pup representations in the MPOA^{Gal} population (*int vs pups*: Vir, 0.73 ± 0.05; D18, 0.92 ± 0.04; D50, 0.89 ± 0.04; *food vs pups*: Vir, 0.728 ± 0.08; D18, 0.948 ± 0.05; D50, 0.86 ± 0.05; *obj vs pups*: Vir, 0.78 ± 0.04; D18, 0.97 ± 0.03; PD50, 0.88 ± 0.02; Fig. 5.7, C). In addition, a similar pattern of pregnancy-induced changes was observed when LDA was performed with neural activity from the second episode of chemo-investigation (Fig. 5.7, E-G). Interestingly, the separability of MPOA^{Gal} population responses to pups versus intruders was negatively correlated with the fraction of pup retrieval-activated neurons, indicating that MPOA^{Gal} activity sparsening and potentially the improved encoding of parental actions are positively correlated with enhanced pup representations (Fig. 5.7, D).

In conclusion, compared to other social and non-social targets, I found that representations of pups in the MPOA^{Gal} population were better separated by LDA

during pregnancy than at virgin condition. I also observed that the enhanced pup stimuli separability positively correlates with the encoding of parental actions, such as pup retrieval.

In summary, this longitudinal, cellular-resolution calcium imaging from MPOA^{Gal} neurons revealed that pregnancy results in a long-lasting increase in neuronal selectivity for, and separability of, pup stimuli, and in a transiently sparsened population activity leading to enhanced encoding of parental actions.

5.4 The role of hormones in re-organising MPOA^{Gal} activity during pregnancy

We established that MPOA^{Gal} neuronal and neural activity re-organisation occurs during pregnancy. Similar to the effects *ex vivo*, the timecourse of the MPOA^{Gal} activity sparsening suggests that this process is instructed by E2. The long-lasting changes to neuronal selectivity suggests that this process is mediated by P4. However, to causally link the observed functional remodelling to pregnancy hormone action, I sought to perform cellular-resolution calcium imaging from Esr1- and PR-deleted MPOA^{Gal} neurons. Unfortunately, at the time of writing this work, this experiment had not been completed yet, and findings presented here pertain to the optimisation steps needed to successfully record calcium transients from these neurons.

To image receptor-deleted MPOA^{Gal} neurons, the MPOA of *Gal-Flp;PR*^{loxP} females was unilaterally injected with 300 nl of a Flp-dependent AAV expressing the calcium indicator GCaMP7s and Cre recombinase (AAV1-hSyn-fDIO-GCaMP7s-2A-iCre at 6×10^{13} GC/ml), and implanted with a GRIN lens (Fig. 5.8, A). Although neurons were present in the the field-of-view (FOV) (Fig. 5.8, B, top), no calcium transients — i.e. spontaneous activity — were detected throughout the imaging session, as indicated by absent ΔF/F changes in the FOV (Fig. 5.8, B, bottom). This suggests that neurons were unhealthy or over-saturated

with the calcium indicator. To minimise potential neurotoxicity, another animal was therefore injected with the same virus at lower titre (1×10^{13} GC/ml). Given that the miniscope acquisition software permits the live preview of ΔF/F in the FOV while animals are freely behaving in the home cage, fluorescence changes during interactions with a conspecific were checked before each imaging session. In instances where the live ΔF/F did not show any social contact-evoked changes, recordings were not performed. This was the case for the animal whose MPOA was injected with a lower-titre virus. Because GCaMP-expressing neurons were present under the lens (Fig. 5.8, C), the lack of activity might still indicate a toxic over-expression of the calcium indicator. Alternatively, because the 2A peptide might leave a few amino-acids at the C-terminus of the upstream GCaMP, thereby affecting its folding and functionality, neurons might still express a detectable, but defective, calcium indicator.

To address these issues, I tested two different viral strategies to (1) separate GCaMP7s and Cre in different constructs, (2) swap GCaMP7s and Cre in the same vector, so that the 2A peptide is at the C-terminus of Cre, and not GCaMP7s (Fig. 5.8, D). Two weeks after the injection, animals were sacrificed. Both (1) and (2) resulted in GCaMP expression limited to the MPOA (Fig. 5.8, E). I therefore proceeded with viral strategy (1) and implanted a new cohort of animals. This resulted in the successful imaging from PR-deleted MPOA^{Gal} neurons, as indicated by the pup sniffing-evoked activity (Fig. 5.8, F-H). With this viral strategy, some receptor-deleted neurons are expected to be GCaMP-negative due to Cre-dependent, but not Flp-dependent, GCaMP expression. Nonetheless, this is not an issue as all GCaMP-expressing neurons are expected to be receptor ablated. In future experiments, it will be exciting to increase the sample size, image from Esr1-deleted MPOA^{Gal} neurons, and explore whether receptor ablation directly affects the activity changes of this neuronal population observed during pregnancy.

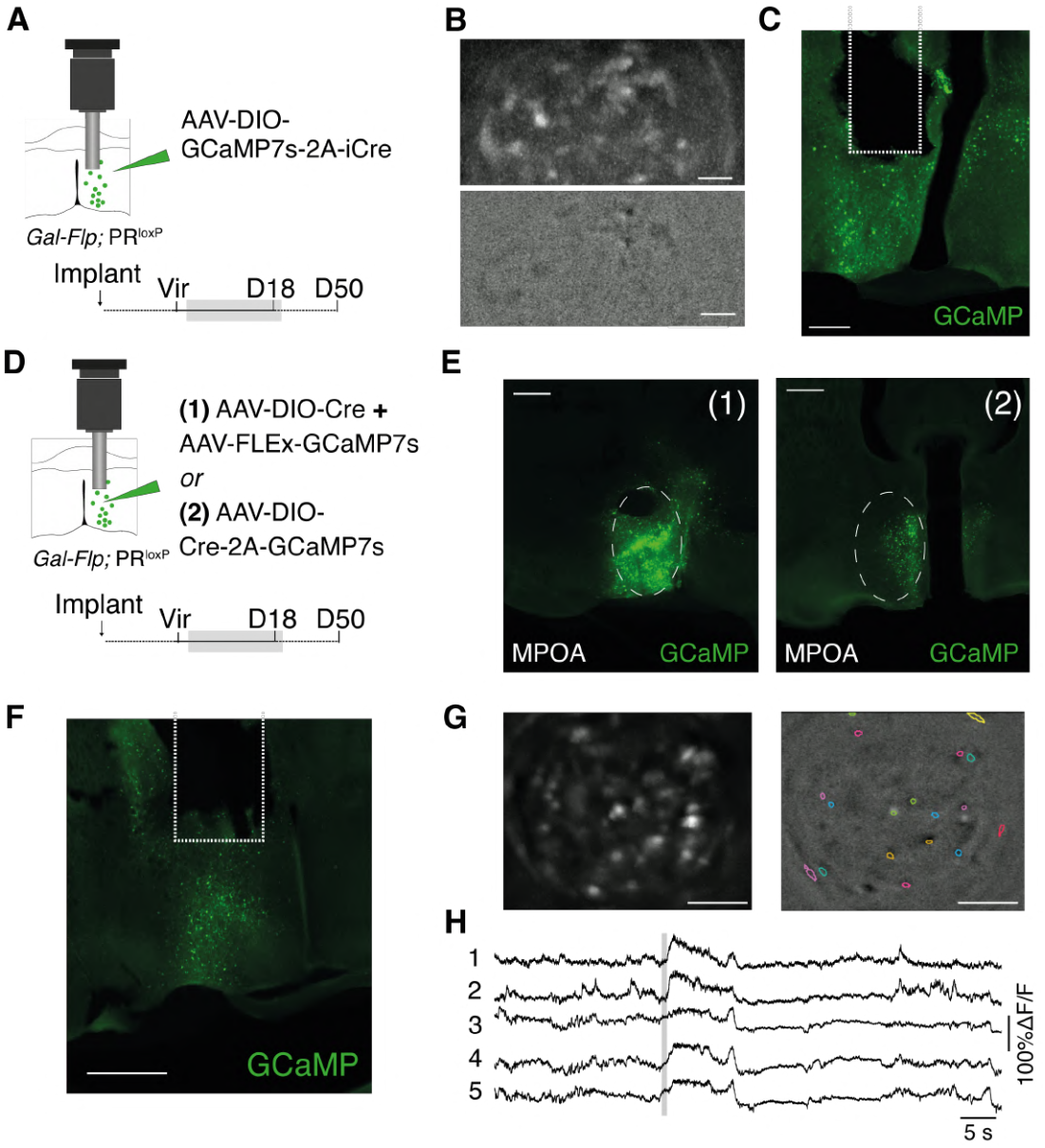

Figure 5.8: **Optimisation steps for imaging from receptor deleted-MPOA^{Gal} neurons**. (**A**) Experimental strategy for imaging from PR-deleted MPOA^{Gal} neurons via Flp-dependent AAV expressing GCaMP7s and Cre in Gal-Flp; PR^{loxP} females. (**B**) Representative FOV after pre-processing and cell detection (top), and ΔF/F from the same FOV (bottom) from a Gal-Flp; PR^{loxp} female injected with AAV-DIO-GCaMP7s-2A-iCre (6×10^{13} GC/ml). Scale bars: 100 μm. (**C**) Example histology of implantation site from a female injected with AAV-DIO-GCaMP7s-2A-iCre (1×10^{13} GC/ml). Scale bar: 100 μm. (**D**) Experimental strategy for imaging from PR-deleted MPOA^{Gal} neurons via (1) double-virus approach to separately express GCaMP7s and Cre, or (2) single-virus approach with swapped Cre and GCaMP7s. (**E**) Example MPOA sections two weeks after injection with strategy (1) or (2). Scale bars: 400 μm. (**F**) Example histology of implantation site following injection with double-virus approach (1). Scale bar: 400 μm. (**G**) Representative FOV after pre-processing and cell detection (left), and ΔF/F activity from the same FOV (right). ROIs indicate automatically detected and manually curated set of neurons. Scale bars: 200 μm. (**H**) Representative neuronal activity traces. Indicated is first pup contact.

5.5 Discussion

In this chapter, we probed single neuron- and population-level MPOA^{Gal} activity *in vivo* to assess pregnancy-induced functional changes that could underlie the observed behavioural adaptations.

I observed a reduction in the number of spontaneously active MPOA^{Gal} neurons during pregnancy. In the post-partum period, numbers of active neurons returned to virgin-like levels. Importantly, the reduction was non-significant in non-specific MPOA neurons, suggesting that MPOA^{Gal} neurons were selectively affected. In addition, the absence of the phenomenon in recordings from virgin females imaged 21 days apart — a time window comparable to gestation — indicates that pregnancy, rather than a decrease in calcium indicator expression over time (or neuronal death), transiently modulates the reduction in MPOA^{Gal} neuronal activity. Correspondingly, I also noted a reversible decrease in the fraction of neurons activated by pup retrieval and a decline in the neuronal tuning to both pup retrieval and grooming, indicating that MPOA^{Gal} activity underlying parental actions becomes transiently sparsened during pregnancy. The MPOA^{Gal} spontaneous activity sparsening seems to mirror the neuronal silencing reported by the slice electrophysiology experiments, suggesting that fewer MPOA^{Gal} neurons are active during pregnancy. I also noted that MPOA^{Gal} neurons encode parental actions more sparsely during pregnancy. Given that parental performance is upregulated during pregnancy, an overall reduction in parenting-associated neural activity might seem counter-intuitive. However, sparse coding has been described as a more economical and efficient neural strategy to represent complex sensory or motor information (Olshausen and Field, 2004; Vinograd et al., 2017). I therefore reason that activity sparsening through neuronal silencing promotes a more efficient encoding of parental actions, thus driving a more robust execution of parenting in response to pups in pregnant females. This hypothesis is further supported by the stronger positive neuronal responses evoked by pup sniffing during pregnancy. Pup-evoked neuronal responses had a higher amplitude in

pregnant females than in virgins. In contrast, response amplitude to other social stimuli was not modulated by pregnancy. This indicates that, during and after pregnancy, MPOA^{Gal} neurons respond more strongly to pups than to other social stimuli. By comparing the distribution of stimulus-evoked MPOA^{Gal} neuronal activity across pairs of sensory stimuli, I found that MPOA^{Gal} neurons were more selective to pups than to other social and non-social stimuli during pregnancy. Heightened selectivity for pup cues was still present in the post-partum period, hinting at the long-lasting nature of this modification. Altogether, activity sparsening, strengthening of pup-evoked responses and increased selectivity for pup stimuli at the level of individual neurons might lead to enhanced population-level separability of neural ensemble activity between pup cues versus other stimuli. Correspondingly, pup stimulus separability was positively correlated with the fraction of retrieval-activated neurons, thereby linking activity sparsening with enhanced pup representation.

Consistent with the neuronal silencing *ex vivo*, population sparsening is transient, and thus likely modulated by E2. I hypothesise that this process promotes the P4-mediated recruitment of additional excitatory synaptic inputs onto spontaneously active MPOA^{Gal} neurons, thus resulting in long-lasting potentiation and heightened selectivity of their responses for pup stimuli. Cellular modifications downstream of Esr1 and PR activation would be an interesting topic of future investigations. In addition, although other factors might contribute to the improved parental performance retained by females weeks after parturition, such as frequent exposure to pups, the MPOA^{Gal} circuit remodelling and resulting reorganisation of its activity might underlie post-partum maternal behaviour. Finally, to causally link hormone action with the functional changes observed here, activity from Esr1- or PR-deleted MPOA^{Gal} neurons will need to be recorded.

In summary, we explored functional changes occurring in the MPOA^{Gal} population during pregnancy. I demonstrated that pregnancy results in sparsened population activity during parental actions and in stronger and more selective neu-

ronal responses to pup stimuli. I also propose that the remodelling of MPOA^{Gal} neuronal and neural activities is enabled by E2- and P4-mediated cellular modifications, which prime this population for a more efficient and robust execution of parental behaviour in response to pups. Whether these preparatory functional changes are the direct consequence of hormone action on MPOA^{Gal} neurons is likely yet remains to be addressed in future work.

5.6 Authors contributions

Francesco Monaca, Rachida Ammari, Mingran Cao and Johannes Kohl designed the experiments and the analysis. Francesco Monaca and Mingran Cao performed surgical procedures for all mice and imaging experiments. Francesco Monaca, Mingran Cao and Rachida Ammari performed behavioural annotation. Francesco Monaca and Mingran Cao performed analysis of calcium imaging data, with supervision and input from Johannes Kohl. Neven Borak provided Python functions for parts of the calcium imaging analysis. Francesco Monaca performed histology.

Chapter 6

Conclusions and future directions

6.1 Summary

In the past decades, rapid progress has been made in uncovering the circuit basis of instinctive behaviours. At the same time, plasticity mechanisms over different timescales have been described. However, establishing a relationship between neural plasticity and instinctive behavioural flexibility has remained challenging. Parenting provides an ideal framework to study how hormones remodel key neural circuits to drive behavioural adaptations in infant-directed interactions. The MPOA has a well-documented role in parenting, and recent studies have described behaviourally-relevant and molecularly-defined MPOA populations. Several lines of evidence, primarily in rats, have also linked hormonal events of pregnancy with increased parental performance. Stimulation of the MPOA with ovarian hormones upregulated during pregnancy robustly induces parental behaviour. It was therefore known that the hormonal events of pregnancy act on the MPOA and induce parental behaviour. However, whether and how pregnancy hormones remodel neurons in the MPOA to induce parental behaviour has remained unclear.

To address this question, I tested the following main hypotheses:

1. Parental behaviour changes already during pregnancy.

2. Steroid hormones mediate changes to pup-directed behaviour by acting on $MPOA^{Gal}$ neurons.

3. The biophysical properties of $MPOA^{Gal}$ neurons are affected by steroid hormone action during pregnancy.

4. Pregnancy affects the population activity of $MPOA^{Gal}$ neurons to support better maternal performance.

In Chapter 3, I tested the first hypothesis by characterising parental behaviours in female mice before, during and after pregnancy. I uncovered preparatory and long-lasting modifications of parental behaviour already during preg-

nancy. These behavioural changes were primarily driven by pregnancy rather than repeated exposure to pups. In Chapter 4, I confirmed that steroid hormone receptors are highly enriched in the MPOA, and that the ovarian hormones E2 and P4 directly act on MPOA^{Gal} neurons via their intracellular receptors to induce changes in infant-directed behaviours. This provided evidence for the second hypothesis. Crucially, E2 and P4 exert distinct, non-overlapping effects on the biophysical and morphological properties of MPOA^{Gal} neurons: while E2 induces a transient silencing and increase in intrinsic excitability of MPOA^{Gal} neurons, P4 mediates the long-lasting recruitment of new synaptic inputs on active MPOA^{Gal} neurons (Fig. 6.1). These results support the third hypothesis. I finally propose that the two hormones operate together to sparsen MPOA^{Gal} population, as well as to permanently increase selectivity for, and discriminability of, pup sensory cues. From both single neuron- and population-level perspectives, this increases the signal-to-noise ratio of pup representations in MPOA^{Gal} neurons. These observations, which I presented in Chapter 5, validate the fourth hypothesis.

These findings suggest that pregnancy orchestrates preparatory adaptations to infant-directed behaviours by reshaping a neural circuit critical for parenting.

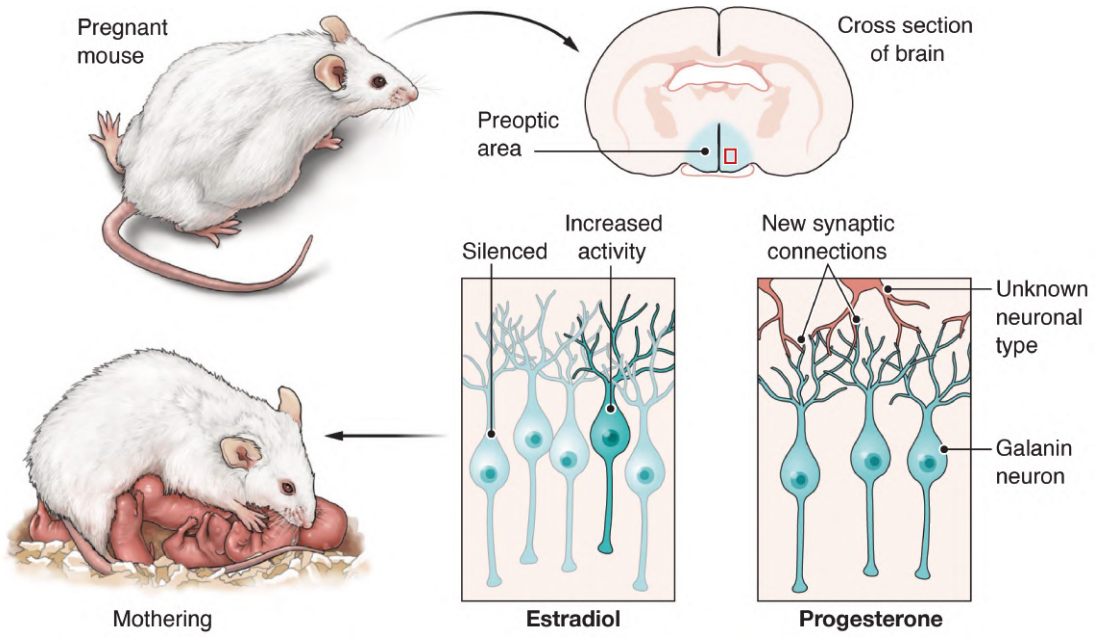

Figure 6.1: **Pregnancy hormones promote parental behaviour**. Graphical abstract summarising the main findings of this work. Adapted from McCarthy, 2023.

6.2 Future directions

This work opens several avenues for future investigation, which I will discuss next.

6.2.1 Hormone-induced changes in local MPOA connectivity

The MPOA^{Gal} population alone consists of several transcriptome types (Moffitt et al., 2018) and functionally distinct pools characterised by projection target. It receives and sends projection to more than 20 brain regions (Kohl et al., 2018). However, the potential relationship between the projection-based classification of MPOA^{Gal} neurons and their transcriptome types remains unclear. Since galanin is expressed in five different inhibitory and two excitatory cell types in the MPOA (Moffitt et al., 2018), an open question is whether transcriptionally-defined neuronal clusters are also functionally specialised. It would also be interesting to explore whether a correspondence between between projection type, transcriptional identity, and activity pattern exists. This would have important conse-

quences. For instance, the increased excitatory synaptic transmission targeting spontaneously active MPOA^{Gal} neurons would differentially affect MPOA^{Gal} post-synaptic partners depending on which molecularly-distinct cell type — excitatory vs inhibitory — is preferentially affected by the phenomenon. By contrast, neuronal silencing would result in disinhibition or enhanced inhibition of downstream areas, depending on whether affected MPOA^{Gal} neurons belong to an inhibitory or excitatory cluster, respectively. Accordingly, it would be exciting to dissect whether there are clusters of MPOA^{Gal} neurons exhibiting divergent responses to pregnancy hormones.

Given that the majority of MPOA^{Gal} neurons are GABAergic, pregnancy nonetheless results in an overall increase of inhibitory tones within this neuronal population, but a finer dissection of the excitation-inhibition balance would further advance our circuit-level understanding of hormone-mediated neural plasticity.

Inputs to MPOA^{Gal} neurons have been mapped (reviewed in Láng et al., 2024), but the source of the increased excitatory inputs is still unclear. Given that extensive reciprocal connectivity has been reported within the MPOA (Kohl et al., 2018), enhanced excitation might originate from glutamatergic MPOA^{Gal} neurons. Glutamatergic MPOA neurons receive direct inhibitory inputs from GABAergic MPOA neurons (Zhang et al., 2021). Though not directly tested, it is equally likely that antagonistic connectivity exists between these two MPOA populations and, by extension, between MPOA^{Gal} neurons. In addition to MPOA^{Gal} neurons, other molecularly-defined MPOA populations might be the pre-synaptic excitatory candidates. For instance, glutamatergic MPOA^{Calcr} neurons, most of which are Esr1-positive, have a crucial role in female caregiving behaviours (Yoshihara et al., 2021). However, because glutamatergic MPOA neurons negatively affect parenting, it is more likely that increased excitatory inputs stem from other parenting-promoting brain regions presumably carrying pup sensory information, such as auditory or somatosensory cortices, although direct projections

from these areas have not been explored yet (Bailey and Isogai, 2022).

The impact of hormonal modulation on local MPOA circuitry and connectivity will be a fascinating topic for further studies.

6.2.2 Experience-dependent upregulation of maternal behaviour

Although female mice exhibit spontaneous caregiving behaviours upon their first exposure to pups, frequent pup exposure further improves their (allo)parental performance (AlsinaLlanes et al., 2015; Numan, 2007; Stolzenberg and Rissman, 2011). Because it occurs in non-pregnant and non-lactating females, as well as in ovariectomised females, this so-called "sensitisation" effect has been seen as hormone-independent (Gandelman, 1973; Rosenblatt, 1967). In absence of gestational hormones, virgins require significantly more exposure time to pups to reach a parental performance comparable to that of post-partum females, suggesting that pregnancy hormones instruct a rapid onset of robust parenting (Fleming et al., 1994; H. S. Mayer et al., 2019). Nonetheless, although it does not induce the full complement of parental behaviours, such as maternal aggression (Martín-Sánchez et al., 2015; A. D. Mayer and Rosenblatt, 1987), sensitisation seems to ultimately results in high levels of maternal responsiveness (Stolzenberg et al., 2014). Similarly, virgin females co-housed with experienced dams and pups exhibit parenting within days, indicating that maternal experience can also be acquired by social transmission (Carcea et al., 2021). Due to the fact that sensitisation-driven alloparenting relies on learned behaviours independently of gonadal hormones (Okabe et al., 2013), current studies on its neural correlates have largely focussed on cortical circuits or have examined the contribution of oxytocin, which is conventionally associated with contact-mediated social bonding (Nagasawa et al., 2012). Oxytocin is synthesised in the PVH and supraoptic (SO) nuclei, acting on receptors distributed across various brain regions, including those within the hypothalamic parenting circuit. The MPOA and VTA express

oxytocin receptors (Oxtr), and estradiol-mediated increases in Oxtr expression have been reported in the MPOA (Pedersen et al., 1994; Peris et al., 2017). Infusion of an oxytocin antagonist into the MPOA and VTA inhibits the induction of parental behaviour in inexperienced females (Okabe et al., 2017; Pedersen et al., 1994; Van Leengoed et al., 1987). Interestingly, while mothers with whole-body oxytocin and Oxtr-knockout exhibit normal maternal behaviour, with the exception of impaired milk ejection (Takayanagi et al., 2005; Tsuneoka et al., 2022; Young Iii et al., 1996), their caregiving behaviours are deficient under stressful conditions (Ng et al., 2023). By contrast, virgin females with whole-body oxytocin knockout do not display parenting under any circumstance (Pedersen et al., 2006). This hints at a facilitatory role of the oxytocin system in mediating the onset of alloparental care (Okabe et al., 2017). Correspondingly, a recent study found that conditional deletion of oxytocin or ablation of oxytocinergic neurons in the PVH and SO of lactating females did not disrupt parenting, whereas virgins failed to develop alloparental behaviours (Hagihara et al., 2023). Similarly, chemogenetic inhibition of PVH oxytocinergic neurons impaired pup retrieval in virgin females that had never performed the behaviour, but not in retrieving virgins (Carcea et al., 2021). Oxytocin action is therefore important for the acquisition of parental behaviour in inexperienced virgin females, while expression of parental behaviour in experienced virgins or dams does not require oxytocin secretion. Oxytocin might exert its experience-dependent modulation on parenting by affecting sensory processing of pup cues. For instance, the left auditory cortex, whose activity has been shown to be strongly correlated with pup calls (Marlin et al., 2015), conveys infant cries to PVH oxytocinergic neurons via the intralaminar nucleus of the thalamus (PIL), and inhibition of the PIL-to-PVH connection has been shown to decrease preference for pup calls (Valtcheva et al., 2023). Enhanced PVH neuronal firing upon pup calls ultimately leads to oxytocin release in the VTA (Valtcheva et al., 2023), thus affecting maternal motivation. In turn, auditory cue-induced oxytocin release might feed back to the

auditory cortex (Mitre et al., 2016; Schiavo et al., 2020), where oxytocin infusion shortens retrieval latencies of virgin females. In contrast, delivery of an Oxtr antagonist to the left auditory cortex of mothers did not affect parenting (Marlin et al., 2015). These experience-dependent effects result from a shift in excitation-inhibition balance of the auditory cortex neuronal activity (Marlin et al., 2015). In addition to the VTA, PVH oxytocinergic neurons contact other downstream targets, such as MPOA$^{\text{Calcr}}$ (Inada et al., 2022), PeFA$^{\text{Ucn3}}$ (Autry et al., 2021) and AHI$^{\text{Oxtr}}$ neurons (K. Sato et al., 2020), indicating that the oxytocin system modulates the activity of neural populations associated with parental behaviour. These observations indicate that, though operating in both virgins and mothers, the oxytocin system might differentially modulate parenting, and orchestrate its acquisition in inexperienced mice, by increasing the salience of pup cues. It has been proposed that in inexperienced females, PVH oxytocinergic neurons act on and remodel parenting-associated downstream circuits to support long-lasting behavioural modifications (Inada, 2024). Although MPOA$^{\text{Gal}}$ neurons do not seem to receive direct inputs from PVH oxytocinergic neurons, they might influence oxytocin release via tyrosine-hydroxylase (TH)-expressing neurons in the AVPe (Kohl et al., 2018; Scott et al., 2015), and a modulatory effect of PVH neurons on MPOA neuronal activity was previously reported (Inada et al., 2022). An outstanding question is therefore whether experience-induced mechanisms and pregnancy-induced hormonal signalling rely on shared neural circuitry to induce parenting. If that is the case, it can be hypothesised that frequent exposure to pups ultimately activates the core parenting neural circuit, which in turn modulates oxytocin release to affect the salience of pup cues. Alternatively, pup exposure might activate PVH oxytocinergic activity, which increases salience of pup cues, thereby driving the core parenting circuits. During pregnancy, hormone action might supplant these mechanisms, relegating the oxytocin system to supporting roles relevant to pregnancy, such as parturition and lactation. Repeated exposure to pups has been shown to upregulate histone acetylation in the

MPOA to induce the expression of genes normally triggered by parturition (H. S. Mayer et al., 2019; Stolzenberg et al., 2012), raising the possibility that hormone- and experience-mediated pathways might ultimately converge on common cellular effectors. However, an anterior cingulate cortex (ACC)-centrolateral thalamic nucleus (CL) circuit controlling the acquisition of parental behaviour exclusively in females was recently described (Glat et al., 2022), suggesting that experience-induced maternal responsiveness might rely on redundant neural circuits. Despite this progress, neural substrates regulating parental behaviour in the absence of pregnancy hormonal stimulation represent a fascinating subject for future studies.

6.2.3 Influence of strain on parental behaviour

Throughout this work, laboratory mice from the C57BL/6 strain were used. Compared to laboratory C57BL/6 female mice, wild mice show markedly different parental behaviour. While laboratory females rarely display pup-directed aggression and tend to be spontaneously parental even before mating and gestation, virtually all wild virgins are infanticidal until parturition (Lonstein and De Vries, 2000; Numan, 2020). Furthermore, unlike laboratory mice, wild females do not retain long-term parental responsiveness, rapidly reverting to infanticidal behaviour after removal or weaning of pups (Lonstein and De Vries, 2000; Mccarthy and Vomsaal, 1985; Soroker and Terkel, 1988). This suggests that wild mice are more strongly relying on hormonal priming than laboratory animals. Despite the presence of spontaneous parenting in virgins, this work reported clear hormone-induced improvement in parental behaviour during late pregnancy. Given the absence of baseline parental care in wild virgins, it can be hypothesised that the same endocrine mechanisms would induce even more pronounced behavioural changes in wild females, as their shift from infanticidal to maternal state likely requires a greater degree of hormonal modulation.

Furthermore, variations in parental behaviour have also been reported across laboratory strains (Shoji and Kato, 2006; Ward, 1980). Observations of five

inbred strains (BALB/c, CBA/Ca, C3H/He, DBA/2, C57BL/6) revealed strain-specific postpartum behaviours. For instance, BALB/c displayed lower levels of pup grooming and were generally slower in retrieving pups worse parental performance compared to the other strains, particularly C57BL/6 and CBA/Ca, which showed more robust parental care (Shoji and Kato, 2006; Ward, 1980).

It would thus be interesting to test the effect of pregnancy hormones on parental behaviour and its neural circuits across strains, and further explore whether these effects vary in parallel with baseline parental responsiveness.

6.2.4 Neural basis of paternal behaviour

Despite the prominent role of mothers in parental provision, a small percentage of mammalian species displays paternal care, including prairie voles (*Microtus ochrogaster*), old-field mice (*Peromyscus polionotus*), house mice (*Mus musculus*) and humans. In contrast to virgin female mice, many of which are spontaneously parental, sexually-naïve males either attack or ignore pups. Pup-directed aggression and infanticide are, however, abolished in sexually-experienced males. Both ejaculation and co-habitation with a pregnant female seem to be necessary for the transition from infanticide to parenting in males (post-mating switch) (Numan, 2020; Vom Saal, 1985; Vom Saal and Howard, 1982). Intriguingly, the duration of the post-mating switch is similar to the female's gestation duration, suggesting that it represents a timekeeping mechanism to ensure that paternal behaviour is directed at given male's potential offspring

Despite the presence of sexually dimorphic circuit elements (for example, Scott et al., 2015 and Inada et al., 2022), the only differences detected so far between sexes are of quantitative nature — i.e., more cells in one sex, or different connection strength. Lesion studies have implicated the MPOA in parenting in male mice and the BNSTrh for the regulation of pup-directed aggression (Tsuneoka et al., 2015). Within the MPOA, ablation of $MPOA^{Gal}$ neurons disrupted paternal behaviour, and a correlation between number of remaining $MPOA^{Gal}$ neurons

and parental performance was observed in fathers (Wu et al., 2014). Similarly, chemogenetic activation of MPOA^{Calcr} neurons reduced infanticide (Yoshihara et al., 2021). Activation of projection-specific MPOA^{Gal} subsets resulted in similar effects in both males and females (Kohl et al., 2018). In addition, in the MPOA, vGluT2-expressing excitatory neurons and vGAT-expressing inhibitory neurons facilitate and inhibit pup-directed aggression in males, respectively; the latter is regulated by projections to the PAG (Zhang et al., 2021). Despite these recent advances, parental behaviour is much less explored in rodent males as compared to females. Since males do not experience pregnancy, the mechanisms underlying the post-mating switch are still unclear. Plasticity changes have been linked to the transition from pup aggression to caring in males. For instance, increased excitatory synaptic transmission has been observed in LHA projections to PVH oxytocinergic neurons in fathers, but not in virgin males, thereby promoting paternal behaviours (Inada et al., 2022). Notably, the same study reported that excitatory synaptic transmission from the LHA returned to virgin levels when males were separated from pups for only 5 weeks, providing a potential cellular correlate to the reversible behavioural change. Moreover, activation of inhibitory BNST-projecting MPOA neurons promotes parenting in male mice (Tsuneoka et al., 2015). Very recently, inhibitory synaptic transmission was found to be increased in the BNSTrh of males following mating and paternal experience (Ito et al., 2023). By contrast, inhibitory inputs from the MeA to the MPOA neurons, most of which were MPOA^{Gal} neurons, were suppressed before pup delivery in males (Ito et al., 2023). This indicates that mating and co-habitation with a late gestational female lead to disinhibition of the MPOA, and increased inhibition of the BNST, thereby suppressing pup-directed aggression. It would be interesting to see whether the enhanced inhibitory drive in the BNSTrh originates from (remodelled) MPOA^{Gal} neurons as a result of their increased excitability. This would suggest that plasticity changes described in this work occur in the MPOA of both males and females. Collectively, these findings indicate that, similar

to females, the MPOA is a key area in the regulation of opposing pup-directed behaviours in males. Specifically, they reveal that inhibitory MPOA activity positively regulates parenting by suppressing infanticide-promoting neurons (Zhang et al., 2021).

In males, the factors triggering neural plasticity and subsequent behavioural changes remain elusive. Despite the absence of a drastic physiological change comparable to pregnancy, hormones might still contribute to the onset of fathering. Conditional deletion of prolactin receptors from the MPOA abolished pup retrieval and grooming in males (Stagkourakis et al., 2020) and secretion of oxytocin from PVH neurons has been reported to positively influence the activity of prosocial MPOA$^{\text{Calcr}}$ neurons, while suppressing infanticide-promoting PeFA$^{\text{Ucn3}}$ neurons (Autry et al., 2021; Yoshihara et al., 2021). Additionally, vasopressin-induced paternal behaviours seem to be mediated in part by oxytocin receptors in the MPOA (Inada et al., 2024). Interestingly, in the MPOA of fathers, the number of Esr1-positive neurons increases with pup exposure and parental experience (Ehret et al., 1993). Continuous stimulation of MPOA with estradiol induced parenting in males which were subjected to an estradiol-progesterone priming regime mimicking pregnancy (Rosenblatt et al., 1998). Males have naturally low levels of circulating estrogens (Nilsson et al., 2015), and these can be converted in the brain from testosterone by aromatase (GarciaSegura, 2008). Estrogens synthesised in the brain have been linked to parental onset in mice (Trainor and Marler, 2002; Trainor et al., 2003). Accordingly, pharmacological inhibition of aromatase decreases pup retrieval in fathers (Akther et al., 2015) and increased aromatase activity has been reported in the MPOA and other limbic regions following paternal experience (Duarte-Guterman et al., 2023). It can therefore be hypothesised that, in the transition to fatherhood, increased aromatase activity in the MPOA promotes estradiol signalling which, in turn, mediates cellular modifications comparable to those we reported in pregnant females. Consistent with this view, current circuit-level evidence seems to suggest that enhanced

MPOA inhibitory activity, possibly via $MPOA^{Gal}$ neurons, plays an important role in suppressing pup-directed aggression in males (Ito et al., 2023). To test this hypothesis, I performed pilot experiments to assess whether whether C57BL/6 males undergo the post-mating switch (*data not shown*). Following a similar experimental design employed for maternal behaviour testing, I longitudinally quantified pup-directed interactions in males exposed to pups at 4-day intervals. Behaviour testing was performed at the same timepoints used in Chapter 3. To maximise the likelihood of behavioural switching, males co-habited with the female they successfully mated with throughout the experiment. Interestingly, while all male mice attacked pups during their first exposure at virgin state, the percentage of males displaying pup-directed aggression dropped drastically when the female was in the final stages of gestation. Males also started retrieving pups to the nest after their birth, and remained highly parental until the female's late post-partum period. Several external factors might influence the observed switch to parental behaviour, including co-habitation with the female and subsequent exposure to pups. Nevertheless, these preliminary findings constitute a robust starting point for future investigations into the neural (and possibly hormonal) basis of the post-mating switch in males.

6.3 Conclusion

By investigating pregnancy-induced neural remodelling underlying parenting onset, this work connected steroid action to neural activity and behavioural adaptations. Understanding the mechanisms of pregnancy-mediated neural plasticity provides conceptual advances into how steroid signalling modulates other instinctive behaviours and prepare animals for future behavioural needs. Excitingly, these findings also have potential implications for human biology. Despite the pervasive idea of "pregnancy brain" and surveys showing that 50-80% of people experiencing pregnancy and childbirth report cognitive impairments, such as memory loss, studies of pregnancy in women were until recently limited to

examining the systemic effects of gestation, disregarding changes in brain function. Recent longitudinal neuroimaging projects have begun to probe neural alterations induced by pregnancy, revealing volumetric changes accruing in the last trimester of gestation (Hoekzema et al., 2022, 2017; Martínez-García et al., 2021). For instance, cortical volumes seems to shrink by nearly 5% during pregnancy. Intriguingly, the magnitude of these changes is correlated with levels of estradiol and other pregnancy hormones (Hoekzema et al., 2022; Servin-Barthet et al., 2024). While most neuroanatomical modifications are transient, their functional consequence seems to be long-lasting. For instance, changes in the default neural network, which is predominantly affected by pregnancy-induced cortical reductions, have been hypothesised to support long-lasting ability of parents to empathise with infants (Servin-Barthet et al., 2024). This suggests that the endocrine events of human pregnancy trigger a neurodevelopmental transition resembling the hormone-mediated neural changes of adolescence. This is reminiscent of the findings of this work, where I hypothesise that, in mice, pregnancy opens a critical window of adult plasticity akin to changes occurring in the developing brain (G. B. Smith et al., 2015). Another curious parallel between rodent and human pregnancy-induced neural adaptations is the timescales of brain changes. In women, cortical adaptations associated with maternal attachment initiate already during pregnancy, before the peripartum period (Hoekzema et al., 2017; Servin-Barthet et al., 2024), similar to what happens in mice. Although parenting is clearly different between humans and rodents, and people are exposed to — and influenced by — a considerably more complicated environmental and socio-psychological blend of factors, similar hormonal signalling-dependent mechanisms underpinning motherhood might be fundamentally conserved across species.

Finally, pregnancy is a period of heightened risk of mental health issues. Periods of neural plasticity likely confer a vulnerability for the development of mental disorders, such as post-partum depression and anxiety (Barba-Müller et al., 2019).

Understanding the mechanisms governing adaptive changes might therefore help us understand the mechanisms underlying maladaptive changes. Recent clinical advances, such as the approval of zuranolone last year — a synthetic progesterone derivative and the first oral treatment for post-partum depression (Deligiannidis et al., 2023) —, make this a particularly exciting time to study the neural bases of parental behaviour, in health and disease.

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