UNIVERSIDADE D
COIMBRA

Catarina de Jesus Martins Pacheco

ALLOPARENTAL BEHAVIOR

THE EFFECT OF PUP EXPOSURE ON NESTBUILDING AND THE ROLE OF SEROTONIN

Tese realizada no *Institut du Cerveau* no âmbito do Mestrado em Biologia Celular e Molecular com especialização em Neurobiologia, orientada pela Doutora Patricia Gaspar e a Professora Ana Luísa Carvalho e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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ABSTRACT

In mammals, the primary offspring caregiver is usually the dam, but alloparental care is also frequently observed. Virgin female mice, after a short exposure to pups, express some behaviors of the maternal repertoire such as pup retrieval, pup licking, and nest building. 5-HT deficient mice show altered maternal behavior and preliminary observations indicated that alloparental care is also perturbed. Here, we focused on nest building, looking at effects of pup exposure in control and 5-HT depleted mice, analyzing behavior and immediate early gene (c-Fos) expression. 8-week-old virgin female mice were exposed to pups (P1-P5) for 30-60 min during 3 consecutive days. Video recordings allowed measuring time spent nesting and interacting with pups. We then performed whole brain immunostaining of c-Fos, clearing, and light sheet microscopy. Neural activation was analyzed using ClearMap.

In my thesis work, we were able to show that repeated pup exposure increased the time spent nesting both in control and 5-HT depleted mice, indicating that serotonin projections from the dorsal raphe are not required for this behavior. Pup exposure elicits activation of brain areas known to be important in maternal behavior, such as the medial preoptic area. To identify new candidate brain areas associated with this specific behavior, we compared the neural activation of high and low nest-builders, that spend, respectively, more 30% or less than 5% of the observed time nesting. We found a higher activation in the ventrolateral periaqueductal gray when females nest for a long time, suggesting a role of this brain regions in alloparental nest building.

Keywords: Alloparental behavior; Nest building; Serotonin; MPOA; vlPAG

RESUMO

Nos mamíferos, o cuidador primário das crias é normalmente a mãe biológica, mas a intervenção de cuidados aloparentais é frequentemente observada. Depois de uma curta exposição a crias, fêmeas virgens de murganho expressam alguns dos comportamentos do repertório maternal, entre os quais recolher as crias para o ninho, lamber as crias e nidificação. Murganhos com deficiências em 5-HT apresentam alterações no comportamento maternal, sendo que resultados preliminares indicam que o comportamento aloparental também é perturbado. Neste estudo, focámo-nos na nidificação, observando o efeito da exposição a crias neste comportamento, quer em animais controlo, quer em animais com deficiências em 5-HT. Para tal, avaliamos o comportamento e a expressão de c-Fos nestes murganhos. Murganhos adultos foram expostos a crias (P1-P5) durante 30-60 min por 3 dias consecutivos. As exposições foram gravadas e as gravações permitiram quantificar o tempo que os murganhos passaram a interagir com as crias e a nidificar. Por fim, realizámos imunocoloração de cérebro inteiro de c-Fos, clareamento e microscopia *light sheet*. A ativação neuronal foi analisada com ClearMap.

Nesta tese, mostramos que várias exposições a crias aumentam o tempo despendido em nidificação nos dois grupos de murganhos testados, controlo e com deficiências em 5-HT, indicando que as projeções de serotonina provenientes do núcleo dorsal da rafe não são necessárias para a expressão deste comportamento. A exposição a crias promove a ativação de várias regiões do cérebro previamente implicadas na expressão de comportamento maternal, como a área pré-óptica medial. De maneira a identificar possíveis regiões cerebrais envolvidas na expressão de nidificação em fêmeas virgens, comparamos a ativação neuronal entre dois grupos destes animais, consoante o tempo despendido a nidificar quando expostos a crias. Um grupo era composto por murganhos que nidificaram durante mais de 30% do tempo, e o outro composto de murganhos que nidificaram durante menos de 5% do tempo. Nesta comparação, verificamos que a sub-região ventrolateral da substância cinzenta periaquedutal apresenta um nível superior de ativação no grupo de fêmeas virgens que nidificaram por um período mais prolongado (mais de 30%), sugerindo que esta região pode estar envolvida na expressão de nidificação em fêmeas aloparentais.

Palavras-chave: Comportamento aloparental; Nidificação; Serotonina; MPOA: vlPAG

LIST OF ABBREVIATIONS

5-HT	Serotonin
5-HTP	5-hydroxytryptophan
AAV	Adeno-associated virus
AOB	Accessory olfactory bulb
AP	Anterior-Posterior
AuC	Auditory cortex
AVP	Vasopressin
BLA	Basolateral amygdala
BMA	Basomedial amygdala
BNST	Bed nucleus of the stria terminalis
COA	Cortical amygdala
DA	Dopamine
DBE	Dibenzyl ether
DCM	Dichloromethane
dPAG	Dorsal periaqueductal gray
DRN	Dorsal raphe nucleus
DV	Dorsal-Ventral
GPCRs	G protein-coupled receptors
IP	Intraperitoneal
KO	Knockout
L-AADC	Aromatic l-amino acid decarboxylase enzyme
lPAG	Lateral periaqueductal gray
MeA	Medial amygdala
ML	Medial-Lateral
MOB	Main olfactory bulb
MPOA	Medial preoptic area
MRN	Medial raphe nucleus
NA	Numerical aperture
NAc	Nucleus accumbens
OXT	Oxytocin
PAG	Periaqueductal gray
PBS	Phosphate buffered-saline

PRL	Prolactin
PVT	Paraventricular nucleus of the thalamus
RT	Room temperature
SERT	Serotonin transporter
TPH	Tryptophan hydroxylase
Tryp	Tryptophan
USVs	Ultrasonic vocalizations
vlPAG	Ventrolateral periaqueductal gray
VMAT2	Vesicular monoamine transporter 2
VP	Ventral pallidum
VTA	Ventral tegmental area

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CHAPTER I

STATE-OF-THE-ART

In most vertebrate species parental behavior is essential for the reproductive success of the species (Leckman & Herman, 2002) and survival of newborns by ensuring their well-being and development (Rosenblatt, 1967). Parental care includes any behavior toward an immature conspecific that increases the probability of survival until maturity. In mammals, due to lactation, the parturient female is the primary caregiver of the offspring which leads to behaviors referred to as maternal. However, in some mammals, both maternal and paternal behavior can be found, and even individuals who are not the biological parents of the newborns can provide care similar to maternal and paternal care, this is referred as alloparental care or alloparental behavior (Numan & Insel, 2003). Parenting is a complex behavior that includes (I) increase in motivation to interact with young; (II) recognition and processing of offspring cues; (III) temporary suppression of other behaviors, such as mating; and (IV) execution of specific motor routines (Kohl *et al.*, 2017; Kohl & Dulac, 2018).

Most of the mechanistic knowledge of the neurobiology of parental care, especially maternal care, have been studied extensively in rodents. Laboratory Norway rats (*Rattus norvegicus*), laboratory mice (*Mus musculus*), California mice (*Peromyscus californicus*), prairie vole (*Microtus ochrogaster*) and mandarin vole (*Lasiopdomys mandarinus*) are the rodents most used to study parental behavior (Kenkel *et al.*, 2017; Kuroda *et al.*, 2011). Newborn rodents (pups) are born "altricial" (immobile at birth), hairless, ectothermic, with their eyelids and ear holes sealed and with poor motor skills. The pups seem to start hearing by the fourth or fifth day, opening their eyes between postnatal day 12-14, and by the sixth day they are completely covered with a thin layer of first hair (Weber & Olsson, 2008). Hence, they depend on their mothers (dam) for the regulation of body temperature, nutrition and protection from harm until weaning (Kuroda *et al.*, 2011).

1. Maternal behavior

During and after pregnancy, the mother's brain undergoes various physiological changes, in order to meet the needs of the offspring. The initiation of maternal behavior, in postpartum female rodents, is stimulated by both internal hormonal priming during pregnancy and the interaction with pups after delivery, and consequent processing of cues associated with the newborns (Olazábal *et al.*, 2013). The female brain is primed, during pregnancy, by hormones such as estrogen, progesterone, prolactin (PRL), vasopressin

(AVP) and oxytocin (OXT) (Bridges *et al.*, 1997; Gandelman, 1973a). After delivery, interaction with the pups induce chemosensory signals, that are important for the maintenance of the maternal behavior repertoire (Gammie, 2005). These hormonal and sensory signals are then transmitted to various neural circuits involved in different aspects of maternal behavior, involving a number of neuromodulators, such as, serotonin (5-HT) (Bridges, 2015; Dulac *et al*., 2014). However, some aspects of maternal care are known to be independent of hormonal priming related to pregnancy and parturition.

1.1. Maternal behavior repertoire

In rodents, maternal care corresponds to a variety of behaviors that can be grouped into two categories, those that are pup directed and those indirectly related to the pups. Pup directed behaviors include placentophagia, pup retrieval, pup licking and grooming, and nursing (crouching and feeding). Non-directed behaviors include nest building and maternal aggression (Bridges, 2015) (**Fig. 1**). Both of these categories of behaviors are considered evidence of maternal motivation and maternal responsivity (Numan & Stolzenberg, 2009).

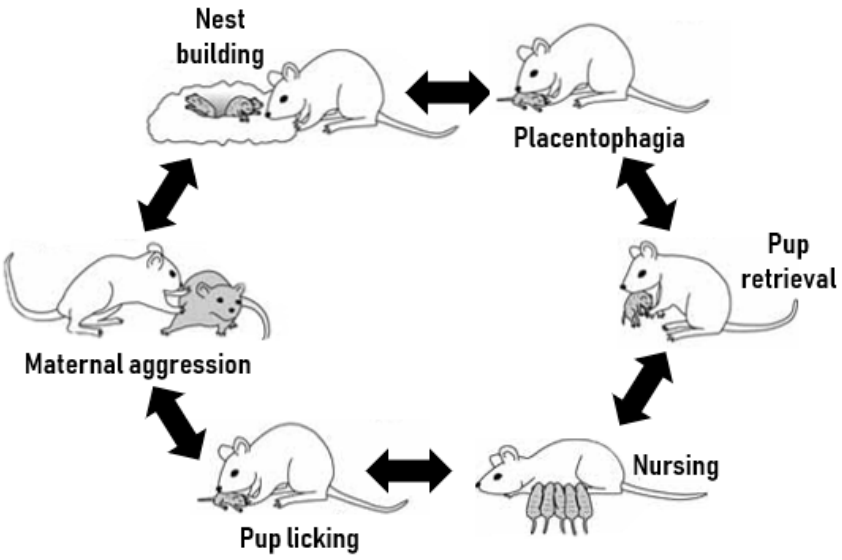

Figure 1 | Maternal behavior repertoire.

The repertoire includes both pup directed behaviors (placentophagia, pup retrieval, nursing and pup licking) and pup indirect behaviors (nest building and aggression against an intruder).

1.1.1. Placentophagia

After delivery of each pup, the dam cleans the pup's body by licking to remove the amniotic fluid and membrane. This behavior ensures that the pup's body is dry and clean, and also maternal licking stimulates respiration of neonates. If this behavior is not done properly, pups can become entwined by remains of the umbilical cords and placentas and their skins adhere to other objects such as bedding (Kristal, 1980).

1.1.2. Pup retrieval

Pup retrieval is essentially driven by hairless pups within the first postnatal week (Kuroda *et al*., 2011). When a pup falls from the nest or when the dam moves the nest to another location, the dam will move toward the pups and will retrieve them back into the nest. This behavior involves sniffing the pup before gently grabbing it with her incisors, bearing it into the nest and placing the pup there. "Retrieval" is when pups are carried over a relatively long distance, "mouthing" refers to short distance movements around the nest (Lonstein & Fleming, 2002).

Among all the maternal behaviors listed, pup retrieval is the most frequently used as an index of maternal responsiveness in rodents (Kuroda *et al.*, 2011; Rosenblatt, 1967).

1.1.3. Nursing

Nursing is a quiescent behavior in which the dam exposes her nipples to pups providing maternal milk to them. This behavior is performed for long periods of time in a variety of nursing postures. The most frequent nursing postures is kyphosis (arched-back posture) and is observed during the first week of lactation (Kuroda *et al*., 2011); it consists in the dam standing over the litter with rigidly spread limbs, which results in a dorsal arch. The prone position, during the second week consists in the dam laying flat on top of the litter (Lonstein & Fleming, 2002).

1.1.4. Pup licking

Dams often spend a large fraction of their time licking their pups. This behavior can be subdivided into two categories: anogenital licking and body licking. Anogenital licking helps the pup urinate and defecate and also has long term effects on their sexual development (Lonstein & Fleming, 2002). Body licking ensures that the pups stay clean (grooming), helps regulate their body temperature and stimulates their activity, helping

them to access the dam's nipples and promoting more productive suckling (Angoa-Pérez & Kuhn, 2015; Champagne *et al.*, 2003).

Pup licking constitutes a form of body contact that provides pups with tactile stimulation having a high impact in pup growth, emotional and social development, and responses to anxiety (Champagne *et al.*, 2003).

1.1.5. Maternal aggression

Maternal aggression refers to the aggressive behavior of a dam defending her litter against a potential infanticidal conspecific. It is linked to a decrease in the dam's anxiety, which has been related to modifications in the hypothalamic-pituitary-adrenal axis during lactation (Angoa-Pérez & Kuhn, 2015).

1.1.6. Nest building

This behavior starts even before parturition and declines after the first two weeks of lactation (Kuroda *et al.*, 2011) and involves transportation and manipulation of bedding in the potential nesting site (Lonstein & Fleming, 2002). Non-pregnant female mice build a flat "sleeping nest" in which they sleep, while pregnant females build a more complex nest called "brood nest". This second type of nest is bigger than the sleeping nest and has completely enclosed edges with one or two entrances (Gandelman, 1973b). The nest's quality is important for the success of the offspring and for the dam-infant interaction, since a good brood nest ensures the preservation of the pup's body temperature and also helps pups access to the dam's ventrum (Bult & Lynch, 1997).

1.2. Neurobiology of maternal behavior

Several brain areas and neuronal circuits have been implicated in the onset of maternal behavior, both in maternal motivation and maternal aggression (**Fig. 2**). The majority of the insights in neuronal circuits underlying maternal behavior is provided from studies performed in rats (reviewed in Dulac *et al*., 2014) but more recently, laboratory mice emerged as an attractive model to study the neurobiology of maternal behavior due to its robust maternal care (Kohl *et al*., 2017).

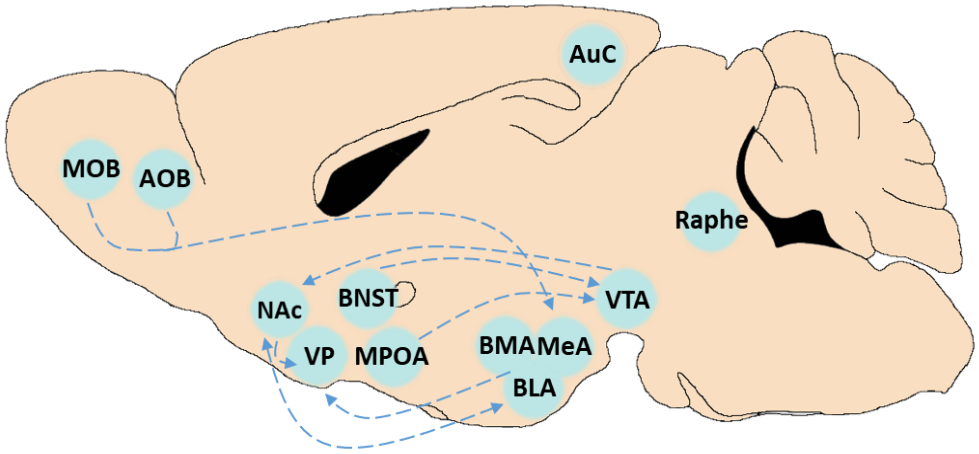

Figure 2 | Schematic representation of the brain areas involved in maternal behavior, both maternal motivation and maternal aggression.

Dashed lines represent known connections between the areas represented that are potentially involved in maternal behavior. The lines and arrows simply denote origins and targets and do not represent actual axon path or excitatory inputs. Not all the known connections and areas are represented. AOB, accessory olfactory bulb; AuC, auditory cortex; BLA, basolateral amygdala; BMA, basomedial amygdala; BNST, bed nucleus of stria terminalis; MeA, medial amygdala; MOB, main olfactory bulb; MPOA, medial preoptic area; NAc, nucleus accumbens; Raphe, Raphe nuclei; VP, ventral pallidum; VTA, ventral tegmental area. (Adapted from Dulac *et al.*, 2014)

1.2.1. Sensory cues in maternal behavior

In rodents, a set of maternal behaviors are regulated by sensory cues, such as olfactory and auditory cues, originated from the pups. Auditory and olfactory cues from the pups are often perceived simultaneously by the dam, therefore, the dam's brain may integrate the contingency between the two type of stimuli to recognize and take proper care of pups (Okabe *et al.*, 2013). Although both stimuli are important for the normal expression of maternal behavior, olfaction may play a bigger role in rodents.

Rodents are macrosmic mammals, this means that olfaction is their primary sensory system. It has been demonstrated that olfaction is important in the regulation of dam-infant interactions (Kuroda *et al.*, 2011). Laboratory strains of rodents (mice and rats) do not only care for their own pups, they generally also retrieve foster pups. Rat dams will retrieve their own pups first before retrieving the foster pups, a preference which is abolished after olfactory bulbectomy (Lévy & Keller, 2009).

In rats, chemosensory information provided by the pups' presence is unattractive to non-pregnant females, the olfactory information from pups is necessary for the pup avoidant reaction (Lévy & Keller, 2009). The elimination of pup odors through the induction of anosmia by olfactory bulbectomy, complete vomeronasal nerve cuts, pharmacological blockade of transmission in the accessory olfactory bulb (AOB), or destruction of the olfactory epithelium by intranasal application of zinc sulfate, abrogates the avoidant reaction and decreases the latency to the initiation of the maternal behavior. Studies where the primary and the vomeronasal olfactory systems are disrupted indicate that both are important for the inhibition of maternal behavior in virgin female rats (Fleming *et al*., 1979). Induction of anosmia by bilateral bulbectomy in female rats before parturition produce impairments in maternal behavior, there is decreased anogenital licking, maternal aggression and total time spent with pups, infanticide, impairments in pup retrieval and incomplete placentophagia. In contrast, when anosmia is induced after parturition, normal onset of maternal behavior is reported in primiparous rats. Taken together, this suggests that, in rats, olfaction facilitates the contact between dam and their pups, but is not crucial for the effective care of pups (Benuck & Rowe, 1975).

In mice, olfactory bulbectomy is reported to cause infanticide and impairments in maternal behavior in both virgin and postpartum females. Bulbectomized primiparous mice show decreases in nursing and licking (Seegal & Denenberg, 1974). The accessory olfactory system cannot be accounted for these impairments since selective vomeronasal organ removal does not alter maternal behavior, although it decreases maternal aggression (Kuroda *et al.*, 2011; Lepri *et al*., 1985).

Rodent pups produce ultrasonic vocalizations (USVs) when they are in distress or hypothermic from being isolated from their mother or littermates, and these two types of USVs can be distinguished by their adult conspecifics. USVs are characterized by frequencies between 30 and 80 kHz and elicits the mother to search and retrieve the isolated pups to the nest (Noirot, 1972). Female mice respond to digitally recorded pup cold USVs, even without olfactory cues. The female approached the speaker and their behavior was similar to their behavior toward cold pups. These behaviors were not verified when synthesized ultrasounds were reproduced (Uematsu *et al.,* 2007). The response toward pups USVs, consequently the retrieving behavior, could be enhance by social experience, such as mating and parenting (Okabe *et al.,* 2010).

1.2.2. Anatomical structures involved in maternal behavior

1.2.2.1. Medial preoptic area and bed nucleus of the stria terminalis

The medial preoptic area (MPOA) and the adjacent bed nucleus of the stria terminalis (BNST) have been found to be important neuronal circuits for the regulation of maternal behavior (Numan, 2007). Neurons in the MPOA/BNST region express high levels of c-Fos, a molecular marker for neuronal activation, when female rats and mice show maternal care or are exposed to pups, indicating that these regions are activated during maternal behavior (Calamandrei & Keverne, 1994; Fleming & Walsh, 1994). It has been hypothesized that these regions activate maternal behavior and eliminate avoidance/fear responses toward pup odors (Numan, 2007).

MPOA/BNST damage, such as bilateral electrical lesions, cell body-specific excitotoxic lesions and bilateral knife cuts which disrupt the lateral neuronal connections of these two brain regions, induce impairments in maternal behavior in both postpartum and pup-sensitized virgin female rats. Pup retrieval and nest building are the behaviors of the maternal behavioral repertoire most affected by the MPOA/BNST lesions (Numan, 1974, 2007).

Different subpopulations of neurons of the MPOA influence specific components of maternal behavior. In a recent study, Wu *et al.*, (2014) demonstrated that MPOA neurons expressing the neuropeptide galanin ($MPOA^{Gal}$) are specifically activated during parenting in mice. Inactivation of $MPOA^{Gal}$ neurons impaired maternal behavior while optogenetic activation triggered pup licking without triggering other components of maternal behavior. In another study, Fang *et al.,* (2018) showed that estrogen receptor alpha (Esr1)-expressing neurons in the MPOA ($MPOA^{Esr1+}$) are important to mediate pup approach and retrieval: inactivation of $MPOA^{Esr1+}$ neurons impairs these behaviors and optogenetic activation of $MPOA^{Esr1+}$ neurons stimulate these behaviors.

1.2.2.2. Amygdalar complex

Several nuclei of the amygdalar complex have been implicated in the onset of maternal behavior. High levels of c-Fos are triggered in some amygdalar nuclei such as medial amygdala (MeA) and cortical amygdala (COA) when female mice show maternal care or are exposed to pup sensory cues (Calamandrei & Keverne, 1994; Fleming & Walsh, 1994).

The medial amygdala (MeA), which receives olfactory information from the primary and accessory olfactory systems (Kang *et al.*, 2011), has been shown to play a role in the suppression of maternal behavior and avoidance reaction in virgin female rats. MeA lesions in virgin female rats facilitate maternal responses (Numan *et al.*, 1993). Some authors hypothesized that during pregnancy and lactation there are changes in the activity of the MeA that decrease fear of pups and facilitate maternal care (Numan, 2007). Also, the MeA is important for the normal expression of maternal aggression. High levels of c-Fos are present in the MeA in highly aggressive lactating mice when exposed to a male intruder (Gammie & Nelson, 2001).

The basolateral and basomedial nuclei of amygdala (BLA/BMA) projections to the ventral pallidum (VP) seems to be important for maternal motivation. BLA/BMA lesions, in rats, produce impairments in pup retrieval and in the operant bar-pressing test when pups are used as the reinforcing stimulus in dams, suggesting that these brain areas are implicated in maternal motivation (Kuroda *et al.*, 2011; Lee *et al.*, 2000).

1.2.2.3. Nucleus accumbens and ventral tegmental area

The nucleus accumbens (NAc) and the ventral tegmental area (VTA) have been implicated in the regulation of reinforcement related to pup stimuli and in pup retrieval (Numan & Stolzenberg, 2009). Lesions in the NAc, in postpartum rats, disrupt pup retrieval while other components of the maternal behavior repertoire are unchanged (Li & Fleming, 2003). Lesions of the VTA dopamine (DA) neurons produce impairments in maternal care in postpartum rats (Gaffori & Le Moal, 1979), such as persistent impairments in pup retrieval (Hansen *et al.*, 1991). The VTA receives inputs from the MPOA, and this circuit is involved in maternal motivation. The VTA DA neurons compose the ascending mesolimbic DA system and project to the NAc. Disruption of DA in the NAc impairs maternal behavior and when the connection between the MPOA-VTA is blocked, postpartum female rats loose interest in their pups. This suggests that MPOA activates DA neurons of the VTA that project to the NAc causing dams to be attracted to pup stimuli (Numan & Stolzenberg, 2009).

1.2.2.4. Raphe nuclei

The raphe nuclei is composed of a number of different brainstem nuclei (B1-B9) some of which have been implicated in the onset of maternal behavior (Angoa-Pérez & Kuhn, 2015).

2. Alloparental behavior

Alloparental behavior, as mentioned earlier, is defined as caregiving behaviors, that are directed toward a conspecific newborn by an individual who is not the newborn's genetic parent. Alloparental care is similar to parental care from the perspective of the infant receiving the care, but different from the perspective of the caregiver (Kenkel *et al.,* 2017). This behavior occurs in a small percentage of mammals (≈ 3%), including rodents and primates. Females are usually the ones that show this type of care, in many cases, they are neither pregnant nor lactating (Rogers & Bales, 2019). Alloparental female behavior exists in rodents but with notable differences between rats and mice.

Nulliparous female rats show maternal care when presented with foster pups for a period of time, a process called sensitization (Lonstein & Fleming, 2002). During the first exposure, they usually avoid pups and start showing maternal care only after 5-8 days of exposure. This process seems to be dependent on hormonal factors since hormonal treatments, such as physiological levels of progesterone and estradiol in conjunction with PRL (Bridges & Ronsheim, 1990), facilitates the initiation of maternal behavior in virgins (Rosenblatt, 1967). In contrast to rats, and virgin female mice of most laboratory strains show spontaneous maternal behavior and only need 15 to 30 minutes of pup exposure to initiate the behavior. The rapid onset of maternal care in female mice suggest that this behavior is independent of hormonal priming (Martín-Sánchez *et al.*, 2015).

Experienced females (primiparous or multiparous) follow a sequence of behaviors when they are presented with pups: the dam goes around the cage and retrieves pups one by one to the nest. After the last pup is retrieved, the dam goes around the cage one more time to make sure that she retrieved all the pups. Then the dam goes back to the nest and licks the pups, crouches over them and nurses when possible. Normally the nest that the dam has is a sleeping nest, so after retrieving and warming the pups, the dam continues building the nest until she achieves a brood nest. Virgin female mice show a similar sequence when exposed to pups (Kuroda *et al*., 2011).

Even though virgin female mice show spontaneous maternal behavior, they respond differently to pups than dams (postpartum females). Regarding the latency to approach and sniff the pups, Martín-Sánchez *et al.*, (2015) found no difference between dams and pup exposed females, which indicates that virgin female mice show no aversion to pups. In the pup retrieval test, pup-sensitized females are slower than the dams during the first

day of testing, but they become faster across tests (Martín-Sánchez *et al.*, 2015; Stolzenberg & Rissman, 2011). Pup exposed females spend more time licking the pups when compared to dams, this overexpression can be due to the fact that pups are a novel stimulus for them, and spend less time crouching over pups since nulliparous females don't produce milk, they can't exhibit true nursing (Martín-Sánchez *et al*., 2015). Nest building was also found to be increased in virgin female mice presented with foster pups. Females showed increase in nest building activity 24 hours following cohabitation with pups and more nests were rated as "brood" (Gandelman, 1973b).

The difference between virgin females and postpartum females tend to disappear as virgin females gain more experience with pups (Alsina-Llanes *et al*., 2015; Martín-Sánchez *et al*., 2015; Stolzenberg & Rissman, 2011).

Some of the same brain areas involved in maternal behavior have been implicated in alloparental behavior. Most of the studies on the neurobiology of these behavior were performed in the monogamous and bi-parental prairie vole and mandarin vole, especially in males. Virgin male prairie voles, after exposure to foster pups, show increased neural activity in some brain areas known to be implicated in maternal behavior such as, AOB, MeA, MPOA/BNST and paraventricular nucleus of the thalamus (PVT) (Kirkpatrick *et al*., 1994). In mice, virgin females exposed to pups for two different periods of time, 15 min or 60 min, showed increased expression of c-Fos in the prelimbic (PL) and infralimbic (IL) cortex, MeA and COA (Alsina-Llanes & Olazábal, 2020).

The fact that initiation of maternal care begins almost immediately after a contact with newborns, in virgin female mice, suggests that the experience of interaction with newborns produces alterations in the neural circuits responsible for increasing maternal motivation and responsiveness, and more repeated cohabitation with newborns elicits more effective maternal care (Martín-Sánchez *et al*., 2015; Stolzenberg & Rissman, 2011).

3. Serotonin

5-HT is a monoamine neurotransmitter that is widely distributed in key brain areas influencing affective state, impulsivity, learning and memory, attention, sleep cycles, aggression and sexual behavior (Pawluski *et al.*, 2019). 5-HT is synthetized by tryptophan hydroxylase (TPH), a rate-limiting enzyme that converts the amino acid tryptophan

(Tryp) into 5-hydroxytryptophan (5-HTP). The TPH enzyme has two isoforms TPH1 and TPH2, the first is mostly found in tissues in the periphery, and the second is the predominant isoform in the brain (Mohammad-Zadeh *et al.*, 2008). 5-HTP is then converted into 5-HT by the aromatic l-amino acid decarboxylase enzyme (L-AADC). Then 5-HT is packed into synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2), to be released into the synaptic cleft. 5-HT released is reuptaken in the presynaptic neurons by the serotonin transporter (SERT) (**Fig. 3**).

5-HT influence almost all the cells in the brain and, as mentioned above, the primary source of serotonin projections is the raphe nuclei and the rostral raphe cell groups (B6-B9) send 5-HT projections to all the brain areas known to be involved in maternal and alloparental care (olfactory areas, MPOA/BNST, amygdalar complex, NAc and VTA) (Mohammad-Zadeh *et al.*, 2008; Muzerelle *et al.*, 2016). Serotonin acts on fourteen subtypes of serotonin receptors, which are grouped into seven families (5-HT1 to 5-HT7) based on their structural and functional characteristics. Only one of these families of receptors is a ligand-gated channel (5-HTR3), the others are all G protein-coupled receptors (GPCRs) (Pawluski *et al.*, 2019).

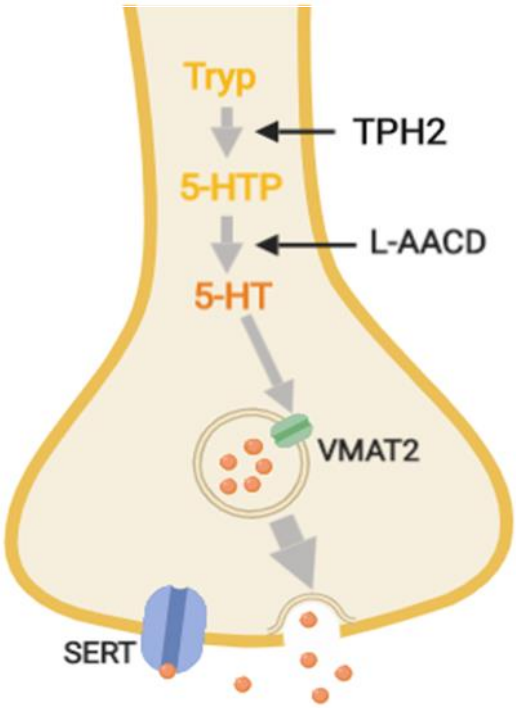

Figure 3 | Serotonin synthesis, packaging, release and reuptake.

Tryptophan hydroxylase (TPH), converts the amino acid tryptophan (Tryp) into 5-hydroxytryptophan (5-HTP) that is then converted into serotonin (5-HT) by the aromatic l-amino acid decarboxylase enzyme (L-AADC). 5-HT is packed into synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2). When released into the synaptic cleft, the excess of 5-HT can be reuptake by the serotonin transporter (SERT).

3.1. Serotonin implications in maternal behavior

Initially, the role 5-HT in maternal behavior was thought to be associated with increases in the release of maternally relevant hormones, since 5-HT and 5-HT receptor agonists stimulate the release of PRL, AVP, and OXT. The release of these hormones is stimulated via some of the 5-HT receptors, at least 5-HT1A, 5-HT2A and 5-HT2C receptors are involved in the release of PRL, AVP and OXT (Bagdy, 1996; Jørgensen *et al.*, 2003). This action of 5-HT is considered an indirect action but recently a direct influence of 5-HT in maternal behavior has been demonstrated.

The serotonergic system, in rodents, seems to be upregulated during pregnancy and early motherhood, and then declines around the time of pup weaning (Harding & Lonstein, 2016; Pawluski *et al.*, 2019). In mice, when comparing virgin to postpartum nonlactating and lactating females, the latter have significantly higher serum levels of 5-HT. However, serotonin levels in the DR, as detected by immunoreactivity, are lower in postpartum versus virgin laboratory mice (Jury *et al.*, 2015).

Lesions to the raphe nuclei, the principal source of 5-HT innervation, produce impairments in maternal behavior (Barofsky *et al.*, 1983; Holschbach *et al.*, 2018). Median raphe nucleus (MRN) lesions in rats cause a transient impairment in pup retrieval, deficits in nursing, pup licking and lactation, and infanticide in postpartum females (Barofsky *et al.*, 1983; Yurino *et al.*, 2001). Some studies found no effects in dorsal raphe nucleus (DRN) lesions (Yurino *et al*., 2001), while others showed that lesions in this region decrease maternal aggression, significantly reduced pup licking and generated aberrant patterns of nursing behavior (Holschbach *et al.*, 2018). The total time that dams spend nursing was not significantly different between lesioned and control females, but lesioned females did not show the anticipated decline in kyphosis across days of testing as the control females did. The lesioned dams also displayed decreased maternal aggression when an intruder was introduced in cage (Holschbach *et al.*, 2018).

Recently, the use of hyposerotonergic mouse models demonstrated that broad serotonin deficiencies impair maternal caregiving. This is based on the analysis of mouse mutants that have defective synthesis or differentiation of serotonin raphe neurons: Pet1-KO, VmaT2 CKO, Tph2-KO (Trowbridge *et al.*, 2011).

Pet-1 is an ETS transcription factor that is restricted to the 5-HT neurons in the brain, Pet-1 is critical for serotonin neuron development and serotonin synthesis since that influences the transcription of TPH2, VMAT2 and the SERT (Hendricks *et al*., 2003). Pet-1 knockout (KO) mice have normal numbers of 5-HT precursors in the developing hindbrain but 80% of these neurons do not reach a mature state and the 5-HT synthesis is greatly reduced (Hendricks *et al*., 2003; Trowbridge *et al*., 2011). Moreover, in this KO the failure in DRN neuronal development, alters not only 5-HT transmission but also its co-neurotransmitters, such as glutamate and various neuropeptides (Deneris & Gaspar, 2018). A study by Lerch-Haner *et al*., (2008) demonstrated that Pet1-KO mice had impairments in maternal behavior. Pet-1 KO dams were described as having deficits in nursing, they spend less time crouching over the pups when compared to control dams, and decreased pup survival although lactation was not impaired since milk pouches were consistently observed. Pet-1 KO dams also show maternal neglect, deficits in nest building and in pup retrieval. When new bedding material was given, Pet-1 KO females failed to retrieve the majority of the pups and instead focused on frequent digging and moving across the cages. Even when the nest was not disturbed, Pet-1 KO dams retrieved fewer pups with higher latency than control dams. The phenotype of Pet-1 KO mice was partly rescued by genetic restitution of Pet-1, with the human ortholog. Another study using Pet-1 KO mice (Scotto-Lomassese *et al*., in prep) found some contradictory results when compared to Lersh-Harner's findings. Pet-1 KO dams show no defects in nest building, pup retrieval, pup licking or placentophagia. Although decrease pup survival and defects in nursing were found in Pet1 dams when compared to controls, the time Pet-1 KO dams with an arched back posture was significantly reduced. Pet-1 KO mice, in this study, was maintained in a C57BL/6 background which could account for some of the differences between both studies.

TPH2 is the TPH isoform responsible for the synthesis of 5-HT in the raphe nuclei. TPH2 KO mice have a reduction of almost 90% in the levels of 5-HT in the brain. Despite the near complete depletion of 5-HT, KO mice are viable but present increased lethality during the first 3 postnatal weeks (Alenina *et al.*, 2009; Trowbridge *et al.*, 2011). TPH2 KO mice show behavioral abnormalities as adults, specifically TPH2 KO female mice have abnormal maternal behavior. TPH2 KO dams display cannibalism, and maternal neglect. Hence pups born to TPH2 dams have a significantly lower percentage of survival and most of them die in the first postnatal days (Alenina *et al.*, 2009; Angoa-Pérez *et al.*,

2014; Scotto-Lomassese *et al.*, in prep). TPH2 KO dams were able to build a nest, after the addition of new nesting material, but when compared to controls the nests built by TPH2 KO dams were incomplete while the nest from the controls were high-walled nests (Scotto-Lomassese *et al.*, in prep). In the pup retrieval test, TPH2 dams were not able to retrieve all pups during the testing time (Scotto-Lomassese *et al.*, in prep) although no differences between the control dams and the TPH2 dams in the time taken to find a hidden cookie, which indicates that this deficit is not caused by impairments in olfaction (Alenina *et al*., 2009). TPH2 damns displayed a decrease in the kyphosis nursing position accompanied by an increase in the less engaged low-arched back nursing, indicating impairments in nursing (Angoa-Pérez *et al*., 2014; Scotto-Lomassese *et al.*, in prep). However, TPH2 KO dams had no impairments in lactation since pups' stomachs were visibly filled with maternal milk (Alenina *et al*., 2009).

Serotonergic neurotransmission is important for homeostatic modulation of neuronal circuits thereby the absence of these throughout lifetime may disrupt the development of neuronal circuits involved in maternal behavior, but it is still unclear whether the maternal impairments observed in hyposerotonergic mice models used so far are caused by altered serotonin neurotransmission during adulthood or by altered brain connectivity and structure which could occur due to the lack serotonin neurotransmission during development (Pawluski *et al.*, 2019).

Recently, a brain-specific conditional and time-specific Tph2 knockout ($Tph2^{f/f}$) mice model was generated (Gutknecht *et al.*, 2008; Kriegebaum *et al.*, 2010). In this model, 5-HT depletion, in $Tph2^{f/f}$ females, was induced after the first full maternal experience. Multiparous $Tph2^{f/f}$ dams show no deficits in all maternal behaviors evaluated (nest building, pup retrieval and nursing), although pup survival was decreased when compared to controls (Scotto-Lomassese *et al.*, in prep). Since the depletion of 5-HT is obtain by viral injection in the DRN (B7), viral recombination and consequent 5-HT depletion can vary between cases. When the relationship between the extent of 5-HT depletion and several measures of maternal behaviors, authors found a significant correlation between the number of 5-HT neurons and pup survival and nursing (Scotto-Lomassese *et al.*, in prep).

Overall, in hyposerotonergic mouse models, Pet-1 KO, TPH2 KO and $Tph2^{f/f}$, dams appear to neglect their pups displaying other non-pup directed activities. This is consistent

with evidence showing that the lack of central 5-HT is correlated with increased impulsivity (Angoa-Pérez *et al.*, 2012).

CHAPTER II

FRAMEWORK AND OBJECTIVES

1. Role of Serotonin in alloparental behavior

All the studies done, so far, to explore the role of 5-HT in parental behavior were done using pregnant and postpartum females. In these studies, not only the neurotransmission of 5-HT but also the effect of 5-HT depletion on the production and realizing of maternal hormones, could account for the deficits observed in these females. To further examine the effect of 5-HT depletion in parental behavior, it is necessary to explore some components of the behavior independently of the hormonal context of pregnancy.

Previous work in my host laboratory has focused on alloparental care in hyposerotonergic, Pet1$^{-/-}$ and Tph2$^{-/-}$, mice models (Scotto- Lomassese *et al*., in prep). In both cases, females were exposed to C57BL/6 pups during 4 consecutive days.

Pet1$^{-/-}$ virgin females showed increase infanticidal behavior when compared to controls (Pet1$^{+/-}$) but the difference between both groups was not significant (**Fig. 4A**). The non infanticidal Pet1$^{-/-}$ females showed a rapid interest for the pups and no difference was found in the number of sessions required for full pup retrieval between Pet1$^{-/-}$ females and controls (**Fig. 4B**). However, not all retrieving Pet1$^{-/-}$ females crouched over the pups whereas this behavior was expressed by all the controls also, Pet1$^{-/-}$ females required more training sessions to initiate this behavior compared to controls (**Fig. 4C**).

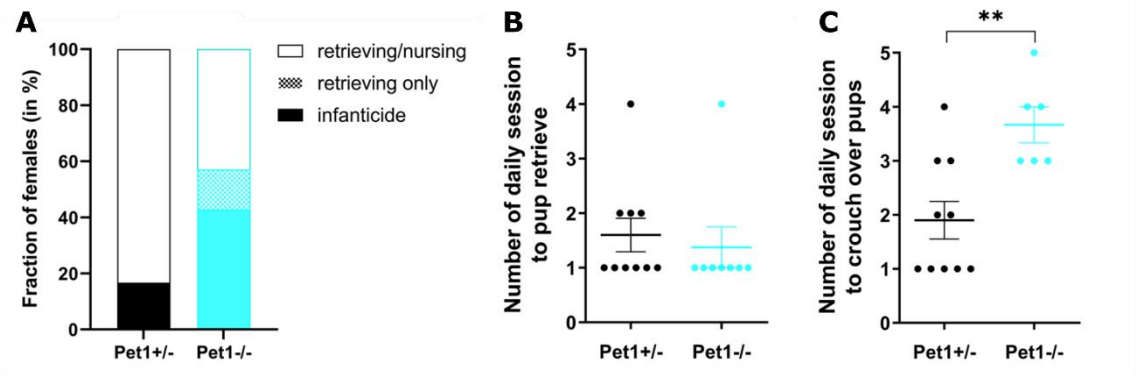

Figure 4 | Alloparental behavior in Pet1 knockout (Pet1$^{-/-}$) virgin female mice.

(A) Virgin female mice exposed to pups, Pet1$^{-/-}$ (n=14) and controls (Pet1$^{+/-}$) (n=12), were classified taking in account their response to the pups on the first day of exposure. **(B)** Number of days of exposure necessary for the virgin females retrieve all 5 pups to the nest during the first 10 min **(C)** Number of days of exposure necessary for virgin females initiate crouching behavior. **p < 0,01; Mann-Whitney's test.

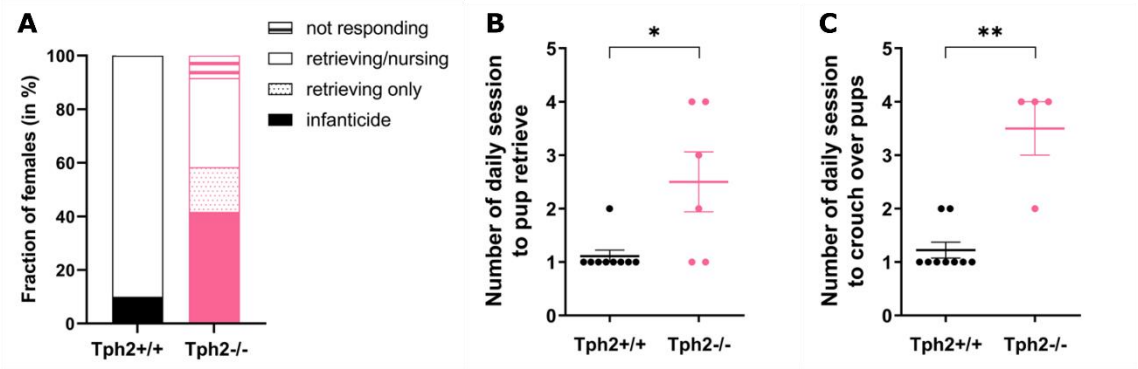

Figure 5 | Alloparental behavior in TPH2 knockout (Tph2$^{-/-}$) virgin female mice.

(A) Virgin female mice exposed to pups, Tph2$^{-/-}$ (n=12) and controls (Tph2$^{+/+}$) (n=10), were classified taking in account their response to the pups on the first day of exposure. **(B)** Number of days of exposure necessary for the virgin females retrieve all 5 pups to the nest during the first 10 min, *p < 0,05; Mann-Whitney's test **(C)** Number of days of exposure necessary for virgin females initiate crouching behavior. **p < 0,01; Mann-Whitney's test.

Tph2$^{-/-}$ virgin females also showed increase infanticidal behavior compared to controls (Tph2$^{+/+}$) but this difference did not reach significance (**Fig. 5A**). Responding Tph2$^{-/-}$ females showed some deficits in pup retrieval: Tph2$^{-/-}$ females required significantly more training sessions to retrieve all pups than controls (**Fig. 5B**). Similarly, to Pet1$^{-/-}$ females, most Tph2$^{-/-}$ virgin females did not crouch over the pups whereas control mice adopted nursing-like behaviors. (**Fig. 5A**); more training sessions were needed to observe a full alloparental behavior (**Fig 5C**).

Taken together, the results obtained from 2 hyposerotonergic mice models, show similar defects in alloparental behavior with increased incidence of aggression to pups and an increased delay in displaying a crouching, nursing-like behavior over the pups. Only Tph2$^{-/-}$ mice seemed to have a small defect in pup retrieval.

The results observed in constitutive models of 5-HT depletion, could be a consequence of altered brain connectivity of parental neural circuits during development, rather than reflecting the requirement of 5-HT neurotransmission for the behavior (Trowbridge *et al.*, 2011; Pawluski *et al.*, 2019). To determine the role of 5-HT transmission in alloparental behavior one would need to perform a selective depletion of 5-HT in adulthood.

2. Effect of pup exposure on nest building behavior in alloparental females

Nest building is a well-documented behavior in rodents that is essential for thermoregulation as well as shelter and protection of the offspring (Deacon, 2006).

Parental nest building can be elicited by an internal state, such as pregnancy, or by an external stimulus, such as the presence of pups. The structural complexity of the nest increases during pregnancy , as mice evolve from the construction of flat nests to the construction of high nests with walls (Bond *et al.,* 2002) and the quality of the nest are further enhanced by active contact with the offspring (Gandelman, 1973b) and maternal experience (Broida & Svare, 1982).

Previous results from our laboratory (unpublished data) have shed light on the effects of pregnancy on nest building **(Fig. 6)**. When nesting material is given to virgin female mice or pregnant female mice (both C57BL/6), the quality of the nest built by pregnant mice, over a 24h period, increase drastically when compared to virgin females **(Fig. 6A and B)**. Plugged mice were found to build nests of higher quality, nests scored as 3 or 4 (brood nests), as fast as 24h following the plug onset, an effect that further increased over time, reaching a plateau at E6, and stagnating until E16, where more than 80% of mice built high quality nests **(Fig. 6B)**. Not only the quality of the nests increased, but also pregnant females spent more time nest building when compared to virgins **(Fig. 6C and D)** and the latency to start the behavior is short in pregnant females **(Fig. 6E).**

While the hormonal control, during pregnancy, of select neurons activity in this behavioral transition is essential (Lisk, 1971; Voci & Carlson, 1973) it is not required for its maintenance throughout lifespan, as the behavior of parental nest-building persists after the pregnancy hormones levels drop to baseline, and presence of pups is sufficient to elicit a shift in nesting behavior (Gandelman, 1973b). Although, little is known about the effects of repeated pup exposure on nest building behavior, specially the neural circuits involved in the behavioral shift observed in virgin female mice.

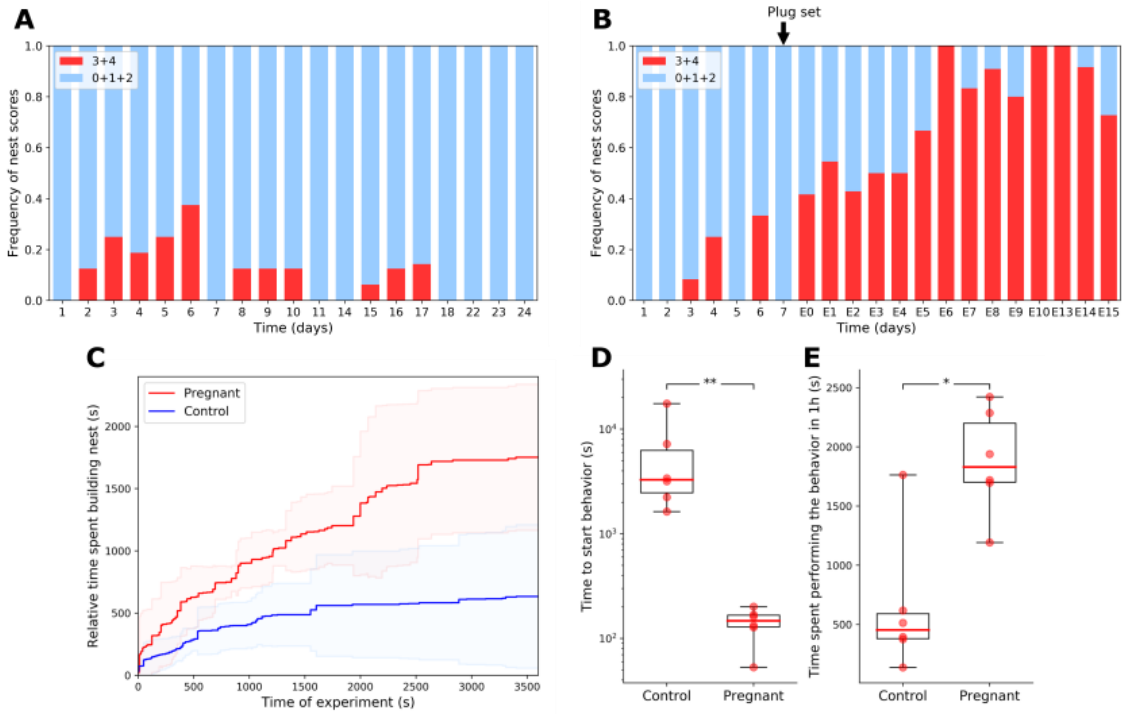

Figure 6 | Pregnancy state increases quality of nest, and time spent building a nest.

(A) Relative frequency of nest scores for each virgin C57BL/6 female mice (control) (n = 16) over a total period of 24 days. **(B)** Relative frequency of nest scores for each pregnant C57BL/6 female mice (n = 12) over a total period of 21 days. The blue part of the charts represents all nest scores ranging from 0 to 2 (flat nests). The red part of the charts represents all nest scores ranging from 3 to 4 (brood nests). **(C)** Cumulative curves of the relative time spent building nest over the course of the experiment for virgin (blue) or pregnant mice (red). The darker lines represent mean values in the 2 groups with a temporal resolution of 1s. The lighter lines represent SD values for the 2 groups. **(D)** Delay to initiate the behavior. **p < 0,01; Mann-Whitney's test. **(E)** Overall time spent building a nest following the initiation of behavior during the time of experiment (1h). *p < 0,05; Mann-Whitney's test.

Given this previous evidence obtained in my host laboratory, my project had 3 main goals:

1. To quantify the effect of pup exposure on nest building in virgin female mice.
2. To identify neural candidates involved in nest building behavior in alloparental care.
3. To analyze the consequences of 5-HT conditional depletion in the dorsal raphe nucleus ($Tph2^{f/f}$ mice model) on nest building.

CHAPTER III

MATERIALS AND METHODS

In this chapter, Catarina Pacheco assisted Dr. Aude Muzerelle and Dr. Patricia Gaspar in stereotaxic surgeries. Tomek Topilko assisted in some animal perfusions.

1. Animal handling and housing

All animal procedures were performed in compliance with the European legislation for animal experimentation (European Community Guidelines and French Agriculture and Forestry Ministry Guidelines for Handling Animals- decree 87849). Animals were housed in groups (3-5 per cage) and maintained under standard laboratory conditions, in ventilated cages, at constant temperature (22°C) and humidity (60%), under a 12-12 hours light-dark cycle and with access to water and food *ad libitum*. Pregnant dams were maintained under the same laboratory conditions but were housed in pairs.

Adult C57BL/6 female mice were obtained from Janvier Labs. The C57BL/6 pups used were obtained from pregnant dams also from Janvier Labs.

The conditional Tph2 ($Tph2^{f/f}$) mice line was generated on a mixed genetic background and maintained on a C57BL/6 background. $Tph2^{f/f}$ mice allows tissue-specific Cre/LoxP mediated gene deletion. The insertion of two LoxP sites targeting the exon 5 result in the elimination of this exon and in a truncated non-functional TPH2 protein by creating a shift in the reading frame (Gutknecht *et al.*, 2008; Kriegebaum *et al.*, 2010). $Tph2^{f/f}$ line was locally bred at the Institut du Fer à Moulin (IFM), Paris.

2. Stereotaxic surgery and viral vector

The same replication-defective adeno-associated virus (AAV) was used in both animal groups, $Tph2^{f/f}$ and C57BL/6. The AAV9.*hSyn*.HI.*eGFPLCre*.WPRE.SV40 (10^{12}-10^{13} genome containing particles/ml Penn Core Vector, USA) virus was used for conditional deletion of Tph2 in the DRN neurons in $Tph2^{f/f}$ animals.

5 week-old female mice were anesthetized with a combination of ketamine (150 mg/kg) and xylazine (10 mg/kg) (Sigma-Aldrich, USA) via intraperitoneal (IP) injection, followed by an analgesic IP injection of flumixine (5mg/kg) before surgery. Mice were placed on a foam board and head-fixed in a stereotaxic frame (David Kopf Instrument, USA). Injections were directed to the DRN B7 subdivision using the following coordinates related to Lambda: AP= 0.5 mm; ML= 0.75 mm; DV= –3.2 mm with a 10º angle; this angle aims to avoid large blood vessels on the midline. Bilateral injections were made. The virus solution was loaded into glass capillaries (30 to 50 µm tip diameter; PCR micropipette, Drummond Scientific company), fixed on hydraulic micromanipulator MO-10 (Narishige, Japan), Each animal received bilaterally 300 nL of virus (1/100 in

saline solution (NaCl 0.9%). After each injection the capillary was left for 5 min in the target site to prevent backflow.

Behavioral assessment was performed 3 weeks after surgery to allow the animals to recover and the sufficient Cre expression and Tph2 gene recombination. [Experiment performed at IFM]

3. Behavior assays and nest scoring

Prior to the exposure, all virgin females were individually housed for 2-3 days.

The night before of the first behavior recording, the females' cages were placed in the experimentation room for habituation. The nesting material was changed the night before of the recordings, 3 new cotton nestlets (5cm square, SAFE, France) were added per cage. 8 week-old naïve virgin female mice were exposed to pups taken from newborn litters (1-5 days old) or to a control object, cubes of rubber with similar size and color as pups. The females' behavior as recorded in a home cage video recording system, 5 min of habituation was recorded before adding the pups. After the addition of the pups, the females were left in the behavior room undisturbed for the entire exposure time. At the end of the exposure, the females were perfused or return to the housing room and pups return to the dams cage. All the recordings were done with dim light.

Video recordings were then manually segmented to annotate each bouts of activity during exposure. The time spent nest building (interacting, actively shredding and moving the nestles onto a nest site), interacting with pups (sniffing, licking and crouching over them) was annotated. Other relevant behaviors were also registered, such as, latency to retrieve all the pups, latency to nest and latency to interact with the pups or the control object.

The nest quality was evaluated before and after exposure and a score was given according to a 0-4 scale, adapted from Deacon (2006): 0 – nestlets intact; 1 – nestlets partially shredded, but no visible nest site; 2 – nestlets mostly shredded but spread around the cage (no clear nest site); 3 –nestlets mostly shredded but the identifiable nest is flat (no enclosed walls); 4 – all the cotton nestlets shredded , nest with enclosed walls, high enough height to cover the entire animal.

3.1. Behavioral assay 1

Three weeks after the viral injection, naïve virgin females, C57BL/6 (n=7) and $Tph2^{f/f}$ (n=8) were exposed to 3 pups during 40 min for 3 consecutive days. All 3 days of exposure were recorded and segmented, and the nest was scored as mention before. Later, females were perfused for depletion analyses and quantification. [Experiment performed at IFM]

3.2. Behavioral assay 2

In the first cohort, naïve virgin C57BL/6 female mice were exposed to 3 pups (n=5) or control object (n=5) during 20 min for 3 days. All 3 days of exposure were recorded and segmented, and the nest was scored as mention before. Before the exposure, a vaginal smear was performed to check the estrous cycle phase of the females. [Experiment performed at IFM].

In the second cohort, naïve virgin C57BL/6 female mice were exposed to 2 pups (n=11) or to control objects (n=5) during 1h for 3 days. In this cohort only the third day was recorded and segmented, the first two days were considered as habituation. Female mice were perfused after the behavior recording.

3.3. Behavioral assay 3

Naïve virgin C57BL/6 female mice were exposed to a pup for a short period of time (less than 1 min) (n=5) or left undisturbed (n=5). Exposed females were allowed to sniff the pup, after the pup was retrieved from the cage and they were left undisturbed. Their behavior was recorded for 1h and after animals were perfused.

4. Vaginal smear and cytology

A vaginal smear was collected before behavior exposure. 20 µL of saline solution was introduced at the opening of the vaginal canal and aspirated back into the pipette. The aspirated saline was then placed on a glass slide and allow to completely dry at room temperature (RT). Once dry, estrous smears were stained. Air dry slides were placed in a coplin jar containing 0.1% crystal violet stain (Sigma-Aldrich, Germany) in distilled water (dH_2O) for 1 min. Slides were then washed twice in dH_2O. After air dried the excess of dH_2O, slides were mounted in Mowiol (10%, Calbiochem, Germany)-Dabco (2.5%, Sigma-Aldrich, USA).

The stage of the estrous cycle was determined based on the presence or absence of leukocytes, cornified epithelial and nucleated epithelial cells (Byers *et al*., 2012).

5. Perfusion and tissue processing

After completing the behavioral observations all mice were perfused for histological analysis. Mice were anesthetized with Pentobarbital (Euthasol, 100mg/kg) via IP and transcardially perfused using a peristaltic pump (Gilson, USA). First, with approximately 20 mL of cold phosphate buffered-saline (PBS), to wash the blood, followed by tissue fixation with approximately 20 mL of 4% paraformaldehyde/PBS (Electron Microscopy Sciences, USA). Whole brains were quickly and carefully dissected to maintain intact the structure and transferred to 4% paraformaldehyde/PBS for overnight post-fixation at 4ºC. On the next day, brains were washed 3 times in PBS to remove the excess of fixative. Finally, brains were stored, until further processing, at 4ºC in PBS with 0.02% sodium azide (NaN_3, Sigma-Aldrich, Germany) to prevent bacterial and fungi growth.

6. Tissue clearing, c-Fos immunolabeling and imaging

Whole brain clearing and immediate early gene, c-Fos, immunostaining was followed the iDISCO+ protocol previously described in Renier *et al.*, (2016).

6.1. Tissue pretreatment

Brain samples were dehydrated in gradual series of methanol (Sigma-Aldrich, France) in water for 1h30min each (20%, 40%, 60%, 80% and 2 washes of 100%). After dehydration, a lipid extraction step was performed via overnight incubation in 66% dichloromethane (DCM, Sigma-Aldrich, Germany) in methanol and then washed twice in 100% methanol for 4h. Samples were then bleached at 4ºC overnight in 1:5 mix of 30% hydrogen peroxide (Sigma-Aldrich) and methanol. Tissue rehydration was performed with incubation of 1h30min in decreasing series of methanol/H_2O (60%, 40% and 20%). Samples were then washed in PBS and twice in PBS containing 0.2% of Triton X-100 (Sigma-Aldrich) for 1h30min each. These steps were performed with gentle shaking at RT, except when otherwise is specified.

6.2. c-Fos immunolabeling

Pre-treated samples were permeabilized at 37ºC for 24h in permeabilization solution, composed by 20% dimethyl sulfoxide (Sigma-Aldrich), 2.3% Glycine (Sigma-Aldrich, USA), 0.2% Triton X-100 in PBS, then blocked for 48h at 37ºC in blocking buffer

composed by 0.2% gelatin (Sigma-Aldrich), 0.2% Triton X-100 in PBS. All buffers were supplemented with 0.02% NaN_3. Whole brain samples were incubated at 37ºC with the primary rabbit anti-c-Fos antibody (Synaptic Systems #226-004, Germany) 1:1000 in the same blocking buffer for a week under constant agitation. Next, samples were washed 4 times for 2h in PTwH buffer, composed by 0.2% Tween, Heparin 10 µg/mL in PBS and left overnight in the same buffer. Then, whole brains were incubated at 37ºC with the secondary antibody Alexa Fluor™ 647 Donkey Anti-Rabbit IgG (Thermo Fisher Scientific #A-31573, USA) 1:1000 in the same blocking buffer used before for a week under constant agitation. These were then washed 4 times in PTwH for 2h each and left overnight in the same buffer.

6.3. Tissue clearing

After immunostaining, samples were again dehydrated in an increasing series of methanol/H_2O for 1h30min each (20%, 40%, 60%, 80% and 2 washes of 100%) and left overnight in 66% DCM in methanol. Remaining methanol was washed out with 2 washes in 100% DCM for 15 min each. Samples were then cleared in dibenzyl ether (DBE, Sigma-Aldrich) used for refractive index matching. Finally, whole brain samples were stored in a full vial of DBE in the dark at RT until light sheet imaging.

6.4. Light-sheet fluorescence imaging

Imaging of the cleared samples was acquired on a LaVision Ultramicroscope II (LaVision, Biotech) equipped with a sCMOS camera (Andor Neo, UK). Samples were positioned in sagittal orientation with the right side facing up.

Brains were imaged twice. A first acquisition was done with a 4X objective lens (MVPLAPO 4X), a 639nm excitation LED laser and a 680/30 emission filter to acquire the c-Fos signal (far-red fluorescence). The acquisition was done in tiling mode. The field of view as cropped in a region of 1000*1000 pixels. A mosaic, of approximately 4*7 tiles, was created in order to fit the whole sagittal view of the brain and the tile overlap was set to 10%. In the second acquisition, a 488nm excitation LED laser and a 525/50 emission filter were used to detect brain tissue autofluorescence, with a 1.3X objective lens (MVPLAPO 1.3X). For both acquisitions, only the left light sheet was used and the stepsize was set to 6 µm. The light-sheet numerical aperture (NA) was set to 0.03 and laser power to maximum (100%).

7. ClearMap analysis

A modified version of ClearMap (https://www.idisco.info) was used to analyze, quantify and register c-Fos positive (c-Fos$^+$) cells. The c-Fos data was acquire in tiling mode which creates different tiles that should be aligned and stitched into a single image, for that a stitching step was performed via interface to TeraStitcher, an open-source tool for stitching of teravoxel-sized tiled microscopy images. After stitching, the data for both light-sheet acquisitions, stitched c-Fos data and autofluorescence data, were aligned onto each other to account for possible movements of the sample that may occur between acquisitions. Next, the auto-aligned data was aligned onto a reference cleared brain atlas. The cell detection was done by detection of the bright fluorescent c-Fos signal after a background subtraction to eliminate signal from the autofluorescence of the tissue. Cells with sizes between 5 to 900 voxels were selected. Finally, samples were registered to the Allen Brain Institute 25µm annotation map (http://alleninstitute.org/). Cells counts of each sample in annotated brain areas were represented in a heatmap and an average heatmap was created for the different groups. The average cell count between different groups were compared using the independent student t-test assuming unequal variances. Statistical tests were performed numerically using the SciPy statistics library (http://www.scipy.org/).

8. 5-HT immunohistochemistry on tissue sections

Fixed brains were transferred to a solution of 30% sucrose in PBS containing NaN_3 for cryopreservation for 2 days. Coronal sections of 50 µm were serially cut using a cryo-microtome (Microm Microtech, France) and sections containing the raphe nuclei were collected in series of 3. The sections were stored in cryoprotectant (30% ethylene glycol and 30% glycerol in 0.12M phosphate buffer) at -20ºC until further processing.

Free-floating section were washed 3 times in PBS for 15 min and permeabilized and blocked in a solution containing 2 g/L gelatin and 0.25 % triton in PBS. After, sections were incubated for 48h in the primary rabbit anti-5-HT antibody (#PC177L, Calbiochem) 1:5000 in the same buffer mention above. The sections were washed 3 times in PBS baths for 15 min, followed by a 2h incubation in the secondary antibody Cy^{TM}5 Donkey Anti-Rabbit IgG (#711-605-152, Jackson Immuno Research, Europe Ltd) 1/200 in the same buffer at RT. Finally, after 3 washes in PBS 3 times for 15 min, sections were mounted in Mowiol.

5-HT stained sections were imaged on a slide scanner, Axio Scan.Z1 (Zeiss, Germany), with x10 objective. Images were exported and analyzed using the Zeiss Zen 3.1 blue edition software (Zeiss, Germany). Total number of 5-HT cells were counted on 3 rostro-caudal levels in the DRN using automated cell counting in ImageJ software. The extent and location of the injection was analyzed for each case.

9. Statistical analysis

Statistical analysis was performed with GraphPad Prism 8 (GraphPad Software, USA). Data are presented as mean +/- S.E.M or, when in box plots, as median + quartiles + minimum and maximum values. Statistical differences are presented as probability levels of $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****). The normal distribution of the data was verified. If the data presented a normal distribution, Unpaired student t-test was used for the statistical analysis, if not, Mann-Whitney test was used. One of these two statistical tests was used for the statistical analysis of all experiments with the exception of some behavioral analysis where two-way ANOVAs were employed. The adequate multiple comparisons test was performed, when applicable.

CHAPTER IV

RESULTS

1. Effect of Pup exposure on nest building in virgin female mice

Previous reports have indicated that pup exposure increases nest building activity (Gandelman, 1973b). However, this effect has not been quantified precisely since only nest scores and nest weight were measured. In order to perform a thorough temporal, quantitative and qualitative analysis, C57BL/6 virgin females were exposed to pups (n=5) or to objects (n=5) for 3 consecutive days during 20 min and filmed; new nesting material was added every night and nests were scored daily before and after the observation period (**Fig. 7A and B**).

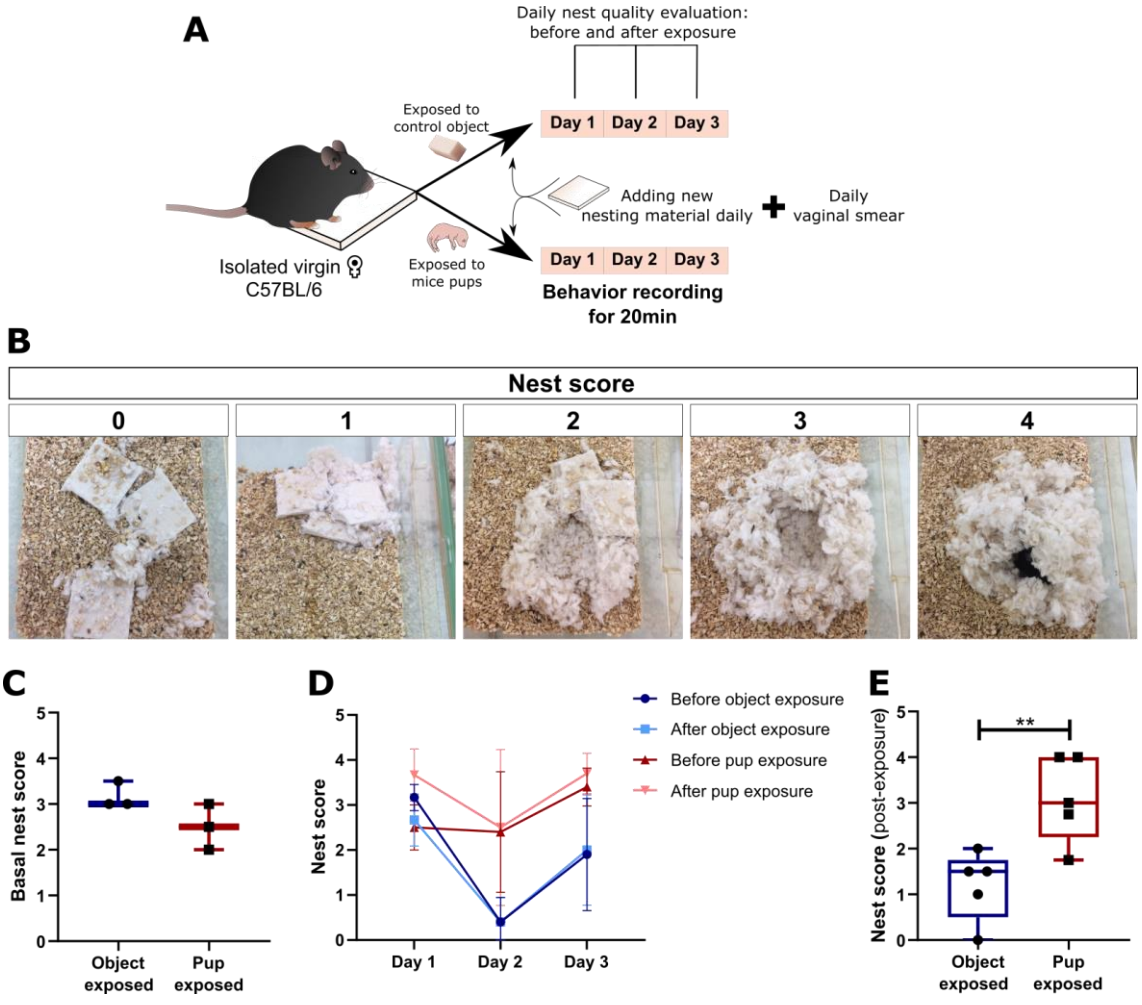

Figure 7 | Pup exposure can increase the quality of nest built by virgin females.

(A) Schematic representation of the experimental paradigm used on this cohort. **(B)** Overview of the nest scoring system used, ranging from 0 to 4. **(C)** Basal nest score for both groups, pup exposed (n=3) and object exposed females (n=3). **(D)** Mean nest scores for pup exposed females (n=5) and object exposed females(n=5), in 3 consecutive days of exposure **(E)** The quality of nest built by the pup exposed virgin female was thighed than object-exposed females (median value over 3 days) . Unpaired two-tailed t-test. **p < 0.01

At the basal level, on day one, nest rating was similar in the two groups:(**Fig. 7C).** Twenty minutes after pup exposure, already at day one, nest quality was increased (**Fig. 7D**). Mean score post-exposure over the 3 days, was significantly higher for pup- exposed compared to object-exposed females (3.0 vs 1.5; Unpaired two-tailed t-test, p= 0.008). Using two-way Anova, there was no effect of time on nest scores (**Fig. 7D**). Overall, these results indicate that pup exposure can elicit an increase in the quality of nest in virgin female mice, and this effect appears upon the first exposure with no clear improvement by repeated exposure.

To obtain a more quantitative evaluation of the mouse behavior. The videos that were recorded during exposure to pups or objects were analyzed and relevant behaviors were manually segmented annotating: 1) interaction time with the pups or the objects, 2) latency to interact 3) nesting time. All pup-exposed females, except one (on day 2), retrieved all pups during the first 10 min of exposure period, already in the first day of exposure. None of the object-exposed females retrieved any object (**Fig. 8A**). The total time spent interacting with objects (28%) or pups (33%) was similar. (**Fig. 8B and C**). However, total latency to approach pups was shorter than latency to approach objects (2s versus 6s, Mann-Whitney U test, p= 0.048). Furthermore, there were longer bouts of interaction with pups than with objects (**Fig. 8A**). The total time spent nesting was significantly increased in females exposed to pups versus objects (0.4% versus 25%, Mann-Whitney U test, p= 0.008; **Fig. 8F**), and more frequent and longer bouts of nesting activity were observed (**Fig. 8A**). Comparison of interaction and nesting times over 3 consecutive days showed no change for interaction time (**Fig. 8B**) but increase in nesting behavior across the exposure days (n.s., **Fig.8E**).

Altogether these data indicated that, C57BL/6 virgin female mice do not show aversion to pups since all pup-exposed females approached, interacted, and retrieved the pups on the first day of exposure. In addition, pup exposure increased the time virgin females spent nesting and this increase was higher upon repeated exposures , resulting in higher quality nests.

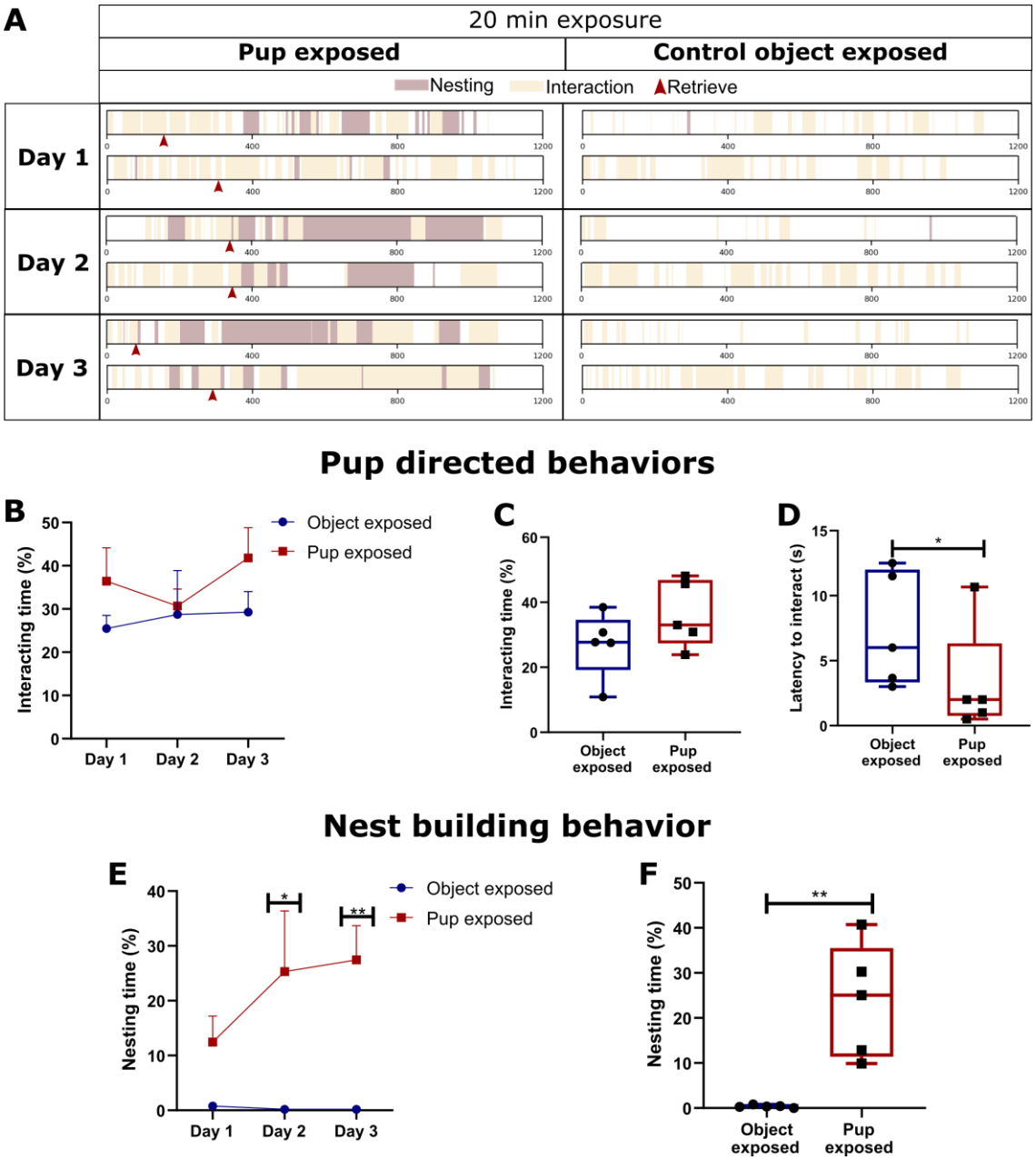

Figure 8 | Pup exposure increase motivation towards nest building behavior.

(A) Representative raster plots of bouts of activity during exposure to pups (left) or object (right) over 3 consecutive exposure days (2 mice per condition are illustrated). Each bout of time spent nest building and interacting, are represented. Red arrow indicates the time when all 3 pups or objects were retrieved into the nest site. **(B)** Mean percentage of time spent interacting with pups or control object for both groups in 3 consecutive days of exposure. Several behaviors were classified as interacting: sniffing, licking and crouching over. **(C)** Average interaction time (%) over 3 exposure days. **(D)** Average latency to interact, with pups or object, for 3 exposure days. Unpaired two-tailed t-test. *p< 0.05. **(E)** Mean percentage of time spent nesting for both groups, pup exposed females (n=5) and object exposed female (n=5), in 3 consecutive days. Two-way ANOVA with Sidak's multiple comparisons test. *p< 0.05; **p < 0.01. **(F)** Average of nesting time (%) of all 3 exposure days. Mann-Whitney U test. **p < 0.01

2. Effect of estrous cycle phase on nest quality

It has been shown that gestation improves nest-building abilities, suggesting a role of hormones. In fact, progesterone administration increases the quality of nest building in mice (Lisk, 1971). Moreover, we noted a variability of nest quality among the virgin females of our experimental groups in basal conditions. This suggested that there could be an effect of estrous cycle on the quality of the nests. To evaluate this, vaginal smears were collected daily in virgin females (n=10) after scoring their nests (<10 am). The estrous cycle phase was evaluated based on the presence or absence of leukocytes, cornified epithelial and nucleated epithelial cells (**Fig. 9A**). Virgin females built nests of similar quality, whether they were in Metestrus, Diestrus, Proestrus or Estrus (**Fig. 9B**). Moreover, individual females had consistent nest scores over a 5 day period (not shown). Thus, the variability of nest building among virgin is not explained by their estrous cycle phase.

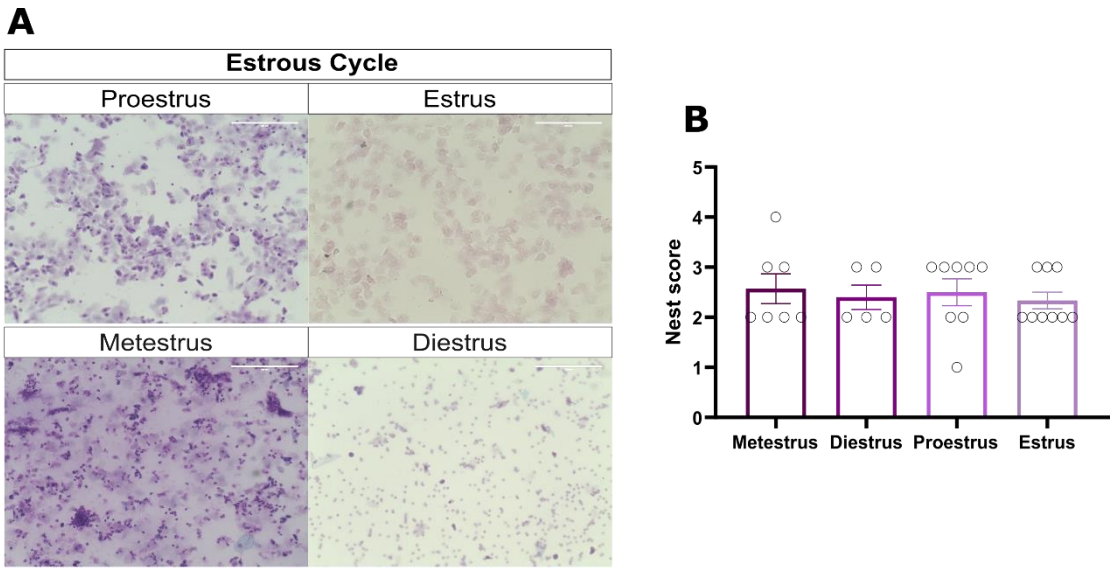

Figure 9 | Estrous cycle phase has no effect on nest quality.

(A) Representative microscope images of the different estrous cycle phases in female mice**. (B)** Mean nest score over different phases of the estrous cycle. The estrous cycle phase was assessed by daily vaginal smear performed before the exposure tests.

3. Effect of short pup exposure on nest building

To assess whether pups trigger improved nest building abilities of whether the continued presence of pups is necessary to elicit this behavioral effect, we exposed females to one pup for a short period of time (less than 1 minute). Females were exposed to one pup and allowed to sniff and lick the pup during the exposure time and after which the pup was removed from the home-cage. Control mice were left undisturbed. Behavior was recorded 1hour after the removal of the pups and analyzed for nesting and sleeping time.

Some bouts of nest building behavior were observed in pup exposed versus undisturbed females (**Fig 10A**). However, the bouts observed were shorter than in the previous experiment (with continuous exposure to pups) (**Fig. 8A**). The mean time spent nesting was higher in short pup exposed females when compared to undisturbed females (0% versus 0.8%, Mann-Whitney U test, p= 0.048, **Fig. 10B**), however the low nesting activity of the control group begs for questions. All short pup exposed females actively interacted with the nesting material after the removal of pups from the cage. Even though only two undisturbed females actively interacted with the nesting material, we can see that latency to interact with the nesting material (latency to nest) was slightly higher in these two undisturbed females when compared to short pup exposed females (n.s., **Fig. 10C**). The time spent sleeping was used as a proxy for arousal. Pup exposed and undisturbed females did not differ in the time spent sleeping during the observation time (n.s., **Fig. 10D**) although, 2 undisturbed females spent a higher percentage of time sleeping.

Overall, these results indicate that a short exposure to pup can elicit nest building activity in virgin females but, not to the same extent as a continuous pup exposure.

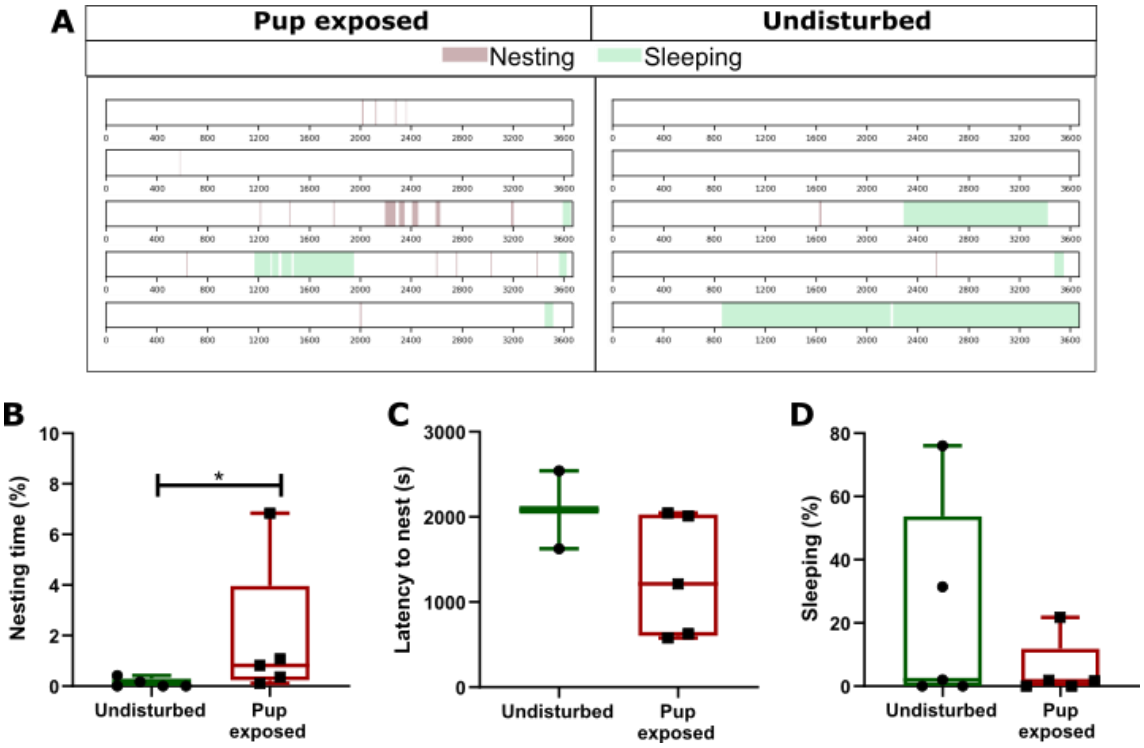

Figure 10 | Short pup exposure can be sufficient to elicit nest building activity.

(A) Raster plots representing each bout of activity, nest building and sleeping, for short pup exposed (n=5) and undisturbed (n=5) females. In short pup exposed females, the behavior was quantified after the pup was removed from the cage **(B)** Percentage of time spent actively interacting with the nesting material (nesting) during 1h. Mann-Whitney U test. *p<0.05 **(C)** Latency, in seconds, to actively interact with the nesting material. **(D)** Percentage of time spent sleeping during 1h.

4. Effect of conditional depletion of 5-HT in alloparental behavior

In order to evaluate the effect of time-specific regional depletion of 5-HT in alloparental behavior, we performed a similar behavior paradigm as above using conditional 5-HT depleted ($Tph2^{f/f}$; n=8) virgin females compared to control (C57BL/6; n=7. All mice underwent surgery with stereotaxic injections of a Cre-GFP-expressing AAV in the DRN (B7). Three weeks after surgery, both groups were exposed to pups for 40 min during 3 consecutive days and new nesting material was added every night before the exposure (**Fig. 11A**). To characterize alloparental care in this model, we studied two types of behaviors: pup directed behaviors (**Fig. 13**), as the time spent interacting and pup retrieval, and nest building behavior (**Fig 11B and C; Fig. 12B, C and D**). Both types of behaviors reflect alloparental motivation and responsiveness.

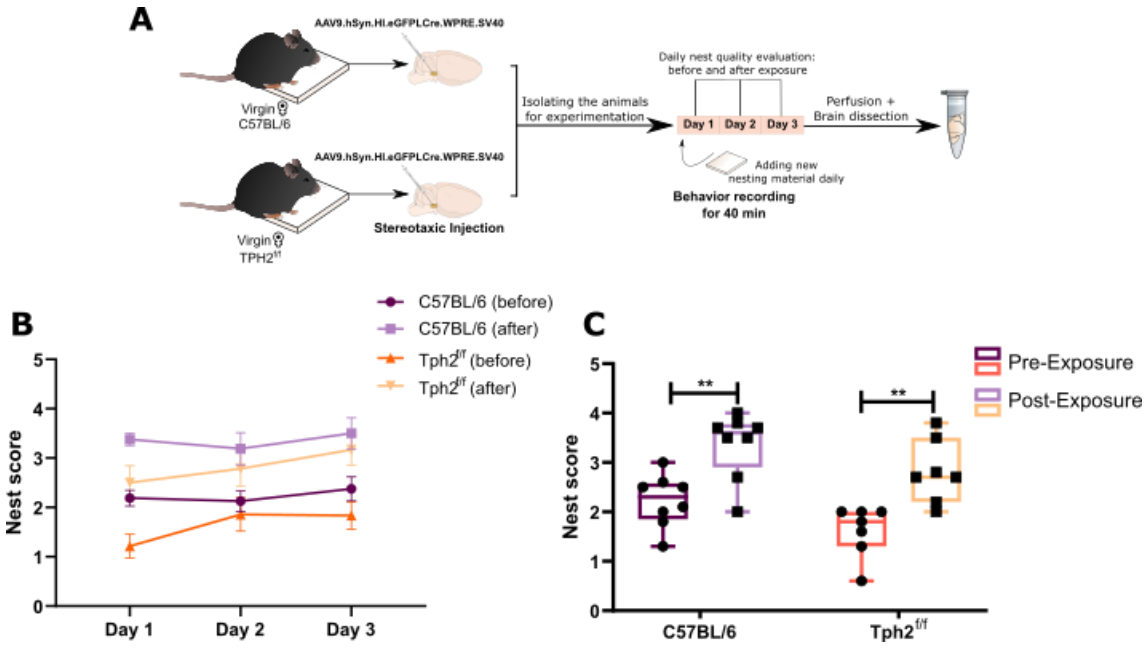

Figure 11 | Pup exposure increases the quality of nests in $Tph2^{f/f}$ virgin females.

(A) Schematic representation of the experimental paradigm used on this cohort. **(B)** Mean nest score for both groups, C57BL/6 (n=7) and $Tph2^{f/f}$ (n=8) females, pre and post-exposure in all 3 consecutive days of pup exposure. **(C)** Average nest score for both groups pre and post-exposure. Unpaired two-tailed t-test, $^{**}p < 0.01$

The nests built by C57BL/6 and Tph2$^{f/f}$ females was scored daily, using the same procedure described above (**Fig. 8B**). All females, from both experimental groups, increased the quality of their nests after pup exposure over the 3 exposure days. The effect was not long-lasting however, since already on the next day, the nest score went back to baseline (**Fig. 11B**). When comparing the average nest score, pre and post-exposure we could see that the increase in nest quality was significant for both groups (C57BL/6: 2.2 versus 3.4, p= 0.002; Tph2$^{f/f}$: 1.6 versus 2.8, Unpaired two-tailed t-test, p= 0.002, **Fig. 11C**). Although, C57BL/6 had slightly higher nest scores than Tph2$^{f/f}$ females, this difference was not significant (**Fig. 11B and C**). Due to the fact that the nest score scale has small range, from 0 to 4, a small difference could be masking a significant difference in nest building. To further explore that, we performed a quantitative analysis of nesting behavior.

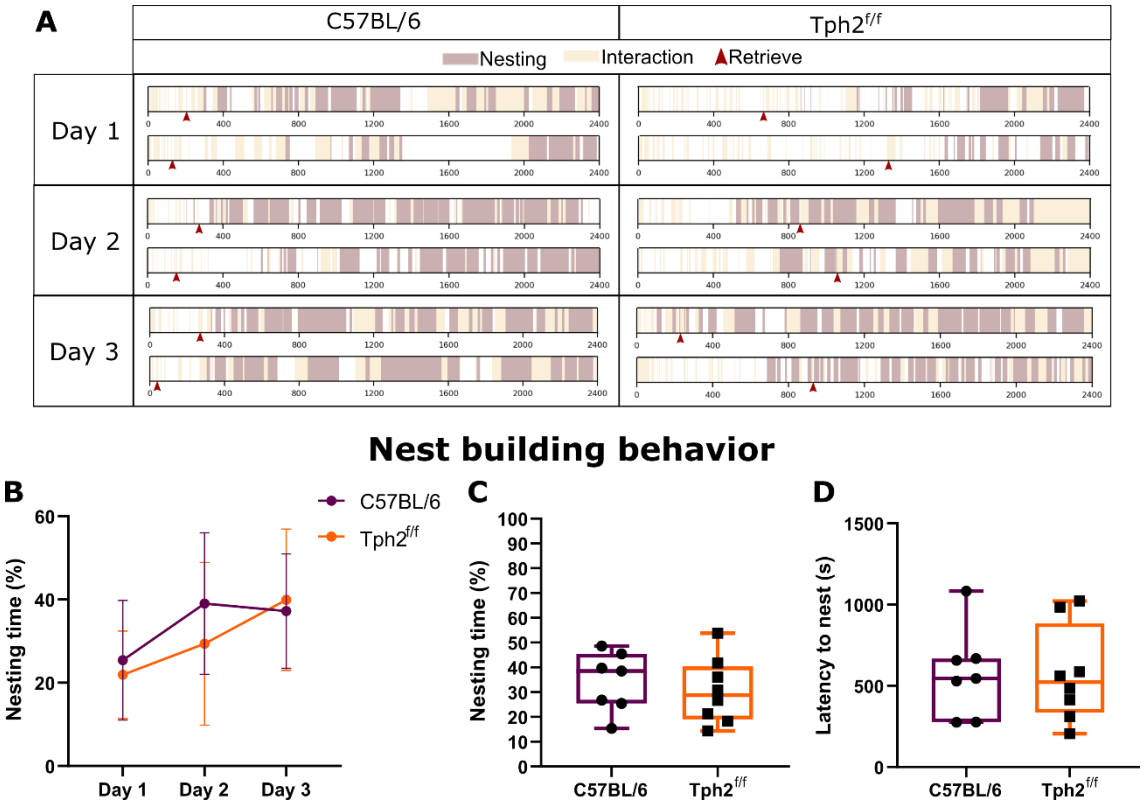

Figure 12 | Tph2$^{f/f}$ females don't have impairments in alloparental nest building.

(A) Representative raster plots exemplifying bouts of activity during exposure time in the 3 consecutive exposure days. Each bout of time spent nest building or interacting with pups are represented. Red arrow indicates when all 3 pups were retrieved into the nest site. **(B)** Mean percentage of time spent nesting for both groups, C57BL/6 (n=7) and Tph2$^{f/f}$ females (n=8), for all 3 consecutive days of exposure, during 40min. **(C)** Average percentage of nesting time, in all 3 exposure days, during 40 min. **(D)** Mean latency, in seconds, to actively interact with the nesting material.

The total time spent building a nest did not vary between controls and Tph2$^{f/f}$ females (39% versus 29%, n.s., **Fig. 12 C**) also, time to initiate the behavior was similar for both groups (545s versus 522s, n.s., **Fig. 12D**). Control females seemed to have longer bouts of nesting activity, but the frequency of nesting bouts was very similar in both groups (**Fig. 12A**). Similarly, the percentage of time actively interacting with the nesting material was similar for both groups in all 3 days of exposure (**Fig. 12B**).

To further explore the effects of conditional depletion of 5-HT in alloparental behavior, we analyzed the pup interaction dynamics. Tph2$^{f/f}$ females interacted the same amount of time with pups, when compared to control females (27% versus 26%, n.s., **Fig. 13A and B**). The percentage of time Tph2$^{f/f}$ females spent actively interacting with pups did not vary significantly with repeated pup exposure, although a small increase was observed on the third day of exposure (**Fig. 13A**). Tph2$^{f/f}$ females had the same latency to approach the pups as control females (3.7s versus 6.5s, n.s., **Fig. 13C**), demonstrating that Tph2^{f7f} females did not show aversion to pups. During all exposures, females from both groups retrieved all pups to the nest site (**Fig. 12A**). C57BL/6 and Tph2$^{f/f}$ females showed a similar latency to retrieve pups over the 3-day observation, despite a small increase in delay on day 2 (**Fig. 13D**). Total latency to retrieve in Tph2$^{f/f}$ females did not differ from that of control females (395s versus 529s, n.s., **Fig. 13E**). Together, our data suggests that a conditional 5-HT depletion, in adulthood, did not produce impairments in 2 different alloparental behaviors in virgin female mice.

Since the efficiency of viral recombination and consequent 5-HT depletion can differ across cases, we performed a histological examination of the DRN. For each case, serial sections of the DRN were immunolabeled for GFP and 5-HT, allowing to visualize the site of injections and the efficiency of 5-HT depletion at different levels of the DRN. In all injected mice, numerous GFP positive nuclei were present in the DRN B7 nucleus (**Fig. 14A3 and A4**). Counts of 5-HT positive (5-HT^{+}) cells in the injected Tph2$^{f/f}$ mice showed a 71.3% reduction, ranging from 64.5% to 79.2%, of 5-HT^{+} cells compared to controls (**Fig. 14A2**). The difference in the number of 5-HT^{+} cells counted, between the both groups, is highly significant (Unpaired two-tailed t-test, p= 0.002, **Fig. 14B**). Although, 5-HT immunoreactivity was strongly reduced in injected Tph2$^{f/f}$, we could observe a faint 5-HT staining in those animals (**Fig. 14A2**); this could be due to the maintained capacity to reuptake and storage pre-existing 5-HT, since the high affinity 5-HT transporters (SERT and VMAT2) are still expressed in DRN neurons.

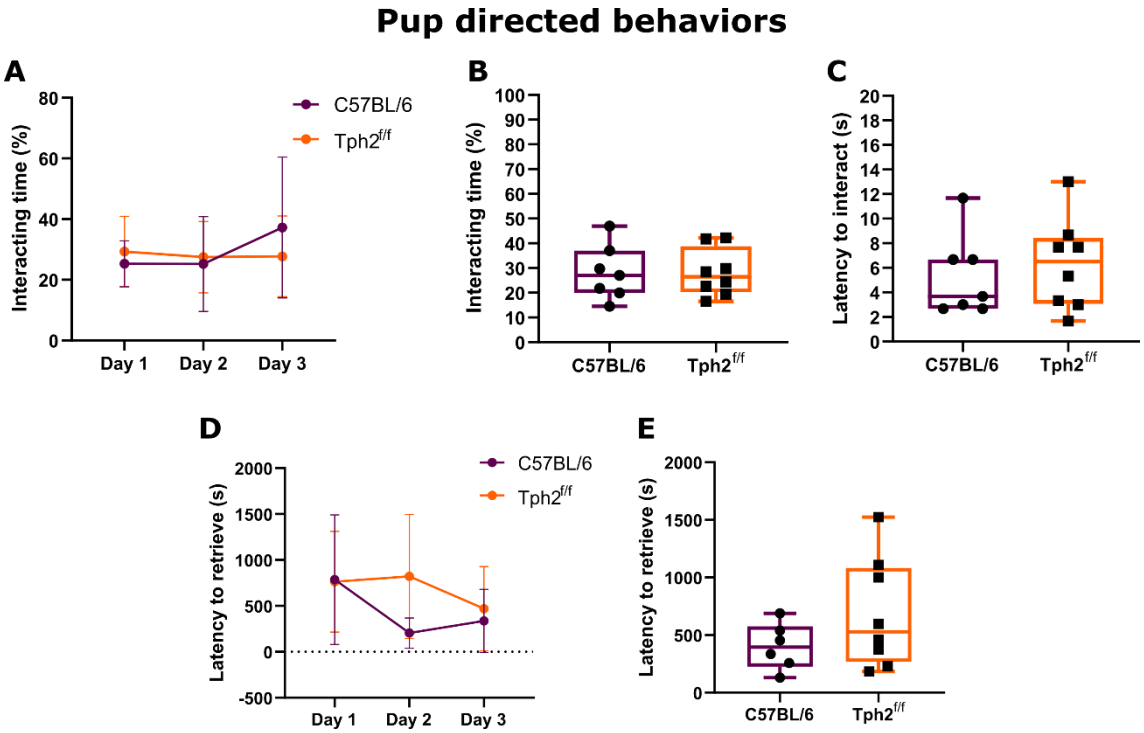

Figure 13 | Virgin Tph2$^{f/f}$ females do not show aversion to pups.

(A) Mean percentage of time spent interacting with pups for both groups, C57BL/6 (n=7) and Tph2$^{f/f}$ females (n=8), for all 3 consecutive days of exposure, during 40min. Several behaviors were classified as interacting: sniffing, licking and crouching over the pups. **(B)** Average of interacting time (%) in all 3 exposure days, during 40min**. (C)** Mean latency, in seconds, to approach and interact with the pups, in all 3 exposure days. **(D)** Mean latency, in seconds, to retrieve all 3 pups to the nest site, for all 3 exposure days. **(E)** Average latency, in seconds, to retrieve all pups, in all exposure days.

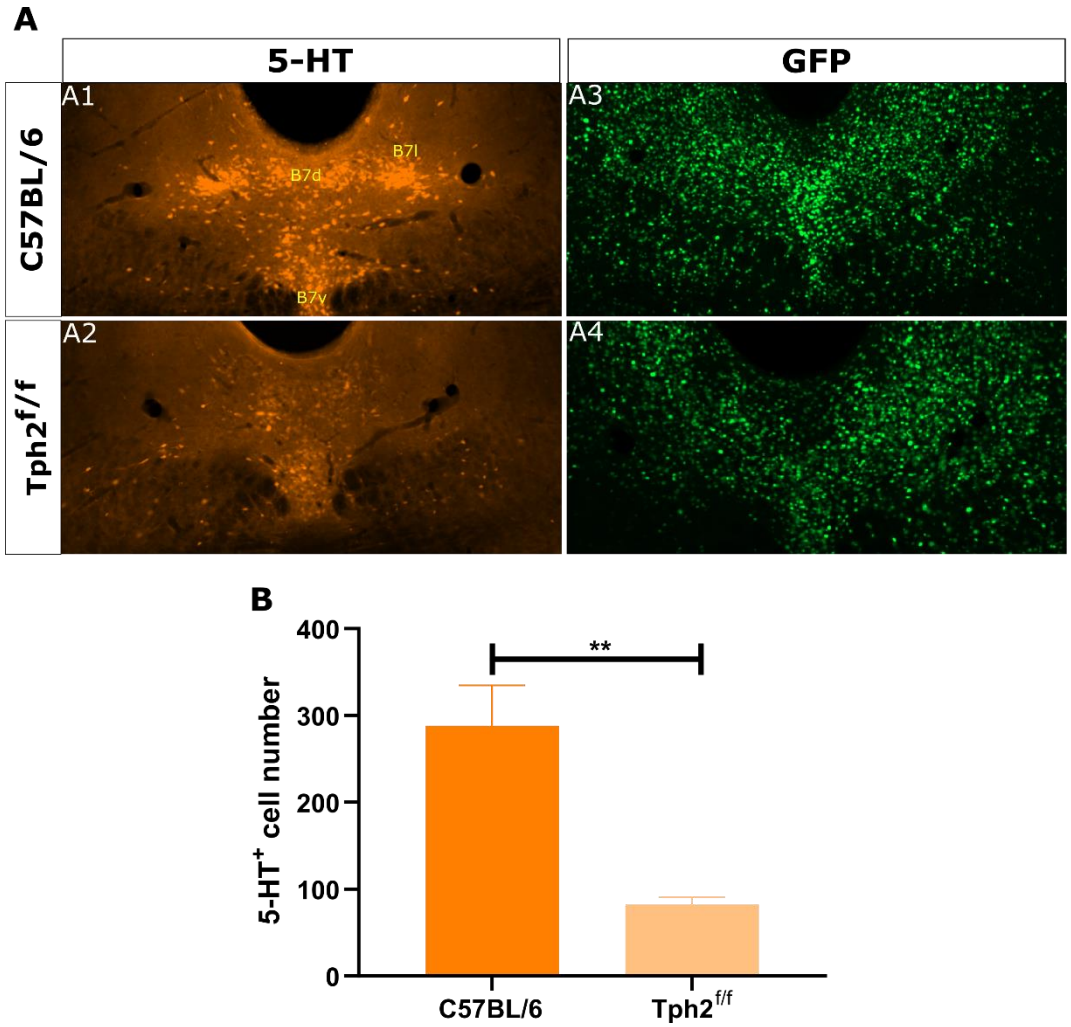

Figure 14 | 5-HT depletion in injected Tph2$^{f/f}$ mice.

(A) Representative images of 5-HT (A1 and A2) and GFP (A3 and A4) immunolabeling in both groups, C57BL76 and Tph2$^{f/f}$ female mice injected with AAV9.*hSyn.HI.eGFPLCre*.WPRE.SV40. Dorsal raphe nucleus B7 at the same level is represented for both cases. Several subcomponents of the DRN are represented in the image, such as dorsal (B7d), ventral (B7v) and lateral (B7l). **(B)** Number of 5-HT positive (5-HT^{+}) cells in both injected groups. Unpaired two-tailed t-test. **p<0.01

5. Brain areas involved in female alloparental behavior

Considering the results obtained in the first behavior paradigm used, we decided to perform a similar paradigm to evaluate the neural activation induced by pup exposure in virgin female mice. In this new paradigm (**Fig. 15A**), we exposed virgin female mice to pups (n=11) or to control objects (n=5) for 3 days, for 1 hour as described previously, except that only the last day of exposure was recorded and segmented. The first 2 days of exposure were considered as habituation, since we observed a higher increase in nest building and interaction with pups of the third day of exposure in our previous results (**Fig. 8B and E**). It should be noted that the time of exposure was higher than in the first experiment and experiment was performed in a different animal facility.

We observed the same effects as in the first experiment used, with one exception: the pup-exposed females showed more and longer bouts of nest building activity when compared to object-exposed females, that had almost no bouts of nest building activity (**Fig. 15B**). Quantitatively, the difference in nesting behavior, between both groups, was highly significant (0% versus 14.3%, Mann-Whitney U test, p= 0.0005, **Fig. 15C**). Quantitative analysis of time spent interacting also showed a significant different between both groups (0.2% versus 50.4%, Mann-Whitney U test, p= 0.0009, **Fig. 15D**). The latency to approach and interact with pups or control objects was significantly shorter in pup-exposed females (86s versus 24s, Unpaired two-tailed t-test, p= 0.04, **Fig. 15E**). We also observed that 2 females exposed to pups did not retrieve all pups during the exposure time. The ones that retrieved all pups did it by the first 10 min with the exception of one female that retrieved the last pup almost at the end of the exposure time (**Fig. 15B**). None of the object-exposed females retrieved any object.

The different results obtained in both experiments could be due to the fact that animals were housed and recorded in different animal facilities. Also, the control object used was somewhat different in the second experiment. So, taking this into account, even though the paradigms were similar, we cannot really compare the results obtained.

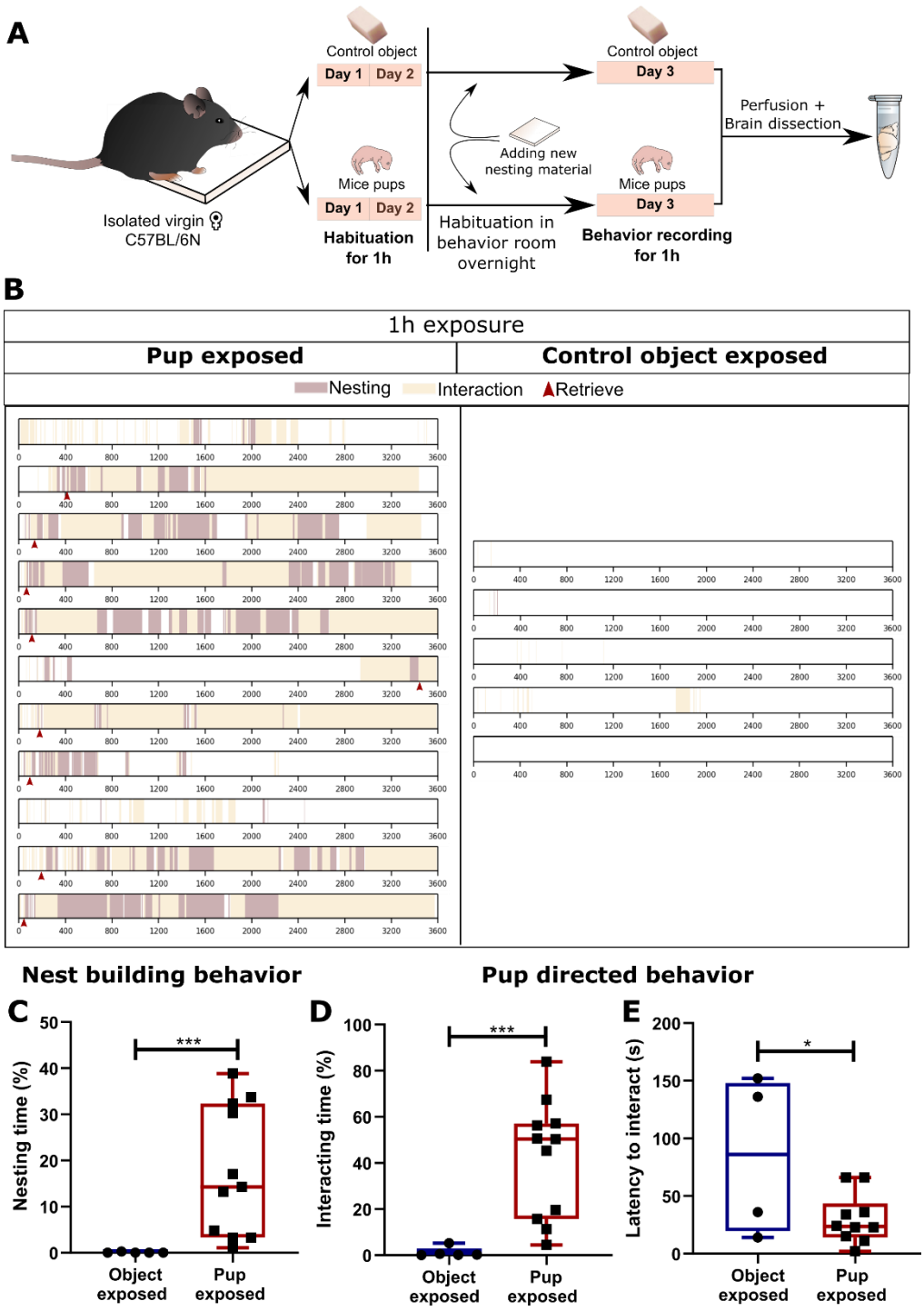

Figure 15 | Pup exposure elicits nest building behavior in virgin female mice.

(A) Schematic representation of the experimental paradigm used on this cohort. In this paradigm, females were exposed to pups or control object for 3 days, but only the third day was recorded for further analysis. The first 2 days were considered as habituation. **(B)** Raster plots showing bouts of activity during the third day of exposure , pups or control object, for 1h. Each bout of time spent nest building or interacting with pups are represented. Red arrow indicates when all 2 pups were retrieved into the nest site. **(C)** Percentage of time spent actively interacting with the nest material (nesting) during 1h. Mann-Whitney U test. ***p<0.005. **(D)** Percentage of time spent interacting during 1h. Mann-Whitney U test. ***p<0.005. **(E)** Latency, in seconds, to actively interact with the exposure subject, pup or control object. Unpaired two-tailed t-test. *p< 0.05.

In order to try to dissect the brain areas activated by alloparental care, we perfused the animals and harvested the brains 1h after exposure to pups or objects. We expected the expression level of c-Fos in neurons active at the onset of the behavior to be at its highest. Dissected whole brains underwent a tissue clearing (iDISCO+) and c-Fos immunolabeling protocol and then were imaged using light-sheet microscopy. Data obtained from microscopy was analyzed by automated 3D mapping and analysis of activated neurons on whole cleared brains using a custom python pipeline (ClearMap), to map the distribution of c-Fos positive cells (c-Fos$^+$) as a proxy for neuronal activity in pup exposed and object exposed virgin females (**Fig. 16A**).

To perform an unbiased analysis, we surveyed the brain looking for differentially activated regions. Few brain areas showed significant differences in neural activation when comparing pup-exposed and object-exposed females. We found a higher number of c-Fos$^+$ cells in the MPOA and adjacent BNST (**Fig. 16B**), in several nuclei of the amygdalar complex (**Fig. 16C**) and in the olfactory tubercle (**Fig. 16D**) when females were exposed to pups. Consequent visualization and analysis of the raw data using Imaris corroborated the results obtained using ClearMap analysis.

All regions identified as having higher expression of c-Fos$^+$ cells in alloparental females have already been implicated in some aspects of parental behavior. Our data therefore suggest that these brain regions are also involved in neural circuits responsible for the expression of alloparental behavior in virgin female mice.

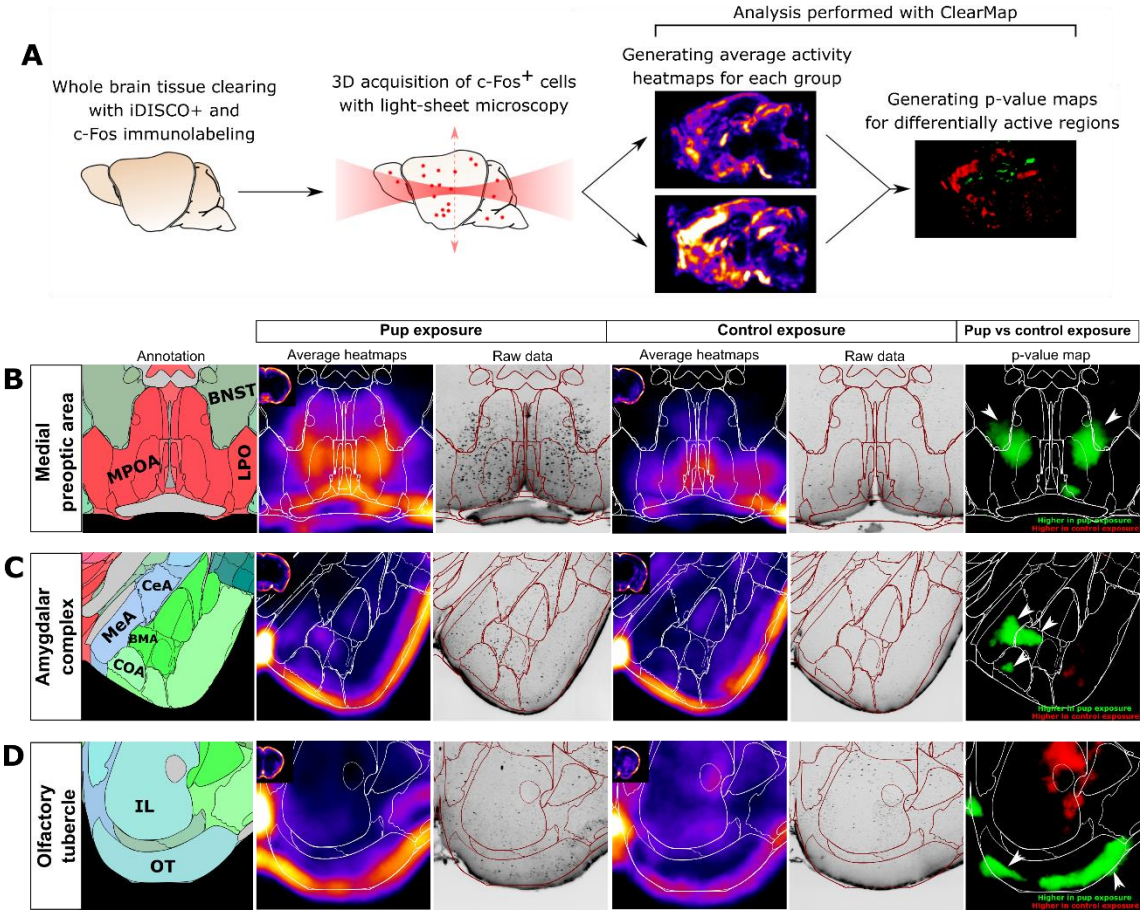

Figure 16 | Neural activation during alloparental care.

(A) Volume mapping of neuronal activity using iDISCO+ and ClearMap. **(B)** Higher neuronal activation is observed in the MPOA and adjacent BNST of pup exposed females when compared to object control exposed females. **(C)** Higher neuronal activity is observed in several nuclei of the amygdalar complex of pup exposed females. **(D)** Higher neuronal activity is observed in the olfactory tubercle of pup exposed females. All groups were composed of pup exposed females (n=8) and object exposed females (n=4). Overlay of the raw c-Fos labeling with the average activity heatmaps and p-value maps in coronal projection for all the groups. BNST, Bed nucleus of stria terminalis; MPOA, Medial preoptic area; LPO, Lateral preoptic area; CeA, Central amygdala; MeA, Medial amygdala; BMA, Basomedial amygdala; COA, Cortical amygdala; IL, Infralimbic area.

6. The neuronal substrate of nest building behavior in alloparental care

When observing the results obtain for the percentage of time spent pup exposed females nest building (**Fig. 15C**), we observed that females could be classified in two different groups. In one group, females spent a high percentage of their time nest building (more than 30%) while in the other group , females spent a very low percentage of time nest building (less than 5%). In order to assess possible neuronal substrates involved in alloparental nest building, we compared the neuronal activation of both groups, high nesting (%) females and low nesting (%) females.

First, we looked into the neural activation of the MPOA and adjacent BSNT. In high nesting females, the MPOA and adjacent BNST had similar activity levels when compared to low nesting females (**Fig. 17A**). Hence, this means that the increase of activity in the MPOA and adjacent BNST observed, during pup exposure, was related to other aspect of alloparental care other than nest building.

When surveying the brain, we focused our analysis on sub-cortical regions, as the activity in the cortex could mostly reflect the general motor and sensory experiences of females during the exposure time. Few regions showed significant differences between high nesting females and low nesting females. Most of the regions found presented higher neural activity when females spent less time nesting. Only one region seemed to be highly activated when females nested for a long period of time. We found that the ventrolateral periaqueductal gray (vlPAG) had a significant increase in its activity in high nesting females, while the dorsal periaqueductal gray (dPAG) seemed to be more active in low nesting females (**Fig. 17B**). The higher neural activation of vlPAG in high nesting females could suggest a role of this structure in alloparental nest building.

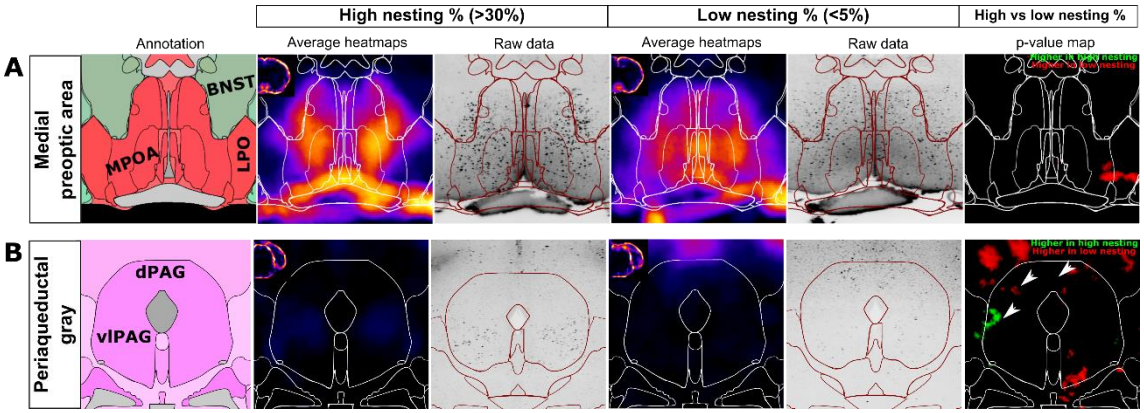

Figure 17 | Neural activation during nest building in alloparental care.

(A) Both groups, females that nest more the 30% of the exposure time and females that nest less than 5% of the exposure time, show similar neural activation in the MPOA and adjacent BNST. **(B)** Differentially neural activation during in different regions of the PAG. Higher neural activity is observed in high nesting (%) females when compared to low nesting (%) females in the vlPAG (green p-value). dPAG show higher neural activation in low nesting (%) females (red p-value). All groups were composed of high nesting (%) females (n=3) and low nesting (%) females (n=3). Overlay of the raw c-Fos labeling with the average activity heatmaps and p-value maps in coronal projection for all the groups. BNST, Bed nucleus of stria terminalis; MPOA, Medial preoptic area; LPO, Lateral preoptic area; dPAG, Dorsal periaqueductal gray; vlPAG, ventrolateral periaqueductal gray.

CHAPTER V

DISCUSSION AND PERSPECTIVES

1. Discussion

In this work, we confirmed that exposure to pups in virgin females elicit the building of higher quality nests and show that this effect is due to an increase in the time spent nesting. We also demonstrate that conditional depletion of 5-HT in the DRN has no effect on alloparental nest-building. Using whole brain mapping of neural activity reporter, we identified the vlPAG as a structure possibly implicated in improved nest building in an alloparental context.

Laboratory female mice show robust alloparental care (Kuroda *et al.*, 2011) but the mechanisms underlying this behavior have been somewhat understudied. Moreover, the few studies that focus on alloparental behavior in mice tend to neglect an important component of alloparental behavior which is nest building. Nest building is an important behavior in parental and alloparental care. It is essential for protecting the young from hypothermia, since most pup rodents are born altricial. It also serves as shelter and protection of the offspring from conspecifics (Brain, 1986). Nest building behavior has been shown to correlate positively with the survival of mouse offspring, since high quality nests are correlated to a higher number of weaned pups (Bult & Lynch, 1997).

In this work, we assessed alloparental behavior in adult C57BL/6 virgin females with special focus on nest building behavior to identify the specifics of behavioral changes caused by pup exposure and to reveal possible neural correlates.

Contrary to female virgin rats, female virgin mice display spontaneous maternal and do not need a sensitization period (Kuroda *et al.*, 2011). We confirmed that C57BL/6 virgin mice exposed to pups show no aversion towards pups since all females approached and interacted with them upon first exposure. Moreover, none of the exposed females attacked the pups. Exposed virgin females approached pups more rapidly than objects. This is likely due to pups' olfactory and auditory cues that act as attractive stimuli leading to a rapid approach and is followed by alloparental care behaviors. Pup retrieval is a proactive behavior and is the mostly used behavior to assess parental motivation (Numan & Stolzenberg, 2009). Almost all pup-exposed virgin females retrieved the pups into the nest site upon first exposure, in contrast with object-exposed females (that did not retrieve the objects).

Regarding nest building behavior, we confirmed observations in the literature showing that virgin female mice build better nests when presented with pups (Gandelman, 1973b).

Our study differs from this study by several points. Our control group is formed by virgin females exposed to a control object to account for the presentation of a new stimulus. Gandelman's evaluation was performed on a different inbred strain and it is well-known that genetic background can potentially influence various behavioral phenotypes in mice (Bothe *et al*., 2004). We recorded the dynamics of the effect of presentation to pups on nest building activity over several days. This quantitative analysis showed that the time spent building the nest is higher in pup-exposed females and this effect increases across exposure days. This last observation point towards a motivational effect of pup exposure towards the execution of this specific behavior. Our study also shows that a very short exposure to a pup can be sufficient to elicit increased nest building activity in some virgin female mice. However, the significance of this result is questionable since this difference is mainly due to one female in a cohort of 5. More studies, with a higher number of animals would be needed to evaluate the effect of short pup exposure on nest building.

The neural mechanisms underlying the onset of alloparental behavior are not well understood in virgin female mice. In the present study, we investigated which brain areas are activated in adult inexperienced females when performing different behaviors of alloparental care. Alloparental behavior is composed of different types of behaviors, such as pup retrieval, nursing-like behaviors and nest building (Kuroda *et al.*, 2011). These behaviors are likely related to different neural circuits and mechanisms. In my study, I focused specifically on the nest building behavior.

Preliminary studies of my host laboratory had shown that mice with 5-HT constitutive depletion , the TPH2 KO, have impairments in alloparental behavior (Scotto-Lomassese *et al.*, in prep). To determine whether these defects are due to reduced transmission of developmental changes of neural circuits caused by constitutive 5-HT depletion, we used a conditional model of 5-HT ($Tph2^{f/f}$) depletion, in which we could target 5-HT depletion to the DRN in adults. Contrarily to the results in TPH2 KO, and Pet-1-KO (Scotto-Lomassese *et al.*, in prep), $Tph2^{f/f}$ :: DRN-Cre virgin females showed no aversion to pups when exposed to them since none of the exposed females attacked the pups. We also did not find any impairments in the two alloparental behaviors that were evaluated. $Tph2^{f/f}$ :: DRN-Cre females retrieved all the pups into the nest site and did not differ from control mice in the latency to retrieve. Regarding nest building behavior, the total time spent building a nest and the time to initiate the behavior was similar for both groups. These results suggest that depletion of 5-HT neurotransmission in the DRN in adulthood has

no effect on the dynamics of pup interaction and nest building in virgin female mice.

The differences found in alloparental care between $Tph2^{f/f}$ and constitutive KO females could be due to the lack of 5-HT neurotransmission during development but also due to the fact that the DRN depletion of 5-HT in the conditional mice model used was incomplete (Gutknecht *et al*., 2008; Kriegebaum *et al*. 2010; Scotto- Lomassese *et al.*, in prep). We found that 5-HT^{+} cells in the DRN of injected $Tph2^{f/f}$ mice showed a 71.3% reduction when compared to controls. The extent of 5-HT depletion in the forebrain was not quantified.

We then used c-Fos immunohistochemistry as a reporter for neuronal activity in pup-exposed versus object-exposed females. c-Fos, is part of the immediate early genes family, is a well characterized proto-oncogene that was shown to be expressed in a restricted temporal window following neuronal stimulation, and thus have been used as a reporter for neuronal activity for decades (Kawashima *et al.*, 2014). Brain-wide activity mapping using c-Fos have been traditionally performed either manually or automatically on brain sections to map neurons activated during natural mouse behaviors such as social interactions (Kim *et al*., 2015). However, in a cleared brain, individual cells can be imaged without slicing, thus preserving the topological integrity of the tissue, which improves the accuracy of the cell registration onto reference atlases (Renier *et al.*, 2014).

Using ClearMap analysis, we found higher neural activation in several brain areas already known to be implicated in maternal care, such as the medial preoptic area (MPOA) and adjacent BNST, several amygdalar nuclei and the olfactory tubercle.

The MPOA and BNST are known to be critical for the initiation and maintenance of maternal behavior in rodents (Numan, 2007). For example, lactating rodents express higher c-Fos expression when performing different maternal behaviors (Calamandrei & Keverne, 1994; Fleming & Walsh, 1994). We found higher number of c-Fos^{+} cells in the MPOA and the adjacent BNST, suggesting a role for these brain areas in alloparental behavior as well. The distribution of active neurons during alloparental behavior was very similar to that observed previously in similar studies (Renier *et al*., 2016; Wu *et al.*, 2014).

The MeA and COA are areas usually associated with the inhibition of parental behaviors. Lesions in these amygdalar nuclei facilitated the induction of parental behaviors in rats (Fleming et al., 1980). However, pup presentation also induces higher expression of c-Fos^{+} cells in parental female mice and prairie voles; and lesions in the

MeA and COA significantly decreased the time male prairie voles interacted with pups (Kirkpatrick *et al*., 1994). In our study, we found an increase c-Fos expression in the MeA and COA in alloparental females. This finding is consistent with previous studies in parental female mice and suggests that in laboratory mice MeA and COA might be important for the induction of alloparental behavior, likely processing olfactory information for the pups (Keshavarzi *et al.*, 2015). We also found increased c-Fos expression in BMA, this amygdala nuclei relays olfactory and somatic sensory information from pups to circuits known to be involved in maternal motivation (NAc and VP circuits) (Numan & Insel, 2003). However, we did not find an increased c-Fos expression in the NAc or VP when comparing alloparental females and object-exposed females.

Another brain area that showed increased expression of c-Fos$^+$ cells when females shown alloparental behaviors was the OT. The OT receives dense innervation from neurons in olfactory bub and processes odor information in a similar manner to the primary olfactory cortex (Payton *et al.*, 2012). Moreover, OT electrical stimulation resulted in the recruitment of neurons within brain structures, including NAc, known to be essential for motivated behaviors (FitzGerald *et al.*, 2014). Although, we did not find increased neural activation in the NAc, increased expression of c-Fos$^+$ cells in the OT suggest a role of this structure in regulating olfactory cues from pup and consequently maternal odor-motivated behaviors.

In our quantitative analysis of nest building activity, we found in both paradigms that alloparental females could be divided into two different groups: high nesting females and low nesting females. When comparing the whole brain neuronal activation of both groups, we found only one brain region, the vlPAG, which was highly activated when females nested for a longer period of time.

The PAG has been implicated in several behaviors of the maternal repertoire. The main known implication of the PAG in maternal behavior is related to nursing arched back posture, kyphosis. Postpartum rats shown increased neural activation in the caudal vlPAG when allowed to interact with their pups. This effect was observed in response to interaction with sucking pups compared with non- suckling pups or no stimulation from pups (Lonstein & Stern, 1997). Lesions of the vlPAG and lateral PAG (lPAG) significantly reduced the time dams crouched over the pups in an arched back posture and increased the time they spent in prone position or just hover over the pups (Lonstein &

Stern, 1997, 1998). Although the focus of these lesions studies was on nursing, authors also found that vlPAG and lPAG lesions produced significant differences in the time lesioned dams spent nest building. Lesioned dams spent significant less time building a nest when compared to sham dams (Lonstein & Stern, 1997, 1998). The lesions in these studies were made in the same regions of the PAG as those in which we saw increased c-Fos expression in high nesting female mice. However, one must bear in mind, when comparing these studies is that they were performed in different animal models (rats or mice), and in different states (virgin or post-partum females).

Our approach to evaluate neural activation using c-Fos expression as a proxy has several limitations. The neural activation observed in female mice, on both analyses, could account for the different behavior other than pup related behaviors or nest building activity. However, females compared for the nest building neural candidate performed the same behaviors in a similar manner with the exception of nest building. With the identification of vlPAG as a possible candidate, more precise tools, such as fiber photometry, can be used to precisely record in-vivo neural activation in this area when alloparental females are performing nest building.

2. Perspectives

The results generated in this work led to several new additional questions that would require further experimentation. Also, due to the temporal constrictions of this thesis we were not able to perform some key experiments and data analysis. As such, several new experiments will greatly enhance our knowledge on the role of vlPAG and on the effect of 5-HT in alloparental nest building.

The vlPAG may play a role in alloparental nest building but whether this region is implicated in the appetitive or consummatory phase of the behavior is unclear. A characterization of vlPAG neurons activated during alloparental nest building should be performed in order to obtain a model for in-vivo recordings of neural activation in the vlPAG, using fiber photometry, in alloparental females performing nest building. We will also like to explore the effect of different pup cues, such as USVs and olfactory cues, in nest building behavior and subsequent role of vlPAG. These experiments will further confirm the role of vlPAG in alloparental nesting and also shed some light in its implication in the behavior.

In our conditional 5-HT depleted mice, we were unable to obtain a complete depletion of 5-HT in the DRN and we did not quantify the extent of 5-HT depletion in different forebrain regions involved in maternal behavior. The quantification of 5-HT$^+$ axons in the forebrain will further elucidate the role of 5-HT neurotransmission in this behavior and to what extend the depletion is effective in forebrain structures. Also, the generation of a conditional mice model with a complete depletion of 5-HT and further evaluation of maternal and alloparental care could explain some of the behavior differences between conditional and constitutive 5-HT depleted models. Overall, these experiments will further contribute to our understanding of the DRN serotonergic neurotransmission mechanisms involved in parental care.

CHAPTER VI

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