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First-response cells initiating experience-induced maternal care behavior in virgin female mice

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Zusammenfassung

Mütterliche Fürsorge ist ein vielschichtiges Sozialverhalten, das für das Überleben und die Fitness des Nachwuchses wesentlich ist und eine entscheidende Rolle für deren kognitive, emotionale und soziale Entwicklung und ihrem Wohlbefinden spielt. Dieses evolutionär konservierte Verhalten wird durch hormonelle Veränderungen während der Schwangerschaft unterstützt, die das mütterliche Gehirn auf die Ansprüche der Versorgung des Nachwuchses vorbereiten. Trotz des Fehlens von neuro-endokrinen Veränderungen, die mit der Schwangerschaft einhergehen, zeigen sexuell unerfahrene weibliche Mäuse Fürsorgeverhalten, wenn sie mit neugeborenen Jungen konfrontiert werden. Diese Studie untersucht den anterior cingulate cortex (ACC) in dieser Form der erfahrungsinduziertern alloparentalen Fürsorge bei sexuell unerfahrene weiblichen Mäusen unter Verwendung des Targeted Recombination in Active Population (TRAP) Systems zur genetischen Markierung aktivierter Neuronen. Unsere Ergebnisse zeigen, dass alle kortikalen Schichten des ACC bei sexuell unerfahrenen weiblichen Mäusen durch Kontakt mit neugeborenen Mäusejungen stärker rekrutiert werden als bei postpartalen Weibchen. Wir validierten das TRAP2-System, indem wir die neuronale Aktivierung in der medial preoptic area (MPOA) und dem bed nucleus of the stria terminalis (BNST) analysierten: Regionen, die bekanntermaßen an der Vermittlung mütterlicher Fürsorge beteiligt sind, aber nicht selektiv bei erfahrungsinduzierter alloparentaler Fürsorge aktiviert werden. In Übereinstimmung mit früheren Studien fanden wir, dass sowohl BNST als auch MPOA auf Säuglingsreize reagieren, ihre Aktivierung jedoch nicht spezifisch Fürsorgeverhalten bei sexuell unerfahrenen Weinchen. Unsere Ergebnisse unterstreichen die wichtige Rolle des ACC in der alloparentalen Fürsorge und bieten eine Grundlage für die Identifizierung und Charakterisierung der Neuronenpopulationen, die dieses Verhalten initiieren.

Abstract

Maternal care is a multifaceted social behavior essential for the survival and fitness of offspring, and it plays a critical role in their mental development and well-being. This evolutionarily conserved behavior is driven by hormonal changes during pregnancy, which prime the maternal brain for caregiving. Despite the absence of neuro-endocrine changes associated with pregnancy, sexually inexperienced female mice can demonstrate parenting behavior, as form of alloparental care, which is triggered by exposure to pups and enhanced by experience. This study investigated the anterior cingulate cortex (ACC) in experience-induced alloparental care in virgin mice using the Targeted Recombination in Active Populations (TRAP) system for genetic tagging of activated neurons. Our findings reveal that all cortical layers of the ACC are more extensively recruited in virgin females compared to postpartum females. We validated the TRAP2 system by analyzing the medial preoptic area (MPOA) and the bed nucleus of the stria terminalis (BNST), regions well-known for their involvement in mediating maternal care, but not selectively activated by experience-induced alloparental care. Consistent with previous studies, we found that while both BNST and MPOA respond to infant cues, their activation is not specific to parenting behavior in virgins. Our results highlight the critical role of the ACC in alloparental care and provide a foundation for identifying and characterizing the neuronal populations initiating this behavior.

Acknowledgement

I would like to express my deepest gratitude to my supervisor, Univ. Prof. Dr. Daniela D. Pollak, for her expert guidance, constant encouragement, and invaluable insights throughout the course of my studies.

Special thanks to Jhun, our animal caretaker, whose assistance ensured the smooth running of the experiments. I would also like to extend my heartfelt thanks to Maureen and Astrid for their crucial roles in keeping the lab running efficiently.

I am forever grateful to my colleagues for creating a warm and supportive environment in our lab: Anna, Laura, Kathi, Karin, Orsi, Kira, Annabelle, and Carina. I truly appreciate your friendship and collaboration.

Introduction

Parental care is a multicomponent social behavior that is critical for the survival and fitness of the offspring. It offers an opportunity to safeguard the young while also preparing them for an autonomous life. This deeply ingrained evolutionary behavior, found across diverse species, plays a pivotal role in fostering the mental development and well-being of the young.

The significance of parental care becomes evident in instances where this behavior is absent or deficient. Inadequate maternal care leaves a lasting mark on the children's lives, especially during critical or sensitive developmental periods. According to longitudinal studies carried out on both human subjects and laboratory animals, neonatal chronic stress exposure through maternal neglect was shown to lead to the dysfunction of endogenous stress response systems, changes in the mesolimbic dopamine system and altered gene expression profiles due to unbalanced histone acetylation patterns [1, 2, 3] . These alterations greatly influence the offspring's long-term mental and physical health.

As such, children growing up without sufficient parental interactions are prone to stress-related disorders, such as anxiety and depression. Neglected children are reported to face a greater risk of developing effective disorders and changes in social behavior [4] . Moreover, a staggering 80% of reported cases of child maltreatment in the United States are linked with major depressive disorder (MDD) [5] .

Most mammalian species exhibit a uniparental care arrangement, wherein mothers assume exclusive responsibility for nurturing the offspring, and male involvement is rather rare. While all mammalian fathers contribute to the development of the offspring by indirect genetic and epigenetic routes, only 3-5% of the species exhibit paternal care, predominantly the species living in social monogamy [6] .

An individual who assumes a parental role for the offspring, distinct from the mother or father, is referred to as an alloparent. This care arrangement is present in less than 3% of mammalian species, and includes humans (*Homo sapiens*), mice (*Mus musculus*), marmosets (*Callitrichidae*) and tamarins (*Saguinus*) [6] .

Alloparental care is observed widely in humans: Taking care of grandchildren, and participation in daycare or educational settings are everyday examples. Adoption, which involves inherent challenges, is another aspect of this phenomenon. Given that mice readily exhibit alloparental care [7] , they offer a viable model organism for studying the neuronal pathways underlying this behavior.

The impact of maternal care on rodent offspring

Maternal care behavior consists of highly conserved, dynamically regulated components [8] . In rodents, this behavior typically involves activities such as nest building, grooming, nursing and protection of the young [9] . Mothers create a safe and warm environment by constructing nests to provide insulation and protection from predators [10] . Grooming involves cleaning of the pups' fur, which not only maintains hygiene but also stimulates circulation and promotes bonding between mother and offspring [11] . Through nursing, mothers provide essential nutrients and warmth to their young [12] . Maternal rodents also exhibit vigilant behaviors to protect their offspring from potential threats [13] . When a pup strays from the nest or becomes separated from the litter, the mother responds by actively searching for and bringing the pup back to the nest [14] . Because of its great reproducibility, pup retrieval is commonly used in research as a proxy for maternal care behavior [15–18] .

In mammals, including rodents, variations in the offspring's behavior manifest based on the quality of maternal care received during early development. For instance, male laboratory rats (*Rattus norvegicus*) demonstrate distinct phenotypes based on the levels of licking/grooming (LG) provided by mothers during the postnatal period [11]. Offspring experiencing low LG are more susceptible to long-term elevated plasma corticosterone levels following stress exposure. They exhibit decreased exploration of new environments [19] , heightened fearfulness [20] , and deficits in learning and memory [21] compared to those receiving high LG. This indicates that early somatosensory stimulation can profoundly shape stress responsiveness and emotional reactivity to novelty.

Female offspring of rat dams with low levels of LG similarly exhibit significant behavioral alterations later in life. These females demonstrate decreased maternal behavior [22] , likely perpetuating a cycle of low LG across generations. Additionally, they display increased sexual behavior [1].

The impact of maternal care on the behavioral phenotype of offspring is mediated through the hypothalamic-pituitary-adrenal (HPA) axis [11]. Downstream epigenetic modifications lead to distinct gene expression patterns in pups exposed to varying levels of maternal tactile stimulation, with low and high levels of care resulting in different behavioral phenotypes [23] . For example, offspring of high LG dams show reduced plasma adrenocorticotropic hormone and corticosterone responses to acute stress and decreased levels of hypothalamic corticotropin-releasing hormone messenger RNA [11] . Overall, these findings highlight the critical role of

maternal care in shaping stress responses and behavioral outcomes in offspring through epigenetic regulation and HPA axis modulation.

The neuroendocrine background of maternal care behavior

The regulation of maternal care is considered a neuroendocrine-controlled process. Hormones from the pituitary gland and other peripheral endocrine tissues (including the ovaries, adrenal glands, and placenta) are essential in providing feedback to central nervous system sites, particularly within the hypothalamus [24] .

In certain species like rats, when virgin females are presented with pups, they exhibit avoidance behavior rather than maternal care [25] . It has been shown that hormonal events during pregnancy support the mother's motivation towards caring for the pups and weaken the neural circuitry responsible for avoidance and defense [26] . A depiction of this phenomenon can be seen in *Figure 1*.

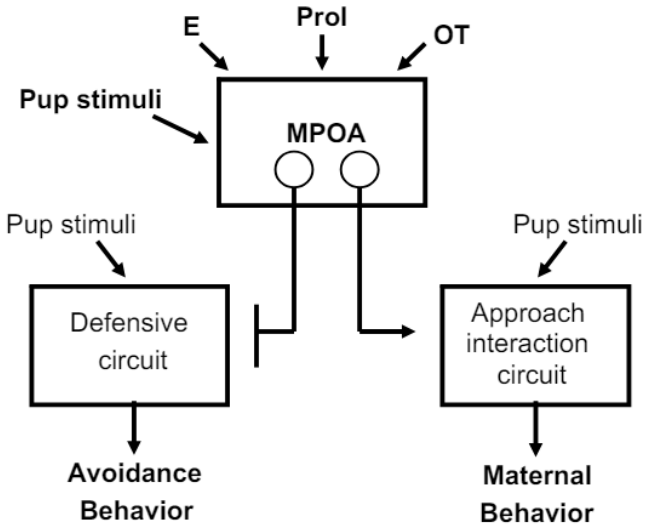

Figure 1. Medial preoptic area interactions with dopamine neural systems control maternal behavior in rats.
Hormones such as estradiol (E), prolactin (Prol) and oxytocin (OT) increase the responsiveness of the MPOA to pup stimuli, inhibit the defense circuitry and strengthen approach circuitry. [27]

The avoidance/defense circuitry as depicted in *Figure 2A* involves the olfactory bulb (OLF), the medial amygdala (MeA), the anterior hypothalamic nucleus (AHN) and the periaqueductal grey (PAG). Olfactory pup cues are relayed towards the MeA through the olfactory bulb. The MeA, an area strongly involved in fear-related behaviors [28] , subsequently projects to the

anterior hypothalamic nucleus and information is then relayed to the PAG to initiate defense behavior toward the pups [6] . Hormonal changes during pregnancy result in modulation of the activity of this circuitry, preventing mothers from exhibiting pup aversive behavior.

In the opposing circuitry depicted in *Figure 2B* the medial preoptic area (MPOA) at the rostral region of the hypothalamus is known as a central hub for the control of maternal care behavior. Several experiments involving lesions of the MPOA have found that such damage completely abolishes maternal behaviors, such as nest building and pup retrieval [29,30]. In contrast, electrical stimulation of the MPOA has been shown to enhance maternal responsiveness in rats [31].

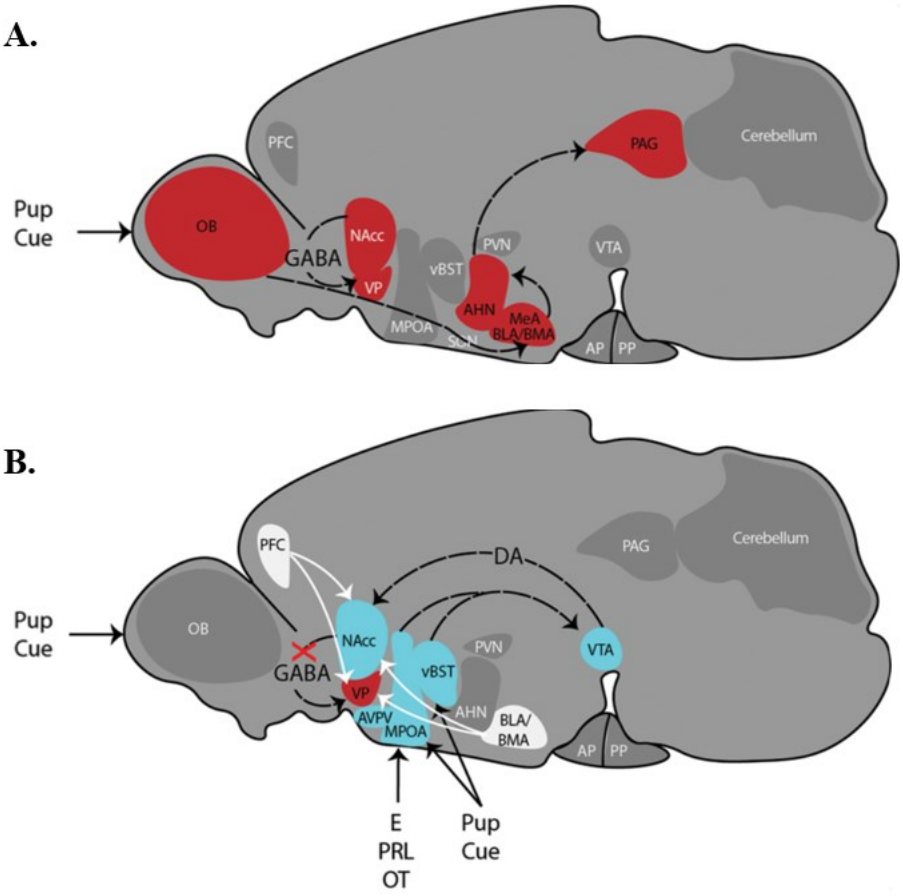

Figure 2. Neural circuitry underlying avoidance and approach towards pups in the rodent brain.
A. *Pup cues activate avoidance and defense circuitry in virgin female rats* ***B.*** *Pup cues activate approach and care behavior in postpartum rats. (Abbreviations: AHN = anterior hypothalamic nucleus; AP = anterior pituitary; BLA = basolateral amygdala; BMA = basomedial amygdala; E = estradiol; MeA = medial amygdala; MPOA = medial preoptic area; NAcc = nucleus accumbens; OB = olfactory bulb; OT = oxytocin; P = progesterone; PAG = periaqueductal gray; PFC = prefrontal cortex; PP = posterior pituitary; PRL = prolactin; PVN = paraventricular nucleus of the hypothalamus;*

SON = supraoptic nucleus of the hypothalamus; vBST = ventral bed nucleus of the stria terminalis; VP = ventral pallidum; VTA = ventral tegmental area)[6]

The central role of the MPOA in maternal responsiveness is further underscored by its high expression of receptors for pregnancy-related hormones and opioids crucial for parenting modulation [32]. Estradiol, progesterone and oxytocin throughout pregnancy and parturition act on the MPOA and dampen the avoidance/defense circuitry while strengthening the circuitry for attraction and acceptance toward pup cues [27].

In the circuitry responsible for maternal care, sensory information from pups reaches the MPOA and the adjacent ventral bed nucleus of the stria terminalis (BNSTv). These regions stimulate dopaminergic neurons located in the ventral tegmental area (VTA), which project to the nucleus accumbens (NAcc), thereby recruiting the core pathway associated with motivation and reward-related processes. GABAergic projections from the NAcc towards the ventral pallidum (VP) are inhibited in mothers, allowing for approach behavior. Further information on pup cues is relayed through the basolateral/basomedial amygdala (BLA-BMA) and the prefrontal cortex (PFC) to the NAcc and VP, allowing for a more intricate control of maternal behavior [27,33].

Experience-induced alloparental care in virgin females

It is important to note that while hormones prepare the maternal brain for interaction with pups, non-hormonal factors also significantly influence these behaviors. Interestingly, maternal care can manifest in the absence of the neuroendocrine changes associated with pregnancy [7]. Prolonged exposure to pup stimuli leads to the development and enhancement of pup-directed behaviors in nulliparous rodent females, a phenomenon called sensitization. In the hallmark study of Rosenblatt et al. non-pregnant intact, ovariectomized and hypophysectomied female rats showed complete maternal behaviors after repeated exposure to pups. This study underscored that while hormonal changes during pregnancy expedite positive responses towards pups, they are not indispensable [34].

In other species, like mice, even nulliparous females that have not experienced mating, pregnancy or parturition show nearly immediate maternal behaviors [7,16] . Another seminal study conducted by Stolzenberg et al. demonstrated that while postpartum females are typically faster to retrieve their pups to the nest and spend more time tending to them than maternal virgin females, by the fourth day of pup exposure the quality of maternal care was comparable between the two groups as depicted in *Figure 3* [7] .

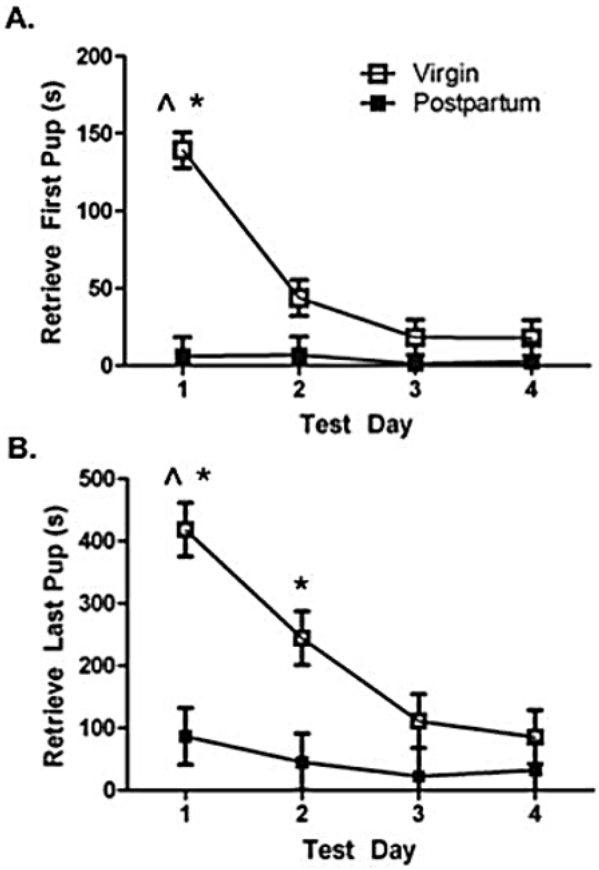

Figure 3. Latency (seconds) to the onset of maternal behavior across test days 1–4. ***A)*** *Latency to retrieve the first pup on test days 1–4.* ***B)*** *Latency to retrieve all pups on test days.* ** Significantly different from the postpartum group. ^ Significant trend across test days.* [7]

These findings suggest that while a fundamental neural framework for maternal behavior exists, this system is heavily dependent on pup cues and can be activated even in the absence of pregnancy-related hormones. Although recent studies have explored the circuitry behind experience-induced maternal behavior, the specific cell population responsible for the first-time response, inducing this behavior remains unidentified.

Rationale and aims of the project

A recent study of our lab suggested that a cortical region could function as an auxiliary component of the maternal care circuitry, initiating and later, mediating pup-induced alloparental care in virgin female mice. The experimental design of the study comprised of virgin females and foster mother mice exposed to pups during the pup retrieval test on three consecutive days. The change in the neural activation patterns from the first day to the last day of exposure was investigated, paralleling the experience-induced improvement in pup retrieval. We discovered that the anterior cingulate cortex (ACC), a subregion of the prefrontal cortex, showed the most significant change in neural activation selectively in virgin females interacting with pups, with the level of activation gradually decreasing over time [16] .

Chemogenetic manipulations further confirmed the importance of this region in maternal behavior in virgin female mice: Activation of the ACC significantly reduced pup retrieval latencies compared to the control groups while inhibition of the same area led to longer pup retrieval times, as shown in *Figure 4* [16] .

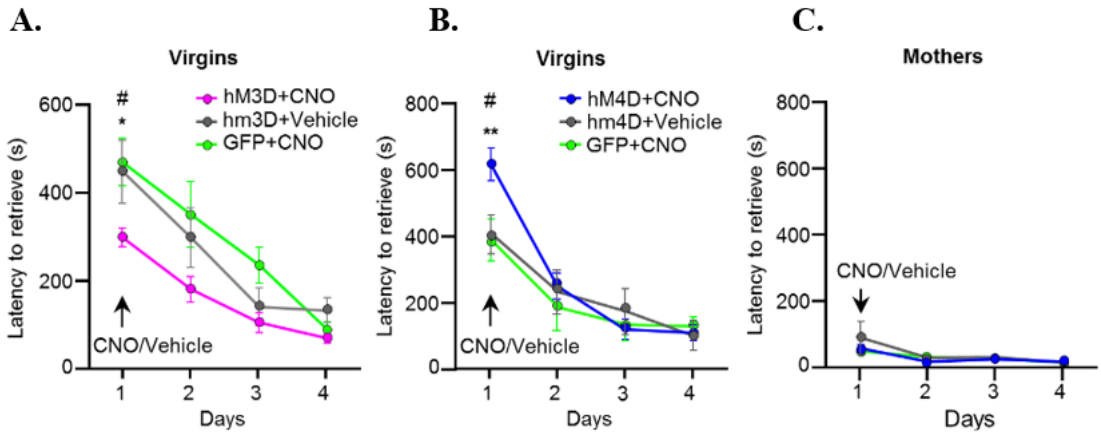

Figure 4. Effects of chemogenetic activation/inhibition of the ACC on pup retrieval latency
A. *Upon activation of the ACC by hM3D construct, virgins retrieve pups faster.* ***B.*** *Upon inhibition of the same area by the hm4D construct latency to retrieve pups increased.* ***C.*** *Chemogenetic manipulations of the ACC did not affect pup retrieval latency in mothers significantly.* [16]

Further investigations revealed that a thalamic subregion, the intralaminar nucleus (ILM), exhibited activation pattern changes mirroring those of the ACC in the same experimental setup, suggesting its involvement in alloparental behavior and its experience-dependent modulation. It has been shown that galanin expressing neurons in the central lateral nucleus of the thalamus (CL) preferentially target excitatory neurons in the ACC during the initiation of pup-induced

maternal behavior in virgin females, shedding light to an accessory prefrontal cortex-thalamus circuitry to the already well-established maternal care pathways [16].

Such work in uncovering the neural circuitry underlying experience-induced maternal behavior is important, as to support therapeutic approaches dealing with maternal care disruptions, such as those experienced by mothers suffering from postpartum mental disorders where bonding with and responsiveness to the child´s needs are impaired [35]. Alleviating the negative effects of conditions such as postpartum depression, anxiety and attachment disorders, all of which greatly impact both the mother and the offspring is thus an important sociomedical topic with relevance beyond generations. Therefore, in this project, we set out to provide the basis for characterizing and molecularly defining the neuronal population responsible for the onset of alloparental behavior in virgin mice. Building on our previous research that highlighted the role of the prefrontal-thalamic feedback circuitry, we focused our investigation on ACC as the main region of interest.

For our ongoing investigations, which involve permanent tagging of activated cells during our behavioral experiments, we opted to use the TRAP2 system [36]. For our downstream studies, we require such a tagging system rather than using immunohistochemical methods, as (although not within the scope of this thesis) our future goal is to investigate the molecular characteristics of TRAPed cells in virgin female mice during pup retrieval using advanced single-cell RNA sequencing technologies.

The TRAP2 construct, presented in *Figure 5*, is a novel tool designed for genetic tagging of activated cells in mice. TRAP stands for "Targeted Recombination in Active Populations," and is based upon using a tamoxifen-inducible Cre recombinase (CreERT2) under the control of the immediate early gene (IEG) promoter cFos. The proto-oncogene c-Fos is expressed in neurons in response to direct stimulation by growth factors and neurotransmitters, and it has been traditionally used as a marker for neuronal activation [37].

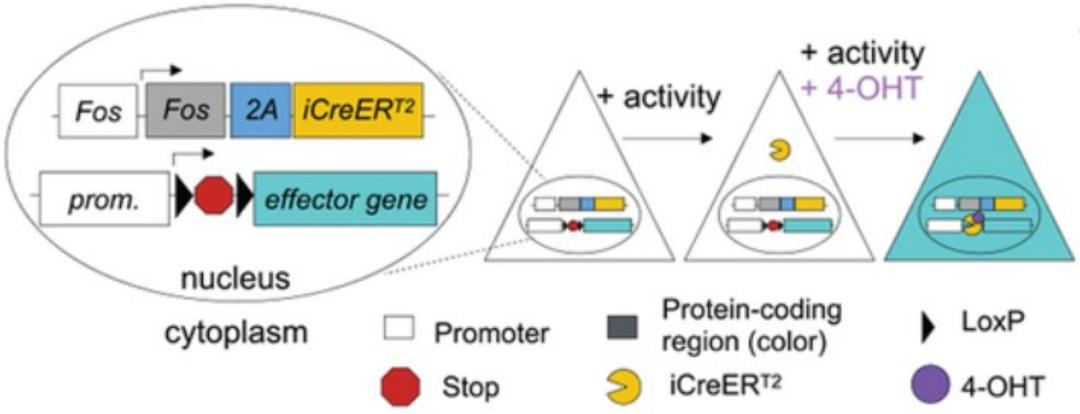

Figure 5. Depiction of the mechanism behind TRAP2 genetic tagging system. *The reporter gene is expressed by the co-incidence of drug availability and neuronal activation.* [36]

When neurons are activated, these IEG promoters drive the expression of Cre recombinase, which, in the presence of tamoxifen translocates to the nucleus and induces recombination at loxP sites. TRAP2 mice were crossed with homozygous tdTomato mice, containing a loxP-flanked STOP cassette followed by the gene for tdTomato. Upon co-incidence of neuronal activation and tamoxifen administration, CreERT2 mediates the excision of the STOP cassette, allowing the expression of the red fluorescent protein tdTomato (= TRAPed cells). If the drug is not present, and the neuron is activated, the Cre recombinase will be expressed but cannot enter the nucleus, therefore no reporter gene is expressed. Based upon this rationale, TRAP2 permanently tags activated cells in the whole brain and allows great temporal precision for tracing neural activation through the timing of the drug injection [36] . Instead of tamoxifen, here we are using its active metabolite, 4-hydroxytamoxifen (4-OHT), which was shown to shorten the TRAPing window around drug injection [38] .

In our attempt to identify the cell population initiating alloparental behavior in virgin female mice, we aimed to examine the neuronal activation of the ACC in greater detail. Specifically, we investigated whether there was a layer-specific activation pattern within the ACC to determine if the responding cell population is restricted to specific layers for targeted selection in later studies. To do so however, we first needed to validate the TRAP2 in our experimental set-up, ensuring that the TRAP signal corresponded to the previously observed pattern of neural activation in cFos antibody staining. To this end we interrogated two brain regions that are key players in maternal care behavior but did not show specific activation in virgin females in our previous study.

Materials and Methods

Subjects

Original breeding pairs of TRAP2 (Fos2A-iCreERT2, JAX mice stock #030323) and Ai14 (here referred to as tdTomato, Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J), JAX mice stock #007914) were obtained from Jackson Laboratories. Transgenic TRAP2tdTomato mice were obtained by breeding homozygous TRAP2 males with homozygous tdTomato females. All animals were kept under a 12:12 h light-dark cycle at 21-24 °C room temperature and 45-65% humidity, with ad libitum access to food and water. Cages were regularly changed once a week. All animal experiments were approved by the national ethical committee of animal care and use in Austria and international laws and policies concerning the laboratory animals were followed adequately. All experiments were planned and conducted to minimize the suffering and keep the number of animals at the minimum level.

Pup retrieval assay

Adult female mice were single housed in standard cages for a week before the onset of experiments. Behavioral testing was conducted during light-phase. Four P4 pups provided by a donor mother were individually placed into the female's home-cage on the side opposite to the nest. Virgin female mice were presented with either four foster pups or four novel objects (Lego® blocks, 1.6 cm X 1.1 cm). Mothers were presented with their own pups. To successfully complete the test, all pups had to be retrieved to the nest in 15 minutes [16] .

Experimental groups

To determine the brain areas engaged in parental behavior during pup retrieval, the following groups were used to include relevant controls for this study.

- Virgin females exposed to pups
- Mothers exposed to pups
- Virgins exposed to novel objects (LEGO® bricks)

Drug preparation and administration

4-hydroxytamoxifen (H6278, Sigma-Aldritch) was first dissolved in absolute ethanol with a concentration of 20 mg/ml at 40 °C. After dissolution, corn oil was added, and ethanol was removed in a vacuum chamber, resulting in a final concentration of 5 mg/ml in corn oil. The drug was intraperitoneally administered less than 10 minutes after the behavioral test, in a concentration of 50 mg per kg of the animal [36] .

Tissue preparation and DAPI staining

Animals were perfused 10 days after behavioral experiments to allow for full expression of the TRAP signal. All subjects were deeply anaesthetized using an intraperitoneal injection of Ketamine (25 mg/mL), Xylazine (10mg/mL) and Sodium chloride (0.9%/mL) and perfused transcardially with 20 mL phosphate-buffered saline (Thermo Fisher Scientific; Massachusetts, USA) followed by 20mL of 4% paraformaldehyde in PBS.

After careful removal of the skull, the brain was transferred into fresh 4% PFA and stored at 4 °C for 24 hours, continued by storage at room temperature in 30% sucrose for 24 hours. Brains were frozen in O.C.T (ScigenTM; Thermo Fisher scientific; Massachusetts, USA) using liquid nitrogen. Brains were cryo-sectioned (Leica® cryostat, Germany) into 50 µm free-floating sections at -20 °C and collected in ethylene glycol based cryoprotectant.

Cryoprotectant was washed away from the 50 µm free-floating slices with 3 changes of PBS. Staining of nucleic acids was performed using DAPI (4′,6-diamidino-2-phenylindole; Sigma D9564, Missouri, USA). Slices were incubated in 1x PBS containing 1:2000 DAPI for 5 minutes.

Microscopy and analysis of labelled cells

Slices were mounted and fixed onto glass slides and covered with cover slips (VWR® Superfrost® Plus Micro Slide). Images were taken on fluorescence imaging microscope with a 20x objective (Zeiss®, Carl Zeiss AG; Oberkochen, Germany).

Images were processed by QuPath and the regions of interests identified according to the ABBA workflow (https://biop.github.io/ijp-imagetoatlas/). The number of tdTomato expressing cells and DAPI+ cells have been counted using ImageJ Fiji software.

Statistical analysis

All statistical analysis was performed using GraphPad PRISM 10. Two-way ANOVA was performed as variance analysis. Correction for multiple comparison analysis was provided by Bonferroni test. The level of statistical significance was set at $P < 0.05$.

Results

Layer-specific analysis of the anterior cingulate cortex confirms the previously defined selective activation of the ACC in response to experience-induced maternal care behavior in virgin female mice in the TRAP2-TdTomato tagging system.

The ACC has been confirmed to play a pivotal role in experience-induced maternal behavior in virgin mice [16,18] . Here, we asked the question whether there was a specificity to cortical layers within the ACC involved in this behavior, which could help narrow down the search for the relevant cell populations. We presented virgin female mice post-partum females with pups for 15 minutes each day on three consecutive days. To account for neuronal activation possibly due to a novelty response, we included a group of virgin mice exposed to objects for the same duration. Active cell populations were permanently tagged by the expression of a red fluorescent protein tdTomato using the TRAP2 system, as depicted in *Figure 6*.

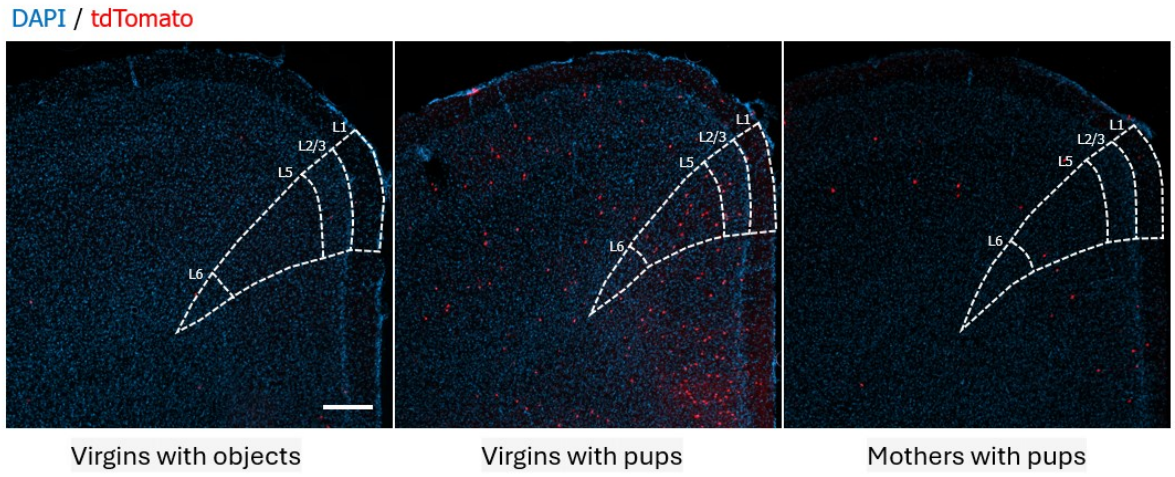

Figure 6. Representative fluorescence pictures of the ACC layers in coronal brain sections. *Dashed lines show the outline of analyzed cortical layers, with tdTomato positive cells marked in red and DAPI positive cells in blue. Images were obtained by a 20x objective, scale bar indicates 250 um.*

We compared the percentage of TRAPed/DAPI-labeled cells in Layers 1, 2/3, 5, and 6 across all experimental groups (n=4 animals per group) as shown in *Figure 7*.

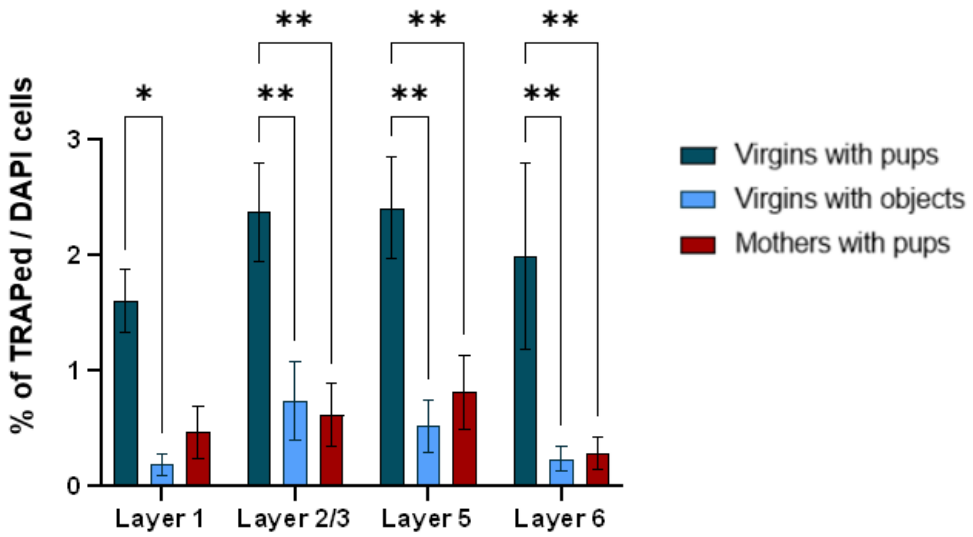

Figure 7. Percentage of TRAPed cells in each layer of the anterior cingulate cortex. *The figure shows Bonferroni's multiple comparisons test results (*P < 0.05, **P < 0.01) of the percentage of TRAPed cells in each layer of the ACC through all experimental groups: virgins with pups (n=4), mothers with pups (n=4) and virgins exposed to objects (n=4).*

The two-way ANOVA identified a significant effect on ACC activation from the experimental group ($F(2, 36) = 27.26$, $P < 0.0001$), however, no significant effects were found for the ACC layers or the interaction between layers and experimental group ($P = 0.2033$ and $P = 0.9703$, respectively).

To explore differences in activation levels across experimental groups, Bonferroni's multiple comparisons test was performed. We found that virgin females exposed to pups exhibited significantly higher neuronal activation compared to mothers and the control group presented with objects. This is in line with our previous observation of the ACC being selectively activated during virgin alloparental care [16].

As revealed by the post-hoc analysis, the percentage of TRAPed/DAPI cells was significantly higher in virgins with pups than in mothers for Layer 2/3 ($P = 0.0039$), Layer 5 ($P = 0.0097$), and Layer 6 ($P = 0.0052$), but not for the most superficial Layer 1, where it was short of statistical significance ($P = 0.0930$). The control group for novelty showed a significantly lower expression of the reporter gene compared to virgins retrieving pups in all layers: Layer 1 ($P = 0.0247$), Layer 2/3 ($P = 0.0078$), Layer 5 ($P = 0.0019$), and Layer 6 ($P = 0.0041$).

Validation of the TRAP2-TdTomato tagging system confirming brain regions not selectively activated by experience-induced maternal care behavior in virgin female mice

The MPOA is the well-established central hub for initiation and execution of maternal behaviors in postpartum females [39]. Together with the BNST, they integrate hormonal and pup-related sensory cues and relay information to the dopaminergic reward system to facilitate approach and care for pups. We asked the question if these two major actors in maternal responsiveness had a role in initiating alloparental care in our experimental paradigm. To achieve higher resolution, we analyzed the ventral, dorsal and posterior regions of the BNST separately.

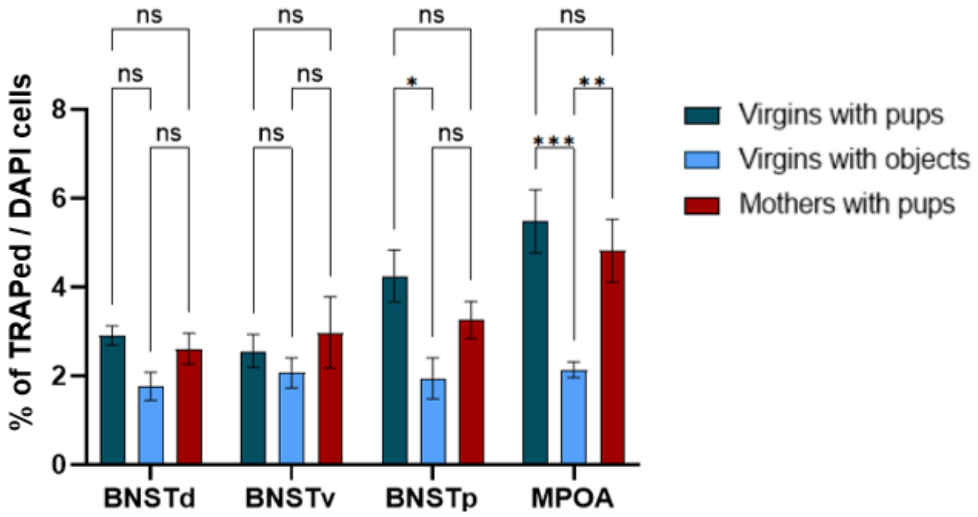

Figure 8. Percentage of TRAPed cells in the BNST and MPOA
*The figure shows Bonferroni's multiple comparisons test results (*P < 0.05, **P < 0.01, ***P < 0.001) of the percentage of TRAPed cells in the dorsal, ventral and posterior bed nuclei of the stria terminalis (BNSTd, BNSTv, BNSTp) and the medial preoptic area (MPOA) through all experimental groups: virgins retrieving pups (n=6), mothers retrieving pups (n=5) and virgins exposed to objects (n=4).*

Two-way ANOVA revealed a significant effect of experimental conditions on neuronal activation levels, with $F(2, 45) = 12.41$ and $P < 0.0001$. Additionally, the brain region analyzed showed a significant effect as well, with $F(3, 45) = 7.112$ and $P = 0.0005$. The interaction between experimental conditions and brain areas was not significant ($F(6, 45) = 1.611$, $P = 0.1663$).

Bonferroni's multiple comparisons test uncovered significant differences in the percentage of TRAPed cells in the posterior BNST and MPOA, while no such differences were observed in the dorsal and ventral BNST regions. In the BNSTp, virgins retrieving pups exhibited significantly higher activation compared to virgins presented with objects ($P = 0.0269$). However, no significant differences were found between virgins and mothers retrieving pups (P

= 0.4542). While the level of activation was higher in mothers than in the control group for social novelty, the difference was not significant (P = 0.2015).

In the MPOA, virgins with pups showed significantly greater activation compared to virgins presented with objects (P = 0.0001), while virgins with objects showed significantly lower activation compared to mothers with pups (P = 0.0026). The level of activation in the MPOA in virgins and mothers retrieving pups was comparable (P = 0.5995), as expected. All results are depicted in *Figure 8*.

Discussion

The TRAP2Tdtomato system is a suitable tool for investigating alloparental care behavior

This study aimed to provide the framework for the identification of the specific neuronal populations within the anterior cingulate cortex responsible for the onset of alloparental behavior in virgin female mice. To this end, we established the use of the TRAP2 system for our expermental design for genetic tagging of activated cells and explored the neuronal activation patterns in the ACC layers to validate the TRAP2 system by examining brain regions well known in the maternal care circuitry but not selectively activated in experience-induced alloparental care. As such, we included the medial preoptic area (MPOA) and the bed nucleus of the stria terminalis (BNST) in our analysis.

Our previous research proved the significance of the entire ACC in relation to alloparental care through immunohistochemical methods [16] , which align with our layer-specific results, serving as a positive control for the TRAP2 system. We also experimentally approached the possible involvement of the MPOA and BNST on first response to pup exposure in virgin female mice. Our results showed no significant differences in activation levels between the experimental groups in the dorsal and ventral BNST, and no specific activation related to alloparental care in the posterior BNST and MPOA. This corroborates with our previous experiments using immunohistochemical methods [16] , and thereby serves as negative control to further validate the TRAP2 tagging system.

Notably, both the posterior BNST and the MPOA exhibited sensitivity to pup-related cues, proving that these regions are specifically tuned to the detection and processing of stimuli related to offspring [27] , which is critical for the regulation of all types of parental care.

The TRAP2 model used in our research presents a mix of advantages and drawbacks that require careful consideration. A key strength is the ability to permanently TRAP activated neurons across the entire brain with precise temporal control around the time of drug injection [36,38] . This provides researchers with the flexibility to design experiments for any behavioral paradigm.

It is important to consider how the drug used to induce recombination in the TRAP2 model might affect the results. Tamoxifen, a nonsteroidal antiestrogen commonly used to treat and prevent estrogen-dependent breast cancer [40] , acts through its active metabolite 4-OHT. This metabolite can interact both as agonist and antagonist with estrogen receptors, depending on the receptor's composition [41]. Since estrogen plays a key role in maternal behavior by

modulating anxiety and approach towards pups [27,42] , the question arises whether injecting 4-OHT might impact alloparental behaviors. A recent study tested TRAP2 virgin female mice for maternal responsiveness a week after 4-OHT or oil injection and found no effect of 4-OHT on any measured aspect [43]. Moreover, they found that endogenous estradiol did not interfere with the TRAPing process, as it is likely selectively driven by a mutated estrogen receptor [43]. These findings together with our results confirm the applicability of the TRAP2 system for studying alloparental care in virgin female mice.

One notable limitation is the use of a single IEG (c-Fos) as an indirect indicator of neuronal activity. Although c-Fos expression is known to be induced by both neuronal and synaptic activity in vivo [44] , IEGs in general do not consistently respond to depolarization [45], which could potentially affect accuracy of our data.

Anterior cingulate layers 2-6 are selectively activated in alloparental care in virgin female mice

We utilized the TRAP2TdTomato system to determine whether the activated cells within the ACC are confined to a specific layer, which would provide additional insights into the identity of the involved cell population. Virgin female mice exhibited significantly higher activation levels across all cortical layers during pup retrieval compared to the novelty control group, indicating that the ACC's involvement is specific to pup-related cues. Neuronal activation in virgins was markedly higher than in mothers retrieving pups in all cortical layers, although significance was not reached in the dorsal most layer 1.

The anterior cingulate cortex, as the rest of the neocortex, exhibits a well-organized laminar structure, each layer with distinct cytoarchitecture as described by Brodmann [46]. While this laminar organization was originally defined by anatomical features, recent research has revealed that thousands of genes exhibit expression patterns that further separate these layers, suggesting a more intricate functional role for each [47]. Additionally, various cell subtypes, which are likely to localize to specific layers [47], could communicate with different areas of the brain.

Layer 1, or the molecular layer, contains a sparse population of neurons primarily involved in local projections and is composed mainly of axons and dendrites from neurons sitting in deeper layers [46,48] . In contrast, layers 2 through 6 are populated by neocortical excitatory cells (ECs). The exception is layer 4 in the ACC, which is present only in the visual, auditory, and somatosensory cortices of the mouse [49]. The ECs, which make up about 80% of all cortical

neurons, are further classified based on their axonal projection targets as reviewed by Kenneth D. Harris and Gordon M. G. Shepherd [50]. For example, intratelencephalic ECs in layers 2 through 6 receive inputs from local ECs, other cortical areas, and the thalamus, and project to local neurons, contralateral cortical areas, and the striatum. Pyramidal tract neurons in Layer 5 receive input from local ECs, the thalamus, and other cortical areas, projecting mainly to subcortical and subcerebral regions such as the brainstem, tectum, spinal cord, thalamus, and basal ganglia. Meanwhile, cortico-thalamic neurons in Layer 6 receive inputs from both higher and lower-order cortical areas and deep-layer local ECs, primarily projecting to the thalamus [50].

Considering our results, the near significance observed in layer 1, combined with the notably higher activation levels across all other layers in the ACC of virgin females, suggests that multiple cell types and subclasses likely contribute to the initiation of alloparental care. This indicates that the ACC's role in alloparental behavior is complex and involves the coordination of multiple brain regions. Previous research has highlighted an important connection with the central lateral nucleus of the thalamus (CL) [16] , but more work is needed to fully understand the entire alloparental circuitry.

While the ACC's role in initiating alloparental care in virgin females is already established, its involvement in parental care behavior extends to both mothers and fathers as well. A study in rats demonstrated that lesions in the ACC disrupted maternal care [51]. Moreover, recent research in mice revealed a sex-dependent activation pattern in the ACC during pup retrieval by both parents. Chemogenetic silencing of excitatory ACC neurons disrupted pup retrieval in mothers during early postpartum days but had no such effect on fathers [18] . Integrating these findings with our results suggests that the ACC's role in caregiving is fundamental, but different neural circuits may be activated depending on both sex and reproductive state.

Research focused on the ACC in the last decades highlighted its diverse roles in cognitive and social functions, such as cost-benefit calculations [52] , motivation and decision-making [53,54] .The ACC evaluates factors like success probability and effort needed to determine how intensely a behavior should be pursued [53] . Parental care behavior is evolutionarily costly: while essential for offspring survival in most mammalian species, it demands constant vigilance and effort from the parent or alloparent and requires diverting resources from oneself to the offspring. One could argue that such a decision may be already facilitated in hormonally primed mothers, which could account for the higher percentage of activated cells in the ACC of non-mother females.

Given its robust functional and anatomical connectivity to both the limbic system and prefrontal cortical areas, the anterior cingulate cortex serves as a critical hub for integrating social cues [55–58]. Empathy, the ability to understand and adopt the feelings of other conspecifics is essential for successful social interactions and was historically thought of as an exclusively human trait. Behavioral studies in animals, however, indicate that they do exhibit empathy-like behaviors, all of which seem to be mediated by the anterior cingulate cortex. Rodents in particular show observational fear learning [55] , social transfer of pain and analgesia [56] and they console their partners after stress exposure [57] . When pups are separated from their mothers, they emit distinct ultrasonic vocalizations that serve to communicate their distress [58] . Considering the ACC's capability to discern the affective states of others, and its observed responsiveness in humans to infant cries [59] , we hypothesize that an upsurge in empathy-like processes in virgin females may be required to initiate caregiving behaviors towards the pups.

To date, the molecular characteristics, genetic profile, and distinct connectivity of cells activated in virgin females but not in mothers in response to first-time pup exosure, therefore responsible for the induction of alloparental care, remain unknown. The current work laid the methodological foundation for future studies utilizing advanced techniques such as single-cell RNA sequencing and connectomics to uncover these details. This knowledge may then pave the way for the development of novel therapeutic strategies for enhancing bonding and care giving behaviors under pathological conditions to mitigate the detrimental effects weighing on both mothers and their offspring.

Abbreviations

4-OHT	4-Hydroxytamoxifen
ACC	anterior cingulate cortex
AHN	anterior hypothalamic nucleus
BMA	basolateral amygdala
BNST	bed nucleus of stria terminalis
BNSTd	dorsal bed nucleus of stria terminalis
BNSTp	posterior bed nucleus of stria terminalis
BNSTv	ventral bed nucleus of stria terminalis
CL	central lateral nucleus of thalamus
CreERT2	tamoxifen-inducible Cre recombinase
EC	excitatory cell
HPA	hypothalamic-pituitary-adrenal axis
IEG	immediate early gene
ILM	intralaminal nucleus
LG	licking / grooming behavior
MDD	major depressive disorder
MeA	medial amygdala
MPOA	medial preoptic area
Nacc	nucleus accumbens
OLF	olfactory bulb
PAG	periaqueductal grey
PFC	prefrontal cortex
TRAP	targeted recombination in active populations
VP	ventral pallidum
VTA	ventral tegmental area

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