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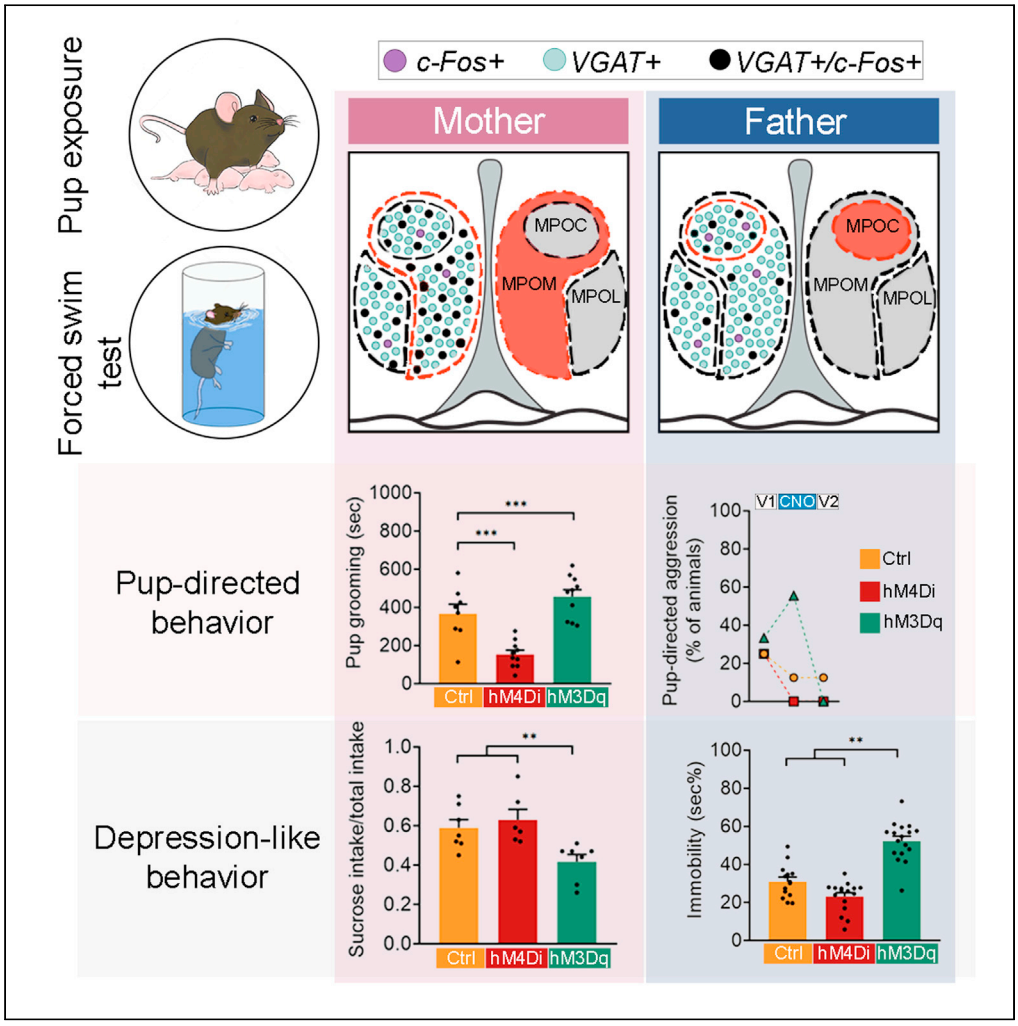

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Highlights

Preoptic GABAergic neurons promote maternal behaviors in females mice

Activation of preoptic GABAergic neurons induces pup-directed aggression in males

Projection pattern of preoptic GABAergic neurons is sexually dimorphic

Depression-like behaviors are provoked by stimulation of preoptic GABAergic neurons

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Sex-specific parenting and depression evoked by preoptic inhibitory neurons

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SUMMARY

The role of preoptic GABAergic inhibitory neurons was addressed in parenting, anxiety and depression. Pup exposure and forced swimming resulted in similar *c-Fos* activation pattern in neurons expressing vesicular GABA transporter in the preoptic area with generally stronger labeling and different distributional pattern in females than in males. Chemogenetic stimulation of preoptic GABAergic cells resulted in elevated maternal motivation and caring behavior in females and mothers but aggression toward pups in males. Behavioral effects were the opposite following inhibition of preoptic GABAergic neurons suggesting their physiological relevance. In addition, increased anxiety-like and depression-like behaviors were found following chemogenetic stimulation of the same neurons in females, whereas previous pup exposure increased only anxiety-like behavior suggesting that not the pups, but overstimulation of the cells can lead to depression-like behavior. A sexually dimorphic projection pattern of preoptic GABAergic neurons was also identified, which could mediate sex-dependent parenting and associated emotional behaviors.

INTRODUCTION

Parental care is a well-defined set of behaviors that increases the offspring's chance of survival. Although parenting is present in all mammals, the different species and sexes exhibit specific strategies (Royle et al., 2014). Although in humans, mother and father can be both involved in care, parenting is sexually dimorphic in most rodents and usually the mother takes care of the infants (Dulac et al., 2014). Interestingly, under laboratory conditions, nulliparous females of most strains display nearly immediate acceptance of pups similar to mothers (Stolzenberg and Rissman, 2011). By contrast, virgin males are known to abandon offspring or even attack them (vom Saal and Howard, 1982). Although maternal care is associated with hormonal changes of internal state and it implies a prolonged interaction with the infants, infanticidal behavior is displayed in short bouts and does not require prolonged hormonal and neuronal changes (Kohl et al., 2017). Still, it has been suggested that the neuronal circuits controlling behavioral responses toward pups are partially shared by both sexes (Wei et al., 2018). In turn, the regulation and the activity of the involved brain regions are altered by the sex and reproductive state of the animal result in the different behavioral outputs.

Profound hormonal and neuronal changes are required to develop appropriate behavioral responses during the postpartum period, which increase the chance that the adaptation of underlying circuits also affect the regulation of emotional states. Some emotional alterations, such as increased anxiety are considered to be adaptive as their role is to make parents more alert to protect their infants. However, sometimes, postpartum period is associated with maladaptive changes like excessive anxiety or reduced maternal motivation, which may adversely affect the socio-emotional and cognitive development of children (Crouch, 1999). Unfortunately, approximately 15% of human mothers cannot properly cope with the infant and suffer from postpartum depression. They exhibit reduced maternal motivation, besides experiencing anxiety and depression (Anokye et al., 2018). The symptoms of postpartum depression do not differ from that of the general depression disorders, thus, they include bad mood, reduced resilience, and anhedonia (O'Hara and Wisner, 2014). Because the occurrence of postpartum depression is linked in time with parenting, it has been suggested that brain alterations required for parental care can somehow contribute to enhanced maternal vulnerability to depression (Pawluski et al., 2017). Feeling hopeless and intolerant with the child is frequently reported in mothers but can also take place in fathers who may also experience depression after the birth of their child (Thiel et al., 2020). The mechanism of the vulnerability to depression during the

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postpartum period is not known yet. We might assume that the pathophysiological impairment of the hypothalamic circuits, which orchestrate the positive and negative aspects of parenting, may cause child abuse instead of maternal care.

The evolutionary conserved medial preoptic area (MPOA) known to be crucial for expression of maternal behavior (Lee et al., 2000; Numan, 1974) and also involved in the control of other social behaviors, including reproduction and aggression (Aleyasin et al., 2018; Kohl and Dulac, 2018; Moll et al., 2012). Despite the anatomical sex differences in MPOA, its role in both maternal and paternal behavior was confirmed. Hormonal (Rosenblatt et al., 1998) and cell specific activation (Wu et al., 2014) of MPOA facilitates immediate caring behavior regardless of sex and reproductive state. By contrast, in laboratory rodents, lesion (Akther et al., 2014), chemical inactivation (Pereira and Morrell, 2009), and cell specific inhibition or ablation (Fang et al., 2018; Wu et al., 2014) of the MPOA cause disruption of maternal motivation, which is a prominent feature of human postpartum depression. These findings advocate that the MPOA promotes parenting. A possible mechanism of the manifestation of parental behavior is the modification of excitatory-inhibitory balance of parenting networks (Marlin et al., 2015). Previous studies showed increased activation of the inhibitory neurons of the MPOA in parenting rodents (Lonstein and De Vries, 2000; Olah et al., 2018; Wu et al., 2014) suggesting that they may promote parental behavior, which has also been supported by recent optogenetics studies (Zhang et al., 2021). The regulatory network of parenting shows similarities between the sexes (Wu et al., 2014). Therefore, it is not known what neuronal mechanisms result in different parenting behavior in males and females. Because it was established that some brain regions receiving projections from MPOA neurons are part of the defensive circuits and inhibit maternal care (Numan and Numan, 1997), in the present paper, we also addressed if projections of inhibitory neurons to these brain regions are sexually dimorphic.

In this study, we used a variety of different experimental approaches to demonstrate the function of inhibitory preoptic neurons in pup-associated behavior in both sexes. First, the activation 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. Unlike previous optogenetics studies in the preoptic area, which did not address depression-like effects following manipulation of preoptic neurons (Fang et al., 2018; Scott et al., 2015; Wu et al., 2014), we aimed to simultaneously examine the different components of pup-directed, anxiety-like and depression-like behaviors using temporally extended manipulation of the cells. For that purpose, chemogenetic tools were applied to induce prolonged changes in the activity of preoptic inhibitory neurons.

Chemogenetic manipulations allow us to mimic closely the physiological mechanisms by modulating cellular excitation to endogenous stimulation instead of directly inducing spiking (Muir et al., 2019). Preoptic inhibitory neurons promoted parenting, anxiety-like, and concomitant depression-like behavior in females. The physiological role of the GABAergic neurons was addressed by not only stimulating but also inhibiting them via chemogenetics. In males, pup-induced activation of preoptic inhibitory neurons was much less extensive. In turn, the experimental stimulation of the preoptic inhibitory neurons evoked aggression toward the pups. Finally, we identified sexually dimorphic projections of preoptic inhibitory neurons to some target brain areas implicating them in sex-dependent caring and depression-like behaviors.

RESULTS

Pup exposure activates GABAergic neurons in the medial preoptic area

First, we investigated the distribution and the pup exposure induced activation of GABAergic neurons in the MPOA of VGAT-ZsGreen1 mice (Figures 1A–1F). The MPOA was identified as the medial preoptic nucleus (MPN) and the adjacent brain region called medial preoptic area (MPA) (Paxinos and Franklin, 2001). The abundance of GABAergic neurons was analyzed by calculating the percentage of NeuN+ cells expressing VGAT and found that 79.4 $\pm$ 2.5% of the preoptic neurons are GABAergic. There is no significant difference in the proportion of GABAergic cells in the different groups ($p = 0.505$) (Figure 1G). The percentage of activated vesicular GABA transporter-positive (VGAT+) preoptic neurons expressing c-Fos was significantly higher in virgin females (22.2 $\pm$ 1.1%, $p < 0.001$) and mothers (25.2 $\pm$ 1.0%, $p < 0.001$) following pup exposure than in the control group (3.3 $\pm$ 0.3%), which females had no interaction with pups. In addition, the ratio of c-Fos+/VGAT+ neurons were significantly elevated in both virgin male (3.4 $\pm$ 0.2%, $p < 0.001$) and father

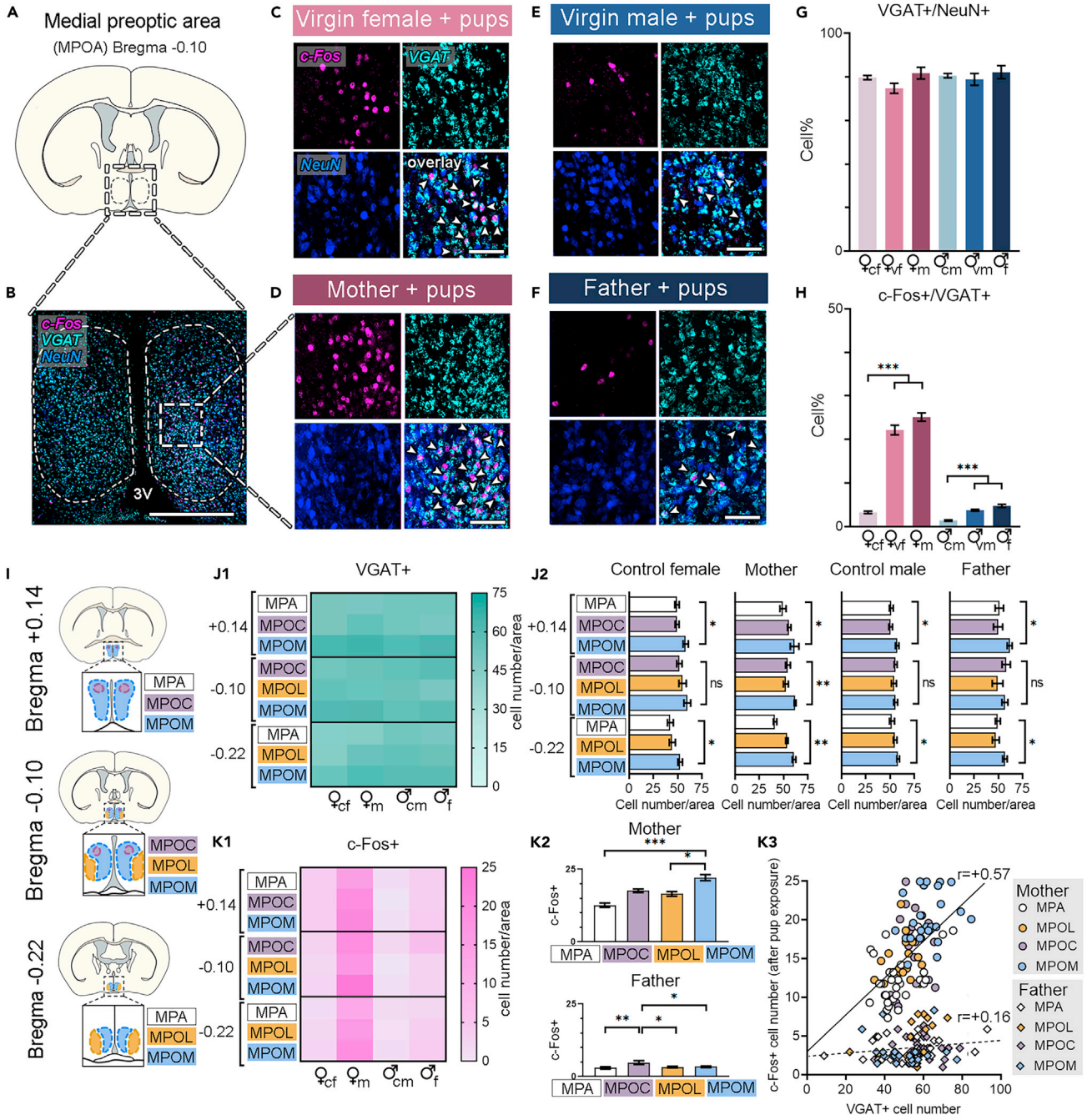

Figure 1. Activation of preoptic GABAergic neurons following pup exposure

(A) Schematic localization of the medial preoptic area (MPOA) in the coronal section indicated by the dashed square. Dashed lines label the border of the MPOA.

(B) Representative micrograph of c-Fos and NeuN immunolabeling in the MPOA of VGAT-ZsGreen1 mother mouse. Dashed lines label the border of the MPOA. V3: third ventricle.

(C–F) Pup-induced c-Fos expression in preoptic VGAT+ neurons of virgin female (C), mother (D), virgin male (E) and father (F) mice. Neurons co-express VGAT and pup-induced c-Fos are marked by white arrowheads.

(G and H) Ratio of the number of VGAT + neurons in NeuN + cells (G) and ratio of the number of c-Fos+ neurons in VGAT-containing cells (H). Data are presented as means ± s.e.m, Mann-Whitney test comparing all the pup interaction groups to fresh bedding controls; ns: not significant, ***p < 0.001. The measurement of cell numbers was performed in randomly selected fields within the different parts of the MPOA.

(I) Schematic drawings of subregions of the MPOA (Paxinos and Franklin, 2001).

Figure 1. *Continued*

(J) (J1) Heatmap of the distributions of the VGAT+ cells among distinct subregions. 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 μm^2. (J2) Comparison of the VGAT+ cell density between subregions of the MPOA. Data are presented as means ± s.e.m, Kruskal-Wallis test; ns: not significant, *p < 0.050, 0.001 < **p < 0.010.
(K) (K1) Heatmap of the distributions of the pup-induced c-Fos+ cells among distinct subregions. 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 μm^2. (K2) Comparison of the c-Fos+ cell density between subregions of the MPOA in mother (upper panel) and father (bottom panel) mice. Data are presented as means ± s.e.m, Kruskal–Wallis test; ns: *p < 0.050, 0.001 < **p < 0.010, ***p < 0.001. (K3) Correlation between the number of pup-induced c-Fos+ cells and the number of VGAT+ cells in the MPOA of mothers and fathers (Spearman correlation; r = 0.62, p < 0.0001 and r = 0.16, P = 0.1372, respectively). Groups are labeled as follows. cf: virgin females after fresh bedding exposure (n = 4); vf: virgin females after pup exposure (n = 3); m: mothers after pup exposure (n = 5); cm: virgin males after fresh bedding exposure (n = 4); vm: virgin males after pup exposure (n = 3); f: fathers after pup exposure (n = 3). Scale bars: 500 μm (B), 50 μm (C–F). The statistics of Results presented in the figure are summarized in supplementary data file (Data S1).

animals (4.8 ± 0.3%, p < 0.001) after pup exposure in comparison with the male control group which had no interaction with pups (1.4 ± 0.2%) (Figure 1H).

Previous findings indicated uneven pup-induced neuronal activation in several subregions within the MPOA in both female and male mice (Tsuneoka et al., 2015). To determine whether there is a connection between the distribution of preoptic GABAergic neurons and pup-linked activation pattern in the MPOA, we investigated the regional changes of both VGAT+ and pup-induced c-Fos+ cells in both parenting and control animals along the antero-posterior axis as well as differentiating the central, medial and lateral regions of the MPN, and we further examined the medial preoptic area (MPA) which localized outside of the MPN (Figure 1I). We revealed significantly elevated number of VGAT+ cells in the medial subdivision of the MPN in control female mice (p < 0.001), mother mice (p < 0.001), control male mice (p = 0.020) and father mice (p = 0.032) compared to central and lateral regions of the MPN and MPA (Figures 1J1 and 1J2). Interestingly, a medial subdivision-specific significant increase of the pup-induced c-Fos+ cells was observed in mothers (p < 0.001), but not in fathers. In contrast, pup exposure resulted in significantly increased number of c-Fos+ cells in the central subdivision of the MPN of father mice (p = 0.031) (Figures 1K1 and K2). In addition, strong correlation between the number of VGAT+ and c-Fos+ cells was detected along the different subdivisions in mother (r = 0.59, p = 0.001), but not in father (r = 0.16, p = 0.137) mice (Figure 1K3).

Chemogenetic modulation of preoptic GABAergic neurons affects maternal behaviors

To establish the functional role of preoptic GABAergic neurons in parenting we employed designer receptors exclusively activated by designer drugs (DREADD)-based technology for 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 (containing only mCherry but no DREADD sequences) or virus expressing hM4Di or hM3Dq receptor (AAV-hSyn-DIO-hM4Di/hM3Dq-mCherry) into the MPOA of VGAT-Cre transgenic female mice (Figure 2A), for which Cre recombinase expression is restricted to VGAT+ neurons (Figure S1A). To validate 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 S1B and S1D–S1F). 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%). In contrast, 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 S1G). The efficacy of virus infection was examined by comparison of medial preoptic mCherry+ and VGAT+ (Figures S1C–S1F) cell numbers of virgin females. On average, 48.2 ± 2.2% of *VGAT*+ neurons were infected by the virus (Figure S1H).

The impact of chemogenetic activation and inhibition of MPOA VGAT+ neurons (Figure 2A) on the pup-evoked place-preference (Figures 2B and 2C) was examined next. After CNO administration, four animals out of seven animals that received hM4Di virus spent more than 60% of their time in pup-independent cage, whereas all animals that received hM3Dq virus spent more than 60% of their time in the pup-associated cage, which we considered as a threshold value for preference. Control virus-injected animals did not show a preference for either cage (Figure 2B). Virgin females, which received control virus, spent 870.1 ± 27.00 s and 929.2 ± 27.00 s of their time in pup-associated and pup-independent cage,

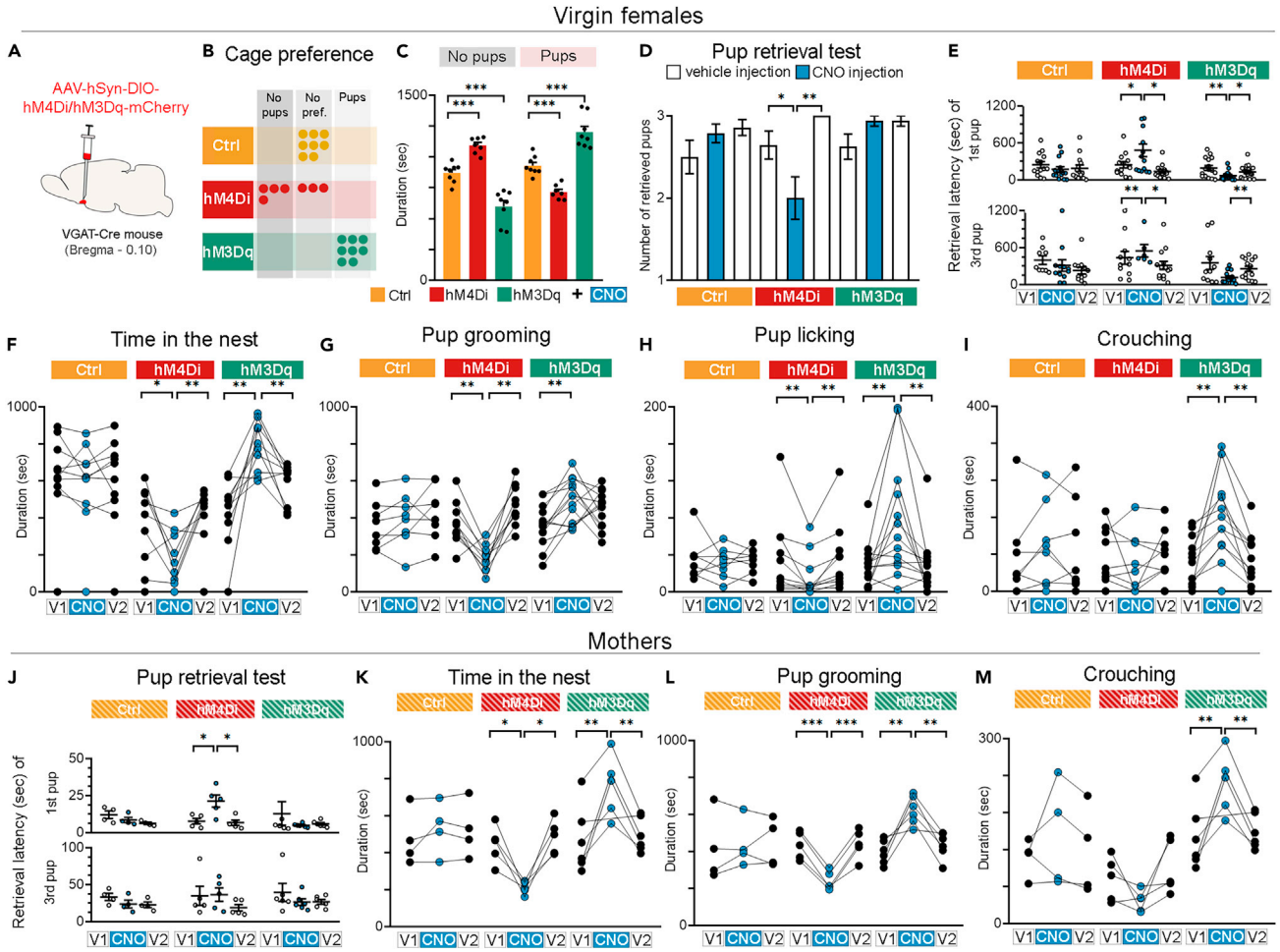

Figure 2. Pup-directed behavior changes after chemogenetic modulation of preoptic GABAergic neurons in virgin female and mother mice

(A) Schematic illustration of viral vector injections 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 (Pups) and animals showed no preference (No pref.). 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) 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) Effect of CNO treatment on latency to retrieve first and third pup in control mothers (Ctrl) (n = 4), hM4Di-injected mothers (n = 5), and hM3Dq-injected mothers (n = 6). Data are presented as mean ± s.e.m., dots represent individual data points, Friedman test followed by Wilcoxon signed-rank test; 0.01 < *p < 0.05.

(K–M) Effect of CNO administration on duration of time in the nest (K), pup grooming (L), and crouching (M) behavior 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 = 16). Repeated measures ANOVA; 0.01 < *p < 0.05, 0.001 < **p < 0.01, ***p < 0.001.

respectively. Inhibition of MPOA VGAT+ neurons resulted in significant preference to the pup-independent cage (1092.6 ± 24.6 s) instead of the pup-associated cage (715.1 ± 22.2 s). In contrast, stimulation of MPOA VGAT+ cells in virgin females resulted in significantly more time spent in pup-associated cage (1201.3 ± 46.7 s) compared to pup-independent cage (598.7 ± 46.7 s) (Figure 2C). We also examined the effects of chemogenetic modulation of MPOA VGAT+ neurons on pup retrieving behavior of virgin females (Figures 2D and 2E). The inhibition of VGAT+ neurons in the MPOA significantly decreased ($p = 0.002$) the number of retrieved pups. In cases of control females ($p = 0.174$) and hM3Dq virus-injected females ($p = 0.224$), there were no significant differences between the numbers of retrieved pups following CNO administration (Figure 2D). Moreover, virgin females with inhibited 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 2E), and spent significantly less time in the nest ($p = 0.030$) (Figure 2F), and with grooming ($p = 0.002$) (Figure 2G) and licking pups ($p = 0.002$) (Figure 2H) 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 2E), spent significantly more time in the nest ($p = 0.003$) (Figure 2F), with grooming ($p = 0.037$) (Figure 2G), licking them ($p = 0.009$) (Figure 2H) and crouching over pups ($p = 0.001$) (Figure 2I). There was no significant difference in the retrieval latency of the first ($p = 0.183$) or third pups ($p = 0.057$) in control females comparing vehicle or CNO injection (Figure 2E). The duration of the time in the nest ($p = 0.368$) (Figure 2F), pup grooming ($p = 0.223$) (Figure 2G), licking the pups ($p = 0.882$) (Figure 2H), and crouching ($p = 0.717$) (Figure 2I) were not affected by CNO treatment in the control group, either.

Similarly, in mother mice, the inhibition of MPOA VGAT+ neurons elevated the retrieval latency of the first pup ($p = 0.022$) (Figure 2J) and decreased the duration of time in the nest ($F(1.152, 4.609) = 25.522$, $p = 0.004$) (Figure 2K) and grooming the pups ($F(1.669, 6.677) = 67.255$, $p = 0.001$) (Figure 2L). After CNO administration, the mothers, which received hM3Dq receptor expressing virus spent significantly more time in the nest ($F(1.638, 8.191) = 25.409$, $p = 0.001$) (Figure 2K), with crouching ($F(1.135, 5.674) = 6.109$, $p = 0.048$) (Figure 2M) and grooming the pups ($F(1.275, 6.376) = 20.121$, $p = 0.003$) (Figure 2L). To determine whether the CNO effect was altered by reproductive stages the behavioral experiments were done in the same animal before pregnancy (virgin) and after giving birth (mother) and found that there were no significant differences in the effect of CNO treatment (Figures S2A–S2D).

We performed correlation analysis to explore if the number of infected neurons influences the magnitude of impact of preoptic GABAergic neurons on maternal behavior. The duration of overall maternal interaction between mothers and their pups positively correlated with the number of infected cells in case of stimulatory virus was used ($r = 0.96$, $p = 0.011$), whereas the correlation was negative when we used inhibitory virus ($r = -0.98$, $p = 0.003$) (Figure S1I). In a separate set of mothers, the repeatability of behavior changes caused by CNO administration was also tested. There were no significant differences between the duration of pup interaction after the first and second CNO treatment (Figure S1J).

Pup-directed aggressive behavior can be elicited by stimulating preoptic GABAergic neurons in males

Next, we examined whether preoptic VGAT+ neurons are involved in the regulation of parental behavior in males. VGAT+ neurons were manipulated in virgin males using the same cell type specific technique as for females. In contrast to the strong effect detected in females, neither inhibition nor excitation of VGAT+ neurons induced significant changes in pup retrieval behavior (Figure 3A). In contrast, chemogenetic activation of VGAT+ neurons in the MPOA evoked pup-directed aggression (Figure 3B), whereas the inhibition resulted in a trend toward increased parenting ($p = 0.07$) (Figure 3C). 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 3D), while the inhibition caused elevated parental care ($p = 0.02$) (Figure 3E).

Depression-like behavior governed by preoptic inhibitory neurons in both sexes

Based on our hypothesis that preoptic inhibitory neurons as main regulators of parenting have a potential role in depression as well, we tested the effect of forced swim test (FST) on neural activity of preoptic VGAT+ cells (Figures 4A–4D). 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%,

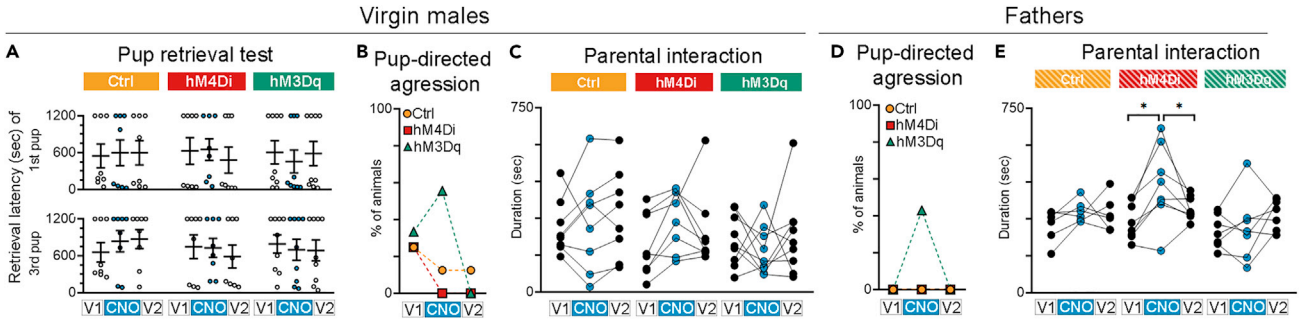

Figure 3. The effect of chemogenetic modulation of preoptic VGAT+ neurons on parenting and aggressive behavior in virgin male and father mice

(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).

$p < 0.000$, respectively) mice after FST compared to the sex-matched control groups (Figures 4E and 4F). *Nota bene*, FST induced significantly higher activation of preoptic GABAergic cells in females than in males ($p < 0.001$) (Figure 4F). 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 4G). 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 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 4H). 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 (Figures 4I–4L). 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 4I, 4J and 4L). 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 4M).

To examine if symptoms of depression other than resilience are also affected by preoptic GABAergic neurons, the hedonic activities following their chemogenetic manipulation was also addressed using sucrose preference test as anhedonia is also known as a core symptom of depression in both rodents and humans (Liu et al., 2018; Rizvi et al., 2017). Indeed, the activation of preoptic VGAT+ neurons resulted in reduced sucrose preference ($p = 0.01$) (Figure 4N), while their inhibition significantly increased the daily consumption ($p = 0.03$) (Figure 4O). Moreover, a strong correlation was found between the duration of maternal care and sucrose preference which further supports the idea neural networks of parenting are at least partly overlapping ($r = -0.5$, $p = 0.04$) (Figure 4P).

Based on the similarities between the activity pattern after pup exposure and FST, we also hypothesized a simultaneous regulatory role of pup-directed behavior and depression-like behavior by preoptic

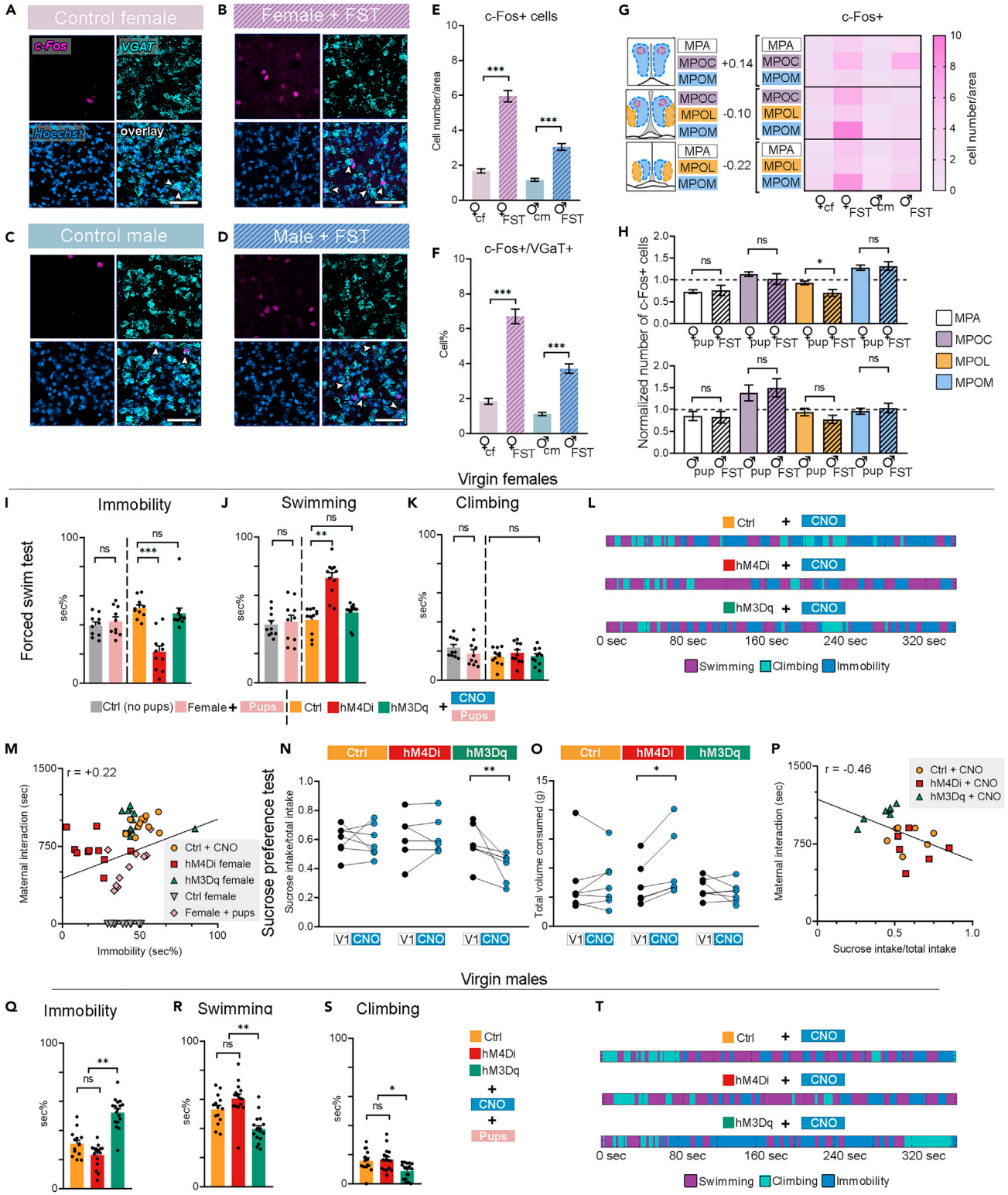

Figure 4. The connection between depression-like behavior and pup-directed behavior under the control of preoptic VGAT + neurons

(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.

Figure 4. *Continued*

(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 μm^2. 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) Representative coding sheets demonstrate the duration of distinct behavior and latency of immobility during the forced swim test of female mice.

(M) 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.

(N) 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.

(O) 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.

(P) 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.

(Q–S) Percentage of immobility (Q), swimming (R) and climbing (S) 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.

(T) Representative coding sheets demonstrate the duration of distinct behavior and latency of immobility during the forced swim test of male mice. Scale bars: 50 μm (A–D).

GABAergic cells in male mice, similar to females. Indeed, chemogenetic 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 FST (Figures 4Q–4T) of male mice after CNO administration.

Overactivation of preoptic GABAergic neurons results in parenting-linked anxiety-like behavior in female but not in male mice

In humans, mothers of young children often feel increased anxiety (Huizink et al., 2017). From animal studies, it has become clear that the high anxiety level is often linked to protective mothering and high maternal motivation (Bosch, 2011). Therefore, 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 level, we performed elevated plus maze (EPM) test following exposure to pups (Figure 5A). 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 5B and 5C). To examine whether pup-induced elevated anxiety level 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 5B and 5C) after exposure to pups and CNO administration. In turn, 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 5D). 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 open field apparatus (12.1 ± 1.8%) (p = 0.04) than control females (28.9 ± 3.1%) (Figure 5F) presented in trajectory maps (Figures 5E1–5E3). We did not observe any difference in either the traveled total distance (Figure 5G) or the velocity (Figure 5H) of the animals during the open field test.

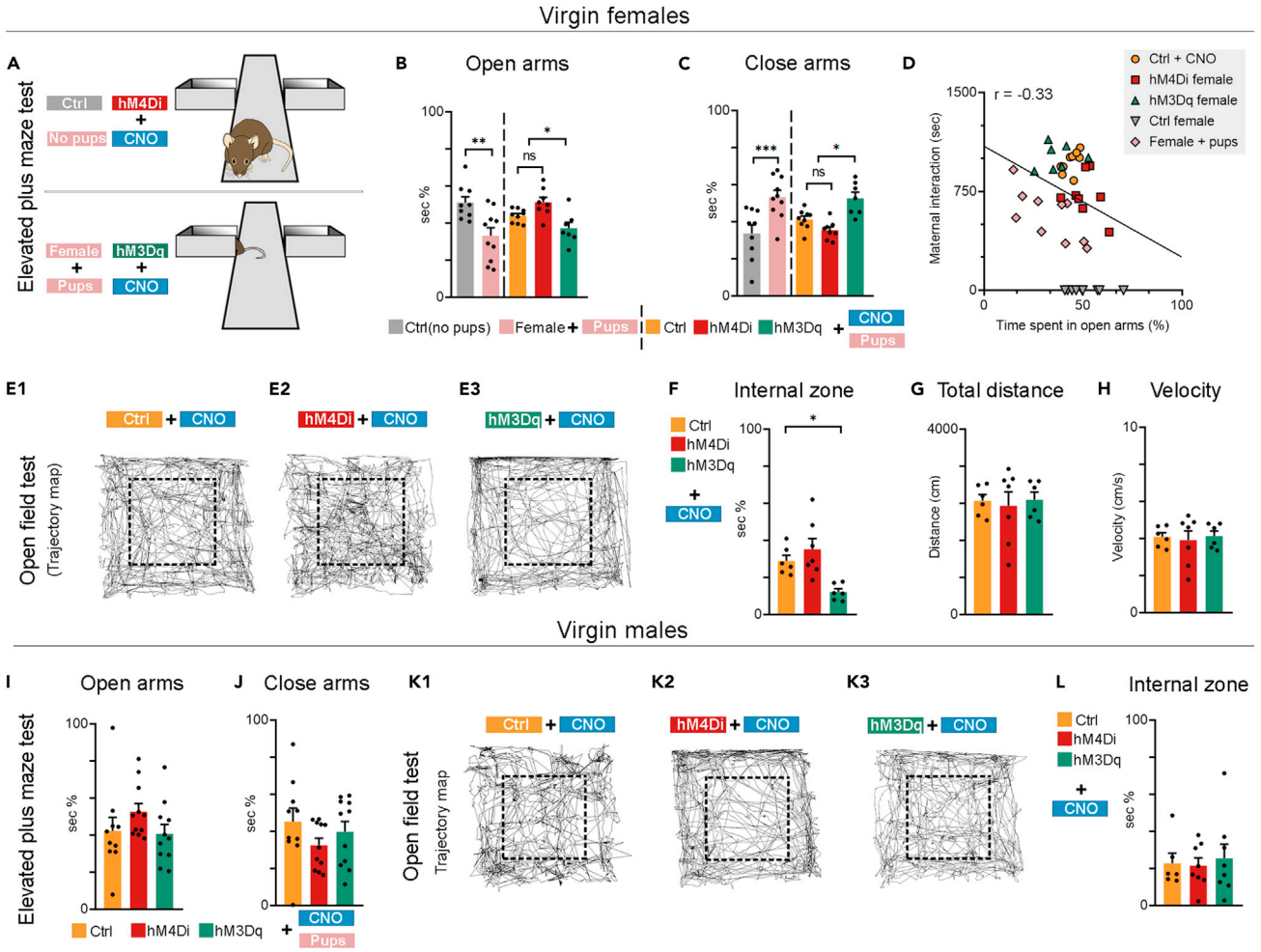

Figure 5. The sex-dependent control of parenting-linked anxiety-like behavior by preoptic VGAT+ neurons in virgin female and male mice

(A) Graphical illustration of the results of elevated plus maze test.

(B and C) Time spent in open (B) and close (C) 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.

(D) 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.

(E) (E1–E3) Representative trajectory 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. Internal zone marked by dashed line.

(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). 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) (K1–K3) Representative trajectory map of control virus-injected (Ctrl) male (K1), hM4Di-injected male (K2), and hM3Dq-injected male (K3) mice after CNO administration in the open field apparatus. Internal zone marked by dashed line.

(L) Time spent in the internal zone of the control virus-injected males (n = 6), hM4Di-injected males (n = 7) and hM3Dq-injected males (n = 6). Mann–Whitney test. Groups are labeled as follows. Ctrl: control virus-injected male mice; hM4Di: hM4Di virus-injected male mice; hM3Dq: hM3Dq virus-injected male mice. 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.

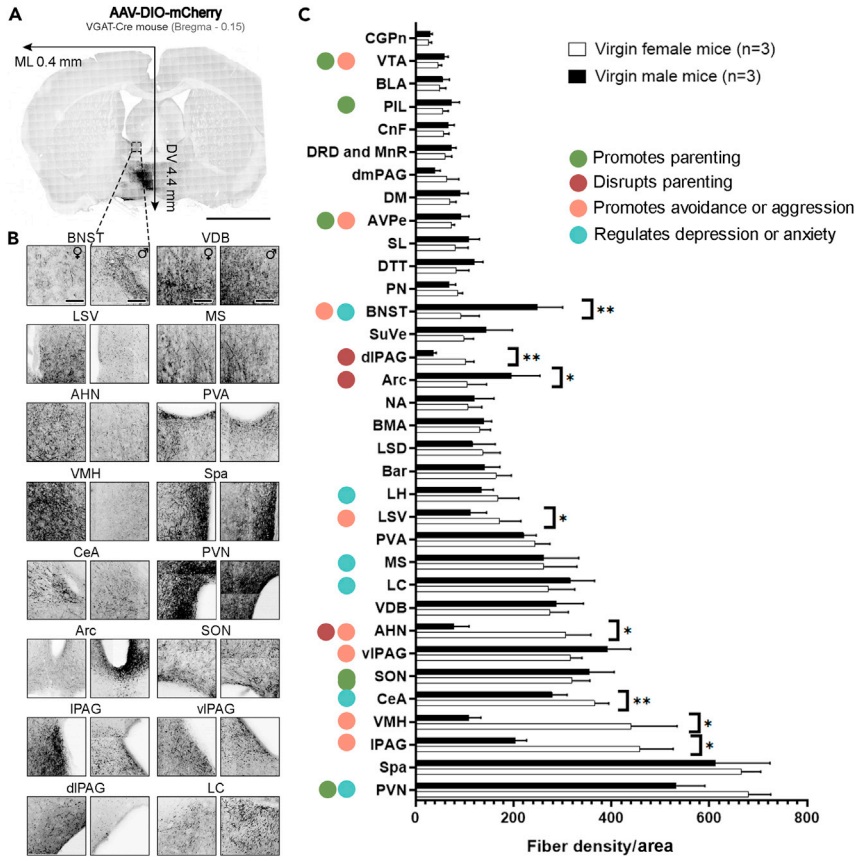

Figure 6. Projections of preoptic VGAT+ neurons

(A and B) Representative confocal micrographs 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 μm^2). Data are arranged in ascending order. Colored dots indicate the revealed function of the projected areas. Data are presented as means + s.e.m. Mann–Whitney test, $0.01 < *p < 0.05$, $0.001 < **p < 0.01$. Abbreviations: PVN = paraventricular nucleus; Spa = subparaventricular zone of the hypothalamus; lPAG = lateral 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; 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 = third ventricle. Scale bars: 2 mm (A), 100 μm (B).

In contrast to virgin females, preoptic VGAT+ neuron-manipulated males did not display changes in anxiety-like behavior either in the EPM test (Figures 5I and 5J) or during open field test (Figures 5K1–5K3 and 5L).

Preoptic GABAergic neurons project brain areas controlling parenting as well as anxiety and depression

To reveal 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 fibers after mCherry-expressing anterogradely spreading virus injection into the MPOA of VGAT-Cre mice (Figure 6A). Immunolabeled fibers were present in a number of brain regions ipsilateral to the injection site. Some contralateral mCherry+ fibers were also present in most target regions but at much lower density

than in the ipsilateral side. 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 found between the sexes in the projections of their preoptic GABAergic neurons to brain areas regulating pup avoidance and aggression, such as anterior hypothalamic nucleus (AHN), ventromedial hypothalamic nucleus (VMH), and periaqueductal gray (PAG) (Figures 6B and 6C). 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 (Figure 6C). In addition, preoptic GABAergic neurons also project to brain regions implicated in anxiety and depression but these projections did not exhibit sexual dimorphism (Table 1). To confirm these findings, we also made a projection map using a peroxidase-based immunohistochemistry technique. Its analysis confirmed the results of the quantitative maps based on fluorescence signal (Figure S3).

DISCUSSION

Although, postpartum neuropsychiatric disorders are major sources of child abuse, only few previous neurobiological investigations attempted to simultaneously examine parenting and anxiety-depression (Maguire and Mody, 2008; Zhang et al., 2021). In this study, we identified preoptic GABAergic cells as an essential neuronal population for mediating pup-directed behaviors and uncovered their simultaneous role in anxiety and depression. Whereas in females silencing of preoptic GABAergic neurons disrupted and overactivation promoted parenting and was also associated with increased anxiety-depression, in males these neurons were rather involved in the regulation of aggression.

First, we confirmed using transgenic VGAT-ZsGreen1 mice that a large portion of preoptic neurons are GABAergic, which has previously been suggested based on GAD67 mRNA expression in both rats and mice (Lonstein and De Vries, 2000; Tsuneoka et al., 2013). In response to pup exposure, a medio-lateral gradient of activation pattern was found within the preoptic area also in agreement with previous data (Tsuneoka et al., 2013). 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. In virgin males and fathers, the activity level was significantly lower and showed different distributional pattern compared to females, in corroboration with previous findings demonstrating increased *c-Fos* expression mostly in the central part of the MPOA in males (Tsuneoka et al., 2015). The ratio of pup-induced c-Fos+ neurons was proportional to the number of VGAT+ neurons in all parts of the female preoptic area, whereas in males, a higher ratio of activated GABAergic neurons was found in the central part of the MPOA. The evenly distributed c-Fos labeling among GABA+ neurons in females suggest that GABA+ neurons are involved in maternal care in all parts of the preoptic area. The increased neuronal activity may be due to neuronal input from the pups (Dobolyi et al., 2014), although hormones may also contribute to neuronal activation in the MPOA (Cao and Patisaul, 2011). Multiple brain regions were reported to project to the MPOA (Chiba and Murata, 1985). Among them, inputs from the amygdala may convey olfactory input from the pups (Chen et al., 2019) evoking caring behavior in females while defensive and withdrawal responses in males. As another example for the promotion of maternal behavior, the posterior thalamus can relay somatosensory input, such as suckling information from the pups (Cservenak et al., 2013).

Although the inputs of preoptic GABAergic cells may provide the basis of the sex-dependent activity pattern, the targets of the same neuronal group allow characterization of potential functional outputs. Analysis of the projection map is in agreement with the hypothesis that one of the main functions of preoptic GABAergic cells is to inhibit the defensive network (Numan, 2006). The projection pattern of preoptic GABAergic neurons was generally similar to that previously described for MPOA neurons (Simerly and Swanson, 1988) suggesting that GABAergic projections constitute a major class of output neurons in the preoptic area. Indeed, it has been recently suggested that the preoptic glutamatergic and GABAergic neurons have similar target areas (Zhang et al., 2021). The most intense projections was found in the paraventricular hypothalamic nucleus, which contains a variety of different cell types, including oxytocinergic, vasopressinergic, corticotropin-releasing hormone-containing, and dopaminergic neurons, which may mediate different aspects of parental adaptations as well as depression-like behavior (Bayerl and Bosch, 2019; Klampfl et al., 2018; Scott et al., 2015; Slattery and Neumann, 2010; Whitley et al., 2020). Another major target area of preoptic GABAergic neurons was the periaqueductal gray. It has long been established that this brain region is responsible for the mediation of kyphosis posture for suckling. Therefore, it is noteworthy that the projection here was more intense in females, which demonstrate suckling. Recently, it has been shown that other aspects of parental behaviors may also be mediated via the periaqueductal gray

Table 1. Summary of the role of preoptic VGAT+ neurons projection areas in parenting and depression

Brain region	Effect on parenting or depression	References
Paraventricular nucleus (**PVN**)	Lesion of PVN disrupts the onset of maternal behavior; neuron loss in the PVN is connected to major depression in humans	Manaye et al. (2005)
Dorsolateral periaqueductal gray (**dlPAG**)	Activation of the dorsal part of PAG depresses maternal behavior, whereas lesion of this area promotes maternal responsiveness	Sukikara et al. (2010)
Lateral periaqueductal gray (**lPAG**)	Activates affective aggression	Canteras (2002)
Ventrolateral periaqueductal gray (**vlPAG**)	Activation of vlPAG suppresses ongoing motivated behaviors and induces defensive behavior	Gross and Canteras (2012)
Ventromedial hypothalamic nucleus (**VMH**)	VMH stimulation induces defensive behavior	Wang et al. (2015)
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	Bridges et al. (1999) and Wang et al. (2015)
Locus coeruleus (**LC**)	Inhibition of LC causes depression-like behavior through decreased noradrenergic signaling	Grimonprez et al. (2015)
bed nucleus of stria terminalis (**BNST**)	BNST lesion inhibits infanticide in male mice; BNST-lesioned animals displays depression-like behavior	Pezük et al. (2006) and Tsuneoka et al. (2015)
Supraoptic nucleus (**SON**)	Pup exposure increases the activity of oxytocin-positive neurons in SON	Okabe et al. (2017)
Medial septal nucleus (**MS**)	glutamatergic neurons in MS exert anorexic effects	Sweeney et al. (2017)
Ventral lateral septal nucleus (**LSV**)	Activation of $GABA_A$ receptors in LSV promotes maternal aggression; inhibition of LSV increases aggression	Wong et al. (2016)
Central amygdala (**CeA**)	Both human and animal studies suggest that CeA regulates anxiety disorders	Gilpin et al. (2015)
Lateral hypothalamus (**LH**)	Lesion of the LH abolishes motivated behaviors, such as food intake or sexual behaviors	Hurley and Johnson (2014)
Arcuate nucleus (**Arc**)	Hunger activated AgRP neurons in Arc inhibit preoptic neurons controlling parenting	Boillot (2019)
Nucleus accumbens (**NA**)	Depressed patients with anhedonia show reduced activation of the NA	Satterthwaite et al. (2015)
Anteroventral periventricular nucleus (**AVPe**)	Stimulation of tyrosine hydroxylase-expressing neurons enhance maternal care in females, while in males suppress inter-male aggression	Scott et al. (2015)
Posterior intralaminar nucleus (**PIL**)	Pup exposure elevates the number of active neurons in PIL	Cservenák et al. (2010)
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	Cohen et al. (2012) and Numan et al. (2009)

The table refers to the most relevant projection areas of preoptic GABAergic neurons regarding their well-known function in parenting, anxiety and depression.

(Zhang et al., 2021). Other brain regions with higher density of inhibitory projections observed in females, such as AHN and VMH play a role in pup avoidance as parts of the rejection circuit (Lee et al., 2014; Li, 2020; Liu et al., 2019; Numan, 2006; Wang et al., 2015). The more intense projections to these sites in females suggest more effective inhibition of pup avoidance in females as compared to males. In addition, the inhibitory projection to the VMH may also be involved in blocking female sexual behaviors as lordosis is mediated by this nucleus (Veening et al., 2014). The potential roles of each projection of preoptic GABAergic neurons are summarized in Table 1.

The functional characterization of the GABAergic neurons was performed by chemogenetics, a method suitable for long-term manipulation of neurons so that parenting and emotional behaviors can be measured immediately after each other in the same animal. The position of the injection site was successfully verified histologically for each animal. A large percentage of DREADD expressing cells were shown to express c-Fos in response to pups in female animals suggesting that the targeted cell group was infected. The ratio of c-Fos expressing DREADD-containing neurons did not depend on the type of virus providing evidence that the stimulation and inhibition affected a similar degree of relevant GABAergic neurons. Functional validation of the chemogenetic manipulations of preoptic GABAergic neurons was provided

by experiments including CNO-induced c-Fos activation in animals expressing the stimulatory DREADD, and also by the disappearance of pup-induced c-Fos expression in neurons containing the stimulated inhibitory DREADD. The repeatability of the CNO-response for both stimulatory and inhibitory viruses demonstrated that the stimulation/inhibition of the preoptic GABAergic neurons was stable and effective. Furthermore, the number of infected GABAergic neurons was proportional to the behavioral response suggesting a similar function of the affected neurons even though local heterogeneity of the inhibitory neurons of the preoptic area is plausible (Moffitt et al., 2018; Tsuneoka et al., 2013). By silencing the preoptic GABAergic neurons, defensive circuits are released from inhibition resulting in more neutral and less caring behavior toward pups. These results also suggest that the presence of pups per se provides substantial stimulation of these cells, and MPOA GABAergic neurons have an endogenous, physiological role in the regulation of maternal behavior. In contrast, the induced activity of preoptic GABAergic neurons evoked marked elevation in different aspects of maternal behaviors and made the animals more vulnerable to depression and anxiety. Although the adaptive effects of parenthood-associated anxiety are known, excessive anxiety, similar to depression occurring in the postpartum period, may have long-term negative effects on the quality of the life of the offspring. Furthermore, a measure of motivational drive using the place preference test (Tzschentke, 2007) conditioned by the pups was also increased by stimulation and decreased by inhibition of preoptic GABAergic neurons suggesting that the activity of preoptic GABAergic cells affected not only the behavioral outputs but also the level of internal motivation.

Based on the results, the activation of preoptic GABAergic neurons by the pups is necessary for maternal behavior, which, however, can also promote depression-like behavior. The increased neuronal activation measured after FST indicated the involvement of the preoptic inhibitory cells in depression-like behavior. Antidepressants reduced the activation of the neurons involved in the regulation of depression-like behavior after FST suggesting the depressive-like effect of the activated cells (Duncan et al., 1996; Silva et al., 2012). Indeed, silencing of preoptic GABAergic neurons resulted in reduced resiliency. Furthermore, one of the most common symptoms of depression is a loss of appetite (Konttinen et al., 2019; Simmons et al., 2016). Increased volume consumption after inhibition of preoptic GABAergic cells highlighted the antidepressant-like effect, while the reduction of sucrose preference induced by overactivation of the same group suggested anhedonia providing further evidence for the involvement of preoptic inhibitory cells in the regulation of depression. The similar activation pattern within the preoptic area by pup exposure and by the FST suggests that overlapping cell populations may be involved in parenting and depression-like behavior. Interestingly, exposure of females to pups, and thereby activating these neurons, was not sufficient to evoke depression-like behavior, which suggests that taking care of the pups normally does not lead to depression, but the neuronal circuit is vulnerable for overstimulation or pathophysiological alterations, which could represent a potential mechanism of postpartum depression. The network is capable of inducing depression-like behavior in males, too. As opposed to the depression-like behavior, anxiety-like behavior was enhanced by not only chemogenetic stimulation of the preoptic GABAergic neurons but also by previous pup exposure suggesting that the presence of pups provides sufficient stimulation for the appearance of basal anxiety-level, which may be adaptive in protecting the pups. Indeed, previous animal studies revealed that high anxiety levels are linked to protective mothering and high maternal motivation (Bosch, 2011).

The opposite effect of manipulating the activity of the preoptic GABAergic neurons was found in males as compared to females, which suggests different roles of these neurons between the sexes. Previous studies identified some preoptic cell types, such as the galanin (Gal) and estrogen receptor alpha (ERα) expressing neurons in the MPOA whose activation increased parenting not only in female, but also in male mice (Wei et al., 2018; Wu et al., 2014). Although, most Gal+ and ERα+ neurons are GABAergic, they represent only a portion of preoptic GABAergic neurons (Cservenak et al., 2017; Wu et al., 2014) suggesting that opposing effect of chemogenetic manipulation of preoptic GABAergic neurons in males and females may be the result of the manipulation of Gal and ERα-negative GABAergic neurons. In addition to the higher number of activated neurons in females, the activation pattern was also different due to the relatively large number of activated neurons in the central part of the medial preoptic nucleus in males. Activation of these cells may be responsible for aggression in male mice. This effect remains present in fathers as well. In fact, these cells could play a role in reduced pup-directed caring behavior of fathers compared to mothers. The role of preoptic GABAergic neurons in aggression is also in line with previous studies, which showed that activation of bed nucleus of the stria terminalis (BNST)-projecting MPOA neurons simultaneously stimulated Gal+ neurons inducing parenting and further GABAergic cells inducing pup-directed aggression resulting in stochastic behavior toward pups in male mice (Tsuneoka et al., 2015).

In conclusion, we identified GABAergic neurons in the preoptic area to increase pup-directed behaviors in females and possibly a separate group of GABAergic preoptic neurons eliciting pup-directed aggression in males. We also demonstrated that GABAergic preoptic neurons responsible for parenting behavior could lead to depression-like behavior if overstimulated.

Limitations of the study

GABAergic neurons within the preoptic area may have different subgroups with potentially different connections and functions, which was not addressed in the present study.

STAR★METHODS

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  - Pup-induced place preference test
  - Spontaneous parental behavior and pup retrieval test of chemogenetically manipulated mice
  - Elevated plus maze test
  - Open field test
  - Forced swim test
  - Sucrose preference test
  - Expression of the c-Fos after exposure to pups
  - Expression of the c-Fos after forced swim test
  - Sample preparation for immunohistochemistry
  - Fluorescent immunolabeling
  - Peroxidase immunohistochemistry
  - Microscopy
  - Anterograde tracing
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Image analysis and cell counting
  - Analysis of projections

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103090.

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AUTHOR CONTRIBUTIONS

DD participated in the design of the experiments, in performing the laboratory work, interpretation of the results and the literature, and writing of the manuscript. GP did some of the histological labeling and analysis, VS performed pup-induced behavioral tests and some c-Fos activation studies, ES participated in virus injections, DZ participated in virus injections, design of the experiments, interpretation of the results, and

correction of the manuscript. AD participated in the design of the experiments, interpretation of the results and the literature, and writing of the manuscript.

DECLARATION OF INTERESTS

There are no competing interests for any author.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chicken Anti-mCherry Antibody	Abcam	Cat# AB205402; RRID: AB_2722769
Mouse Anti-NeuN Antibody	Merck Millipore	Cat# MAB377; RRID:AB_2298767
Rabbit Anti-c-Fos Antibody	Santa Cruz@Biotechnology	Cat# SC-166940; RRID:AB_10609634
Alexa Fluor 594 AffiniPure Donkey AntiRabbit IgG	Jackson@ImmunoResearch	Cat# AB_2340621; RRID:AB_2340621
Alexa Fluor 594 AffiniPure Donkey AntiChicken IgY	Jackson@ImmunoResearch	Cat# AB_2340377; RRID:AB_2340377
Alexa Fluor 488 AffiniPure Goat AntiMouse IgG	Jackson@ImmunoResearch	Cat# AB_2338840; RRID:AB_2338840
Biotin-SP (long spacer) AffiniPure Donkey AntiChicken IgY (IgG)	Jackson@ImmunoResearch	Cat# AB_2313596; RRID:AB_2313596
Bacterial and virus strains		
pAAV-hSyn-DIO-hM3D(Gq)-mCherry	Krashes et al., 2011	Addgene AAV2; 44361-AAV2
pAAV-hSyn-DIO-hM4D(Gi)-mCherry	Krashes et al., 2011	Addgene AAV2; 44362-AAV2
Experimental models: Organisms/Strains		
Mouse: VGAT-IRES-Cre knock-in mice (*Slc32a1*$^{tm2(cre)Lowl}$/J)	The Jackson Laboratory	Stock No: 016962
Mouse: Ai6(RCL-ZsGreen (B6.Cg-*Gt(ROSA)26Sor*$^{tm6(CAG-ZsGreen1)Hze}$/J)	The Jackson Laboratory	Stock No: 007906
Software and Algorithms		
ImageJ	M. D. Abràmoff, P. J. Magalhães, and S. J. Ram. Image Processing with ImageJ. 2004	https://imagej.nih.gov/ij
IBM SPSS Software	IBM Corp. Released@891 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.	https://www.ibm.com/analytics/spss-statistics-software
Solomon Coder	Peter A. Solomon Coder (Version Beta: 17.03.22): A Simple Solution for Behaviour Coding. 2017	https://solomon.andraspeter.com
QuPath	Bankhead et al., 2017	https://qupath.github.io
Other		
Hoechst 33342 Solution	Thermo Fisher Scientific	Cat# 62249
VECTASTAIN® Elite ABC-HRP Kit, Peroxidase	Vector Laboratories, Burlingame, CA, USA	Cat# PK-6100
DAB Substrate Kit, Peroxidase (HRP), with Nickel, (3,3'- diaminobenzidine)	Vector Laboratories, Burlingame, CA, USA	Cat# SK-4100
Aqua-Poly/Mount	VWR	Cat# 87001-902
DePeX mounting medium	Sigma-Aldrich	Cat# 06522

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Arpad Dobolyi (dobolyi.arpad@ttk.elte.hu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data reported in this paper will be shared by the lead contact upon request.
- No custom code was used in the analysis of the data.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Procedures involving mice were carried out in accordance with the Hungarian Ministry of Agriculture's Animal Hygiene and Food Control Department guidelines for experimental protocols (PEI/001/37-4/2015) and with EU Directive 2010/63/EU for animal experiments. The experiments were implemented with the permission from the Ethical Board of Eötvös Loránd University (ELTE MÁB 02/2014).

The offspring (12 females and 10 males) of VGAT-IRES-Cre and GtROSA26Sor_CAG/ZsGreen1 mice (The Jackson Laboratory; stock numbers: 016962 and 007906) were used for histology analysis of pup exposure experiments and forced swim test (aged 10-16 weeks). The expression of VGAT was verified by comparing the expression pattern of ZsGreen fluorescent protein to that of GAD2 in GAD2-IRES-Cre mice in the Allen Brain Atlas. For behavior tests and further histological analyses, we used VGAT-IRES-Cre transgenic mice (8-16 weeks at the start of experiments). Based on the previous publications, neither health issue, nor behavioral deficit was reported in the mouse line, in which Cre recombinase was inserted into the vesicular GABA transporter (VGAT) locus (Vong et al., 2011). Similarly, no behavioral or health issue was found in VGAT-Cre mice crossed with reporter mice. Mice were maintained on a 12h light/dark cycle with lights on at 7am at 22 $\pm$ 1°C. Food and water were available ad libitum unless otherwise specified.

Viral vectors

We employed Cre-dependent adeno-associated viral vectors for chemogenetic activation (AAV2-hSyn-DIO-hM3Dq-mCherry; Addgene, cat. #44361-AAV2) and inhibition (AAV2-hSyn-DIO-hM4Di-mCherry; Addgene, cat. #44362-AAV2) as well as control viral vectors (AAV2-hSyn-DIO-mCherry; Addgene, cat. # 50459-AAV2) developed and used previously successfully (Krashes et al., 2011).

METHOD DETAILS

Animal surgeries

VGAT-IRES-Cre mice 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 placed in a stereotaxic frame (Leica Biosystems). 20 nl of AAV-hSyn-DIO-hM4Di/hM3Dq-mCherry virus was injected per side at a flow rate of 57 nl per min into the MPOA 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) using nanoinjector (Nanoinject2010 by WPI). After the injection was completed, the glass capillary was held still for 10 min to allow the diffusion of the virus. Then, glass capillary was withdrawn slowly. Animals were allowed to recover for 2–3 weeks from surgeries before subsequent behavioral tests.

Chemogenetic manipulation

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

Behavior tests

At least two weeks after surgeries, about a week prior to tests the mice were placed into new cages and kept individually. All experiments were performed in the first stage of the light phase under controlled light (60 LUX) and temperature (22 $\pm$ 1°C) conditions. 45 min before the experiments, mice were intraperitoneally injected with vehicle (0.5% DMSO solution) or CNO-injection (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 manually and 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 a Table S2.

Pup-induced place preference test

The experiments were performed using three groups of animals: the control group received control virus (AAV-hSyn-DIO-mCherry), while the second and third groups were injected by AAV-hSyn-DIO-hM3Dq-mCherry and AAV-hSyn-DIO-hM4Di-mCherry viruses, respectively. The conditioned place preference apparatus consists of two chambers (55 cm x 40 cm x 27 cm) connected in the middle with a removable transparent tube. The two chambers have different wall textures (one with a wide red ribbon, the other without that). For additional sensory cues, we placed two different objects into the chambers (an orange round bowl into the signed-wall one and a purple square shaped bowl into the other one). 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. We varied the combination of cues as well as the location of pups to make sure that any potential bias by the apparatus is counteracted. On the testing day, animals were intraperitoneally injected by CNO-solution 45 min prior to the test. During this phase, the pup-associated chamber and non-pup-associated chamber were connected with a tube to allow free access to the entire apparatus. At the beginning of the test, the experimental mice were placed into the pup-associated chamber to allow free exploration in the whole apparatus for 30 min. The behavior of the animals was video-recorded, and the time spent in each chamber was determined using Solomon Coder software.

Spontaneous parental behavior and pup retrieval test of chemogenetically manipulated mice

10-16 weeks old virgin females and males were housed individually for about a week before the test. 24 hours prior the behavior assay paper dumplings were placed into the cage for building nest. Each virgin animal was tested with three 3-6 days-old pups. Behavior assays were stopped if the adults hurt the pups. The pregnant mice were separated from males before parturition and kept together with their infants. On P3-P6, after removing the litters from the mother, three of the pups were re-introduced into the home cage and pup retrieval and parental behavior were measured for 30 min. Father mice lived together with the pregnant females during delivery and the postpartum period, so the fathers were exposed to their pups. 12 hours prior to the test, fathers were separated from their offspring and then three of the pups were re-introduced into the home cage of the fathers and both retrieving and parental behavior were recorded for 30 min.

The test period took 5 days for each animal. To confirm the repeatability of the effect elicited by the CNO administration, an additional animal group was used and tested for 9 days (Table S2). 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). The experimental protocol allows self-controlled as well as between groups evaluation. In addition, the repeated experiments demonstrated that the effect induced by CNO can be eliminated and repeated. 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. Return of the adult to the cage marked the beginning of the assay. All observations were recorded in the same room, at the same time of the day, using a camera located laterally to the clear Plexiglass cages. Recorded videos were analyzed by Solomon Coder software.

The following behaviors were scored in 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 of attack the pups), parental interaction (duration of the cumulative time spent grooming and licking the pups in the males).

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).

Elevated plus maze test

The elevated plus maze is a widely used behavioral assay for anxiety behavior of mice. 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. For this purpose, we used individually housed (separated from their littermates two days prior to the test) VGAT-Cre virgin females and individually housed VGAT-Cre females after 30 min exposure to pups. 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 behaviors were recorded and the video was analyzed by Solomon Coder. The following behaviors were scored: time spend and number of entries in the open and closed arm, frequency to enter into all arms. After each trial, the maze was cleaned with 70% ethanol and then with dry wipe.

Open field test

The open field test is another commonly used test to measure the activity as well as the anxiety-like behavior of mice. The open field box was made from polypropylene (40 x 40 x 60 cm). The animals were intraperitoneally injected by CNO or vehicle. 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 both measured by SMART program. The amount of time in the center and periphery of the arena was analyzed manually and by the SMART program, too. After each experiment, the open field arena was cleaned with 70% ethanol and then with dry wipe.

Forced swim test

The forced swim test is commonly used for the measurement of resiliency component of depression-like behavior. 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. For this purpose, we used individually housed (separated from their littermates two days prior to the test) VGAT-Cre virgin females and individually housed VGAT-Cre females after 30 min exposure to pups. Each experimental mice (virus-injected females, VGAT-Cre females, which had no interaction with pups, VGAT-Cre females after exposure to pups and virus-injected males) were placed in a transparent cylindrical tank (made from glass, 20 cm height x 15 cm diameters) for 6 min. The water level was 15 cm from the bottom and the temperature of the water was 25 $\pm$ 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 by Solomon Coder from a video recording.

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 (2nd day 8 AM to 4th day 8 AM). On the 4th day, we removed all bottles from the home cage from 8 AM to 8 PM. Each mouse was intraperitoneally injected by vehicle injection (DMSO solution) 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 8 PM, each mouse got an intraperitoneal injection, but in this case, all mice were injected with CNO. At 8 PM, 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.

Expression of the c-Fos after exposure to pups

10-16 weeks old control females, control males, experimental females and experimental males were placed into fresh-bedding containers and kept individually for two days before the perfusion (control females and males) or pup exposure (experimental females and males). 3-6-day-old VGAT-Cre pups were used in the experiment. On the day of the test, five pups were introduced to the home cage of experimental females and males for 30 min. Mother animals were kept with their pups constantly from parturition to transcardial

perfusion (they were not separated) to reveal the natural response of preoptic GABAergic neurons to motherhood. In 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, which took place 3-6 days after the birth of the pups. Control females and males were anesthetized and perfused two days after separation. In case of experimental virgin animals, 90 min after the introduction of the pups, all animals were anesthetized and transcardially perfused, while mothers and fathers were anesthetized and perfused 3-6 days after the birth of pups. The measurement of cell numbers was performed in fields, which were randomly selected. All the different subdivisions of the MPOA, shown in Figure 1I. In Figures 1C–1H, were represented in a way that equal number of pictures were selected from each subdivision.

Expression of the c-Fos after forced swim test

10-16 weeks old control females, control males, experimental females and experimental males were placed into fresh-bedding cages and kept individually two days before the perfusion (control females and males) or forced swim test (experimental females and males). On the test days, all experimental animals were exposed to the forced swim test described above (STAR Methods: Forced swim test). 90 min after the start of the test all experimental animals were anesthetized and perfused. Control animals were anesthetized and perfused two days after separation.

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.

Fluorescent immunolabeling

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 S1) 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. After several washes in TB, sections were mounted onto glass slides and coverslipped using mounting medium (Aqua-Poly/Mount).

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 S1) 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 more 2 hours. Then, the brain sections were incubated in 3,3′-diaminobenzidine (DAB) with 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 (Sigma).

Microscopy

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. Images were adjusted using the ZEN Software. Full resolution of the images was maintained until the final versions, which were adjusted to a resolution of 300 dpi.

Anterograde tracing

To reveal the projection map of preoptic VGAT+ neurons, we used 4-4 female and male VGAT-IRES-Cre mice. The mice were anesthetized with ketamine/xylazine-hydrochloride in 0.9% saline (16.6 and 0.6 mg/ml,

respectively, 10ml/kg body weight i.p.) mixture and injected 20 nl of AAV-hSyn-DIO-mCherry unilaterally into the MPOA (anteroposterior: +0.6 mm from Bregma; ventral: 5.4 mm from dura; medio-lateral: 0.4 mm from midline). Four weeks after the surgery, mice were deeply anesthetized and perfused transcardially with saline followed by 4% PFA. The brains were postfixed in 4% PFA for 24 h and then stored in 0.1 M phosphate-buffer until sectioning into three series of 40 μm sections on a vibratome (Leica). Two series (every 2 out of 3 sections) were immunolabeled for mCherry to map the anterograde projections using DAB immunoperoxidase and fluorescent signal, respectively.

QUANTIFICATION AND 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 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 (for example in 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. We used Pearson correlation test to measure linear correlation between two variables. 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 $p \leq 0.05$ ($0.01 < *p < 0.05$, $0.001 < **p < 0.01$, $0.001 > ***P$)

Image analysis and cell counting

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 as well. The colocalization between two signals were determined manually. The cell number was determined by adding the number of counted cells on all the sections. All processes were performed by experimenters blind to the sex and the treatment of the animals.

Analysis of projections

After immunolabeling, 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) (Bankhead et al., 2017) to evaluate the fluorescent immunoreactivity signal. Machine learning based pixel classification feature was applied to automatically measure the area density of the anterogradely labelled nerve fibers in the distinct regions across the whole brain. For statistical analysis, Mann-Whitney test was used.