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# Simultaneous control of pup-directed and depression-like behavior by preoptic inhibitory neurons in mice

**Ph.D. thesis**


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**Table of Contents**

**List of abbreviations**

| | |
|---|---|
| BSA | bovine serum albumin |
| DREADD | designer receptor exclusively activated by designer drugs |
| EPM | elevated plus maze |
| ERα | estrogen receptor alpha |
| FST | forced swim test |
| GA | glutaraldehyde |
| GABA | γ (gamma)-aminobutyric acid |
| GAD | glutamate decarboxylase |
| Gal | galanin |
| IEGs | immediate early genes |
| MeA | medial amygdala |
| MPOA | medial preoptic area |
| OFT | open field test |
| OT | oxytocin |
| OXTR | oxytocin receptor |
| PAG | periaqueductal grey |
| PB | phosphate buffer |
| PFA | paraformaldehyde |
| PRL-R | prolactin receptor |
| pSTAT5 | phosphorylated transcription activator protein 5 |
| Pth2R | parathyroid hormone 2 receptor |
| SDN | sexual dimorphic nucleus |
| SPT | sucrose preference test |
| TB | tris buffer |
| TBS | tris buffer with saline |
| TH | tyrosine hydroxylase |
| Tip39 | tuberoinfundibular peptide 39 |
| TTB | tris buffer with Triton-X100 |
| VGAT | vesicular GABA transporter |
| VTA | ventral tegmental area |

## 1. Introduction

Parenting can be viewed as an evolutionary programmed reproductive behavior whose purpose is to increase the likelihood that the immature offspring will survive to maturity. Parental care is a special and enduring form of prosocial interactions with a long-term impact not only on the mental and physical development of the offspring but also on their reproductive behavior, making the neurobiology of parental care a particularly important topic from an evolutionary point of view [1,2]. Investigating the mechanisms underlying the physiological responses towards infants in nonhuman animals can provide a framework for understanding how parental circuits may become dysfunctional leading to child abuse or neglect by human mothers. Despite the extensive characterization of the hormonal and nonhormonal background of these complex behaviors, the underlying neurobiology of the potential connections between motherhood and postpartum disorders is in its infancy.

### 1.1. Diversity of parental behavior

In mammals, raising offspring requires significant investment that is exclusively performed by a single parent in species demonstrating a uniparental care system, or by both parents in species exhibiting biparental care. In the case of the uniparental care system, maternal behavior is the dominant form of parenting, since fertilization ensures maternity but not paternity, and only the female lactates [3]. Across the species, the infant-directed interactions of males demonstrate high variabilities ranging from ignorance and aggression to paternal behavior[4,5].

Alloparental behavior is an alternative and cost-effective form of parenting, in which a related or unrelated group member in cooperation, or independently from the biological parents assist and invest substantially in the caretaking behaviors directed towards the infants. The evolution of alloparental behavior is mainly limited to the species with altricial young. The reason for the exclusive emergence of this unique form of parenting is twofold. Achieving developmental milestones in slow-maturing, large-brained apes and humans is a time- and energy-consuming process. According to currently accepted views, in the early stage of primate evolution, cooperative support for the survival of an individual was one of the critical steps of their reproductive success [6]. From a different perspective, under natural conditions, newborns are not capable to move

from one nest to another thus minimizing the chance of offspring getting mixed up and erasing the need for selective attachment mechanisms. To support this phenomenon, under laboratory conditions, the lack of forming selective attachments to their own offspring in mice makes it possible the alloparental behavior including cross-fostering or exchanging the young between litters contributes to the success of the breeding [7,8].

In addition to fostering behavior, as a result of experimental selection of laboratory mice, a novel and naturally not seen form of alloparental behavior has emerged. Sexually unexperienced feral or wild mice typically avoid or even attack when exposed to young, while under laboratory conditions, nulliparous females exhibit relatively prompt acceptance towards pups, similar to mother animals [9]. The immediate caring behavior shown by laboratory virgin females in the absence of priming the brain with the endocrine events associated with pregnancy suggests that the environment and selection can alter the brain mechanisms involved in processing infant cues. Exploring the neurobiological mechanisms underlying alloparental behavior may help to understand how maternal behavior can be relatively emancipated from hormonal control allowing for the adoption of children in human culture[10].

### 1.2. The motivational model of pup-directed behaviors in rodents

Typical motivated behaviors include feeding behavior, prosocial behaviors, such as sexual or parental behavior, and avoidance of pups. They are driven by the internal processes, which direct the behavior of an animal toward a particular goal. All of the abovementioned behaviors can be separated into appetitive or goal-directed and terminated or consummatory components.

During the goal-directed phase, the main motivation of the animals is to make a contact and react to the stimulus, even if the stimulus is aversive. The term goal-directed behavior refers to both approach responses toward a pleasant stimulus, and rejection or avoidance responses evoked by an unpleasant or noxious stimulus. The aversive stimulus can induce approaching behavior aiming to actively reject or attack the stimulant, as well as avoidance or escape responses. Concerning pup-related behavior, a good example of the two types of goal-directed responses is the behavior outputs of maternal females and the wild virgin mice following the exposure to young. Maternal female mice will seek out their offspring and immediately retrieve them back to the nest from a novel and potentially dangerous environment [11]. The conditioned place preference test is another

paradigm in which the goal-directed aspects of maternal behavior can be measured. During the training days, pups with their mother are placed in one distinct compartment of the two-compartment cage, while the other compartment is not associated with any rewarding stimulus [12]. On the test day, mothers spend significantly more time in compartments previously containing pups suggesting that they are searching for their pups serving as a reward. In contrast to mothers, virgin females usually avoid even well-known places if the pups are placed there [12]. Virgin males typically approach or chase the immature conspecifics if they appeared within their territory [13]. The major driving force behind the rejection behaviors exhibited by virgin males towards alien pups is to maximize their reproductive success and transmit more of their genes to future generations by decreasing the chance of the survival of the offspring derived from another male.

Consummatory components are made up of those actions that are performed once the desired goal is achieved. Well-defined consummatory components of maternal behavior are the crouching behavior aiming to keep the helpless newborns warm and provide them milk, the grooming behavior, and the genital licking to facilitate urination and elimination. In the case of virgin males, the territory owner immediately bites and attacks the alien infants upon close contact occurs [13]. However, drawing a sharp boundary between the goal-directed and consummatory phases is controversial.

Considering the potential neuroanatomical background of the two components of motivated behaviors, distinct brain regions may be involved in the regulatory processes. While the appetitive behaviors require higher cognitive mechanisms and can be influenced by experience and learning that is usually associated with telencephalic activity [14], the consummatory phase is more reflexive and potentially underpinned by the brainstem controlling stereotyped, instinct behavior elements [15]. One of the critical factors of the successful regulation of complex behaviors is an integrating center fine-tuning multimodal information about the inner and outer world and initiating motor outputs throughout their connections to brain regions involved in the control of motivated behaviors. The hypothalamus is a great candidate for this unique task due to its complex role including its regulatory influence over the autonomic nervous system and endocrine system and its brain-wide reciprocal connections to telencephalic regions as well as to the brainstem.

## 1.3. Neurobiology of parenting

Although the maternal behavior and motivation of humans and rodents are emancipated from the hormonal events to a varying degree, laboratory virgin females exhibit a relatively prompt caretaking behavior directed towards the conspecific young, similar to the perfectly normal maternal behavior of nonpostpartum women [9]. What these examples suggest is that even in the absence of hormonal priming, the physiological functioning of the intact maternal neuronal network is sufficient to induce and maintain maternal behavior and motivation. The core aspects of caretaking behavior are likely to be regulated by evolutionary conserved hypothalamic circuits in all mammals making it possible to explore the neurobiological basis of parenting using laboratory mice.

### *1.3.1. The essential role of the medial preoptic area in caretaking behavior*

Among the numerous hypothalamic nuclei involved in the regulation of parenting, the rostrally located medial preoptic area (MPOA) has a prominent function as a node and integrated center of this complex behavior. Lesion of the MPOA performed during pregnancy or the postpartum period disrupts the expression and maintenance of maternal care, respectively [16,17]. Furthermore, pharmacological depression of the neuronal activity of preoptic cells using gamma-aminobutyric acid type A (GABAA) receptor agonists results in deficits in all components of maternal behavior, including goal-directed retrieving behavior and consummatory grooming behavior [18]. In further support for the involvement of MPOA in maternal motivation and behavior, receptors binding sex steroid hormones, oxytocin, and prolactin are highly expressed by preoptic neurons [9]. In particular, following the direct administration of estradiol, all components of parental behavior are activated in both male and female rats [19,20]. The relevant functions of the estrogenic compounds in parenting are primarily regulated by the estrogen receptor alpha (ERα) nuclear receptor subtype. Reversible inactivation of ERα-expressing preoptic neurons leads to impaired retrieval behavior, while optogenetic activation of the same neuronal population immediately induces pup retrieval through their connections to the ventral tegmental area (VTA) [21].

In addition to the involvement of behavioral responses towards pups, the estrogen-ERα complex also serves as a transcription factor regulating the expression of numerous preoptic genes. Above all, estrogen binding to ERα significantly increases the expression

level of oxytocin receptor (OXTR) making preoptic neurons more receptive to sensory stimuli from pups near the time of parturition [22]. The involvement of oxytocin (OT) and its action on OXTR located in the MPOA in the induction of maternal behavior has been shown to be indispensable. Genetically modified animals with OXTR deficiency exhibited defects in lactation and maternal care [23], similar to primiparous parturient rats who received OXTR antagonist microinjection directly into the MPOA [24].

Interestingly, the regulatory role of estrogen-ERα binding is not restricted to the expression level of OXTR. Administration of estradiol to ovariectomized female rats dramatically increases the mRNA level of prolactin receptor (PRL-R) presumably through its action on ERα nuclear receptor expressed by prolactin-responsive neurons [25]. The high degree of colocalization between the prolactin-induced phosphorylated transcription protein-5 (pSTAT5) and ERα immunoreactivities in lactating animals further supports the idea of the estrogen-dependent expression of PRL-Rs [26,27]. The importance of prolactin and its action on PRL-Rs in the control of maternal care has been confirmed using mice with a knockout mutation of the PRL-R gene. In the lack of the PRL-R gene, virgin female and mother mice showed significant deficits in all aspects of maternal behavior [28]. More precisely, conditional knockout of PRL-R from preoptic cell population results in failure of milk ejection proposing that the reduced maternal motivation may connect to the inability of pup feeding [29].

A newly discovered neuronal pathway is also assumed to be involved in the regulation of lactation through its projection to the MPOA. The tuberoinfundibular peptide 39 (Tip39) is highly expressed in lactating animals by neurons located in the posterior intralaminar complex (PIL) of the thalamus [30]. The potential function of Tip39-expressing neurons in the transmission of suckling information has been supported by using immunohistochemistry, tracer techniques, and a pharmacological approach. A significant portion of neuronal population expression Tip39 is activated following suckling stimuli [30]. Furthermore, injection of anterograde tracer to the PIL revealed that one of the major outputs of neurons located in the PIL is the MPOA, which is abundant in the receptor binding the Tip39 protein, namely the parathyroid hormone 2 receptor (Pth2R) [31]. Pharmacological inactivation of Pth2R by administration of its antagonist almost completely blocks the suckling-induced prolactin release, providing further

evidence for the involvement of Tip39-expressing neurons in suckling information transmission to the MPOA.

Apart from the important role of hormone-receptor interactions in parenting-dependent gene regulation and behavior control, increased expression of immediate early genes (IEGs) in association with pup exposure significantly contributes to the expression of parental care. IEGs are transiently and rapidly transcribed in response to a wide variety of stimuli, providing an indirect readout of neuronal activation. The induction of these genes can occur without the need to synthesize new transcriptional factors and requires only the post-translational modification of pre-existing factors, allowing the immediate response [32]. The earliest identified and widely used transcription factor encoding the IEGs family is the *fos* family. In maternal animals, the expression of the *fos* gene is significantly elevated in the MPOA following exposure to pups [16]. Interestingly, in genetically modified mice lacking the *fos* gene, nurturing behavior is entirely disrupted leaving other cognitive functions undisturbed. The radical manifestation of knockout mutation of the *fos* gene in caretaking behavior proposes the crucial involvement of IERs in altering the MPOA phenotype to assist in the expression of parenting [33].

In addition to the functions of IEGs in the onset of maternal behavior, both the genes and their protein products can be used to identify neuronal groups recruited by pup exposure. In 2000, Lonstein and De Vries have reported that more than half of the total population of pup-induced c-Fos-immunoreactive neurons expressed glutamate decarboxylase (*GAD*), the synthesizing enzyme for the inhibitory neurotransmitter GABA, making it functionally possible that one of the functions of certain preoptic GABAergic neurons is to inhibit brain regions involved in defensive behavior [34]. Since the MPOA projects to brain regions that are known as the nodes for avoidance and aggression, namely the anterohypothalamic nucleus (AHN), ventromedial nucleus (VMN) and medial amygdala (MeA), the projection map of preoptic neurons provides considerable insight into the idea that MPOA outputs may inhibit the action of rejection responses to novel pup stimuli in mice exhibiting caretaking behavior [35,36,37]. Notably, the MPOA is not a homogenous population. By combining single-cell RNA-sequencing and imaging-based *in situ* cell type identification, more than 70 distinct neuronal populations were characterized with selective activity pattern following social interactions in mice [38]. This incredibly diversity place the MPOA in a position to mediate the large scale of pup-

directed behaviors from infanticide to caretaking. A recent study draws the attention of the scientific community to the temporal changes of the excitability of the preoptic neurons with the reproductive state, assuming that the heterogeneity of this brain region is not only relies on its genetics, but also influenced by the current needs [39].

### *1.3.2. The defensive-rejection circuit and its inhibition*

The olfactory system has an essential role in driving both social and antisocial behaviors, including pup-directed caregiving or rejection. While the function of the main olfactory system is primarily connected to prosocial behavior and acceptance [40], the vomeronasal system is rather involved in eliciting infanticide and suppressing parenting behavior [41]. Ablation or genetic inactivation of the vomeronasal system significantly reduces the aggressive bouts exhibited by virgin males and evokes parental care [42]. Information from both olfactory systems converges in the medial amygdala (MeA). The regulatory role of the MeA in pup-directed aggressive behavior has been proven by a large body of literature. Neurons located in the MeA are more highly activated in virgin males compared to fathers following exposure to pups, indicating their involvement in infanticide behavior [43]. Furthermore, both electrical and excitotoxic amino acid lesions of these brain regions evoke immediate acceptance in virgin female rats [44,45]. In contradiction with findings in female rats, more recently, it has been proposed that GABAergic neurons in the MeA control parenting behavior in a sexually dimorphic manner, hence their increased activity evokes enhanced maternal behavior in females while inducing pup-directed aggression in male mice [36]. The bidirectional role of MeA in the regulation of parenting makes it possible that acceptance and defensive responses are mediated by alternate amygdaloid circuits, through their projections to the AHN/VMN or MPOA to produce rejection or caregiving, respectively.

Regarding the defensive neuronal network, both AHN and VMH send glutamatergic projections to the periaqueductal grey (PAG), which brain region plays a critical role in motivating behavior including sexual behavior and parenting, as well as in behavioral responses to unpleasant or potentially threatening stimuli [46,47,48.] The PAG has been anatomically divided into dorsomedial, dorsolateral, lateral, ventrolateral, and lateral subregions with well-characterized functions for each part. According to the current knowledge, while the dorsal subregions of PAG mediate avoidance and aggressive behavior, the ventral parts are rather involved in promoting immobility and

freezing responses [49]. In support, experimentally increased activity of the dorsal PAG disrupts the maternal behavior, which was exhibited by female rats previously, while lesion of the same brain region induces responsiveness towards pups [46,50].

Another amygdaloid output that potentially controls infant-directed behavior responses is its projections to the MPOA. Given that the vast majority of preoptic neurons are GABAergic [51] and numerous brain regions targeted by preoptic neurons are known to be involved in avoidance, rejection, and aggressive behavior [52], MPOA could be a good candidate for the inhibitory regulatory node of the defensive circuit in parenting animals. Studies performing cell-specific ablation and optogenetic activation of a subset of GABAergic neurons in the MPOA provide additional support for the idea that MPOA output might directly inhibit brain regions promoting pup-directed rejection. More concretely, most galanin-expressing cells located in the MPOA can be characterized as GABAergic inhibitory neurons based on their *GAD* content and are highly responsive to pup exposure [42,53]. Genetic ablation of galanin (Gal) neurons impairs parental behavior independently from the reproductive states and sex, while their stimulation significantly reduces the aggressive behavior demonstrated by virgin males [42]. More recent evidence confirms the supporting role of preoptic GABAergic neurons in healthy parenting behavior and investigated a previously unknown function of this neuronal population regarding stress coping and anxiety [54].

Based on the previous findings, a hypothetical working model can be drawn focusing on the inhibitory functions of the MPOA and defensive network (Figure 1.). Olfactory stimuli from pups converge in the MeA and activate neurons projecting to brain regions involved in avoidance and aggression, or neurons promoting parenting through their efferents to the MPOA. Typically, in virgin males and wild females, the antisocial circuit is activated following pup stimuli, while in mothers, fathers, and laboratory females the defensive network is inhibited by GABAergic preoptic inputs. Inhibitory neurons located in the MPOA may serve as a node for the intricate interplay between endocrine events and multimodal sensory information.

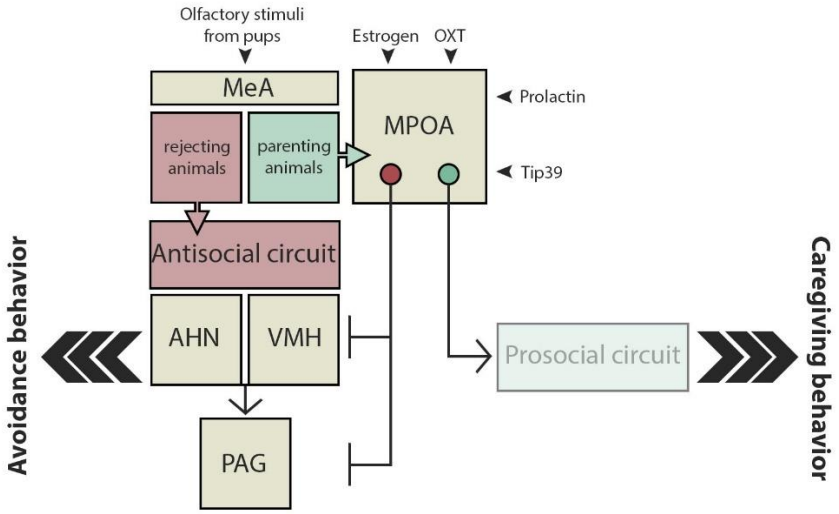


***Figure 1. Hypothetical working model of brain circuits mediating behavior responses towards pups.*** *Olfactory stimuli are differently processed in MeA of animals not showing (rejecting animals) and animals showing (parenting animals) parental care. In parenting animals, MeA activates the MPOA to promote parenting, while in rejecting animals MeA stimulates antisocial circuits to evoke defensive or aggressive behavior. Estrogen, oxytocin (OXT), prolactin, and Tip39 act on preoptic neurons to increase the responsiveness to pups. The efferents of MPOA simultaneously inhibit the antisocial circuit and stimulate the prosocial circuit for immediate acceptance behavior.*

### 1.4 Overview of postpartum depression

The physiological caretaking behaviors of parental animals and humans are underlined by precise circuit-level changes associated with parenthood, including the enhanced activity of social networks and diminished activity of the antisocial circuit. However, if something went wrong with the balance between the excitatory and inhibitory valence, it could have a long-termed impact both on the quality of parental behavior and the parent-infant relationship, potentially leading to the impaired physical and mental development of the offspring [55].

Both pregnancy and the postpartum period are associated with numerous hormonal and neuronal changes, making the mothers especially vulnerable to emotional ups and downs. Immediately after the birth, the first incidents of being parents may present challenges and are often accompanied by mood swings, irritability, sadness, and crying, lasting for a few days to two weeks. Unfortunately, about 10-20% of mothers around the world experience more intense and longer lasting symptoms that eventually interfere with their ability to take care of their children, which condition can be diagnosed as postpartum depression [56]. Postpartum depression is an understudied psychiatric disorder demonstrating one of the underestimated global social problems with suicide

accounting for approximately 20% of postpartum deaths [57]. The high prevalence of maternal suicide and the increased rate of onset of depression in postpartum women compared to nonpregnant women [58] indicates that the development of depression during the peripartum and postpartum period should be distinguished from major depression and requires a more effective treatment plan. Thus, understanding the neurobiological mechanisms contributing to the pathological phenotype of parenting is imperative.

### *1.4.1. Neuronal background of postpartum depression*

Clinical and experimental studies provide evidence for both hormonal and neuronal influence in postpartum depression. Regarding the hormonal impact, the crucial and broad role of ERα as a mediator of endocrine changes in connection to parenting makes it a good candidate for postpartum depression studies. Genetical screening of human mothers revealed that polymorphisms in ERα are associated with symptoms of postpartum depression [59]. The involvement of estrogen and not only its receptor in the development of depression is supported by experiments with laboratory animals. Withdrawal of estrogen evokes depression-like behavior in rats, while administration of estrogen has an antidepressant-like effect in animal models of depression [60,61]. Another interesting candidate for studying the neurobiological basis of postpartum depression is oxytocin, given the well-known role of this peptide in the regulation of social bonds, attachment, breastfeeding behavior, and emotion. Clinical investigations have shown that the single nucleotide polymorphisms in the gene encoding OXT or OXTR, similar to the decreased plasma level of OXT were predictive of the progress of postpartum depression [62,63]. The imbalance in the signaling of classical neurotransmitters, especially the main fine-tuning inhibitory GABA and the primary excitatory neurotransmitter glutamate have also been implicated in the underlying pathophysiology of postpartum depression [64,65].

### *1.4.2. Measuring depression in rodents*

One of the major limitations to revealing the precise neurobiological background of distinct psychological disorders is the uncontrollable factors when working with human post-mortem brain samples, such as medication history, age, the circumstances of the death, or the time between the death and tissue collection. All of these limitations can be overcome by using animal models in a well-controlled environment. Furthermore,

working with animals enables genetic modification to identify molecular candidates that may have therapeutic value.

Behavior tests were developed to measure the depression-like behavior of the laboratory animals taking to account the symptoms of human depression, including hopelessness, loss of interest, anxiety, or irritability. The forced swim test (FST) is a widely used paradigm to measure escape latency from an aversive, unpleasant environment [66]. Anhedonia can be assessed by the sucrose preference test (SPT), in which a healthy animal shows a preference for sucrose solution, while an animal exhibiting depression-like behavior does not show interest in normally pleasurable stimulus [67]. Open field test (OFT) and elevated plus maze test (EPM) make it possible to establish the level of anxiety and locomotor activity by measuring the amount of time the rodents spend in the less fenceless part versus the open part of the apparatus. Although rodents generally avoid open spaces, a healthy rodent explores all parts of the apparatus due to the natural exploratory instinct, while anxious animals cling to the walls [68]. Finally, considering the etiology of depression, most animal models established to study depression have been developed on the basis of acute or chronic stress exposure, thus the neurobiology of depression, anxiety, and stress cannot be sharply separated from each other [69].

## 2. Objectives

Taking into consideration the gap in knowledge about the potential neurobiological connection between motherhood and mood disorders, we aimed to reveal a previously unknown player in the development of depression by expanding our interest in the potential involvement of preoptic inhibitory neurons. We set four specific goals that cover the possible sexual differences, which can address major questions regarding the diverse nature of parental behavior across mammalian species and may provide an insight into sex-specific regulation of psychiatric disorders.

Our first aim was to map the distribution of preoptic GABAergic neurons activated by pup exposure in both female and male mice with different reproductive states to explore whether the pup-induced activity of preoptic inhibitory neurons shows sexual dimorphism or internal state-dependent pattern. Within the confines of this goal, we took the advantage of the stimulus-evoked rapid expression of c-Fos protein and analyzed the percentage of c-Fos-expressing GABAergic cells visualized by fluorescent immunolabeling against the c-Fos in genetically modified mice expressing ZsGreen fluorescent protein driven by the vesicular GABA transporter (VGAT) promoter.

Since the preoptic GABAergic neurons are highly activated following pup exposure, we hypothesized that the activity changes of these cells are manifested in behavior outputs. To test our hypothesis, we performed different behavior tests measuring behavior responses towards pups following the cell type-specific activation or inhibition of preoptic GABAergic neurons using designer receptor exclusively activated by designer drugs (DREADD)-based technology. This way, we had the opportunity to explore the effect of bidirectional changes of neuronal activity of preoptic GABAergic cells on pup-directed behaviors in both sexes with different internal states and investigate to the understudied neuronal control of infant-directed aggression exhibited by male mice.

The next question aimed to reveal how the preoptic GABAergic neurons are embedded in the circuit underlying pup-directed responses. Both the hormonal and non-hormonal factors affecting the activity of preoptic inhibitory cells were examined using a variety of techniques in order to provide not only anatomical but physiological evidence for the integratory role of this neuronal population. Furthermore, by mapping the projections of preoptic GABAergic neurons we sought to anatomically support the role of these cells in connection to parental behavior.

Last, but not least, considering the high prevalence of mood disorders during peri- and postpartum period, we aimed to examine the activity changes of preoptic GABAergic neurons following stress exposure and the potential involvement of the preoptic inhibitory circuit in both anxiety and depression-like behaviors in connection to parenting by correlative studies.

## 3. Methods

### 3.1 Animals

The offspring of GtROSA26Sor_CAG/ZsGreen1 and VGAT-IRES-Cre mice were used for histology analysis of pup exposure experiments and forced swim test (10-16 weeks). For behavior tests and further histological analyses, we used VGAT-IRES-Cre transgenic mice (8-16 weeks at the start of experiments). For electron microscopical investigation, four mother rats were used (16 weeks). Rodents were maintained on a 12h light/dark cycle with lights on at 7 am at 22 ± 1°C. Food and water were available ad libitum unless otherwise specified.

### 3.2 Animal surgeries and viral vectors

VGAT-IRES-Cre mice were anesthetized with ketamine/xylazine-hydrochloride in saline (16.6 and 0.6 mg/ml, respectively, 10 ml/kg body weight i.p.) mixture and placed in a stereotaxic frame (Leica Biosystems). 20 nl of Cre-dependent AAV2-hSyn-DIO-hM4Di/hM3Dq-mCherry virus was bilaterally injected at a flow rate of 57 nl/min into the MPOA of VGAT-IRES-Cre mice (anteroposterior: -0.6 mm from Bregma; ventral: 5.4 mm from dura; medio-lateral: 0.4 mm; Paxinos and Franklin mouse brain atlas, 2nd edition) by using nanoinjector (Nanoinject2010 by WPI). Before the glass capillary was withdrawn slowly, it was held for 10 min to allow the diffusion of the virus and prevent the leaking. Animals were allowed to recover for at least 2 weeks from surgeries before subsequent behavioral tests or histological analysis.

### 3.3 Chemogenetic modification of preoptic GABAergic cells

We injected AAV2-hSyn-DIO-hM3Dq virus bilaterally into the MPOA of VGAT-IRES-Cre mice for activation of VGAT+ neurons. For inhibition, we used AAV2-hSyn-DIO-hM4Di-mCherry virus. Control animals with the same genetic background received AAV2-hSyn-DIO-mCherry virus. 45 min prior to behavior tests animals were intraperitoneally injected with clozapine-n-oxide (CNO) (1mg/kg).

### 3.4 Behavior tests and analysis

At least two weeks after surgeries (intracranial administration of AAV-viruses described above), a week prior to tests the mice were placed into new cages and kept individually. All experiments were performed between 8:00-14:00, under controlled medium light

intensity (60 LUX) and temperature (22 ± 1°C) conditions. 45 min before the experiments, mice were intraperitoneally injected with vehicle (0.5% DMSO solution) or CNO solution (1 mg/10 ml/kg, dissolved in 0.5% DMSO solution). 3-6 days-old pups were used in all behavior assays. Each test was video recorded (SJCAM SJ4000 FULLHD action camera). The behaviors were scored blind to the type of the injected virus. For manual behavior analysis, we used Solomon Coder software (https://solomon.andraspeter.com/), otherwise the open-field tests were scored by SMART video tracking software (PanLab Harvard Apparatus, MA, USA). The protocols of the behavior tests are summarized in Table 1.

*Table 1. Summary of the behavior protocols*

| | | |
|---|---|---|
| **Pup-directed behaviors:** each animal was tested with three 3-6 days-old pups. Behavior assays were stopped if the adults hurt the pups. | **Pup-induced place preference test** | The conditioning phase took 3 days. Each mouse spent 2 hours in the cage containing pups (pup-associated cage) and further 2 hours per day in the cage without pups (non-pup-associated cage) before the testing phase. Ont he testing day, the pup-associated chamber and non-pup-associated chamber were connected with a tube to allow free access to the entire apparatus. |
| | **Pup retrieval test and spontaneous parental behavior test** | The test period took 5 days for each animal. The animals were injected with vehicle on the first and the fifth (and the ninth) day, and with CNO on the second (and the sixth) day (in order to eliminate the potential residual effect of CNO, there were 2 days of rest after CNO treatment). Immediately before the start of the test, the adult mice were placed outside of their home-cage, then their nests were destroyed, and three pups were placed at the farthest corner from the original place of the resting nest. The following behaviors were scored in pup retrieval test: latency to retrieve the first and third pup (the moment when adults picked up a pup and carried it to the nest). The following behaviors were scored in the spontaneous parental behavior test: time in the nest (duration of time spent in the nest with pups); crouching (duration of the nursing-like posture); pup grooming (duration of the caring interaction with pups); pup licking (anogenital licking of pups); in addition, maternal interaction consists of time in the nest, crouching, pup grooming and pup licking behavior; pup-directed aggression (onset attacking the pups), parental interaction (duration of the cumulative time spent grooming and licking the pups in the males). |
| **Anxiety-like behaviors** | **Elevated plus maze test** | The apparatus consisted of two open arms (50 cm x 10 cm) and two closed arms of the same dimensions with walls 40 cm high. 45 min prior to the test the animals were intraperitoneally injected by CNO or vehicle. Additional experiments were performed with non-injected females to establish the effect of pup exposure on anxiety-like behavior. To start the test, mice were placed in the center of the maze, facing a closed arm, and then allowed to explore for 10 min. The following behaviors were scored: time spend and number of entries in the open and closed arm, frequency to enter into all arms. |

| | | |
|---|---|---|
| | **Open field test** | The open field box was made from polypropylene (40 x 40 x 60 cm). Each animal was placed in the center of the arena 45 min after the injection, and then allowed to explore it for 10 min. Overall activity and the velocity were measured also. |
| **Depression-like behavior** | **Forced swim test** | Each virus-injected animal was treated with CNO 45 min prior to the test. Additional experiments were performed with non-injected females to reveal the effect of pup exposure on depression-like behavior. Each experimental were placed in a transparent cylindrical tank for 6 min. The water level was 15 cm from the bottom and the temperature of the water was 25 ± 1 °C. The duration of the immobility (when the mouse is floating passively), the swimming (when the mouse is moving), and the climbing (when the mouse displayed forceful movements against the wall) were determined. |
| | **Sucrose preference test** | Sucrose consumption was measured to assess hedonic behavior using 1% (wt/vol) sucrose solution. Prior to the test, the mice were adapted to sucrose. On the 1st day of the experiment, we removed the water and food for 12 hours. On the 2nd day, we gave back only the water tube and a similar tube filled with sucrose solution, then left the mice with these two bottles continuously for 48 hours. On the 4th day, we removed all bottles from the home cage from for 12 hours. Each mouse was intraperitoneally injected by vehicle injection 45 min prior to the test, and then we measured the weight of both bottles and gave them back to the mice. After 12 hours (on 5th day morning), the measurement was repeated to calculate the consumption. Then, we removed again both water and sucrose solution tubes for 12 hours before the second test. Like the first test condition, 45 min prior to the test, each mouse was injected by CNO. At the end of the test, after weighing the bottles, all mice received back the water and sucrose solutions. On the 6th day, we removed all the bottles from the cages and weighed them again. The preference for the sucrose solution was calculated as the ratio of sucrose intake to the total fluid intake. |
| **Intermale social behavior** | **Resident-intruder paradigm** | Male mice were isolated for 7 days and then for another 7 days without change of the bedding, since territoriality is based on the presence of olfactory cues. Each resident mouse was subjected to a single test against a male intruder of the same age and less weight. Following the CNO administration, the unfamiliar male was placed into the resident-cage at the start of the test. The resident-intruder test lasted for 10 min. The following behaviors were scored: the latency to initiate the first attack (including biting, mounting or arrest) and the first social interaction (close contact to any parts of the body of the intruder by the frontal region of the resident male), the cumulative time spent for attack and social interactions. |

### 3.5 Induction of c-Fos following different stimuli

10-16 weeks old control females, control males, experimental females, and experimental males were placed into fresh-bedding containers and kept individually for 48 hours before the perfusion (control females and males) or exposures to pup or forced swim test (experimental females and males). 3-6-days-old VGAT-Cre pups were used in the pup exposure experiment. On the day of the test, five pups were introduced to the home cage of experimental females and males for 30 min. 90 min after the introduction of the pups, all experimental animals were anesthetized and perfused (described below). Mother animals were kept with their pups constantly from parturition to transcardial perfusion to reveal the natural response of preoptic GABAergic neurons to motherhood. In the case of mother mice, the transcardial perfusion took place 3-6 days after giving birth. Similar to mothers, fathers were kept with their litters until the transcardial perfusion. In the case of experimental animals exposed to forced swim test, all animals were anesthetized and perfused 90 min after the exposure. The calculation of cell numbers was performed in randomly selected fields.

### 3.6 Sample preparation for immunohistochemistry

Animals were anesthetized with ketamine/xylazine-hydrochloride in 0.9% saline (16.6 and 0.6 mg/ml, respectively, 10 ml/kg body weight i.p.) mixture and then transcardially perfused with 100 ml saline followed by 150 ml ice-cold 4% paraformaldehyde (PFA) solution prepared in phosphate buffer (PB). After 12 h post-fixation in the same fixative solution, brains were sectioned in the coronal plane at 40 μm thickness using a vibratome (VT1000S, Leica). Sections were collected in PB containing 0.05% sodium azide and stored at 4 °C.

### 3.7 Fluorescent and peroxidase immunohistochemistry

After washing with TRIS buffer (TB), free floating brain slices were blocked in 5% bovine serum albumin (BSA) in TB containing 0.1% Triton X-100 (TTB) for 1 hour at room temperature and then incubated with appropriate primary antibodies (Table 2.) in 2.5% BSA-TTB for overnight. After incubation sections were washed with TB and incubated with the appropriate secondary antibodies in 2.5% BSA-TTB for 3 hours at room temperature. Sections were then washed with buffer containing Hoechst 33342 dye for 15 min. After several washes in TB, sections were mounted onto glass slides and

coverslipped using mounting medium (Aqua-Poly/Mount). In the case of peroxidase immunohistochemistry, firstly, the slices were treated with 3% $H_2O_2$ for 15 min to block the endogenous peroxidase and then washed with TB. Following the blocking with 5% BSA-TTB the sections were incubated in the appropriate primary antibody (Table 2.) in 2.5% BSA-TTB overnight. After the incubation, sections were washed with TB and then incubated with biotin-conjugated secondary antibody dissolved in 2.5% BSA-TTB for 4 hours, and after that avidin-biotin-peroxidase complex (ABC) was applied for additional 2 hours. Then, the brain sections were incubated in 3,3'-diaminobenzidine (DAB) with (Ni-DAB) or without nickel-sulphate solution until achieving the desired staining intensity. After the reactions, the slices were washed, mounted onto glass slides and coverslipped using DePeX mounting medium.

### 3.8 Microscopy, image analysis, and cell counting

Sections were examined using a Nikon Eclipse light microscope equipped with fluorescent epi-illumination. Images were captured at 2048×2048 pixel resolution with a SPOT RT3 camera (Diagnostics Instruments) using 4–40× objectives. Confocal images were acquired with Zeiss LSM800 confocal microscope using 20-63x objectives at an optical thickness of 2–10 μm. Brain areas were identified based on ventricles and white matters, with the assistance of mouse brain atlas from Paxinos and Franklin. The cell numbers were counted in ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018) with custom-written macro scripts and manually for reliability. The colocalization between two signals were determined manually. All processes were performed by experimenter blind to the sex and the treatment of the animals.

### 3.9 Electron microscopy

#### *3.9.1 Sample preparation and pre-embedding immunolabeling*

Two rats were deeply anesthetized and transcardially perfused with saline for 30 sec followed by 500 ml of 4%PFA, 0.05% glutaraldehyde (GA), and 0.2% picric acid in PB for 20 min (velocity: 25 ml/min). After perfusion, the brains were removed from the skull and washed extensively in PB. 50 μm-thick sections were cut with a vibratome and cryoprotected in 30% sucrose in TB with saline (TBS) overnight. Next day the samples were freeze-thawed over liquid nitrogen for three times. After washes and treatment with

1%$H_2O_2$, the sections were processed for immunostaining using the following protocol: quenching in 50–50 mM ammonium chloride and glycine dissolved in TBS for 30 min; blocking 2 %BSA for 1 hour; anti-TIP39 antiserum (1:3000) (Table 2.) diluted in 0.5% BSA-TBS for 48 hours; biotin-conjugated goat anti-rabbit secondary antibody at 1:500; and ABC at 1:500. The immunoperoxidase reaction was developed with Ni-DAB. After the first reaction, the sections were processed for a second immunostaining. The same protocol was carried out except that the primary antiserum was mouse anti-galanin antibody (1:200) (Table 2.), followed by biotin-conjugated goat anti-mouse secondary antibody at 1:500. The second immunoperoxidase reaction was developed with DAB. All sections were post-fixed in 0.5% $OsO_4$ containing 3.5 %glucose in 0.1 M Na-cacodylate. This was followed by *en bloc* staining with half-saturated aqueous uranyl acetate, dehydration in a series of ethanol at increasing concentrations and acetonitrile, and then embedding in Durcupan. Sections were embedded on slides and cured for 48 hours at 60 °C. Small pieces from the MPA-containing galanin-immunoreactive cells were reembedded and resectioned at 70 nm. Sections were collected on pioloform coated single slot copper grids and stained with lead citrate for 1 min.

### *3.9.2. Sample preparation and post-embedding immunolabeling*

Two mother rats were transcardially perfused for 30 sec followed by fixative containing 2 % PFA and 0.5% glutaraldehyde dissolved in PB for 20 min (velocity: 25ml/min). Then, the brains were removed from the skull and sectioned at 50 μm on vibratome. Brain sections containing the MPOA were processed for low temperature embedding: free aldehydes were quenched with a solution that contained 50 mM ammonium chloride and 50 mM glycine dissolved in TBS. Then, blocks were washed three times in maleate buffer (0.05 M, pH 5.5) before post-fixation in 1% uranyl acetate dissolved in maleate buffer for 180 min in the dark. This was followed by three washes in maleate buffer. Subsequently, blocks were dehydrated in graded series of acetone while progressively lowering the temperature. Samples were then infiltrated with pure LR White alone for 12 hours, at −20 °C followed by pure LR White containing 2% benzoyl peroxide. Small pieces from the MPOA were reembedded and resectioned at 70 nm. Sections were collected on formvar-coated single slot nickel grids allowing immunoreaction on one side of the sections. To demonstrate the presence of TIP39 and glutamate in the same terminal only, two sections were collected on one grid. Four grids carrying consecutive serial sections were

immunolabeled; therefore, terminals were present on multiple sections and could be treated with different antibody solutions on adjacent sections. All immunoreactions were carried out on humidified parafilm-coated 96-well plates. TBS was used for all washes and dilutions. Briefly, the following procedure was carried out: 5% hydrogen peroxide for 3 min; wash in double distilled water; 1% sodium borohydride and 50 mM glycine dissolved in TBS for 2 min; wash in TBS; 1%BSA for TIP39 and 1 % ovalbumin for glutamate reaction for 30 min; rabbit anti-TIP39 antiserum (Table 2.) in 1:100 or rabbit anti-glutamate antibody (Table 2.) in 1:2000 dilution in 0.5% ovalbumin-TBS containing 0.05% sodium azide for 12–18 hours; washes in 0.5% ovalbumin-TBS; 10 nm gold-conjugated goat anti-rabbit secondary antibody 1:50 in TBS with 1% ovalbumin for 4 hours; wash in TBS; 2% glutaraldehyde in TBS for 10 min; wash in biDW; air drying; staining in half-saturated aqueous uranyl acetate for 30 min followed by lead citrate for 30 sec; and air drying.

### *3.9.3. Electron microscopic image acquisition*

For correlated light and electron microscopy, candidate galanin-immunoreactive cells were photographed with a light microscope before reembedding. Electron micrographs were taken by a side-mounted Morada CCD camera (Olympus Soft Imaging Solutions) connected to a JEOL 1011 or with a Gatan UltraScan 1000 CCD camera fitted to a Philips CM100 electron microscope. Due to the low contrast of the specimen, brightness and contrast were adjusted when necessary in the whole digital images of immunogold labeling using Adobe Photoshop CS2 (Adobe Systems).

*Table 2. List of the primary antibodies*

| Target | Host animal | Dilution | Producer |
|---|---|---|---|
| TIP-39 | Rabbit | 1:3000 | Midwest Biomolecules |
| Galanin | Mouse | 1:200 | Biorbyt |
| mCherry | Chicken | 1:2000 | Abcam |
| NeuN | Mouse | 1:2000 | Merck Millipore |
| c-Fos | Rabbit | 1:2000 | Santa Cruz Biotechnology |
| Oxytocin | Mouse | 1:1000 | Abcam |
| Tyrosine hydroxylase | Rabbit | 1:500 | Abcam |
| Estrogen receptor alpha | Mouse | 1:500 | ThermoFisher Scientific |

### 3.10 Anterograde and retrograde tracings

To examine the projection map of preoptic VGAT+ neurons, we used VGAT-IRES-Cre mice. The inputs of the MPOA were revealed by pAAB-Efl1a-mCherry-IRES-Cre retrogradely spreading virus. The mice were anesthetized with ketamine/xylazine mixture and injected 20 nl of AAV-hSyn-DIO-mCherry unilaterally into the MPA. Four weeks after the surgery, mice were deeply anesthetized and transcardially perfused with saline followed by 4% ice-cold PFA. We post fixed brains in the same fixative solution for 24 hours and then stored them in 0.1M phosphate-buffer until sectioning into three series of 40 μm sections on a vibratome. Two series (every 2 out of 3 sections) were immunolabeled for mCherry using DAB immunoperoxidase and fluorescent signals, respectively.

### 3.11 Analysis of the anterograde and the retrograde tracings

After immunolabelling, sections were captured by Zeiss CellObserver microscope equipped with a Yokogawa CSU-X1 spinning disk module using 20x objective. For analysis, we used a free bioimage analysis software QuPath (Qupath, University of Edinburgh, Edinburgh, UK) to evaluate the fluorescent immunoreactivity signal. Machine learning based pixel classification feature was applied to automatically measure the area density of the labelled nerve fibers or cells in the distinct regions across the whole brain.

### 3.12 Electrophysiological measurements

Brain slice preparation from adult VGAT-IRES-Cre/Gt (ROSA)26Sor_CAG/ZsGreen1 female mice (n=3 for each group) was carried out as described: after deep isoflurane anesthesia, the head was decapitated, the brain was removed from the skull and immersed in ice-cold low-Na cutting solution, continuously bubbled with carbogen, a mixture of 95% $O_2$ and 5% $CO_2$. The cutting solution contained the following (in mM): saccharose 205, KCl 2.5, $NaHCO_3$ 26, $MgCl_2$ 5, $NaH_2PO_4$ 1.25, CaCl2 1, glucose 10. Hypothalamic blocks containing the preoptic area were dissected, and 220 μm-thick coronal slices were prepared from the MPOA with a VT-1000S vibratome (Leica Microsystems, Wetzlar, Germany) in the ice-cold low-Na oxygenated cutting solution. The slices were transferred into artificial cerebrospinal fluid (aCSF) (in mM): NaCl 130, KCl 3.5, $NaHCO_3$ 26, $MgSO_4$ 1.2, $NaH_2PO_4$ 1.25, $CaCl_2$ 2.5, glucose 10 bubbled with carbogen and left in it for 1 hour to equilibrate. Equilibration started at 33°C and it was let to cool down to room temperature. Recordings were carried out in oxygenated aCSF at 33°C. Axopatch-200B patch-clamp amplifier, Digidata-1322A data acquisition system, and pCLAMP 10.4 software (Molecular Devices Co., Silicon Valley, CA, USA) were used for recording. VGAT-ZSGreen neurons were visualized with a BX51WI IR-DIC microscope (Olympus Co., Tokyo, Japan) after identifying them by their fluorescent signal. The patch electrodes (OD = 1.5 mm, thin wall; WPI, Worcester, MA, USA) were pulled with a Flaming-Brown P-97 puller (Sutter Instrument Co., Novato, CA, USA). Firing activity of VGAT-ZSGreen neurons was recorded in whole-cell current clamp mode. In order to evoke action potentials even in silent neurons (approx. 30 % of the neurons measured) +10 pA holding current was applied during the recordings. Measurements started with a control recording (3 min), then oxytocin (100 nM-10 μM, Tocris, UK) was pipetted into the aCSF-filled measurement chamber containing the brain slice in a single bolus and the recording continued for subsequent 5 min. Each neuron served as its own control when drug effect was evaluated. Recordings were stored and analyzed off-line. Event detection was performed using the Clampfit module of the PClamp 10.4 software (Molecular Devices Co., Silicon Valley, CA, USA). The firing rate was calculated as the number of action potentials (APs) divided by the length of the corresponding time period (3 min or 5 min). Mean values of the control and treated part of the recording were calculated from these frequency values. All the experiments were self-controlled in each neuron: percentage

changes in the firing rate were calculated by dividing the value of the parameter in the treated period with that of the control period.

### 3.13 Statistical analysis

All statistical calculations were carried out using IBM SPSS Software (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). Data were first tested with Shapiro-Wilk test for normality. If the data set were normally distributed, we used Student's t-test for two groups. In case of the comparison more than two groups, we used one-way ANOVA. When the groups were related to each other (like in the case of self-control measurement of parental behavior) we used repeated measures ANOVA. Otherwise, if the data came from a non-normal distribution, we performed Mann-Whitney test for two groups and Kruskal-Wallis test for more groups comparison. Friedman test was performed when the data were non-normally distributed and not independent. In the case of parametric distribution, we used Pearson correlation test to measure linear correlation between two variables. Otherwise, Spearman correlation was utilized. We used general linear model to describe interactions between sex, physiological conditions and the number of activated GABAergic cells after pup exposure. General linear model allows us to test the simultaneous effects of multiple variables, including mixtures of categorical (sex and reproductive stages) and continuous (number of activated neurons) variables. We listed the degrees of freedom for the main effect, the degrees of freedom for error, the F value, and the p value in all cases. Statistical analyses were considered significant for $p \leq 0.05$ ($0.01 < *p < 0.05$, $0.001 < **p < 0.01$, $0.001 > ***P$).

## 4. Results

### 4.1 Neuronal activity changes of preoptic GABAergic cells in response to pup exposure

We first investigated the pup exposure induced activation of GABAergic neurons in the MPOA of VGAT- ZsGreen1 mice (Fig. 2A-F). The percentage of c-Fos+ (activated) neurons co-expressing vesicular GABA transporter (VGAT+) was normalized to control groups, which females (n = 4) and males (n = 4) had no interaction with pups. The ratio of activated preoptic neurons expressing VGAT was significantly higher in virgin females (713.7% ± 8.1%, P<0.001) and mothers (876.1% ± 5.0%, P<0.001) compared to control female group. In addition, the ratio of VGAT+/c-Fos+ cells were significantly increased in both virgin males (245.1% ± 8.4%, P=0.001) and fathers (318.3% ± 8.5%, P=0.001), in comparison with male control group (Fig. 2G). The other way around, the percentage of VGAT+ preoptic neurons expressing pup-induced c-Fos was significantly elevated in virgin females (142.0% ± 2.2%, P<0.001), mothers (154.1% ± 1.0%, P<0.001), virgin males (119.6% ± 4.1%, P<0.001) and fathers (126.5% ± 3.0%, P<0.001) compared to the sex-matched control group (Fig. 2H). In addition, general linear model revealed the effect of sex and reproductive stage on pup-induced activation of preoptic GABAergic neurons. The percentage of double labelled cells in mothers and virgin females was significantly higher than in fathers and virgin males $F(1,242)=40.33$, P<0.001). Moreover, the main effect of reproductive stage on percentage of double labelled cells was significant ($F(1,242)=11.44$, P=0.001). The interaction between sex and reproductive stage was also significant ($F(1,242)=5.11$, P=0.025). The abundance of GABAergic neurons in the MPOA was analyzed by calculating the percentage of NeuN+ cells expressing VGAT and found that 78.4± 1.9% of the preoptic neurons are GABAergic. There is no significant difference in the proportion of GABAergic cells in the different groups (P=0.4) (Fig. 2I). Previous findings indicated uneven pup-induced neuronal activation in several subregions within the MPOA in both female and male mice [70]. To determine whether there is a connection between the distribution of preoptic GABAergic neurons and pup-linked activation pattern, we investigated the regional changes of c-Fos activation in preoptic VGAT+ neurons along the antero-posterior axis as well as differentiating the central, medial and lateral regions in comparison with control group (Fig. 2J-L). The distribution of VGAT+ neurons was homogenous in all groups,

though a larger density of VGAT+ neurons seemed to appear in the medial subdivision. In line with previous results, the clusters of c-Fos+ and double labelled cells in the control mice are less dense than in parenting mice. Interestingly, the distribution of pup-induced c-Fos+ cells and c-Fos+ cells expressing VGAT was different in mother and father mice. In mothers, at the level of +0.14 and -0.10mm from Bregma, the number of c-Fos+ cells was significantly higher in the medial part of MPOA (MPOC) than other parts (P=0.043 and P=0.027, respectively) (Fig. 2J,K). In contrast, in males, the neuronal activation marked by c-Fos cell positivity was significantly decreased in the same subregions and increased in the central part (P=0.043 and P=0.020, respectively) (Fig. 2J, K).

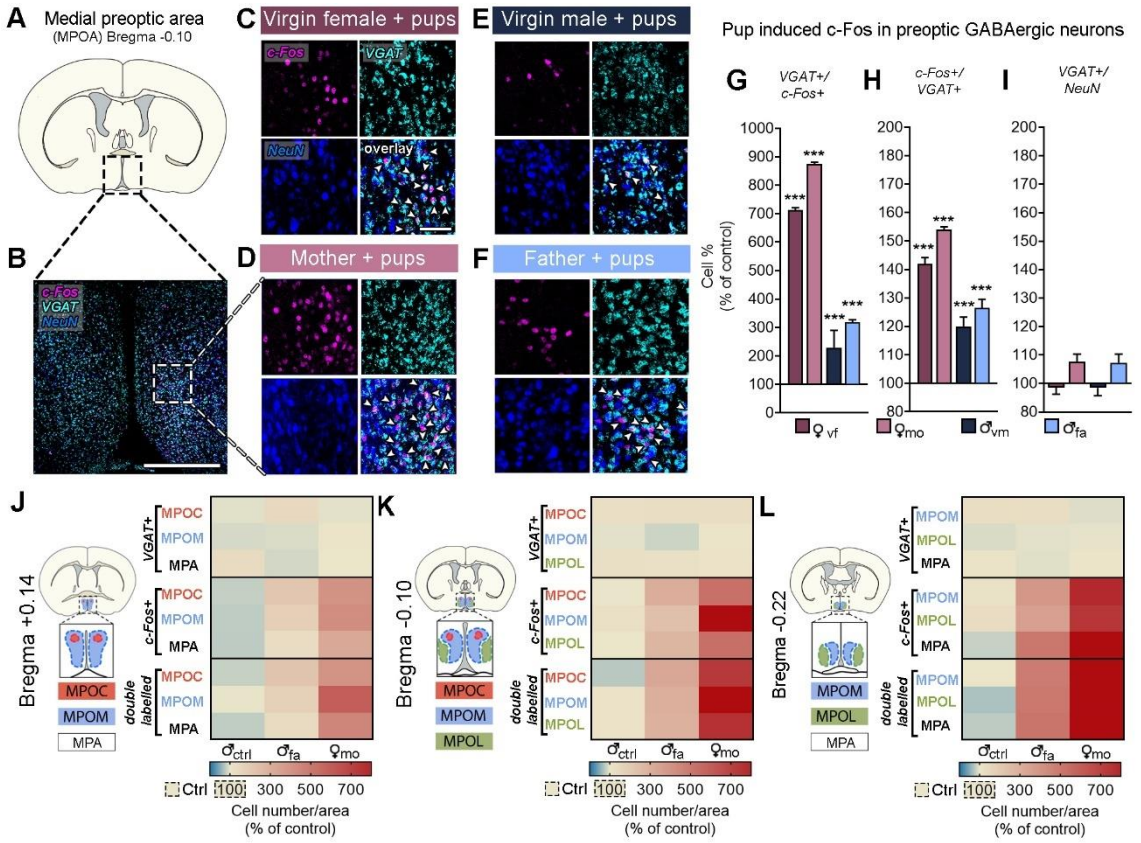


***Figure 2. Activation of preoptic GABAergic neurons following pup exposure.*** *(A) Schematic localization of the medial preoptic area (MPOA) in coronal section. (B) Representative micrograph of c-Fos and NeuN immunolabelling in the MPOA of VGAT-ZsGreen1 mother mouse. (C–F) Pup-induced c-Fos expression in preoptic VGAT+ neurons of virgin female (n = 3) (C), mother (n = 5) (D), virgin male (n = 3) (E) and father (n = 3) (F) mice. Co-expression of VGAT and pup-induced c-Fos is marked by white arrowheads. (G) Enrichment of VGAT in c-Fos+ neurons. (H) Enrichment of c-Fos+ in VGAT+ neurons. (I) Percentage of VGAT NeuN-containing cells normalized to the control group. vf: virgin female; mo: mother; vm: virgin male; fa: father. Data are presented as means ± s.e.m, Mann-Whitney test; ***<0.001. (J-L) Heatmaps of the VGAT+, pup-induced c-Fos+ and double labelled cells distribution at the level of +0.14 (J), -0.10(K) and -0.22 (L) from Bregma; Area = 30,000 μm2. MPA: medial preoptic area; MPOC:*

*central part of the MPOA; MPOM: medial part of the MPOA. Scale bars: 500 μm (B), 50 μm (C-F)*

## 4.2 The bidirectional control of infant-directed behavior by preoptic inhibitory neurons

### *4.2.1. The stimulatory effect of preoptic GABAergic cells on parenting in female mice*

To investigate whether the preoptic GABAergic neurons are functionally involved in the regulation of maternal behavior, we employed designer receptors exclusively activated by designer drugs (DREADD)-based technology. This widely used approach allowed both the selective inhibition and activation of VGAT+ neurons located in the MPOA of female mice. We bilaterally injected Cre-dependent, mCherry-tagged adeno-associated control virus (which does not contain the DREADD sequence) or virus expressing either hM4Di or hM3Dq receptor (AAV-hSyn-DIO-hM4Di/hM3Dq-mCherry) into the MPOA of VGAT-Cre transgenic female mice (Figure 3A-B), for which Cre recombinase expression is restricted to VGAT+ neurons. For validation of the DREADD-based activation and inhibition of VGAT+ neurons we performed c-Fos and mCherry double fluorescent labeling after intraperitoneal injection of clozapine-N-oxide (CNO, the ligand of DREADD, 1 mg/10 mL/kg) (Figures 3C-F). The efficacy of virus infection was examined by comparison of medial preoptic VGAT-positive and mCherry-positive (Figures 2C–-F) cell numbers of virgin females. On average, 48.2 ± 2.2% of VGAT+ neurons were infected by the virus (Figure 2G). Furthermore, the percentage of c-Fos+/mCherry+ neurons was markedly elevated (93.6 ± 0.6%) in hM3Di virus-injected mice compared to the control virus injected group (34.4 ± 1.5%), while only 2.1 ± 0.3% of mCherry+ neurons showed neuronal activation in mice injected by viruses expressing hM4Di receptors. Because there were no significant differences between the percentage of pup-activated VGAT+ neurons in control females without CNO injection and the activated mCherry+ neurons in control virus injected mice after exposure to pups and

CNO, we assume that CNO administration *per se* did not influence the neuronal activation (Figure 3H).

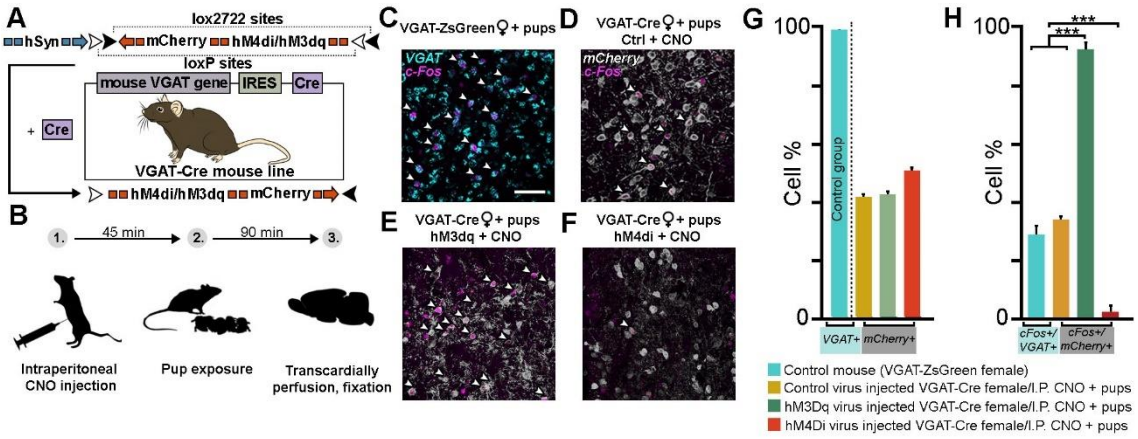


***Figure 3. Validation of designer receptors exclusively activated by designer drugs (DREADD) technique.*** *(A) Schematic illustration of virus constructs before and after Cre-dependent recombination. (B) Experimental design of CNO and pup-induced c-Fos detection. (C) Representative micrograph of c-Fos immunolabeling in the MPOA of VGAT-ZsGreen1 female after pup exposure. (D-F) Representative c-Fos and mCherry immunoreactivity within the MPOA of control virus-injected (D), hM3Dq expressing virus-injected (E) and hM4Di virus-injected (F) VGAT-Cre females after CNO administration and interaction with pups. Co-expression of mCherry and c-Fos is marked by white arrowheads. (G) Comparison of the number of VGAT+ neurons in the MPOA of VGAT-ZSGreen1 females (n = 3) and the number of mCherry+ cells of control virus-injected (n = 4), hM3Dq expressing virus-injected (n = 4) and hM4Di expressing virus-injected (n = 4) VGAT-Cre females. (H) Enrichment of c-Fos in VGAT+ neurons of VGAT-ZsGreen1 female and mCherry+ neurons of virus-infected VGAT-Cre females. Data are represented as means ± s.e.m., Kruskal-Wallis test followed by Dunn-Bonferroni post-hoc test, ns: not significant, *p < 0.05, 0.001 < **p < 0.01, ***p < 0.001. (H) Data are presented as means ± s.e.m.; area = 0.12 mm2. Scale bars: 50 µm (C-F).*

The impact of the bidirectional modification of the MPOA VGAT+ neuronal activity (Figure 4A) on the pup-evoked place-preference (Figures 4B and 4C) was compared first. The cumulative time spent in the pup-associated cage was significantly decreased in mice received hM4Di-coding virus (39.73%), while increased in mice injected by hM3Dq-coding virus (66.74%). There was no preference in control group (51.62%) (Figure 4B). Virgin females, which received control virus, spent 870.1 sec ± 27.00 sec and 929.2 sec ± 27.00 sec of their time in pup-associated and pup-independent cage, respectively. Inhibition of MPOA *VGAT*+ neurons resulted in significant preference to the pup-independent cage (1092.6 sec ± 24.6 sec) instead of the pup-associated cage (715.1 sec ± 22.2 sec). Contrary, stimulation of MPOA VGAT+ cells in virgin females resulted in significantly more time spent in pup-associated cage (1201.3 sec ± 46.7 sec) compared to pup-independent cage (598.7 sec ± 46.7 sec). We also examined the effects

of chemogenetic modulation of MPOA *VGAT*+ neurons on pup retrieving behavior of virgin females (Figure 4D-I). The silencing of *VGAT*+ neurons in the MPOA significantly decreased (P=0.001), while their excitation significantly increased the number of retrieved pups. In case of control females, there was no significant difference between the numbers of retrieved pups following CNO and vehicle administration (P=0.174) (Figure 4D). Moreover, virgin females with silenced MPOA *VGAT*+ neurons retrieved the first pup (F(1.207, 15.692)=10.384, P=0.004) and third pup (F(1.931, 25.101)=17.867, P=0.001) with longer latency (Figure 4E), and spent significantly less time in the nest (P=0.030) (Figure 4F), and with grooming (P=0.002) (Figure 4H) and licking (P=0.002) (Figure 4I) pups than without chemogenetic modulation. On the contrary, after the activation of MPOA *VGAT*+ neurons, virgin females retrieved the first (F(1.702, 25.532)=9.831, P=0.001) and third pup (F(1.864, 27.961)=8.228, P=0.002) faster (Figure 4E), spent significantly more time in the nest (P=0.003) (Figure 4F), with crouching over pups (P=0.001) (Figure 4G), grooming (P=0.037) (Figure 4H) and licking them (P=0.009) (Figure 4I). There was no significant difference in the retrieval latency of the first (P=0.183) and third pup (P=0.057) in control females (Figure 4E). The duration of the time in the nest (P=0.368) (Figure 4F), crouching (P=0.717) (Figure 4G), pup grooming (P=0.223) (Figure 4H) and licking the pups (P=0.882) (Figure 4I) were not affected by CNO treatment in the control group.

Similarly, in mother mice, the inhibition of MPOA *VGAT*+ neurons elevated the retrieval latency of the first pup (P=0.022) and decreased the duration of time in the nest (F(1.152, 4.609)=25.522, P=0.004) and interaction with pups (F(1.669, 6.677)=67.255, P=0.001) (Figure 4J-M). After CNO administration, the mothers, which received hM3Dq receptor expressing virus spent significantly more time in the nest (F(1.638, 8.191)=25.409, P=0.001), with crouching (F(1.135, 5.674)=6.109, P=0.048) and interacting with pups (F(1.275, 6.376)=20.121, P=0.003) (Figure 4J-M). To determine whether the CNO effect was altered by reproductive stages we repeated the behavioral experiments before pregnancy and after giving birth and found that there were no significant differences in the effect size of the CNO treatment (Figure 4N-Q).

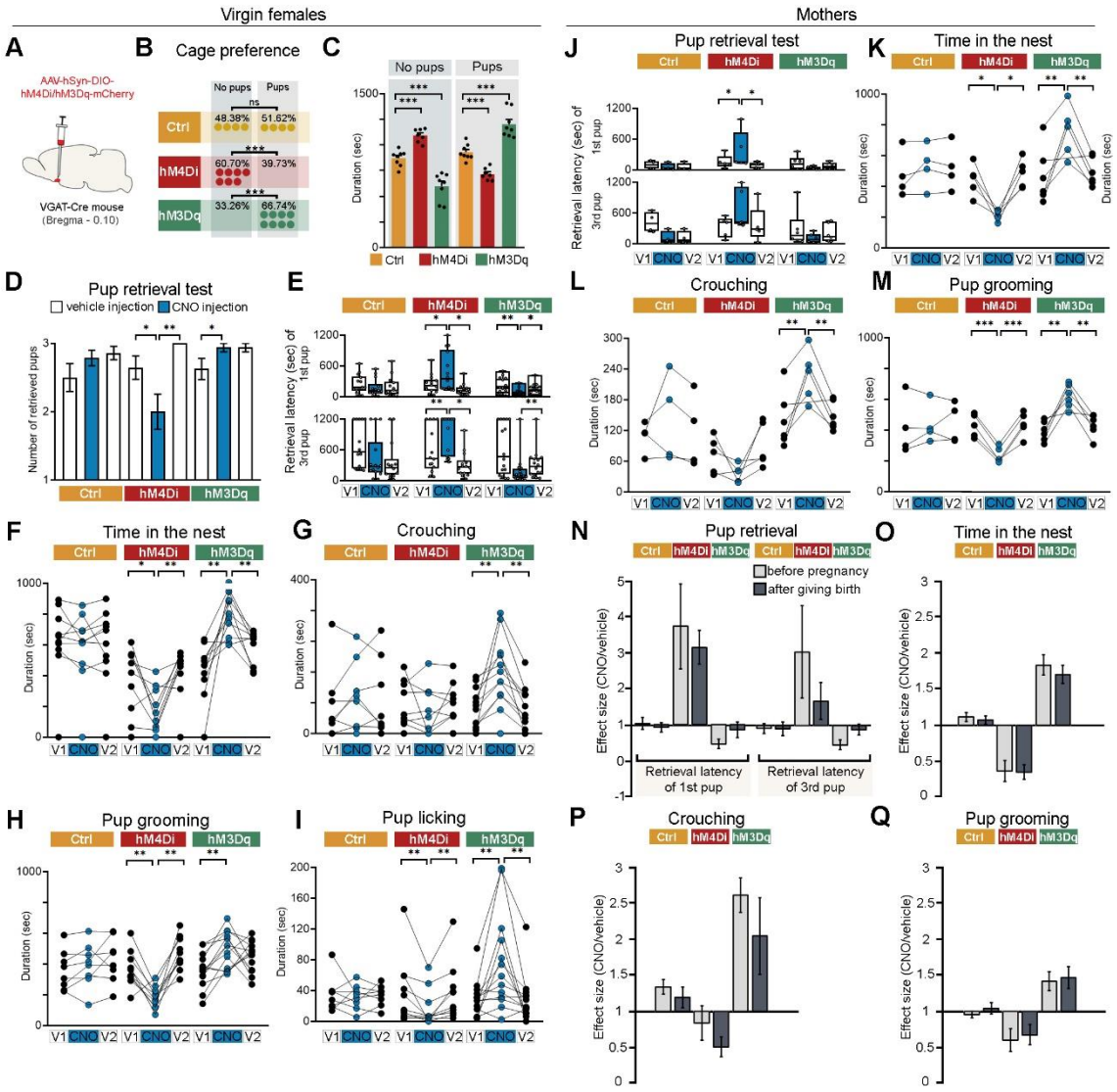


***Figure 4. Changes of pup-directed behavior following chemogenetic modulation of preoptic GABAergic neurons in virgin female and mother mice.*** *(A) Schematic illustration of intracranial injection of viral vector to modulate preoptic GABAergic neurons. (B) Number of animals spent 60% of their time or more in the pup-independent cage (No pups) or spent 60% of their time or more in pup-associated cage (Pup). Each dot represents one animal. Ctrl: control virus injected females (n = 8); hM4Di: inhibitory virus injected females (n = 7); hM3Dq: excitatory virus injected females (n = 8). (C) Behavior response of control virus injected females (Ctrl) (n = 8), inhibitory virus injected females (hM4Di) (n = 7) and excitatory virus injected females (hM3Dq) (n = 8) to place-preference conditioned with and without pups after clozapine-N-oxide (CNO) administration. Data are presented as means ± s.e.m. dots represent individual data points, Mann–Whitney test; ∗∗∗p < 0.001. (D) Number of pups retrieved by each mouse after first vehicle injection, CNO injection and repeated vehicle injection. Data are presented as means ± s.e.m., repeated measures ANOVA; 0.01< ∗p < 0.05, 0.001< ∗∗p < 0.01. (E) Effect of CNO treatment (CNO) and vehicle injections (V1 and V2) on latency to retrieve first and third pup in control group (Ctrl) (n = 14), hM4Di-injected group (n = 14), and hM3Dq-injected group (n = 16). Data are presented as mean ± s.e.m., dots represent individual data points, repeated measures ANOVA; 0.01 < ∗p < 0.05, 0.001 < ∗∗p < 0.01. (F) Time spent in the nest of Ctrl females (n = 10), hM4Di-injected females (n = 9), and hM3Dq-injected females (n = 10) after vehicle (V1 and V2) and*

*CNO injections. (G–I) Effect of CNO administration on duration of pup grooming (G), pup licking (H), and crouching (I) behavior compared to the effect of vehicle injections (V1 and V2) in Ctrl females (Ctrl) (n = 8), hM4Di-injected females (n = 10) and hM3Dq-injected females (n = 13). Individual data points are presented, Friedman test followed by Wilcoxon signed-rank test; ns: not significant, 0.01< ⁎p < 0.05, 0.001 < ⁎⁎p < 0.01. (J) a Retrieval latency of 1st and 3rd pup in control mothers (Ctrl) (n = 4), hM4Di-injected mothers (n = 5) and hM3Dq-injected mothers (n = 6). Friedman test followed by Wilcoxon signed-rank test, *P<0.05.(K-M) Effect of CNO administration on duration of time in the nest (K), crouching (L) and maternal interaction (M) compared to the effect of vehicle injections (V1 and V2) in control mothers (Ctrl) (n=4), hM4Di-injected mothers (n=5) and hM3Dq-injected mothers (n=6). Repeated measures ANOVA, *P<0.05, **P<0.01, ***P<0.001. (N-Q) Comparison of the CNO effect size before pregnancy and after parturition. Data are represented as means ± s.e.m. Mann-Whitney test.*

### *4.2.2. The regulatory role of preoptic inhibitory neurons of aggressive behaviors in male mice*

To establish the role of preoptic GABAergic neurons in paternal care as well, we used the same cell type-specific technique as described above to induce both increased and decreased activity in preoptic inhibitory neurons. The pup-directed behavior outputs of male mice following CNO administration stands in sharp contrast to the strong effect detected in females. Neither inhibition nor excitation of VGAT+ neurons induced significant changes in pup retrieval behavior (Figure 5A). However, chemogenetic activation of VGAT+ neurons in the MPOA evoked pup-directed aggression (Figure 5B), whereas the inhibition resulted in a trend toward increased parenting ($p = 0.07$) (Figure 5C). Despite the well-known differences between the parenting of virgin males and fathers, the selective manipulation of preoptic VGAT+ neurons resulted in similar behavior toward pups in the two groups. Namely, similarly to virgin males, in fathers, the activation of GABAergic neurons in MPOA evoked pup-directed aggression (Figure 5D), while the inhibition caused elevated parental care ($p = 0.02$) (Figure 5E).

To further investigate the role of the preoptic VGAT+ neurons in aggressive behavior we performed resident-intruder assay (Figure 5F) to test the effect in intermale aggression. No significant difference was detected in the duration or latency of intermale social interaction between control and preoptic VGAT+ neurons manipulated groups (Figure 5G). In contrast, the high-level male-directed aggression (158.1 sec ± 31.9 sec) caused by the activation of preoptic VGAT+ neurons compared to the control group (36.8 sec ± 15.5 sec) is consistent with our previous result detected in parenting behavior. Even though there is no statistical difference in the latency of male-directed aggression (p =

0.053), hM3Dq-injected males showed a clear trend in latency of male-directed aggression (105.4 ± 33.8 sec) than control males (297.8 ± 74.2 sec) (Figure 5H).

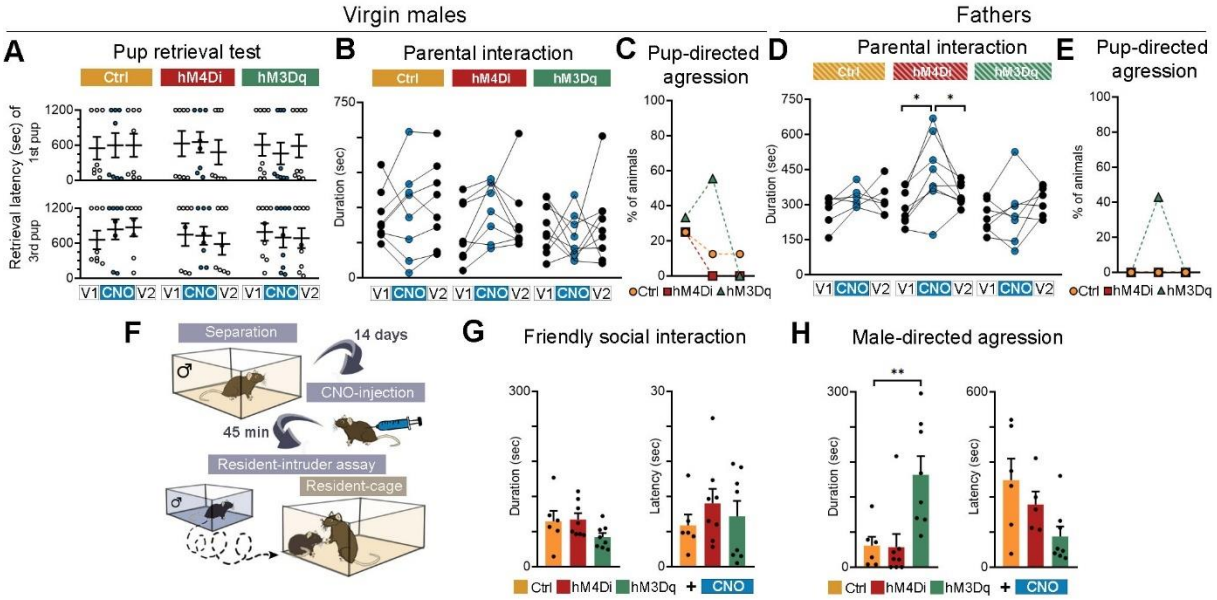


***Figure 5. The effect of chemogenetic modulation of preoptic VGAT+ neurons on parenting and aggressive behavior in virgin male and father mice.*** *(A) Effect of CNO treatment vs vehicle injections (V1 and V2) on latency to retrieve the first and third pup in control virus-injected virgin males (Ctrl) (n = 8), hM4Di-injected virgin males (hM4Di) (n = 8) and hM3Dq-injected virgin males (hM3Dq) (n = 9). Data are presented as means ± s.e.m. dots represent individual data points (animals, which did not retrieve pups, were plotted for maximum duration). Friedman test followed by Wilcoxon signed-rank test. (B) Percentage of control virus-injected (Ctrl) virgin males (n = 8), hM4Di-injected virgin males (n = 8) and hM3Dq-injected virgin males (n = 9) showed pup-directed aggression.(C) Comparison of the duration of parental interaction after the CNO administration (CNO) and vehicle injections (V1, V2) of control virus-injected (Ctrl) virgin males (n = 8), hM4Di-injected virgin males (n = 8) and hM3Dq-injected virgin males (n = 9). Individual data points are presented. Friedman test followed by Wilcoxon signed-rank test. (D) Percentage of control virus-injected (Ctrl) fathers (n = 6), hM4Di-injected fathers (n = 8) and hM3Dq-injected fathers (n = 7) showed pup-directed aggression. (E) The effect of CNO administration on duration of parental interaction compared to the effect of vehicle injections (V1 and V2) in control virus injected fathers (n = 6), hM4Di-injected fathers (n = 8) and hM3Dq-injected fathers (n = 7). (F) Experimental design of resident-intruder test. (G-H) Duration and latency of the friendly social interaction (G) and intermale aggression (H) of the Ctrl males (n = 6), hM4di-injected males (n = 8) and hM3dq-injected males (n = 8) after the CNO administration. Data are presented as means + s.e.m., Mann-Whitney test, **<0.01.*

## 4.3 The potential neuroanatomical background of the pup-related behavior mediated by preoptic inhibitory neurons

### *4.3.1 Projection map and the targets of the preoptic GABAergic neurons*

To understand how preoptic *VGAT*+ neurons may mediate their actions, mapping of their neuronal projections was performed based on the fluorescent density of mCherry immunoreactivity signal of neuronal fibres after mCherry-fused anterogradely spreading

virus injection into the MPOA of VGAT-Cre mice (Figure 6A). Immunolabelled fibres were present in a number of brain regions ipsilateral to the injection site. Some contralateral mCherry+ fibres were also present in most target regions but at much lower density than in the ipsilateral side (*not shown*). Because of the well-known difference in parenting behavior of virgin females and males, we established the projections in both sexes. Indeed, a remarkable difference was revealed between the sexes in the projections of their preoptic VGAT+ neurons to brain areas regulating pup avoidance and aggression, such as anterior hypothalamic nucleus (AHN), ventromedial hypothalamic nucleus (VMH) and periaqueductal grey (PAG) (Figure 6B, C). The markedly more intense projections to these brain regions in female mice suggest a more profound inhibition of these brain regions in virgin females by preoptic *VGAT*+ neurons as compared to virgin males. In addition, preoptic *GABA*ergic neurons also project to brain regions implicated in anxiety, like hypothalamic brain areas including paraventricular nucleus (PVN) and lateral hypothalamic nucleus (LH), as well as to bed nucleus of stria terminalis (BNST) and central amygdaloid nucleus (CeA). To understand the role of projections from preoptic *VGAT*+ neurons in the regulation of parenting, we identified some of the cell types in the target areas. Since oxytocinergic and dopaminergic neurons play an established role in the regulation of parenting and parenting-linked emotional state, we examined the relation between the GABAergic projections of MPOA and these cell types. Double immunolabelling of mCherry with oxytocin or tyrosine hydroxylase (as marker of dopaminergic neurons) indicated that mCherry+ axon terminals closely apposed oxytocinergic neurons in the PVN (Figure 6D) and also in the supraoptic nucleus (SON) (Figure 6E), similar to its connection with dopaminergic neurons in the hypothalamic arcuate and periventricular nuclei (Figure 6F-G).

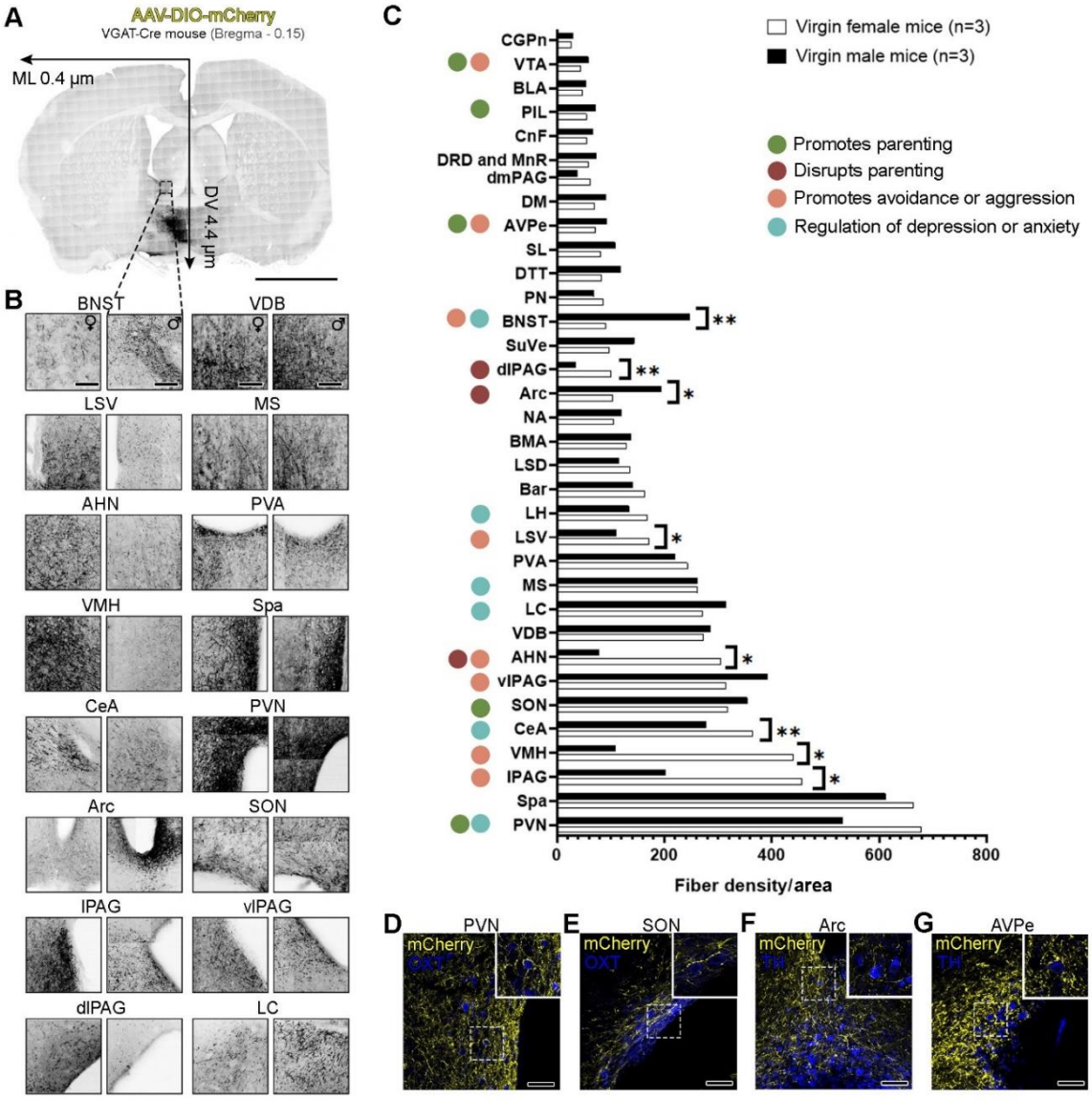


***Figure 6. Brain region and identified neuronal populations received projections from the preoptic GABAergic cells.*** *(A-B) Representative confocal micrograph of the injected site (A) and projected areas by preoptic VGAT+ neurons in virgin female and male mice (B). c Fiber density of preoptic VGAT+ neurons within distinct brain areas (area = 2500 µm2). Colored dots indicate the revealed function of the projected areas. Data are presented as means. Mann-Whitney test, *<0.05m **<0.01. (D-E) Double labelling of oxytocin (OXT) neurons and VGAT+ fibers (mCherry) in paraventricular nucleus (PVN) (D) and supraoptic nucleus (SON) (E) of virgin female mice. (F-G) Double labelling of dopaminergic tyrosine hydroxylase positive neurons (TH) and VGAT+ fibers (mCherry) in arcuate nucleus (Arc) (F) and anteroventral periventricular nucleus (AVPe) (G) of virgin female mice.*

*Abbreviations: PVN = paraventricular nucleus; Spa = subparaventricular zone of the hypothalamus; lPAG = dorsolateral periaqueductal gray; VMH = ventromedial hypothalamic nucleus; CeA = central amygdaloid nucleus; SON = supraoptic nucleus; vlPAG = ventrolateral periaqueductal gray; AHN = anterior hypothalamic nucleus; VDB = nucleus of the vertical limb of the diagonal band; LC = locus coeruleus; MS = medial septal nucleus; PVA = paraventricular thalamic nucleus; LSV = lateral septal nucleus, ventral part; LH = lateral hypothalamic nucleus;*

*Bar = Barrington nucleus; LSD = lateral septal nucleus, dorsal part; BMA = basomedial amygdaloid nucleus; NA = accumbens nucleus; Arc = arcuate hypothalamic nucleus; dlPAG = dorsolateral periaqueductal gray; SuVe = superior vestibular nucleus; BNST = bed nucleus of stria terminalis; HN = PN = paranigral nucleus; DTT= dorsal tenia tecta; SL= semilunar nucleus; AVPe = anteroventral periventricular nucleus; DM = dorsomedial hypothalamic nucleus; dmPAG = dorsomedial periaqueductal gray; DRD = dorsal raphe nucleus; MnR = medial raphe nucleus; CnF = cuneiform nucleus; PIL = posterior intralaminar nucleus; BLA = basolateral amygdaloid nucleus; VTA = ventral tegmental area; CGPn = central gray of the pons; 3V = 3rd ventricle. Scale bars: 2 mm(A), 100 μm (B), 50 μm (D-G)*

### *4.3.2. Neuronal inputs of preoptic inhibitory neurons*

Given that our results revealed the strong connection between preoptic VGAT+ neurons and oxytocinergic neurons located in the PVN and SON throughout inhibitory projection of MPOA, we next investigated whether this connection is *vice versa*. We injected retrograde virus fused with mCherry into the MPOA of VGAT-ZsGreen1 mice (Figure 7A). Whole-brain analysis identified multiple regions which are projecting to the MPOA including brainstem (locus coeruleus), thalamic areas (parafascicular thalamic nucleus, interpeduncular nucleus, ventral tegmental area, substantia nigra, posterior intralaminar nucleus), hypothalamic areas (ventromedial hypothalamic nucleus, arcuate nucleus), amygdaloid nucleus (central, medial and basolateral amygdaloid nucleus), olfactory cortex (cortical amygdala and piriform cortex) and most importantly PVN and SON (Figure 7B, C, D, F). To gain a more detailed insight into the connection between oxytocinergic neurons and MPOA, we then performed double immunolabelling within PVN and SON in the mice injected by retrograde virus into the MPOA. Significantly more OXT+ neurons co-expressed with mCherry in the SON than in the PVN, moreover, almost all retrogradely labeled neurons were OXT+ in the SON (Figure 7E).

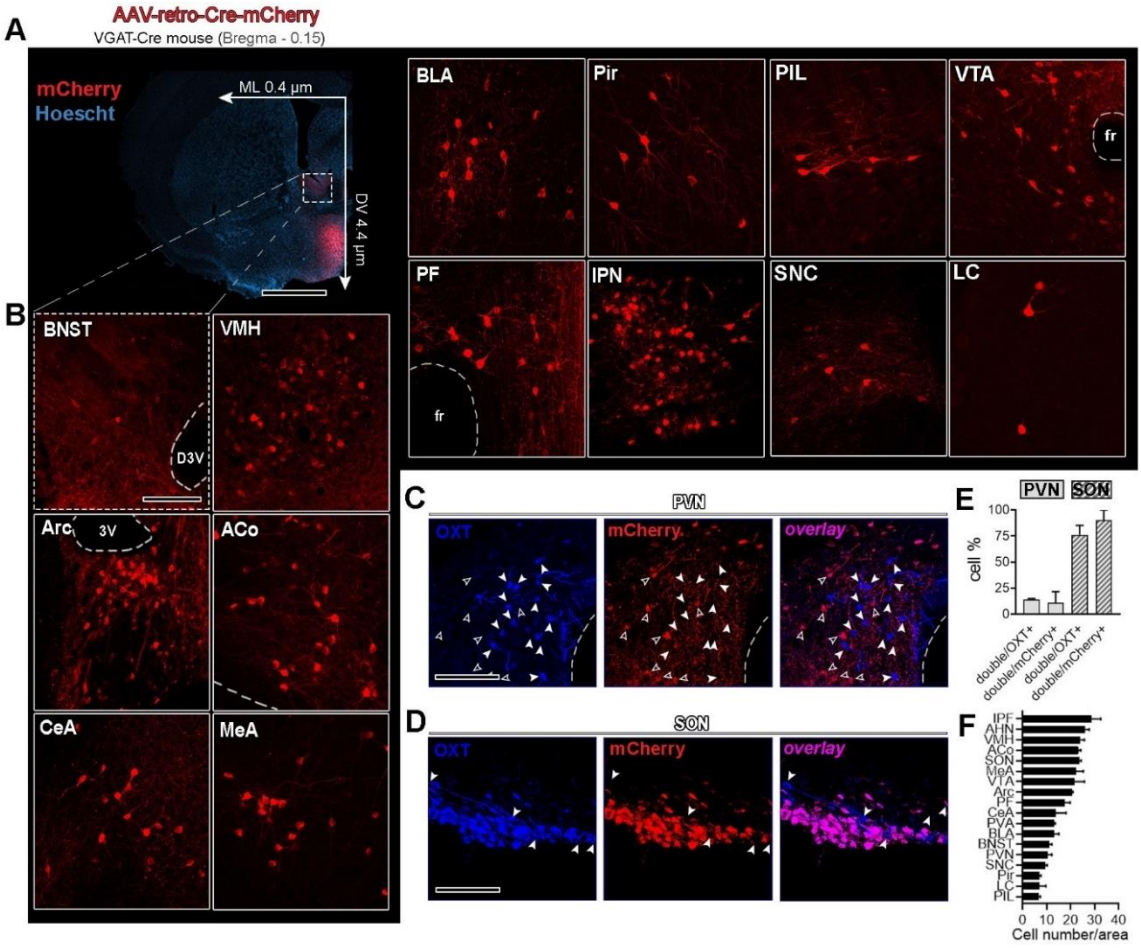


***Figure 7. Identification of MPOA-projecting areas and oxytocin action on preoptic VGAT+ neurons.*** *(A) Representative micrograph of the injected preoptic area. (B) Representative images of input regions with mCherry+ cells. (C-D) Double immunolabelling of OXT and mCherry in the PVN (C) and SON (D). (E) Percentages of double labelled neurons. (F) Number of retrogradely labelling mCherry+ neurons within the distinct areas.*

*Abbrevations: ML = mediolateral; DV = dorsoventral; BNST = bed nucleus of stria terminalis; VMH = ventromedial hypothalamic nucleus; ACo = anterior cortical amygdaloid nucleus; CeA = central amygdaloid nucleus; MeA = medial amygdaloid nucleus; BLA = basolateral amygdaloid nucleus; Pir = piriform cortex; PF = parafascicular thalamic nucleus; IPN = interpeduncular nucleus; PIL = posterior intralaminar nucleus; VTA = ventral tegmental area; SNC = substantia nigra, compact part; LC = locus coeruleus; PVN = paraventricular nucleus; SON = supraoptic nucleus. Scale bars: 2mm (A), 100 µm (B-D)*

### *4.3.2. Hormonal and nonhormonal actions on VGAT-expressing cells located in the medial preoptic area*

Next, we investigated the potential hormonal inputs of preoptic inhibitory neurons. More specifically, to reveal whether the anatomical connections between the OXT-expressing and preoptic GABAergic neurons are physiologically functional, the firing rate of preoptic inhibitory neurons was examined following local administration of

OXT. The fluorescent neurons were recorded using whole-cell patch-clamp mode in acute preoptic brain slices of adult female VGAT-ZsGreen mice. Administration of oxytocin at 100 nM resulted in no significant change in the firing rate (0.9.5 ± 4.41% while the baseline value was 0.78 ± 0.054 Hz in 10 neurons; p= 0.8429, t=0.2040, df=9 Student's t-test) (Figure 8A). Application of higher concentrations, however, triggered significant elevation in the firing rate in 10-10 neurons (1 μM: by 240.0 ± 27.01%, p= 0.0001, t=8.885; 10 μM: by 711.6 ± 51.54%, p=0.0001, t=13.81; df=9, Student's t-test at both doses) (Figure 8B-D). Zoomed periods (0.1 min) before and after the oxytocin administration of the recording seen in Figure 8B can be observed in Figures 8B1 and B2. Another important hormone regarding the parenting is the estrogen. In addition to the regulatory role of estrogen in parenting, the expression pattern of its nuclear receptor ERα shows sexual dimorphism, proposing the role of these neurons in sex-dependent regulation of pup-directed behaviors [71]. Fluorescent immunolabeling in mice expressing ZsGreen fluorescent protein under the VGAT promoter (Figure 8E) revealed that 50.7%± 3.5% of preoptic GABAergic neurons expressed the gonadal steroid receptor ERα in females, while the enrichment of ERα is significantly lower in fathers and virgin males

5.2±0.8% and 1.16%±0.29%, respectively, p<0.000) (Figure 8F). Further, most ERα+ cells are GABAergic (~73.3%±7.5% and ~91.2%±1.5%, respectively). (Figure 8G).

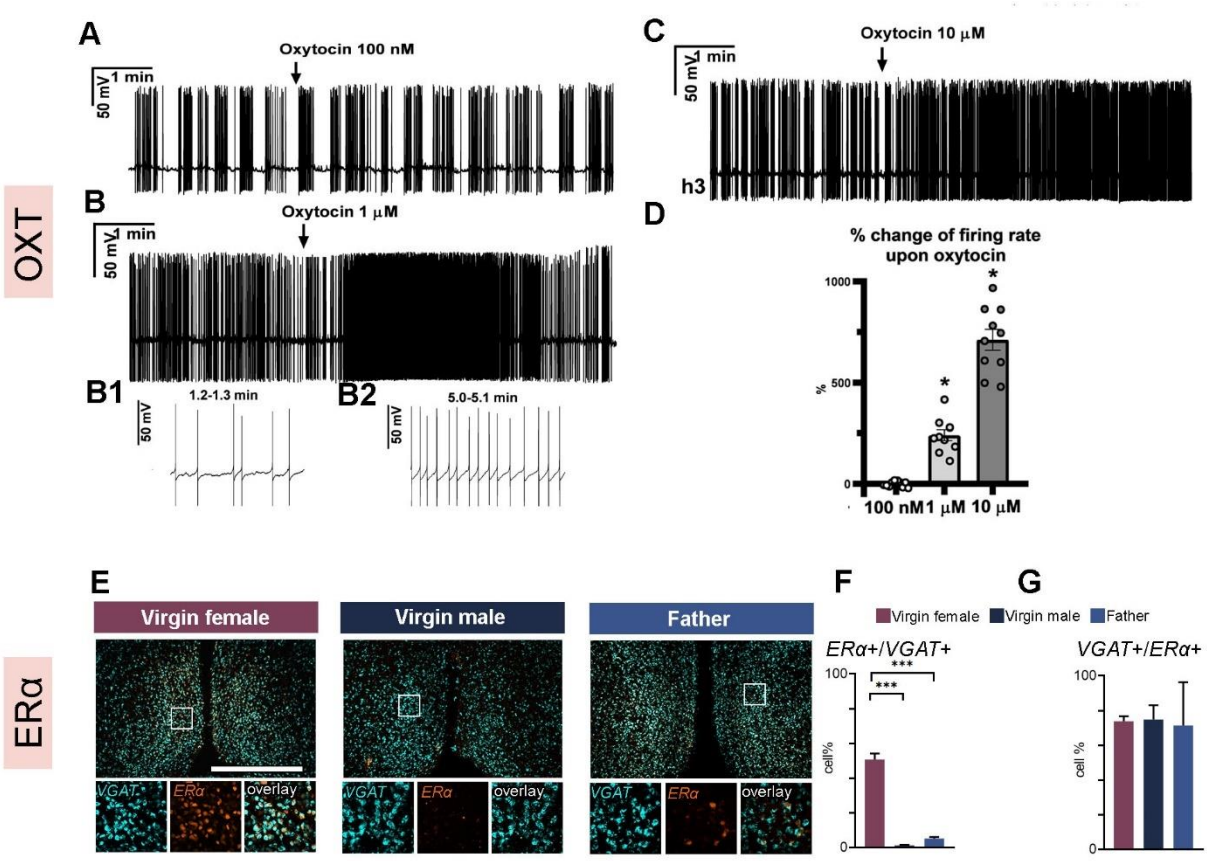


***Figure 8. Hormonal inputs of the preoptic GABAergic neurons.*** *(A-C) Oxytocin dose-dependently (100 nM-10 μM) elevates firing rate in VGAT-ZSGreen neurons of the MPOA in acute brain slice of adult female mice. B1 and B2 are 0.1 min zoomed periods of (B) before and after oxytocin application. (D) Bar graph demonstrating the individual data points that were measured following the application of OXT at different concentration. (E) Representative micrograph of estrogen receptor α (ERα) immunostaining in the medial preoptic area of virgin female, virgin male, and father VGAT-ZsGreen1 mice. (F-G) Percentage of preoptic GABAergic neurons expressing ERα (F) and ERα+ neurons expressing VGAT (G). Data are represented as means + s.e.m. Kruskal-Wallis test followed by Dunn-Bonferroni post-hoc test, ***<0.001. Scale bar: 1mm.*

Based on our previous findings about the crucial role of Tip39 action on the neurons located in the MPOA, we performed fluorescent immunostaining against Tip39 peptide in female mice expressing ZsGreen fluorescent protein in VGAT-containing cells to reveal whether the preoptic GABAergic neurons are targeted by Tip39-positive fibers. High magnification confocal micrograph showed that nerve terminals containing Tip39 are abundant around the VGAT-expressing preoptic neurons, suggesting that these cells receive information from the Tip39-positive cells, that are involved in the transfer of

suckling information during motherhood (Figure 9A). Given that the neuronal circuits underlying this essential element of maternal behavior are highly evolutionary conserved, we took the advantage of correlated light and electron microscopy in order to prove that the Tip39-containing fibers form synapses with preoptic inhibitory neurons in mother rats (Figure 9B1-3). A significant subset of preoptic inhibitory neurons was visualized by using a galanin antibody and peroxidase-based DAB immunohistochemistry. Our approach revealed that galanin-expressing neurons located in the MPOA receive multiple conventional asymmetrical synapses from Tip39-positive axon terminals (Figure 9B) according to the morphological analysis of 17 Tip39-positive boutons. To support the potentially excitatory nature of the asymmetrical synapses, we performed post-embedding immunogold labeling of consecutive serial sections for Tip39 and the stimulatory neurotransmitter, glutamate (Figure 9C-D). Post-embedding immunolabeling for Tip39 and glutamate of adjacent sections revealed that boutons shown to be immunoreactive for Tip39 in previous consecutive sections (Figure 9C1-2) also contain a high density of immunogold demonstrating glutamate content (Figure 9D1-2).

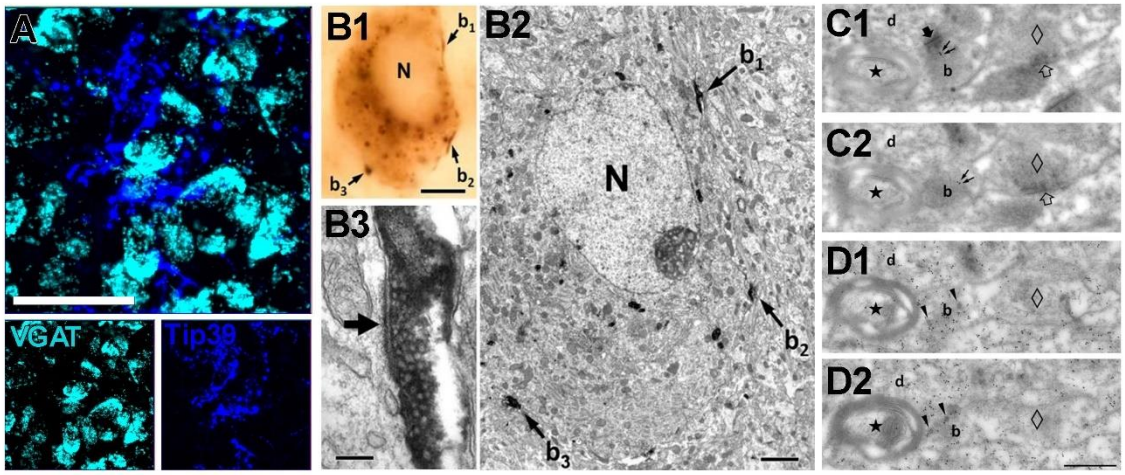


***Figure 9. Presence of the excitatory Tip39-positive fibers around the preoptic inhibitory neurons in mice and rats.*** *(A) A confocal image demonstrates the close somatic apposition of Tip39-expressing fibers on preoptic GABAergic neurons in a mouse brain. (B1-B3) Correlated light (B1) and electron micrographs (B2, B3) of the galanin-expressing neuron receiving three asymmetrical synapses (b1-b3) from Tip39-positive fibers visualized by Ni-DAB histochemistry. The Tip39-positive boutons are indicated by black arrows. N: nucleus. Scale bars: 5 µm (B1), 2 µm (B2), and 200 nm (B3). (C-D) Demonstration of the glutamate content of Tip39 terminals in the MPOA using consecutive serial electron micrograph sections. (C1,2) Consecutive sections labeled for Tip39 by 10 nm gold particles indicated by black arrows in boutons (b) establishing asymmetrical synapse. (D1,2) post-embedding immunogold labeling glutamate by 10nm gold particles demonstrates that glutamate is enriched in the Tip39-positive boutons seen in panel C1,2. Nota bene, myelinated axons (labeled by black stars) and symmetrical synapses (labeled by*

*empty diamond) do not contain glutamate. Scale bars: 20 μm (A), 5 μm (B1), 200 nm (B2), 2 μm (B3), 500 nm (C1-D2).*

## 4.4 The involvement of preoptic GABAergic cells in the regulation of depression- and anxiety-like behaviors

### *4.4.1. Anatomical and functional link between parenting and depression-like behavior*

Based on our hypothesis that preoptic inhibitory neurons as main regulators of parenting have a potential role in depression as well, in addition to our findings about the inputs and outputs of these neurons, we tested the effect of FST on neural activity of preoptic VGAT+ cells (Figure 10A–D). Both the number of c-Fos+ cells and the ratio of c-Fos+/VGAT+ cells significantly increased in female (6.6 ± 0.3, $p < 0.000$, 7.4 ± 0.4%, $p < 0.000$, respectively) and male (3.6 ± 0.2, $p < 0.000$, 4.6 ± 0.3%, $p < 0.000$, respectively) mice after FST compared to the sex-matched control groups (Figure 10E-F). *Nota bene*, FST induced significantly higher activation of preoptic GABAergic cells in females than in males ($p < 0.001$) (Figure 10F). Interestingly, similar to the activity pattern of pup-induced c-Fos+ cells, significantly more FST-induced c-Fos+ cells located in the medial subdivision of the MPN of females ($p < 0.001$), whereas the number of c-Fos+ cells were significantly higher in the central part of the MPN in males after FST ($p = 0.015$) (Figure 10G). In addition, we normalized the regional number of both pup and FST induced c-Fos+ cells to the average number of c-Fos+ cells after exposure to pups and FST, respectively. Comparison of the normalized pup-induced c-Fos+ cell and FST-induced c-Fos+ cell revealed a significant difference in only the lateral subdivision of the MPN of females ($p = 0.040$), whereas the activity levels induced by pup and FST, indicated by the number of c-Fos+ cells, were similar within other regions and in male mice (Figure 10H).

To test whether there is a connection between pup exposure and depression level, we performed FST following pup exposure. Pup exposure did not have an effect on the time of immobility (42.4 ± 3.1%, $p = 0.481$), swimming (41.7 ± 4.5%, $p = 0.970$) and climbing (18.3 ± 2.7%, $p = 0.143$) during the FST compared to control group (39.5 ± 2.4%, 39.9 ± 2.7%, 22.4 ± 2.2%, respectively) which had no interaction with pups (Figure 10I–K). To reveal the potential regulatory role of preoptic GABAergic neurons in the depression-like behavior, both FST and sucrose preference test were performed after modulation of preoptic inhibitory cells by the aforementioned DREADD technique.

Females injected by hM4Di virus and treated with CNO demonstrated more swimming ($p = 0.001$) and spent significantly less time in immobility ($p = 0.001$) than control females (Figures 10I, J, and 10K). Finally, we correlated the duration of maternal interactions with the time of immobility. This demonstrated that time spent with pups did not affect the depression levels of animals ($r = 0.22$, $p = 0.113$) (Figure 10L).

To examine if symptoms of depression other than resilience are also affected by preoptic GABAergic neurons, the hedonic activities following their chemogenetic manipulation were also addressed using the sucrose preference test as anhedonia is also known as a core symptom of depression in both rodents and humans [72]. Indeed, the activation of preoptic VGAT+ neurons resulted in reduced sucrose preference ($p = 0.01$) (Figure 10M), while their inhibition significantly increased the daily consumption ($p = 0.03$) (Figure 10N).

The activation of preoptic GABA+ neurons induced pup- and male-directed aggressive behavior in males, contrary to females. According to human data, depression often leads to aggressive behaviors, but the underlying mechanisms are poorly understood [73]. In order to reveal whether there is a connection between these two behaviors under the manipulation of the preoptic inhibitory system we measured the depression-like behavior and correlated the data with the conspecific male-directed aggressive behavior. Excitation of preoptic VGAT+ neurons significantly increased the immobility time ($P=0.001$) and decreased the duration of active behaviors (swimming and climbing) ($P=0.03$ and $P=0.02$) in the forced-swim test (Figure 10P-R). Moreover, a significant correlation was found between immobility time as an indicator of depression-like behavior and intruder-directed aggressive behavior ($r = 0.5$, $P=0.02$) (Figure 10S).

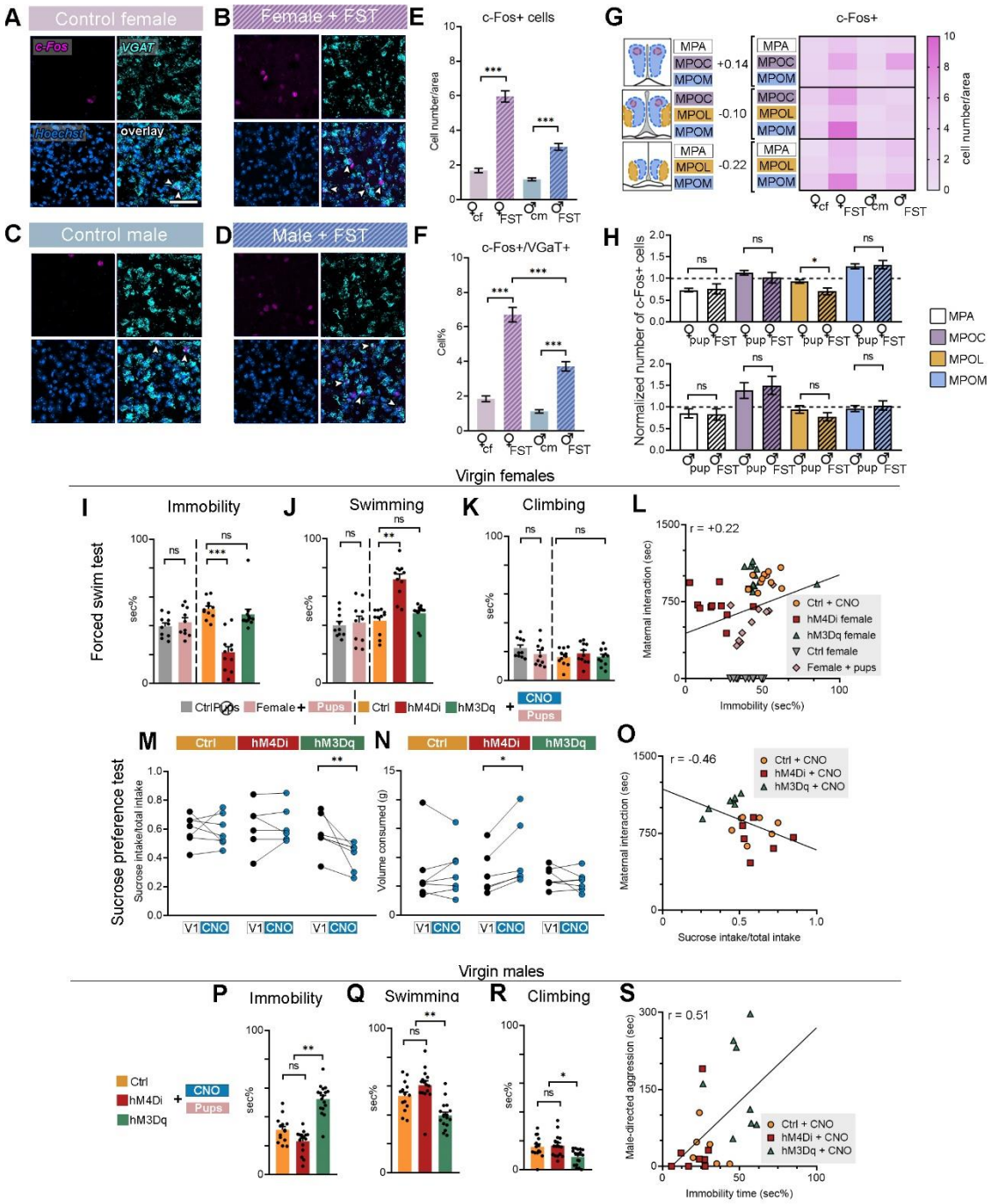


***Figure 10. The connection between depression-like behavior and pup-directed behavior under the control of preoptic VGAT + neurons.*** *(A–D) c-Fos expression after fresh bedding exposure (A, C) and forced swim test (FST) (B, D) of virgin female (A, B) and male (C, D) mice in the medial preoptic area. Co-expression of VGAT and pup-induced c-Fos is marked by white arrowheads. (E) Comparison of the number of c-Fos + cells in the medial preoptic area after forced swim test. (F) Ratio of c-Fos+ cells in VGAT+ cells after forced swim test. Data are presented as means ± s.e.m., Mann–Whitney test; 0.01 < *p < 0.05, 0.001 < **p < 0.01, ***p < 0.001. (G) Heatmap of the distributions of the c-Fos+ cells among distinct subregions after forced swim test. MPA: medial preoptic area; MPOC: central part of the medial preoptic nucleus; MPOM: medial part of the medial preoptic nucleus; MPOL: lateral part of the medial preoptic nucleus. Area = 30,000 μm2. Groups are labeled as follows. cf: control virgin females after fresh*

*bedding exposure (n = 4); cm: control virgin males after fresh bedding exposures (n = 4); FST: virgin females (n = 4) or males (n = 3) after forced swim test. (H) Top: normalized number of c-Fos+ cells after pup exposure (female pup) and FST (female FST) within distinct subregions. Bottom: normalized number of c-Fos+ cells after pup exposure (male pup) and FST (male FST) within distinct subregions. Data are presented as means ± s.e.m., Mann–Whitney test; ns: not significant, ∗p < 0.05. (I–K) Immobility (I), swimming (J) and climbing (K) time in the forced swim test of the control virus-injected females (n = 11), hM4Di-injected females (n = 11) and hM3Dq-injected females (n = 11) after CNO administration (CNO) and pup exposure (pups). Data are presented as means + s.e.m., dots represent individual data points, Mann–Whitney test; ns: not significant, 0.001 < ∗∗p < 0.01, ∗∗∗p < 0.001. (L) Correlation between the percentage of immobility and time spent with maternal care of Ctrl virus-infected females (n = 11), hM4Di-injected females (n = 11), hM3Dq-injected females (n = 11), control virgin females after fresh bedding exposure (n = 9) and virgin females after pup exposure (n = 10). Pearson correlation, r = 0.51, P = 0.001. (M) Comparison of daily consumption of the control virus-injected females (n = 7), hM4Di-injected females (n = 6) and hM3Dq-injected females (n = 6) after vehicle injection (V1) and after CNO administration (CNO). Paired-sample T Test, ∗∗p < 0.01.(N) Comparison of sucrose preference of Ctrl females (n = 7), hM4Di-injected females (n = 6) and hM3Dq-injected females (n = 6) after vehicle injection (V1) and after CNO administration (CNO). Individual data points are presented, paired-sample T-Test, ∗p < 0.05. (O) Correlation between the time spent with maternal care and sucrose preference of Ctrl females (n = 7), hM4Di-injected females (n = 6) and hM3Dq-injected females (n = 6) after CNO administration. Pearson correlation, r = −0.46, p = 0.04.(P–R) Percentage of immobility (P), swimming (Q) and climbing (R) of the control virus -injected males (n = 14), hM4Di-injected males (n = 16) and hM3Dq-injected males (n = 17). Data are presented as means + s.e.m., dots represent individual data points, Mann–Whitney test; ns: not significant, 0.001 < ∗∗p < 0.01. (S) Correlation between immobility time and the duration of inter-male aggression of control virus-injected males (n = 6), hM4Di-injected males (n = 8) and hM3Dq-injected males (n = 8). Pearson correlation, r = 0.51, p = 0.02. Scale bars: 50 μm (A–D).*

### *4.4.2. The sexually dimorphic control of parenting-linked anxiety-like behavior by preoptic inhibitory neurons*

The changes in anxiety level across the transition to parenthood is an adaptive response during the perinatal period and help coping with challenges [74]. However, approaching children with extensive anxiety has a considerable impact on parents-infant relationship and elevates the risk of development psychiatric disorders in both parents and children [75]. Here, we addressed the potential connection between anxiety-like behavior and nursing behavior under the control of preoptic inhibitory neurons. To test the effect of the presence of pups on anxiety levels, we performed elevated plus maze (EPM) test following exposure to pups. Virgin females after exposure to pups spent significantly less time in the open arms (33.1 ± 4.3%, p = 0.004) and more time in the close arms (53.3 ± 0.3%, p = 0.004) compared to the control group (51.0 ± 3.2%, 33.70 ± 4.6%, respectively), which had no interaction with pups (Figures 11A, B). To examine whether pup-induced elevated anxiety levels could be modified by inhibition or excitation

of preoptic GABAergic neurons, we also performed EPM test following pup exposure in CNO-treated mice. The hM3Dq virus-injected virgin female mice, which showed increased maternal motivation, spent significantly less time in the open arms (12.4 ± 1.6; $p = 0.05$) and more time in close arms (51.6 ± 3%) than control virus-injected mice (47.9 ± 1.9, 41.3 ± 1.7%, respectively) (Figures 11A, C) after exposure to pups and CNO administration. Contrary, no significant difference was found in the anxiety levels, indicated by time spent in open arms, in hM4Di virus-injected females (51.2 ± 2.7%) compared to control females suggesting that inhibition of preoptic GABAergic neurons prevented the effect of pup exposure. Furthermore, we found a negative correlation between the anxiety level indicated by the time spent in open arms during the EPM test and the duration of maternal caring behaviors exhibited in the spontaneous pup caring behavior test ($r = -0.33$, $p = 0.03$) (Figure 11C). The effect of overactivation of preoptic VGAT+ neurons on anxiety-like behavior, independently from pup exposure, was confirmed by the open-field test. After CNO administration, females with activated preoptic GABAergic neurons spent significantly less time in the internal zone of the open field apparatus (12.1 ± 1.8%) ($p = 0.04$) than control females (28.9 ± 3.1%) (Figure 11F) presented in both trajectory and activity maps (Figures 11D1–D3, E1-D3, respectively). We did not observe any difference in either the traveled total distance (Figure 11G) or the velocity (Figure 11H) of the animals during the open field test. Contrary to virgin females, preoptic VGAT-manipulated males displayed no changes in anxiety (Figure 11 I, J) in elevated plus maze test. In addition, there was no correlation between male-typical pup-directed aggression and anxiety level (Figure 11K). To confirm our results, we performed open field test too and found no significant differences between control virus injected group and treated groups neither in the duration of time spent in the internal zone (Figure 11N) nor total distance (Figure 11O) or velocity (Figure 11P) as trajectory maps (Figure 11L1-L3) and activity maps (Figure 11M1-M3) presents.

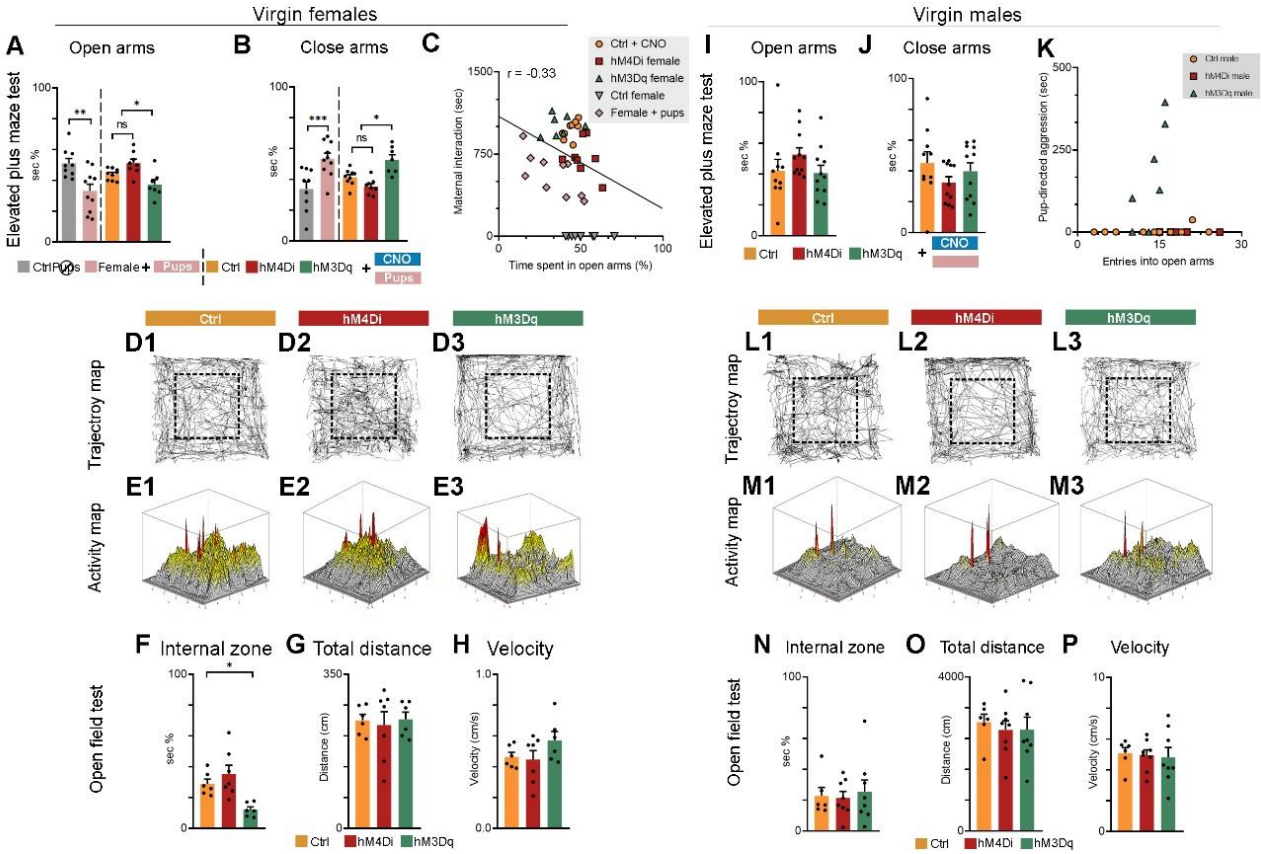


***Figure 11. The sex-dependent control of parenting-linked anxiety-like behavior by preoptic VGAT+ neurons in virgin female and male mice.*** *(A-B) Time spent in open (A) and close (B) arms of control virgin females after fresh bedding exposure (Ctrl (no pups)) (n = 9) and virgin females after pup exposure (Female + Pups) (n = 10); control virus-injected females (Ctrl) (n = 9), hM4Di-injected females (hM4Di) (n = 8), hM3Dq-injected females (hM3Dq) (n = 8) after CNO administration (CNO) and pup exposure (Pups). Data are presented as means + s.e.m., dots represent individual data points, Mann-Whitney test; ns: not significant, 0.01 < ⁎p < 0.05, 0.002 < ⁎⁎p < 0.01, ⁎⁎⁎p < 0.001. (C) Correlation between the number of entries into open arms and duration of maternal interaction of control virus-injected females (n = 9), hM4Di-injected females (n = 7) and hM3Dq-injected females (n = 7), control virgin females after fresh bedding exposure (n = 9) and virgin females after pup exposure (n = 10). Pearson correlation, r = −0.33, p = 0.011. (D) (D1–D3) Representative trajectory map of control virus-injected (Ctrl) female (D1), hM4Di-injected female (D2), and hM3Dq-injected female (D3) mice after CNO administration in the open field apparatus. Internal zone marked by dashed line. (E1-E3) Representative activity map of control virus-injected (Ctrl) female (E1), hM4Di-injected female (E2), and hM3Dq-injected female (E3) mice after CNO administration in the open field apparatus. Red color represents the high activity level, yellow color represents the medium activity level and grey color represents the low activity level. (F–H) Time spent in the internal zone of the control virus-injected females (n = 6), hM4Di-injected females (n = 7), and hM3Dq-injected females (n = 6) (F), total distance traveled in the open field arena by the control virus-injected females (n = 6), hM4Di-injected females (n = 7), and hM3Dq-injected females (n = 6) (G), average velocity in the open field arena of the control virus-injected females (n = 6), hM4Di-injected females (n = 7), and hM3Dq-injected females (n = 6) (H). Data are presented as means + s.e.m., dots represent individual data points, Mann–Whitney test, 0.01 < ⁎p < 0.05. Groups are labeled as follows. Ctrl + No pups: virgin female mice after fresh bedding exposure; Female + Pups: virgin female mice after pup exposure; Ctrl: control virus-injected mice; hM4Di: hM4Di virus-injected mice; hM3Dq: hM3Dq virus-injected mice. (I–J) Time spent in open (I) and close*

*(J) arms of control virus-injected males (n = 10), hM4Di-injected males (n = 11), and hM3Dq-injected males (n = 11). Data are presented as means + s.e.m., dots represent individual data points, Mann-Whitney test; ns: not significant, 0.001 < ∗∗p < 0.01, ∗∗∗p < 0.001.(K) Correlation between the number of enters into open arms and duration of pup-directed aggression of Ctrl males (n = 10), hM4Di-injected males (n = 9) and hM3Dq-injected males (n = 7). Pearson correlation.(L1-L3) Representative trajectory map of control virus-injected (Ctrl) male (L1), hM4Di-injected male (L2), and hM3Dq-injected male (L3) mice after CNO administration in the open field apparatus. Internal zone marked by dashed line. (M1-M3) Representative activity map of control virus-injected male (M1), hM4Di-injected male (M2) and hM3Dq-injected male (M3) mice after CNO administration in the open field apparatus. Red color represents the high activity level, yellow color represents the medium activity level and grey color represents the low activity level. (N) Time spent in the internal zone of control virus-injected males (n =6), hM4Di-injected males (n = 8) and hM3Dq-injected males (n = 8). (O) Total distance travelled in the open field arena by control virus-injected males (n =6), hM4Di-injected males (n = 8) and hM3Dq-injected males (n = 8). (O) Average velocity in the open field arena of control virus-injected males (n =6), hM4Di-injected males (n = 8) and hM3Dq-injected males (n = 8). Data are presented as means + s.e.m., dots represent individual data points, Mann-Whitney test.*

## 5. Discussion

Coping with the challenges associated with the postpartum period is a milestone in every mother's life, which unfortunately is not always smooth sailing. Excessive maternal anxiety and depression often have significant long-term negative effects on the mother, offspring, and family, but the underlying neurobiological mechanisms are not properly understood. The aim of the current study was to precisely delineate the fine-tuning role of the inhibitory circuit of the MPOA which through this neuronal population block the processing of multimodal sensory information from infants as aversive stimuli thus preventing abusive or neglectful behavior.

### 5.1 The involvement of preoptic GABAergic neurons in the sex-specific control of pup-directed behaviors

In contrast to our expanding understanding of the neurobiological basis of maternal behavior, the neuronal circuits underlying infanticidal behavior in virgin males and how these circuits are rewiring during parenthood are still poorly understood. Interestingly, in species demonstrating paternal care including the well-studied California mice or the monogamous prairie voles, with the exception of lactation, all other components of parenting episodes can be captured by observing the infant-directed behaviors of father animals [76]. The similar phenotype of behavior responses towards pups across sexes proposes that the neuronal circuits involved in the regulation of motor outputs following exposure to pups are at least partially shared. Experiments performed with laboratory male mice who normally exhibit parenting when becoming fathers, provide direct evidence for the overlapping nature of the neural network. More specifically, genetic ablation or cell-type specific activation of a pup-responsive preoptic inhibitory neuronal population leads to exactly the same behavior outputs in both father and mother animals [42]. Intriguingly, non-sexually dimorphic neuronal populations with specific functions show similar activity patterns following the triggering stimulus in both sexes, thus the behavior outputs are coded by the neuronal activity, according to the Hebbian theory [77]. However, investigating the pup-directed behavior of virgin animals highlights the potential differences in the parenting circuits, since virgin male mice typically attack pups, while virgin females exhibit almost immediate acceptance behavior [5]. The sexually dimorphic features of the MPOA have been known to be for a long time,

but its sex-dependent regulatory role in parenting has not been completely uncovered yet [78,79].

### *5.1.1. Preoptic GABAergic neurons react to pup exposure in a sex-dependent manner*

In this study, we successfully addressed the previously unknown sex-specific activity pattern and level of preoptic VGAT-expressing neurons evoked by exposure to pups, thus providing indirect physiological evidence for their involvement in the regulation of sexually dimorphic display of behaviors. Consistent with this notion, the central subdivision of the MPOA is found by our c-Fos study to be more active compared to other parts of the MPOA in male, but not in female mice. Interestingly, in rats, central subdivision of the MPOA is a crucial component of the sexual dimorphic nucleus (SDN) mediating the male-typical behavior including copulation and aggression. Although the presence of the SDN in mice has not been confirmed yet, the increased expression of calbindin, the reliable marker for the SDN is highly restricted to the central part of the MPOA in male, but not in female mice [80]. In virgin female and mother mice, a mediolateral gradient of activation pattern was in good agreement with the previous findings [70]. The pup-induced different activity pattern of preoptic inhibitory neurons points to the likelihood that while the medial subdivision of the MPOA is involved in the regulation of female-typical caregiving behavior, the central subdivision may mediate the rejective responses. Furthermore, although the percentage of activated preoptic neurons increased significantly in male mice exposed to pups compared to male mice without pup stimuli, only a small portion of preoptic GABAergic neurons activated, suggesting that under natural conditions the pup exposure does not reach the stimulus threshold of certain preoptic inhibitory cells to initiate an activity response.

### *5.1.2. The supportive role of the preoptic inhibitory circuit in caretaking behavior exhibited by female mice*

Given that the vast majority of preoptic neurons are expressing VGAT, there is a definite probability that the MPOA express its effect on parenting through their inhibitory projections to brain regions initiating pup avoidance or infanticidal behavior. Thus, it is presumable that the increased activity of preoptic GABAergic neurons enhances caretaking behavior but considering the variation of activity patterns across sexes, it is conceivable that in a different way. Recent techniques aiming to cell-type specific

modulation of neuronal activity provide the opportunity to delineate the role of distinct neuronal population in certain behaviors [81]. By applying chemogenetic approaches with the performance of numerous behavior tests measuring both the appetitive and consummatory aspects of parenting we found a striking difference between the pup-directed behavior in female and male mice. Notably, the experimentally induced increased activity of preoptic GABAergic neurons cannot entirely correspond to the stimulation caused by the pup exposure, however, the percentage of reactive neurons approximately mimic the proportion of activated neurons experienced under natural conditions according to our results. These findings also suggest that the presence of pups *per se* provides substantial stimulation of these cells, and preoptic GABAergic neurons have an endogenous, physiological role in the regulation of parental behavior. There is a controversy over which subregions of the MPOA is responsible for the goal-directed and which parts are involved in the consummatory aspects of parenting. However, targeting the neurotransmitter content and genetical profile instead of a region-specific perspective uncovered that the preoptic inhibitory neurons modulate caretaking behaviors exhibited by female mice through silencing defensive circuits allowing the expression of all aspects of parental behavior, independently from their subregional distribution. To assist the general inhibitory function of the MPOA in female mice, by selective repression of neuronal activity of GABAergic neurons located in the MPOA, the rejection networks are presumably released from inhibition leading to the disruption of caretaking behavior, regardless of reproductive states. The similar degree of activation in females and mothers is in line with similar level of caring behavior in them and supports the relative emancipation of maternal behavior from hormonal control.

### *5.1.3. Regulation of infant-directed and intermale aggression by the same preoptic neuronal population*

Conversely, as might have been expected based on the distinct activity pattern, chemogenetic stimulation of the same neuronal population in male mice initiates pup-directed aggressive behavior, while inhibition of preoptic inhibitory neurons only has a little effect on paternal behavior in fathers. The stimulatory effect of inhibition of preoptic GABAergic cells on the duration of paternal interactions with pups observed in fathers, but not in virgin males, suggests that this neuronal population may be involved in the transition from infanticide to paternal behavior. The opposite effect of the activity of the

preoptic VGAT-expressing neurons in males was a striking finding, which suggests sexually dimorphic control of parenting in the MPOA. Previous studies identified both Gal and ERα expressing neurons in the MPOA whose activity increased parenting not only in female but also in male mice [42,82]. Although most Gal-expressing neurons are GABAergic, the proportion of Gal-positive cells is small in comparison with the abundance of GABAergic neurons [42], suggesting that the opposing effect of chemogenetic manipulation of preoptic VGAT-expressing neurons in males and females is the result of the manipulation of Gal and ERα-negative GABAergic neurons. Activation of these cells may be responsible for aggression in male mice. This suggestion is also in line with some previous studies, which showed that activation of BNST-projecting MPOA neurons simultaneously stimulates Gal-positive neurons inducing parenting and further GABAergic cells inducing pup-directed aggression resulting in stochastic behavior towards pups in male mice [83]. Intriguingly, male-directed aggression can also be evoked by the activation of preoptic GABAergic neurons, similar to infanticide behavior. The co-occurrence of both infant- and male-directed aggression following increased activity of preoptic GABAergic neurons indicate that circuits underlying infant-directed aggression are not entirely separated from those mediating intermale aggression, contrary to previous views [9].

### 5.2 The circuit function of the preoptic inhibitory neurons in the regulation of pup-directed responses

To precise understand, how the preoptic inhibitory neuronal population is embedded in the circuit underlying pup-directed behavior, it is essential to explore which brain areas are potentially connected to this cell group. Injection of cell type-specific anterogradely spreading virus to the MPOA revealed that the projections pattern of preoptic GABAergic neurons was generally similar to that previously described for MPOA neurons in the rat [84]. However, we first identified sexually dimorphic projections in some of the targeted areas. The local heterogeneity of the inhibitory neurons of the preoptic area [38], may be in line with the variability of their projections. The higher density of inhibitory projections observed in females to brain areas that play a role in pup avoidance, such as AHN and VMH as the parts of the rejection circuit may lead to altered behavioral manifestations as compared to males. In addition to the abovementioned areas, preoptic inhibitory neurons are projecting to numerous other brain regions that are

involved in the regulation of pup-directed behavior, according to previous findings (Table 3.)

*Table 3. Summary of the role of preoptic VGAT+ neurons projecting areas in parenting*

| **Brain region** | **Effect on parenting** | **References** |
|---|---|---|
| paraventricular nucleus (**PVN**) | lesion of PVN disrupts the onset of maternal behavior | [85] |
| dorsolateral periaqueductal grey **(dlPAG**) | activation of the dorsal part of PAG depresses maternal behavior, while lesion of this area promotes maternal responsiveness | [86] |
| lateral periaqueductal grey (**lPAG**) | activates affective aggression | [87] |
| ventrolateral periaqueductal grey (**vlPAG**) | activation of vlPAG suppresses ongoing motivated behaviors and induces defensive behavior | [88] |
| ventromedial hypothalamic nucleus (**VMH**) | VMH stimulation induces defensive behavior | [89] |
| anterior hypothalamic nucleus (**AHN**) | bilateral lesion with N-methyl-D-aspartic acid results in rapid onset of maternal behavior in rats; activation of AHN elicits avoidance | [89, 90] |
| bed nucleus of stria terminalis (**BNST**) | BNST lesion inhibits infanticide in male mice, activation of the BNST ERα cells induced infanticide in female mice | [91, 83, 39] |
| anteroventral periventricular nucleus (**AVPe**) | stimulation of TH-expressing neurons enhances maternal care in females, while in males suppress intermale aggression | [92] |
| supraoptic nucleus (**SON**) | pup exposure increases the activity of oxytocin-positive neurons in SON | [93] |
| medial septal nucleus (**MS**) | glutamatergic neurons in MS exert anorexic effects | [94] |
| lateral septal nucleus (**LSV**) | activation of $GABA_A$ receptors in LSV promotes maternal aggression; inhibition of LSV increases aggression | [95] |
| lateral hypothalamus (**LH**) | lesion of the LH abolishes motivated behaviors such as food intake or sexual behaviors | [96] |
| arcuate nucleus (**Arc**) | hunger activated AgRP neurons in Arc inhibit preoptic neurons controlling parenting | [97] |
| posterior intralaminar nucleus (**PIL**) | pup exposure elevates the number of active neurons in PIL | [30] |
| ventral tegmental area (**VTA**) | inactivation of dopaminergic neurons in VTA disrupts retrieval of pups, but not nursing behavior; cell activity is increased in VTA after aversive stimulus | [98] |

The dense projections to brain regions regulating parenting confirmed the crucial regulatory function of the preoptic GABAergic neurons in connection to pup-directed behaviors. In addition, given that oxytocinergic and dopaminergic neurons play an established role in the regulation of parenting, we examined the relation between the GABAergic projections of MPOA and these cell types. Double immunolabeling of

mCherry with oxytocin or TH indicated that mCherry-positive axon terminals closely apposed oxytocinergic neurons in the PVN and also in the SON but not dopaminergic neurons in the hypothalamic Arc and AVPe nuclei. The abundance of preoptic VGAT-positive fibers around the oxytocinergic neurons worth mentioning considering the essential role of oxytocin in social bonding, parenting and emotional regulation. The reason for this rather contradictory result is still not completely clear, but it makes it possible that the inhibitory connection between the MPOA and oxytocinergic system may be involved in other regulation of emotional behaviors than the mother-infant relationship, thereby implying that the maternal networks may overlap with other circuits. Contrary, the lack of the direct contact between the preoptic inhibitory neurons and TH-expressing cells located in the hypothalamic nuclei is in a good agreement with previous findings. The physiologically increased activity of dopaminergic neurons of AVPe is indispensable for the expression of maternal behavior in female mice, while their ablation results in faulty in maternal care [92].

Examining the inputs of the preoptic GABAergic neurons helps to understand via which mechanisms these cells are activated upon exposure to pups. Our findings would seem to show that the increased neuronal activity may be partly due to neuronal input from the pups. Retrogradely spreading virus injection to the MPOA revealed MeA as one of the major brain regions projecting to the preoptic nucleus. MeA has a well-known function in the converging both olfactory and auditory information and is involved in the modulation of motoric responses towards pups [99]. Moreover, projections derived from the piriform cortex may also contribute to the pup-induced neuronal activity of preoptic GABAergic neurons as the main cortical area processing olfactory stimuli [100]. A few retrogradely labelled neurons were located in the PIL. PIL, as a thalamic nuclei relay and modulate suckling information incoming from the nipple to the preoptic GABAergic neurons, thus potentially influence the activity of this neuronal population. In support, electron microscopic investigation provided anatomical evidence for the functional synaptic relationship between the excitatory Tip39-positive fibers and galanin-expressing neurons located in the MPOA. In general, our retrograde study revealed that multiple brain regions project to the MPOA, which is consistent with previous findings in rat [101]. However, we identified a novel, particularly strong afferent connection: the oxytocin neurons located in the supraoptic nucleus. The functionality and excitatory nature of this

projection was demonstrated by patch-clamp experiments, which suggest that preoptic GABAergic neurons may be activated by oxytocin. In addition to oxytocin action, the high expression of ERα of preoptic VGAT-positive neurons indicate that estrogen also modulate the activity of these cells and may contribute their sexually dimorphic function [71,102].

### 5.3 The potential connection between parenting and depression from a neurobiological perspective

Although, postpartum neuropsychiatric disorders including extensive anxiety and depression have a long-lasting negative effect on the mental and physical development of children, only a few neurobiological investigations attempted to explore the potential neuronal link between the mood disorders and motherhood. Most of our knowledge on the potential connection between postpartum period and emotional alterations has been obtained from human studies. The findings from human diagnostic imaging techniques suggest that basal forebrain regions specifically related to strong affiliative motivations and emotions including the preoptic area, are highly responsive to both positive and negative stimuli [103]. More specifically, several studies have recorded increases in MPOA responses when mothers view images or videos of their own infants as well as when hearing the cry of their babies [104–106]. It can thus be conceivably hypothesized that some of the brain regions involved in the perception and modulation of infant-directed cues may serve as an interface for the interaction of positive and negative stimuli and react according to the internal state. Furthermore, such studies investigating to understanding of neurobiology of postpartum psychiatric disorders are primarily correlational in nature. Therefore, in our studies with laboratory animals, we also utilized correlational analysis to establish whether there is a relationship between parenting, depression-like behavior, and anxiety-like behavior under the control of preoptic inhibitory neurons.

In order to measure the parenting and emotional behaviors immediately after each other in the same animal, we took the advantage of chemogenetic approach, a method suitable for long-term manipulation of neurons [107]. Following the correlational analysis, we found that the increase of the preoptic neuronal activity simultaneously induces enhanced maternal care and anhedonia in female mice, while the pup exposure itself does not evoke depression-like behavior indicated by the immobility time during the FST. In a nutshell, preoptic GABAergic circuit is involved in the regulation of both parenting and

depression-like behavior but taking care of the pups normally does not lead to depression. Furthermore, the preoptic inhibitory neuronal circuits are presumably vulnerable for overstimulation or pathophysiological alterations, which could represent a potential mechanism of postpartum depression. In further support for the parenting-independent regulatory role of preoptic GABAergic neurons in depression-like behavior, chemogenetic stimulation of preoptic inhibitory cells significantly increased the time spent immobile of virgin male mice, who normally do not show extensive parental care.

Investigating to anxiety-like behavior in connection to parenting we found that strong inhibition or release from inhibition in the projected areas upon chemogenetic modification of preoptic GABAergic neurons resulted in two opposite behaviors: caring anxious female vs. avoiding, less-anxious female behavior. The combined behavioral effect evoked by manipulations of preoptic inhibitory neuron is in good agreement with a previously validated model in rodents [75,108]. As proposed by the strong correlation between the anxiety-like state and parental care discovered in female mice, nor the excitation, not the inhibition of preoptic GABAergic neurons affect the anxiety-like behavior of male mice. However, our assumption is based on correlations and therefore cause-effect relationship cannot be proven.

In addition to findings derived from behavioral investigations, the striking overlap between the pup-induced and the depression test-induced activity pattern of preoptic GABAergic neurons also highlights the possibility that the preoptic inhibitory neurons are shared between the parenting and depression circuit. A further important implication can be drawn from the projection map of preoptic inhibitory neurons. Some of the brain regions receiving information from the preoptic VGAT-positive neurons have a well-known role in the development of depression or anxiety. More concretely, selective robust reduction in the number of neurons in the PVN, but not in the SON was found in patient suffering from major depression and bipolar disorder [109]. In this regard, it is worth mentioning that oxytocin neurons in the SON, which we revealed to project heavily to the MPOA, received much less input from the MPOA than paraventricular oxytocin neurons suggesting a dominantly unidirectional flow of information between preoptic GABAergic neurons and differentially located oxytocin neurons. Since the role of oxytocin in social binding, parenting but also in the reduction of depression is established [110–112], the possibility of indirect regulation of depression-like behavior through inhibition

of oxytocin system is plausible. Moreover, inhibition of neurons located in the locus coeruleus initiate depression-like behavior through decreased noradrenergic signaling [113]. The reduced activity of nucleus accumbens can be connected to the development of depression and anhedonic behavior, too [114]. The inhibitory projections of preoptic GABAergic neurons to brain regions that diminished activity correlates psychiatric disorders makes anatomically possible that the inhibitory neuronal population of the MPOA involved in the initiation of depression.

### 5.4 A hypothetical working model for the simultaneous regulatory role of preoptic GABAergic neurons

According to our results, we can draw an expanded hypothetical working model for the involvement of preoptic GABAergic neurons in the regulation of the parental care and emotional states (Figure 12.). Our retrogradely spreading virus injection to the MPOA revealed that preoptic neurons received information from brain regions modulating pup stimuli including olfactory, auditory and somatosensory information. Next, the projection map of the preoptic GABAergic neurons shows that these neurons send projections to brain regions that are involved in the initiation of pup avoidance and aggression, thus supporting the expression of caretaking behavior. The analysis of the projection of the preoptic inhibitory neuronal population also uncovered a surprising connection between the preoptic GABAergic cells and oxytocin-expressing neurons. We cannot rule out that the role of oxytocin in both parenting and emotional behavior is essential, therefore the inhibition of this system may cause deficits in both behaviors [115]. For this reason, we hypothesize that one of the functions of certain preoptic inhibitory neurons is to inhibit those GABAergic projecting neurons that targeting oxytocin system in order to prevent the inhibition of oxytocin-expressing cells and repression of social bonding and normal mental health. This unexpected relationship between the preoptic inhibitory neurons and oxytocinergic system makes it possible that the preoptic inhibitory circuit module mood disorders via its projection to oxytocin-expressing neurons during motherhood, when the activity of this network especially heightened due to endocrine events and stimuli from infants. Intriguingly, despite of the dense projection of preoptic GABAergic neurons to PVN, we found significantly more retrogradely labeled neurons in the SON, suggesting a dominantly unidirectional flow of information through oxytocinergic system. Finally,

we identified two additional potential influencers on the activity of preoptic GABAergic neurons, the estrogen and Tip39.

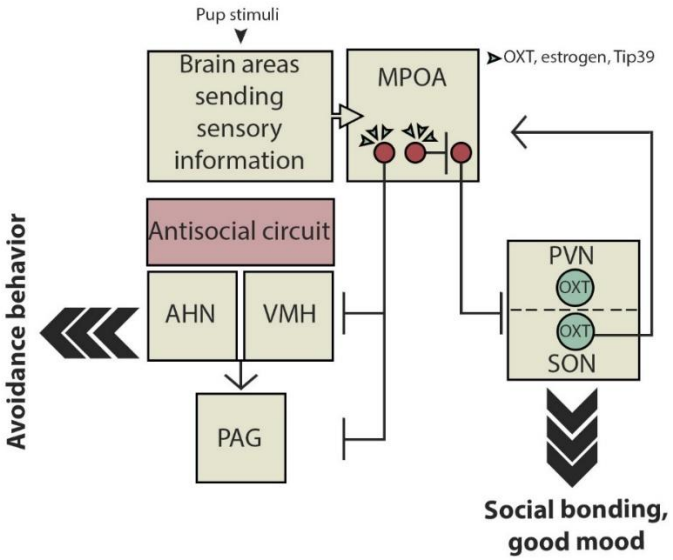


***Figure 12. The expanded hypothetical working model focusing on the inhibitory functions of the MPOA for the initiation of parenting and normal mental health.*** *Pup stimuli and hormonal actions influencing the activity of preoptic GABAergic neurons. Most, but not all preoptic inhibitory cells expressing receptors for OXT, estrogen and Tip39. Projecting GABAergic neurons inhibit brain regions that are involved in rejection towards pups and oxytocinergic system. Oxytocin-positive neurons located in the SON send extensive projection to the MPOA. In a healthy mother the oxytocin-projecting GABAergic neurons are inhibited by local inhibitory interneurons allowing the expression of the positive effect of oxytocin on social bonding and mood.*

## 6. Conclusions

In the current thesis, I raised the possibility of simultaneous control of pup-directed behavior and emotional behavior by the medial preoptic inhibitory neurons via examining the behavior outputs following chemogenetic modification of this neuronal population. Moreover, based on the anatomical and physiological connections of preoptic GABAergic neurons, I provided a hypothetical working model of how these cells may exert their effect.

First, the evidence from our c-Fos investigation implies that the distinct behavior repertoire displayed by males and females towards pups are coded by the different hypothalamic engrams of preoptic GABAergic neurons.

Furthermore, the difference between the pup-induced activity pattern of preoptic GABAergic neurons manifests at the behavior level, as the experimentally increased neuronal activity of preoptic VGAT-expressing cells opposingly controls the pup-directed behavior in male and female mice, independently from their reproductive states. More specifically, while the stimulation of preoptic inhibitory neurons induces enhanced parenting in females, it evokes aggressive behavior towards pups in males.

Exploring the projections of the preoptic GABAergic neurons assumed that this neuronal population may exerts its effect through the inhibition of brain regions that are involved in the defensive behavior. The sex-dependent density of preoptic VGAT-expressing cells at certain brain regions further support the sexually dimorphic role of these cells. In order to influence the other brain regions, preoptic GABAergic neurons should be activated by both pup stimuli and hormonal actions. The reciprocal relationship between oxytocinergic system and preoptic GABAergic neurons may be the link between the regulation of parenting and emotional behaviors, as the peptide hormone oxytocin is well known for its ability to control mood and maternal behavior simultaneously.

Finally, the overlap between the activity patterns evoked by pups and depression test suggest that preoptic GABAergic neurons are involved in the regulation of both pup-directed and depression-like behavior. The increased level of depression following the chemogenetic activation of pup-responsive preoptic inhibitory neurons propose that the impairment of the preoptic GABAergic circuit would cause disinterest instead of caretaking behavior in association with the development of depression.

In conclusion, we identified a crucial component of the caring behavior at the circuit level as GABAergic neurons in the preoptic area were shown to affect pup-directed behaviors in a sexually dimorphic manner. We also demonstrated that the same neurons are responsible for depression-like behavior providing potential explanation why the occurrence of depression is elevated during the postpartum period.

## 7. Summary

Since the occurrence of depression is often linked in time with parenting, it has been suggested that brain alterations required for parental care may somehow contribute to make mothers more vulnerable to depression, however, the underlying mechanisms are poorly understood. In this thesis, I aimed to provide anatomical, physiological and direct neurobehavioral proof for the action of inhibitory preoptic neurons in pup-associated behavior and also highlight that the impairment of this network could potentially contribute to depression.

First, the activity pattern of inhibitory GABAergic neurons within the preoptic area was determined in response to pup exposure and a test of depression. Because the distribution of activated neurons was similar in case of both stimuli, we addressed the role of the GABAergic preoptic neurons in parenting as well as in depression. Intriguingly, we found that activation of preoptic inhibitory neurons elicited parenting in female, while evoke aggression in male mice, that correlate with sexually dimorphic display of parenting in mice. To elicit long-term changes in the activity of preoptic inhibitory neurons, chemogenetic tools were applied. In contrast to the sex-dependent role of preoptic inhibitory network in parenting, it exerts the same effect on both males and females in depression-like behavior, supporting the parenting-independent regulatory role of preoptic inhibitory circuit in depression. To reveal the neuronal circuits, by which preoptic inhibitory neurons may influence pup-associated and emotional behaviors, their neuronal projections were also described in both sexes. Preoptic VGAT-expressing neurons project to brain regions that are involved in the regulation of parenting, infanticide, and emotions in sex-dependent manner. Finally, our anatomical and physiological investigations revealed *vice versa* relationship between preoptic inhibitory system and oxytocinergic neurons. In addition to oxytocin, other hormonal and non-hormonal factors like estrogen and the suckling stimulus activated Tip39 may also influence the activity of preoptic GABAergic neurons according to our histological investigations.

Altogether our findings uncover the previously unknown sexually different role of preoptic inhibitory system in caregiving and provide neurobehavioral and anatomical evidence for the preoptic inhibitory neuronal control over anxiety and depression in mice.

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## 9. Bibliography of the candidate's publications

**Publications related to the thesis:**

Dimén, D., Puska, G., Szendi, V., Sipos, E., Zelena, D., & Dobolyi, Á. (2021). Sex-specific parenting and depression evoked by preoptic inhibitory neurons. iScience, 24(10), 103090. https://doi.org/10.1016/j.isci.2021.103090

Cservenák, M., Kis, V., Keller, D., Dimén, D., Menyhárt, L., Oláh, S., Szabó, É. R., Barna, J., Renner, É., Usdin, T. B., & Dobolyi, A. (2017). Maternally involved galanin neurons in the preoptic area of the rat. Brain structure & function, 222(2), 781–798. https://doi.org/10.1007/s00429-016-1246-5

**Publications unrelated to the thesis:**

Barna, J., Dimén, D., Puska, G., Kovács, D., Csikós, V., Oláh, S., Udvari, E. B., Pál, G., & Dobolyi, Á. (2019). Complement component 1q subcomponent binding protein in the brain of the rat. Scientific reports, 9(1), 4597. https://doi.org/10.1038/s41598-019-40788-z

Udvari, E. B., Völgyi, K., Gulyássy, P., Dimén, D., Kis, V., Barna, J., Szabó, É. R., Lubec, G., Juhász, G., Kékesi, K. A., & Dobolyi, Á. (2017). Synaptic proteome changes in the hypothalamus of mother rats. Journal of proteomics, 159, 54–66. https://doi.org/10.1016/j.jprot.2017.03.006

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