Sex-dependent neural activity underlying parenting behavior in mice

A Dissertation Presented by

Alberto Corona

to

the School of Biological Sciences

at

Cold Spring Harbor Laboratory

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy in Biological Sciences

Cold Spring Harbor Laboratory

April 2022

“The scariest moment is always just before you start.”

-Stephen King

Acknowledgements

First, I would like to thank my research mentor Dr. Stephen Shea. In many ways, you have shown me what it means to be a good mentor. Your kindness is an inspiration for me to always be supportive of trainees. Since the start of my doctoral training, you gave me the intellectual freedom to explore the questions that excited me. Of course, you always provided guidance and support along the way. Honestly, you were always too nice. Most importantly, your work ethics and respect for others is remarkable and part of a legacy that I would like to take with me. I want to thank you for all of the long weekly meetings to talk about science and life in general. It has been a privilege knowing and learning from you, as well as experiencing your optimism, drive, and excitement in the lab. After working with you, I know that your science and lessons will be an inspiration to those fortunate enough to one day work and learn from you.

I want to acknowledge former and current members of the Shea lab for always providing a fun environment to do science in. Dr. Alexandra Nowlan, Alexa Pagliaro, Dr. Roman Dvorkin, Dr. Yunyao Xie, Jane Choe, Dr. Luqun Shen, Dr. Deborah Rupert, and Dr. Fernanda Duque Mendoza thank you for helpful conversations and lab meetings. Clancy Kelahan, special thanks to you for setting up the fiber photometry rig in the Shea lab. I would also like to thank all of my classmates from the 2016 entering class: Luqun Shen, Shaina Lu, Briana Bibel, Christopher Krasniak, David Johnson, Martyna Sroka, Olya Spassibojko, Ran (Rena) Yan, Kathryn O'Neill, and Chengxiang (Charlie) Yuan.

I also want to thank my thesis committee members for all of their support during my training. Dr. Jessica Tollkuhn, thank you for agreeing to be the chair of my committee and for providing insightful feedback at critical points during my training. Dr. Dave Jackson, thank you for being a great academic mentor and always checking on my progress. Also, nice try attempting to convince me that plants have brains too and thanks for all of the good career advice over a cup of coffee or a nice cold beer at Blackford Bar. Dr. Pavel Osten, special thanks to you for the great support with the brain-wide activity screen that, without you, would not have been possible. Dr. Sandeep Robert Datta, thank you for taking the time to be my external examiner.

I want to thank everyone in the Cold Spring Harbor School of Biological Sciences for always supporting all of our student needs. Dr. Alyson Kass-Eisler, thank you for being a time wizard and always making time for the students. Dr. Alex Gann, thank you for the funny speeches during recruitment weekend and all those Dean's teas. Kimberly Creteur, thank you for all of the exciting recruitment activities. Going to ABRCMS with you every year made my time at CSHL more enjoyable. I will miss recruiting with you. Kimberly Graham, thank you for all of the work you do for the students. I will always be grateful for your time and patience scheduling my committee meetings. Dr. Monn Monn Myat, thank you for your help with my outreach activities at LaGuardia Community College during my PhD training.

To my family, thank you for being proud of me and for always encouraging me. One of the most difficult parts of this journey has been to be away from all of you. I especially want to thank you mom for always being there for me, and for teaching me to be a strong person and always be kind to others. That was a life lesson that has helped me in every step

along my career. Dad, thank you for encouraging me to learn to play guitar. Music has opened many doors for me, and it provided me with opportunities that I would not have had otherwise. To my sister Valeria, thank you for being proud of me and dealing with my frequent calls just to bother you. To my brother Carlos, thank you for all of your support and your help at home. I really miss you. To my little brother Leo, I know that you would be very proud of me. My thesis is dedicated to you.

To my beautiful wife Michele, thank you for always cheering for me. You have always supported me and pushed me to always go the extra mile. In fact, sometimes you pushed me too much, and I achieved things that otherwise I would not have done. I could talk about how much you mean to me, or many of the happy moments I have shared with you. Instead, I want to emphasize the difficult times throughout the course of my PhD training. I will be forever grateful to you for supporting me and walking with me through those dark days when things did not work or grant rejections came in. Thank you for listening to me complain about quitting science every time I went through scientific disappointments. Especially, because you knew that the next day I would be even more passionate about science than I was before. I could not have done better without you. Also, thank you for giving me a second family. Ray, you are always an inspiration for me. I am very lucky to have your support during my PhD. Thank you for dealing with your crazy Mexican scientist son-in-law. Christine and Allison, those Sunday game nights are always fun. Thank you for always being so supportive of me and Michele.

To my friends, thank you for all of the good memories during my time at CSHL. Dr. Luqun Shen, thanks for all of those times discussing science over a nice beer at Blackford Bar. Also, thank you very much for attending the graduate student symposium for me every time I unfortunately had to miss it. Dr. Christopher Krasniak, thanks for all the good memories from our time in the Knight House. Dr. Shaina Lu, thank you for always reaching out to catch up. Agustin Mendoza, thank you for always being there to listen to me all the way from California. Thank you for visiting me a couple of times during my PhD. To Lena, Osher, and Noam, thank you for hosting delicious dinners at your house. I always look forward to seeing you. Noam and Osher, thank you for always putting a smile on my face.

Table of Contents

List of figures

List of Abbreviations

USVs	Ultrasonic vocalizations
AC	Auditory cortex
A1	Primary auditory cortex
MOB	Main olfactory bulb
AOB	Accessory olfactory bulb
MOE	Main olfactory epithelium
VNO	Vomeronasal organ
ZnSO4	Zinc sulfate
MMZ	Methimazole
MPOA	Medial preoptic area
VTA	Ventral tegmental area
LC	Locus coeruleus
DBH	Dopamine beta hydroxylase
BNST	Bed nucleus of the stria terminalis
ACA	Anterior cingulate area
ACAv	Anterior cingulate area, ventral part
ACAd	Anterior cingulate area, dorsal part
CRF	Corticotropin-releasing factor
PVN	Paraventricular nucleus
SON	Supraoptic nucleus
mPFC	Medial prefrontal cortex
NMDA	N-methyl-D-aspartate
PAG	Periaqueductal gray
AVPe	Anteroventral paraventricular nucleus
TH	Tyrosine hydroxylase
MeA	Medial amygdala
BLA	Basolateral amygdala
TIDA	Tuberoinfundibular dopamine
dmArc	Dorsomedial arcuate nucleus
NAc	Nucleus accumbens
PND	Post-natal day
IEGs	Immediate early genes
ROIs	Regions of interest
MEZ	Medial hypothalamic zone
BMA	Basomedial amygdala
CeA	Central amygdala
MSC	Medial septal complex
ILA	Infralimbic area
GRN	Gigantocellular reticular nucleus
SSp	Somatosensory primary area
DN	Dentate nucleus
DG	Dentate gyrus
TU	Tuberal nucleus
EPv	Endopiriform nucleus, ventral part
HIP	Hippocampal formation
PIR	Piriform area

TTd4	Taenia tecta, dorsal part
IPN	Interpeduncular nucleus
SNr	Substantia nigra, reticular part
PARN	Parvicellular reticular nucleus
RAmb	Midbrain raphe nuclei
DCO	Dorsal cochlear nucleus
AId	Agranular insular area, dorsal part
ORB	Orbital area
SUMm	Supramammillary nucleus, medial part
PVHp	Paraventricular hypothalamic nucleus, parvicellular division
PVHd	Paraventricular hypothalamic nucleus, descending division
DMHa	Dorsomedial nucleus of the hypothalamus, anterior part
VPL	Ventral posterodorsal nucleus of the thalamus
CS	Superior central nucleus raphe
VP	Ventral posterior nucleus of the thalamus
RT	Reticular nucleus of the thalamus
AON	Anterior olfactory nucleus
PPT	Posterior pretectal nucleus
PAA1	Piriform-amygdalar area
COApl	Cortical amygdalar area, posterior part, lateral zone
VMHc	Ventromedial hypothalamic nucleus, central part
MEV	Midbrain trigeminal nucleus
AHNa	Anterior hypothalamic nucleus, anterior part
PF	Parafascicular nucleus
AUD	Auditory areas
RH	Rhomboid nucleus
PRE2	Presubiculum, layer 2
ENTl	Entorhinal area, lateral part
ENTm	Entorhinal area, medial part
PL	Prelimbic area
VIP	Vasoactive intestinal peptide
DREADDS	Designer Receptors Exclusively Activated by Designer Drugs
CNO	Clozapine-N-oxide
AAV	Adeno-associated virus
EPM	Elevated plus maze
NA	Noradrenaline

Chapter 1. Introduction

Sex differences in behavior are widespread across the animal kingdom, but how they are manifested in underlying neural circuits remains elusive. For example, it is unclear whether they result from differences in neuronal circuitry and/or differences in neuronal activity within the same circuitry. While understanding sexually dimorphic neural differentiation and behavior is fascinating from a basic science perspective, it is also important as it would provide insight into our understanding of some behavioral pathologies and neuropsychiatric disorders. For example, the reasons why postpartum depression affects more than 10% of new mothers and only about 5% of new fathers in the US (Wisner et al., 2013; O'Brien et al., 2017) is not known. Moreover, why attention-deficit hyperactivity disorder is more common in men (Martin et al., 2018) while addiction and schizophrenia are more commonly reported in women (Becker et al., 2017; Li et al., 2017) remains poorly understood.

One fascinating example of a sexually dimorphic behavior is that of parental care. Parental displays are observed across many animal species and are essential for the fitness of the offspring. In humans, parenting involves complex interactions between both parents, siblings, and even grandparents. However, depending on the species, the role of males and females can vary dramatically. For example, in the meadow vole, the mother takes responsibility for the young, but in its close relative, the prairie vole, both parents share responsibility for their offspring (Lonstein and De Vries, 1999). In the majority of mammals, females care for the young, while males frequently ignore or attack their offspring. Although the majority of mammals show uniparental care by the female parent, there is also individual or context-dependent variability within a species. Interestingly, 5-10% of

mammalian species display paternal care (Lonstein et al., 2000; Lukas and Huchard, 2014) raising the possibility to study how the neural pathways underlying parenting are differentially regulated in males and females.

Much emphasis has been placed on understanding the maternal brain in rodents, but the neural circuits underlying paternal care are less well studied. A goal in the field is to determine whether paternal care relies on distinct or identical neural circuits as compared to maternal care. In this chapter, I would like to give a brief overview about the neural circuitry underlying parental care in male and female rodents and the evidence we have for overlapping neural circuitry in males and females. An alternative hypothesis is the existence of sexually dimorphic neural networks and mechanisms that govern parenting in males and females. However, there is very little evidence to support this hypothesis. Although the neural networks that regulate parental care are tightly linked to the networks controlling infanticidal behavior and maternal aggression, I will only focus on the positive regulation of parental behaviors.

1.2 The maternal brain in rodents

Mammalian species are characterized by in utero gestation and nursing of infants providing an inevitable bond between a mother and her offspring. Mice, like most mammals, are born altricial (Numan and Insel, 2003). Parental care in mice involves a variety of stereotyped behaviors such as nesting, nursing, pup grooming, pup licking, and pup retrieval, that require: **1) the detection of infant cues, 2) parental motivation, 3) decision making, and 4) execution of the appropriate behavioral response**. For example, when pups are born, they cannot regulate their body temperature and if they get separated

from the nest, they emit ultrasonic distress vocalizations (USVs) that along with pup smells motivate immediate search, typically by the mother. Although pup retrieval behavior is also observed in laboratory male mice, I will discuss it in a later section.

1.3 Detection of offspring cues by females:
1.3.1 Sensory processing of infant auditory cues

In the typical female mammal, the sensory perception of infant cues can change dramatically depending on the female's state. In rodents for instance, brain plasticity shifts virgin females away from avoiding infants toward displaying rewarding responses after parturition when mothers usually seek interactions with their offspring and would even learn to press a lever to gain access to pups (Hauser and Gandelman, 1985). When mice pups are separated from their littermates, they emit distress USVs (approximately 50 – 80kHz), and the mother gathers them to the nest in order to keep them safe and warm – a behavior called pup retrieval (Sewell 1970; Noirot 1972; Ehret and Bernecker 1986; Ehret 1987; Liu and Miller 2003; Ehret 2005). The identification of pup USVs suggested the involvement of the auditory cortex (AC) in facilitating maternal retrieval behavior, and it was later demonstrated that expression of the immediate early gene *c-fos* increases in AC when mothers listen to pup USVs compared to *c-fos* expression in virgin females listening to the same sounds (Fichtel and Ehret, 1999). Several groups have then tested the necessity of AC for optimal pup retrieval behavior. For example, bilateral AC lesions with ibotenic acid disrupt the acquisition of maternal behavior in surrogate mothers (virgin females that co-habitate with a dam and her pups) (Krishnan et al., 2017), and AC lesions with muscimol disrupted maternal behavior in experienced surrogate mothers (Marlin et al., 2015).

Furthermore, G. Ehret's work suggested that pup USVs were preferentially recognized by the left hemisphere (Ehret, 1987) and Marlin et al., later demonstrated that the

left AC is required for pup retrieval behavior but not the right AC. This lateralization process occurs through balancing cortical inhibition via oxytocin signaling with oxytocin receptors preferentially expressed in the left AC (Marlin et al., 2015). Interestingly, during the transition into motherhood, neurons in the primary auditory cortex (A1) undergo plasticity and integrate pup odors and sounds, enhancing the detection of USVs emitted by pups (Cohen, et al., 2011). The mechanisms for this multisensory integration are still understudied, but some evidence suggests that a pathway from the basal amygdala integrates olfactory cues from the pups and relays information to the auditory cortex to facilitate maternal behavior (Nowlan, et al., 2022). I will discuss responses to pup odors in the next section.

1.3.2 Sensory processing of infant olfactory cues

It is well known that in rodents, tactile, auditory and olfactory cues from the offspring are involved in facilitating efficient maternal behavior. While in rats auditory cues provide the dam with directional information to locate her pups, it was shown that mice use both, auditory and olfactory cues, to locate pups that have been displaced from the nest (Smotherman et al., 1974). However, in a series of classical experiments from the 1970s and 1980s, it was demonstrated that olfaction was an essential component for efficient maternal behavior in mice. First, removing the olfactory bulb from laboratory mice disrupted maternal behavior in dams and virgin females with the majority of bulbectomized females killing the pups (Gandelman et al., 1971). However, this first study did not assess the contribution of the main olfactory bulb (MOB) or the accessory olfactory bulb (AOB) individually. Briefly, in the majority of mammalian species, there are two chemosensory systems within the nasal cavity. The main olfactory system is involved in processing volatile

compounds directly from the main olfactory epithelium (MOE) to neurons in the MOB. On the other hand, the accessory system is involved in processing nonvolatile chemosensory cues such as pheromones through vomeronasal organ (VNO) signaling to the AOB (Halpern and Martinez-Marcos, 2003; Dulac and Wagner, 2006). Second, intranasal application of zinc sulfate (ZnSO4), which selectively destroys the MOE, was shown to disrupt maternal behavior (Vandenbergh, 1973) and VNO removal did not have an effect on maternal behavior (Lepri, et al., 1985; Bean and Wysocki 1989). Additionally, it was recently shown that application of methimazole (MMZ), which selectively destroys the MOE, in virgin females who have learned and shown maternal responses to foster pups, disrupts maternal behavior in mice of the CBA/CaJ strain (Nowlan, et al., 2022).

Genetic tools in recent years have allowed for molecularly defined manipulations of specific genes within the olfactory system. For example, *cnga2* and *trpc2* are cation channels that are exclusively expressed in the MOE or VNO, respectively, and can selectively label specific cell populations within the olfactory system (Mandiyan, et al., 2005; Liman, et al., 1999). *cnga2* null mutants showed deficits in pup retrieval behavior, and *trpc2* null mutants did not, suggesting that the MOE is essential for efficient maternal care and the VNO is not (Fraser and Shah, 2014). It is worth noting here that the *cnga2* gene is also expressed in the brain, and it is possible that the behavioral effect in the null mutants was not purely sensory dependent. Furthermore, Wu et al., showed that genetic ablation of *trpc2* has no effect in female pup retrieval behavior, but it switches males from displaying infanticide to showing paternal care (Wu et al., 2014). I will discuss these findings in males in more detail in a later section.

1.3.3 Sensory processing of tactile cues

Maternal contact has been shown to play an important role for the well-being of the offspring (Poindron 2005; Sullivan et al. 1986). For example, pairing feeding bouts with tactile stimulation and the presentation of an olfactory cue elicits a behavioral state in rat pups via the noradrenergic system. That is, when pups were exposed to the olfactory cue alone after the conditioning phase, the pups oriented and showed attraction to the smell and also showed an increase in huddling and feeding behaviors (Sullivan et al. 1986; Sullivan et al., 1989). All mammalian species are characterized by the nursing of infants, so suckling behavior is characteristic among all mammals (Blass and Teicher, 1980). In rats, suckling behavior is facilitated by depositing saliva on the nipples (Blass and Teicher, 1980). It was then established that suckling stimulation from pups elicited an increase of electrical activity in neurons in the paraventricular nucleus evoking the release of oxytocin during milk ejection in anesthetized mother rats (Wakerley and Lincoln, 1973). Interestingly, oxytocin has also been shown to be required for social learning with appetitive social cues (Choe et al., 2015). I will discuss more on the role of oxytocin in maternal behavior in the next section.

1.4 Maternal motivation

In his recent book (Numan, 2020), Michael Numan provides a simple definition for motivation: "it is an internal process that modifies the way an organism responds to the same external stimulus," and discusses how it applies to parental behavior. For example, virgin rats reject or avoid pups, but after parturition they exhibit maternal care in response to the same stimuli (Numan, 2020). In the case of males, the change is more striking; this definition can apply to the physiological changes that occur after mating and extended exposure to the female, shifting the virgin male from killing the pups to showing paternal behavior. Numan then goes on to divide this goal-directed behavior in two phases: an initial

appetitive phase, which is characterized by reward seeking behaviors to obtain a desired goal, and a consummatory phase which is composed of the behaviors elicited in response to the desired goal or outcome. With respect to parental behavior in rodents, the appetitive phase includes behaviors such as pup retrieval and returning to the nest after a period of absence, and the consummatory phase includes behaviors such as nursing, which are elicited in response to tactile pup stimuli such as suckling behavior (Numan, 2020). In this section, I will focus on motivated appetitive goal-directed behaviors and the corresponding neural mechanisms.

1.4.1 The role of the medial preoptic area (MPOA) in maternal behavior

For decades, the medial preoptic area (MPOA) of the hypothalamus has been known to be an essential positive regulator for the emergence and maintenance of the appetitive components of parental behavior in rodents (Numan et al., 1977; Numan and Numan, 1994.; Pedersen et al., 1994; Kohl, et al., 2016; Wu et al. 2014; Kuroda et al. 2007; Numan 2020). For example, it was demonstrated that lesions in the MPOA in virgin female rats prevent the acquisition of maternal behavior and lesions of the MPOA in lactating females disrupts the maintenance of maternal behavior (Numan, et al., 1977; Numan 2020). Mice and rats can learn to press a lever to gain access to pups as they find it rewarding (Hauser 1985; Lee et al., 1999). Remarkably, rats with MPOA lesions will continue to seek food rewards, but not pup related rewards suggesting that the MPOA modulates the rewards specific to parental behavior (Lee et al., 1999). Interestingly, inactivation of the MPOA not only disrupts appetitive parental behaviors, but it can also affect nursing behavior by decreasing the amount of time mothers engage in this behavior and in some cases completely abolishing nursing behavior (Bosch and Neumann, 2008).

Additionally, the MPOA contains populations of neurons that highly express receptors for pregnancy related hormones (Kohl et al., 2016; Numan 2020), and it has been demonstrated that hormonal treatments directly in the MPOA in rats can stimulate maternal behavior in hysterectomized-ovariectomized females (Rosenblatt et al., 1998). For instance, estrogen plays an important role in the regulation of maternal behavior. Estrogen stimulation in the MPOA enhances pup retrieval responses (Rosenblatt et al., 1998; Rosenblatt and Siegel, 1975.; Siegel and Rosenblatt 1975) and bilateral infusions of prolactin in the MPOA in nulliparous rats stimulate maternal behavior (Bridges et al., 1990). Modern techniques have allowed for the targeting of molecularly defined cell populations in the MPOA. For example, estrogen receptor-alpha expressing cells in the MPOA were shown to modulate pup approach and retrieval behavior in mice (Fang et al., 2018), and a population of inhibitory cells that express the neuropeptide galanin in the MPOA has been shown to be essential for the regulation of parenting in male and female mice (Wu et al., 2014). All of these observations motivated the identification of MPOA inputs and outputs to start dissecting specific components of the circuits that govern parental behavior (Simerly and Swanson, 1986; Kohl et al., 2018).

1.4.2 The role of the ventral tegmental area (VTA) in maternal behavior

Many animals find social contact rewarding (Krach et al., 2010). One characteristic of parental behavior is the reward-seeking phase or the state of attraction toward close interactions with offspring. It has been proposed that this component of the behavior is facilitated by the dopaminergic system (Numan and Stolzenberg 2009; Stolzenberg and Numan 2011; Fang et al. 2018; Kohl et al. 2018; Xie et al., 2022.; Rogers and Bales 2019). The neural substrates and the role of dopamine in classical reward processing (i.e. drugs of

abuse or food rewards) have been studied extensively. However, the mechanisms by which animals process social rewards are less well understood. Interestingly, the dopaminergic system has been associated with the maternal motivation to engage in pup retrieval behavior (Curry et al. 2013; Stolzenberg et al. 2010; Fang et al. 2018; Henschen et al., 2013; Numan et al. 2005; 2009; Xie et al., 2022). The projections from the MPOA to the ventral tegmental area (VTA) in the midbrain modulate the approach component in retrieval behavior (Fang et al., 2018). Specifically, estrogen receptor alpha expressing cells in the MPOA project to and inhibit non-dopaminergic cells in the VTA. This projections facilitate pup approach and the reliable activation of dopaminergic cells in the VTA during pup retrieval behavior (Fang et al., 2018). Additionally, it was shown that galanin expressing cells in the MPOA project to the VTA, and activation of this circuit using optogenetics increased the animals motivation to interact with pups (Kohl et al., 2018). The activation of dopaminergic cells in the VTA during pup retrieval enables the emergence of reliable maternal behavior through a reinforcement learning mechanism in which the contact with the pups is the rewarding component (Xie et al., 2022).

1.4.3 The role of the noradrenergic system in maternal behavior

The locus coeruleus (LC) is a small cluster of neurons in the pons, and it is the main source of noradrenaline in the brain. Noradrenergic LC neurons send extensive projections to many regions in the brain including most of the cortex (Schwarz et al., 2015; Kebschull et al., 2016). Firing of LC neurons is typically categorized in two types of firing: sustained slow firing "tonic firing" and rapid bursts of activity "phasic firing" (Aston-Jones and Cohen, 2005). The LC is an important regulator of stress, arousal, and state-dependent cognitive processes (Berridge and Waterhouse, 2003).

However, the contribution of LC during natural social behavior remains poorly understood. Interestingly, noradrenaline has been linked to establishing memories related to maternal behavior (Sullivan et al.,1989; Sullivan et al. 1986), and in sheep, noradrenaline has been implicated in imprinting the offspring smells for maternal selectivity (Numan, 2020). Moreover, dopamine beta hydroxylase (dbh) is the enzyme that converts dopamine into noradrenaline. Mutant mice lacking the dbh gene showed a deficit in maternal behavior, and most of the pups died after being neglected by the mothers. This deficit was restored by restoring noradrenaline signaling before birth (Thomas and Palmiter, 1997). Recently, it was shown that pup retrieval behavior can elicit precise phasic bursts of activity in pup-exposed virgin mice (surrogate mothers) (Dvorkin and Shea, 2022).

Dvorkin and Shea observed that LC neural activity during pup retrieval was highly correlated with subsequent pup retrieval behavior. That is, the peak of the ca^{2+} signals preceded the peak of the velocity of the female's return to the nest with the pup in her mouth. This dynamic was not observed in other behaviors, so they concluded that phasic bursts of LC activity is implicated in shaping goal-directed behavior (Dvorkin and Shea, 2022). Neuroanatomical tracing experiments have demonstrated noradrenaline innervations from LC in important regions for the emergence and maintenance of parental behavior such as the bed nucleus of the stria terminalis (BNST), MPOA, and the anterior cingulate area (ACA). It would be informative to assess the contribution of noradrenaline signaling in these downstream regions. Particularly, in chapter 5, I will provide some evidence of sex-dependent noradrenaline release in the ACA during pup retrieval behavior in mice that may be associated with decision-making in goal directed-behaviors.

The BNST is adjacent to the MPOA. Neuropeptide and steroid receptors for prolactin, estradiol, and oxytocin, which have been shown to establish and maintain

maternal care in rats, are highly expressed in the BNST (Pfaff and Keiner, 1973; Freund-Mercier et al., 1987; Bakowska and Morrell, 1997). Additionally, pup exposure suppresses expression of *c-fos* in the BNST in lactating females compared to virgin females, and excitotoxic lesions of the BNST disrupt maternal behavior in rats (Numan and Numan, 1996; Matsushita et al., 2015). One of the possible roles of the BNST in modulating maternal behavior is thought to be in providing an anxiolytic state in postpartum females by reducing the release of corticotropin-releasing factor (CRF) in the BNST during anxiety-inducing situations (i.e pups in distress) (Klampfl et al., 2014). Another possible role for the BNST in modulating maternal behavior is through the noradrenergic system. The BNST receives very dense noradrenaline innervations (Fendt, 2005), and noradrenaline has an inhibitory effect in 70% of neurons in the BNST (Casada and Dafny, 1993). Interestingly, increases in BNST noradrenaline release impaired pup retrieval behavior in dams, but elimination of the noradrenergic input to the BNST was not sufficient to induce maternal care in virgin rats suggesting that noradrenaline signaling is important for the modulation of maternal care (Smith et al. 2012).

1.4.4 The role of the paraventricular nucleus (PVN) in maternal behavior

The role of oxytocin in the regulation of maternal care has been studied extensively (Valtcheva and Froemke, 2018; Numan, 2020). The neuropeptide oxytocin is mainly produced in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) in the hypothalamus (Stoop, 2012). Lesions of the PVN in rats did not disrupt the maintenance of maternal behavior suggesting that this region is not essential for efficient maternal care (Numan and Corodimas, 1985). However, PVN lesions in virgin females disrupted the onset

of maternal behavior suggesting that oxytocin may be important for the initiation of maternal care (Insel and Harbaugh, 1989).

In laboratory mice, the role of oxytocin has been studied with the use of mouse genetic lines with null mutations for the oxytocin gene or the oxytocin receptor gene. Null mutants for the oxytocin gene showed no deficits in maternal pup retrieval behavior, nest building, and other behaviors. However, these mice lack the ability to eject milk, and the pups could not feed (Nishimori et al., 1996). In contrast, null mutants for the oxytocin receptor gene showed longer latencies to retrieve and crouch over the pups (Takayanagi et al., 2005). However, when oxytocin receptor null mutants are tested in their home cages there are no deficits in maternal behavior (Yoshihara et al., 2017). These results suggest that oxytocin may be important in modulating parental behavior in stressful or challenging conditions. In section 1.4.1, I mentioned that galanin expressing neurons in the MPOA are essential for maternal behavior. These neurons project to the oxytocin PVN neurons preferentially in females compared to males (Kohl et al., 2018). It is possible that those projections are important for the modulation of maternal behavior.

1.5 Maternal decision-making

As mentioned before, parental behaviors require the detection of infant cues and motivation to then select and execute an appropriate behavioral response. During this decision-making process, animals need to evaluate the situation, integrate the distress of the offspring, and decide whether or not to engage in a specific behavior such as pup retrieval. The medial prefrontal cortex (mPFC) has been implicated in social cognition, decision making, cost-benefit analysis, attentional selection and goal directed behavior, vicarious fear learning, and sensitivity to the distress of other conspecifics (Smith et al., 2021; Jeon et al.,

2010; Bicks and Morishita 2015; Rudebeck et al. 2006; Peters et al., 2005). All of these observations make the mPFC a likely candidate to be playing a role in parental decision making. Interestingly, the mPFC has also been shown to play a role in modulating maternal responses in rodents. For example, electrolytic lesions of the anterior cingulate area (ACA) – a sub-region of mPFC – disrupted maternal pup retrieval behavior in early post-natal days in rats (Slotnick, 1966). Furthermore, mPFC chemical lesions with tetrodotoxin disrupted maternal pup retrieval behavior in lactating rats (Febo et al., 2010). Alfonso et al., showed that excitotoxic lesions of the mPFC before pregnancy can also alter maternal care in rats (Afonso et al., 2007). Moreover, Alsina-Llanes and Olazábal examined whether neurotoxic lesions in the mPFC had an effect on maternal behavior and infanticidal behavior. They found that NMDA neurotoxic lesions delayed the onset of maternal behavior, but had no effect on infanticidal behavior in pup naïve females (Alsina-Llanes and Olazábal, 2021). These results suggest that the mPFC plays an important role in the rapid decision to whether or not to engage in parental behavior possibly by reducing anxiety in the first encounters with pups. I will come back to this observation in chapter 4 where we show that the anterior cingulate area exhibits stronger activation during pup retrieval behavior in early postnatal days compared to later days. This possibility makes the mPFC an interesting target to further investigate decision making in sexually divergent behaviors such as parenting differences between males and females. One aspect that makes this decision process particularly interesting is that during parental interactions with infants, there is not an immediate benefit for the caregiver, so it may present an experimentally tractable model to reveal the neural computations that underlie decisions between pro-social and selfish behaviors. Again, I will come back to this observation in chapter 4 where I discuss sexually

divergent activation of the anterior cingulate area (ACA) during pup retrieval behavior in males and females.

1.6 Execution of parental behavior

Efficient parental behaviors require the ability to coordinate motor components that lead to a desired behavioral outcome. It has been proposed that MPOA neurons that project to the periaqueductal gray (PAG) coordinate some of the motor components of parenting behavior (Kohl, et al., 2017; Numan 2020). Galanin expressing cells that project to the PAG were shown to be activated during pup grooming in males and females and artificial activation of these cells had no effect in virgin females but suppressed infanticidal behavior in virgin males (Kohl et al., 2018). Interestingly, inhibition of these cells decreased pup grooming behavior, but it had no effect in other aspects of parenting (Kohl et al., 2018). These results are consistent with the involvement of the PAG in motor aspects of grooming behavior without affecting the motivation and behavioral performance in other behaviors. Additionally, neurons in the reticular formation have also been implicated in motor coordination in parenting behavior (Kohl et al., 2017).

1.7 Other brain regions implicated in parental care

Other brain regions have been associated to maternal behavior, but their role is less well understood. For example, peptidergic neurons in the Edinger-Westphal nucleus have recently been implicated in preparatory nest building behavior by priming pregnant female mice to build better nests compared to virgin females (Topilko et al., 2022). Another brain region that was implicated in maternal behavior was the anteroventral paraventricular nucleus (AVPe) in the hypothalamus. Scott et al, identified a population of tyrosine

hydroxylase (TH) expressing neurons in the AVPe that is more numerous in females compared to males. Inhibition of these neurons was shown to disrupt maternal behavior but had no effect on paternal behavior (Scott et al., 2015) In contrast, activation of this population of neurons increased maternal behavior and circulating oxytocin levels.

1.8 The paternal brain in rodents

Mammalian species are typically uniparental with the majority of species exhibiting maternal care and only approximately 5-10% displaying paternal care (Lonstein et al., 2000; Lukas and Huchard, 2014). While the maternal brain in rodents has been studied extensively, the neural mechanisms that facilitate paternal behavior remain poorly understood. Paternal behavior in rodents is observed in the wild in biparental species such as the California mouse (*Peromyscus californicus*) (Horner, 1947; Dudley, 1974) and prairie voles (*Microtus ochrogaster*) (Oliveras and Novak, 1986). On the other hand, rats and laboratory mice (*Mus musculus*) do not exhibit paternal behavior in the wild. Typically in nature, males and virgin females of these species exhibit infanticidal behavior (vom Saal and Howard, 1982; Soroker and Terkel, 1988; Numan, 2020). However, in the laboratory, selective breeding caused virgin female mice to spontaneously show maternal behavior and virgin males still exhibit infanticidal behavior. Interestingly, after mating, if the male remains with the female mate throughout the pregnancy, the sire exhibits paternal behavior after parturition and infanticidal behavior is almost completely inhibited (Tachikawa et al., 2013; Liu et al., 2013). Given that laboratory conditions enable the expression of paternal behavior in mice, it provides an opportunity to examine whether the neural substrates and mechanisms that mediate parental care are the same in males and females.

1.9 Detection of offspring cues by males

To my knowledge, there have not been studies that investigate how males respond to auditory cues emitted by the pups. For example, it is clear that in females, brain plasticity in the auditory cortex during the transition to motherhood enhances the detection of USVs emitted by pups. It is not known if males exhibit similar changes in the transition to fatherhood, but that goes beyond the scope of this thesis work. Interestingly, female mice of the ICR strain emit 38 KHz USVs that can induce parental behavior in males suggesting that the male's behavior can be modulated not only by the pup cues but also cues from the female mate (Liu et al. 2013).

Olfactory cues are important for the modulation of maternal behavior (see section 1.3.2). Exposure to pups increased *c-fos* expression in the olfactory bulb of male prairie voles (Kirkpatrick et al., 1994a). In bi-parental species, removing the olfactory bulb in males has the same effect as in females. For example, removing the olfactory bulb in prairie voles induces infanticide in males that otherwise would exhibit paternal responses (Kirckpatrick et al. 1994b). Removal of the VNO in females has no effect on maternal behavior. In contrast, removing the VNO in virgin male mice suppressed infanticidal behavior and promoted paternal care similar to sires. VNO removal has no effect in sires' parental behavior (Wu et al. 2014; Tachikawa et al., 2013) suggesting that vomeronasal signaling promotes infanticidal behavior but it is not necessary for the maintenance of paternal behavior. Furthermore, Isogai et al., showed that chemosensory cues from the pups activate the VNO and promote infanticidal behavior (Isogai et al., 2018).

Additionally, the medial amygdala (MeA) receives inputs from the AOB (Tachikawa et al., 2013), and it detects chemosensory cues in both sexes (Unger et al., 2015). GABAergic neurons in the MeA promote parental behavior in males and females. However, the same

population of neurons in males can promote infanticidal behavior depending on the level of activation (Chen et al., 2019). In female mice, there is evidence that suggests that the BLA can integrate olfactory information and facilitate maternal care (Nowlan et al., 2022). Interestingly, BLA lesions in male California mice have been shown to disrupt paternal behavior suggesting that the same neural networks are present in both males and females (Lee and Brown 2007).

1.10 The role of the medial preoptic area (MPOA) in paternal behavior

In section 1.4.1, I provided evidence that supports the hypothesis that the MPOA is an essential regulator of maternal behavior. Given all of the evidence about the importance of the MPOA in maternal behavior, early work on paternal behavior focused on the contribution of the MPOA to parental care in males. For example, lesions of the MPOA in male rats prevented the acquisition of paternal behavior suggesting that, as in females, the MPOA is essential for the emergence of parenting in males (Rosenblatt et al., 1996). Additionally, ablating the MPOA in male rats that have been sensitized to pups and have shown paternal behavior disrupts ongoing paternal behavior suggesting that the MPOA is also important for the maintenance of paternal behavior (Sturgis and Bridges, 1997). Estrogen stimulation directly in the MPOA was also shown to facilitate paternal behavior in rats providing more evidence of convergent mechanisms mediating parenting in males and females as the same treatments also stimulate maternal care (Rosenblatt and Ceus, 1998).

In mice, there is an increase of *c-fos* expression in the MPOA of males after pup exposure in uniparental and bi-parental species (Lambert et al., 2013). Galanin-expressing neurons in the MPOA were also shown to be active during parental behavior in male mice and inhibition of these cells disrupted parental behavior in both males and females (Wu et

al., 2014; Kohl et al., 2018). Interestingly, Stagkourakis et al., identified a population of tuberoinfundibular dopamine (TIDA) neurons located in the dorsomedial arcuate nucleus (dmArc) of the hypothalamus that regulate prolactin secretion depending on their oscillation patterns. These neurons regulate paternal behavior by activating prolactin receptor galanin neurons in the MPOA in mice (Stagkourakis et al., 2020). These oscillation patterns are opposite in non-paternal rats, and by artificial optogenetic modulation of these oscillation rhythms, paternal behavior can be induced in rats.

1.11 Summary of network controlling parental behavior in rodents

Briefly, in female mice, auditory and olfactory offspring cues access the brain through the auditory cortex (AC) and the main olfactory epithelium (MOE). This information is relayed to other brain regions such as the BLA and the MeA. The MPOA is an essential regulator of maternal behavior and integrates all of this information. Projections from the MPOA orchestrate different components of maternal behavior. For example, projections to the VTA modulate dopaminergic activity which shapes maternal behavior through reinforcement learning and reward pathways to the nucleus accumbens (NAc). The MPOA projects to oxytocinergic cells in the PVN which can also regulate the VTA. It is possible that projections from the VTA and the LC to the prefrontal cortex influence parental decision making by shaping goal directed behavioral responses. Finally, the information reaches the motor components of the circuit such as the PAG and the reticular formation to facilitate a behavioral outcome. Most of the evidence I presented suggests that paternal behavior is modulated by the same neural substrates as maternal behavior with some examples of sexually divergent activation of the same circuitry giving rise to different behaviors in males and females.

1.12 Thesis Research Summary

The goal of this thesis work was to identify brain regions that give rise to sexually dimorphic patterns of parental behavior. Sex differences in behavior are widespread across the animal kingdom, but how they are manifested in underlying neural circuits remains elusive. For example, it is unclear whether they result from differences in neuronal circuitry or differences in neuronal activity. Laboratory mice display pronounced sex differences in offspring directed behaviors. We compared the networks that underlie parenting behavior in male and female mice by using *c-fos* to map whole-brain activity at cellular resolution after pup interactions, and we identified brain regions that responded to pup-directed behaviors. Here, we focused on the anterior cingulate area (ACA) because it is sensitive to distress in conspecifics, and it is reciprocally connected with locus coeruleus (LC) which likely modulates maternal behavior. We used fiber photometry to monitor activity in ACA and LC of dams and sires during free interactions with pups. Excitatory ACA neurons were differentially activated in males and females during pup retrieval behavior, and inhibitory ACA neurons showed reciprocal activity. We also confirmed that LC supplies a robust noradrenergic projection to ACA, and we observed sex differences in both LC neuronal activity and NA release in ACA during pup retrieval. Finally, chemogenetic inactivation of ACA output disrupted pup retrieval behavior in early postnatal days and decreased motivation to interact with pups in distress. We propose that ACA maintains differential sensitivity to pup distress in dams and sires. Therefore, in this case, behavioral differences between males and females are sub-served by divergent activation of shared circuitry.

Chapter 2: Sex- and Context- dependent Modulation of Parental behavior in Mice

2.1 Introduction

Maternal behavior has been extensively studied in rodents, but the neural circuits underlying paternal care are less well understood, and even less is known about how sex differences in the brain drive and modulate sex-specific behaviors. As mentioned in the previous chapter, a particular example of a sexually dimorphic behavior in rodents is that of parental care with females being maternal and a fraction of males infanticidal and some paternal. However, it is unclear whether these differences in behavior result from differences in neuronal circuitry and/or differences in neuronal activity.

Parenting is a social behavior that requires the detection of offspring cues to choose an appropriate behavioral response. In laboratory mice, females usually care for the offspring by feeding and providing a safe environment for their pups. For example, when pups are separated from the nest, in order to return to safety and warmth, they will emit a high frequency call known as the ultrasonic distress vocalization (USV) and the mother will retrieve them to the nest. Males exhibit different behaviors toward the young compared to females. Although males also retrieve pups, their behavior is inconsistent and more variable across days compared to females, and a fraction of males attack and kill the pups, due to pup-directed aggression through vomeronasal organ (VNO) signaling (Isogai et al., 2018; Tachikawa et al., 2013; Wu et al., 2014). Interestingly, paternal behavior is subject to additional contextual regulation as compared to maternal behavior (Liu et al. 2013). Therefore, we wanted to observe male and female interactions with pups in different contexts and examine differences in behavior.

As mentioned before, virgin males often attack and kill the pups. In order to avoid infanticidal behavior, we performed our experiments using only fathers, mothers, and surrogate mothers of the CBA/CaJ strain. Surrogate mothers are virgin females that have been co-housed with a pregnant female for at least 3 days before parturition and with the pups throughout the experimental period.

2.2 Sex differences in contextual regulation of parental behavior

To assess parenting behavior in male and female mice of the CBA/CaJ strain, we exposed mice to their pups in different contexts. First, we chose to examine pup retrieval behavior in the home cage to assess the efficacy of pup gathering using a pup retrieval behavioral assay (Figure 2.1A) previously used in our lab (Krishnan et al., 2017). Mothers and surrogate mothers exhibit robust behavioral performance and time dependent improvement whereas fathers show unreliable and inconsistent behavior across multiple days and a higher mean latency to gather pups in their home cage (Figure 2.1B). Previous research showed that males of the ICR strain do not gather pups in a novel environment (clean cage) (Liu et al., 2013). We therefore tested animals in the pup retrieval behavioral assay in a novel cage to investigate contextual regulation of parental behavior. Interestingly, the novel cage disrupts time dependent improvement in fathers and surrogate mothers, but not in the mothers (Figure 2.2A). Fathers showed poorer pup retrieval performance than females in both contexts, and a two-way ANOVA with Holm-Sidak's multiple comparisons test revealed a significant interaction between sex and context: DF=2; ****$p<0.0001$.

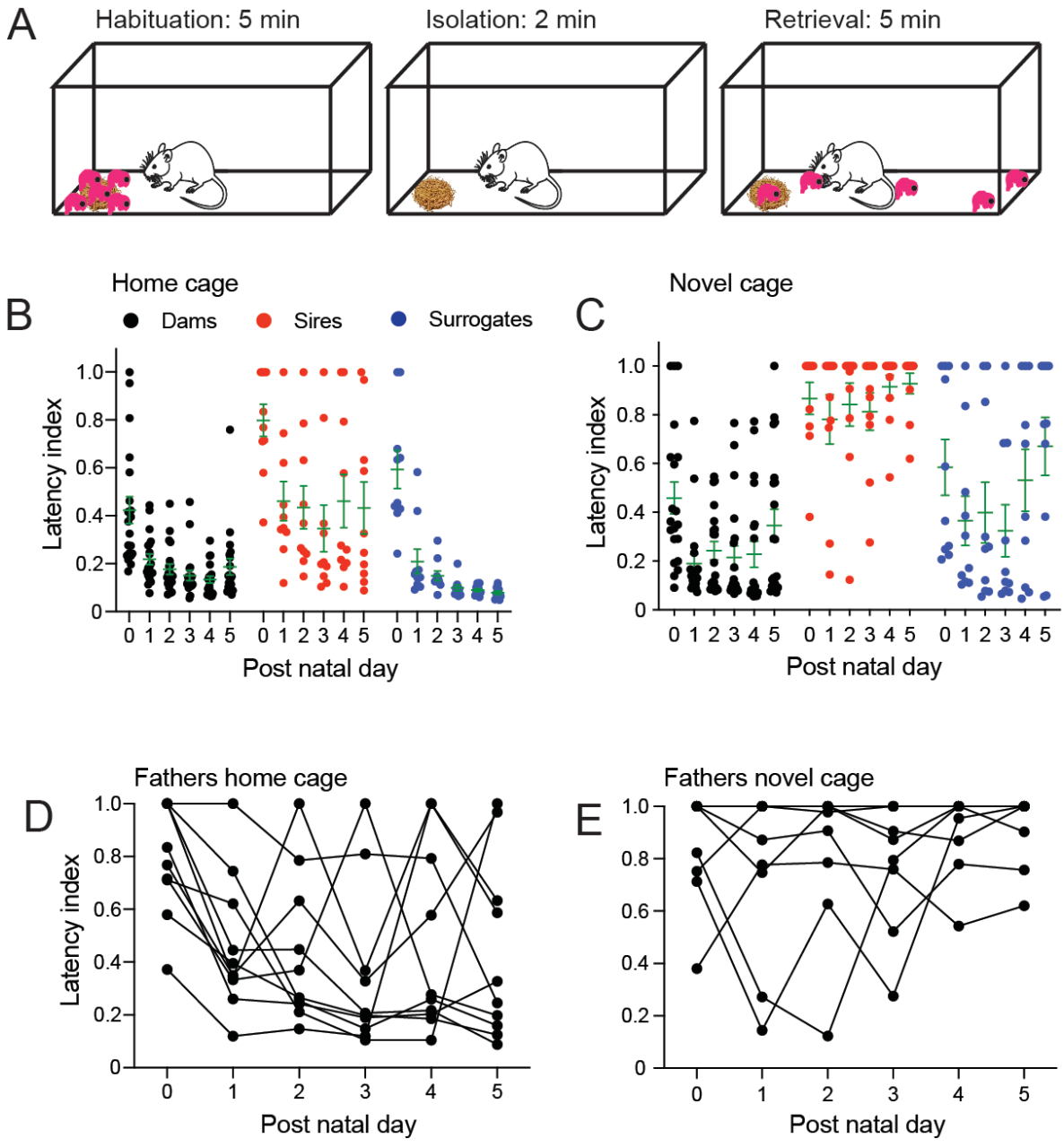


Figure 2.1 Fathers exhibit inconsistent behavior across days and additional contextual behavioral regulation compared to mothers.

A) Schematic of behavioral paradigm. **B,C)** Scatter plots showing a normalized measure of latency to gather pups for mothers (n=20 per context), fathers (n=10 per context), and surrogate mothers(n=10 per context) in their home cage or a novel cage, respectively, at postnatal days 0 – 5. Lines represent mean ± s.e.m. **D)** Father's behavioral performance in the home cage across days showed inconsistent behavior. Lines track the individual's performance across PNDs 0 – 5**. E)** Father's behavioral performance in a novel cage across days showed inconsistent behavior. Lines track the individual's performance across PNDs 0 – 5.

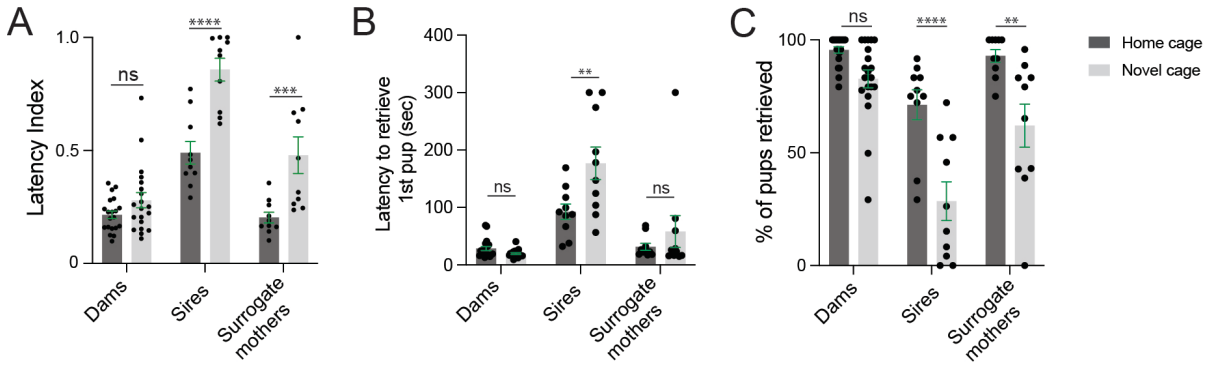


Figure 2.2. Quantification of behavioral performance in a pup retrieval assay reveals sex dependent contextual modulation of behavior.

A) Mean performance at PND 0 – 5 for mothers, fathers, and surrogates as measured by normalized latency. Lines represent mean ± s.e.m. (n=10 fathers, 20 mothers, and 10 surrogate mothers). **B)** Mean latency to retrieve the first pup at PNDs 0 – 5. Lines represent mean ± s.e.m. **C)** Percent of pups retrieved averaged across PNDs 0 – 5 in the home cage and a novel cage. Lines represent mean ± s.e.m. (n=10 fathers, 20 mothers, 10 surrogate mothers).

The multiple comparison test showed significant differences across contexts in fathers and surrogate mothers, but not in mothers (Figure 2.2A). The multiple comparison test also revealed significant differences between sexes (Figure 2.3).

Additionally, we measured the latency to retrieve the first pup, and we found that fathers take longer than females to engage in pup retrieval behavior in both contexts (Figure 2.2B). A Two-way ANOVA with Holm-Sidak's multiple comparisons test showed a significant interaction between sex and context: DF=2; ****$p<0.0001$. We also quantified the percent of pups retrieved from post-natal days 0 – 5 and fathers retrieve less pups than females in both contexts (Figure 2.2C). A Two-way ANOVA with Holm-Sidak's multiple comparisons test showed a significant interaction between sex and context: DF=2; ****$p<0.0001$.

2.3 Effects of nesting material from home cage on a novel cage pup retrieval paradigm

One possibility for the fathers' poor behavioral performance in the novel cage is that the olfactory cues from the home cage are not present. We tested if the olfactory cues from the home cage nest were sufficient to improve males' behavioral performance in a novel cage. The nest from the home cage was moved to a novel cage prior to the assay and the test subject was habituated with the pups in the nest for 5 minutes. The pups were then removed for 2 min, and then scattered in the novel cage. The test subject was allowed to gather the pups back to the nest for 5 min. The fathers' latency to retrieve the pups was lower in the home cage, followed by the novel cage with the nest from the home cage, and lastly in the novel cage (Figure 2.4 B-D). The multiple behavioral conditions tested did not affect mothers' behavior suggesting that there is more flexibility in the circuitry mediating this behavior in males (Figure 2.4 A-C).

2.4 Effects of corncob bedding material on parental behavior in males and females

In addition to physiological changes, parenting behavior is subject to environmental factors such as stress and housing conditions, which can in turn, affect hormone levels (Wisner et al., 2013). Estrogen levels surge during late pregnancy, and this surge is essential in regulating the onset of maternal behavior (Rosenblatt and Siegel, 1975; Siegel and Rosenblatt, 1975). Additionally, estrogen receptor-alpha expressing cells in the MPOA were shown to modulate pup approach and retrieval behavior (Fang et al., 2018). Interestingly, it has been reported that housing mice using corncob bedding reduces estrogen receptor-alpha expression in the brain and decreases aggressive behaviors in the California mouse (Landeros et al., 2012). Corncob bedding is widely used in the animal facilities at Cold Spring Harbor Laboratory. Given the importance of estrogen levels for proper maternal behavior, we wanted to investigate the effect of housing our mice using corncob bedding in our pup retrieval assay in dams and sires. We did not find any significant differences in the animals' behavior between the two conditions (Figure 2.5).

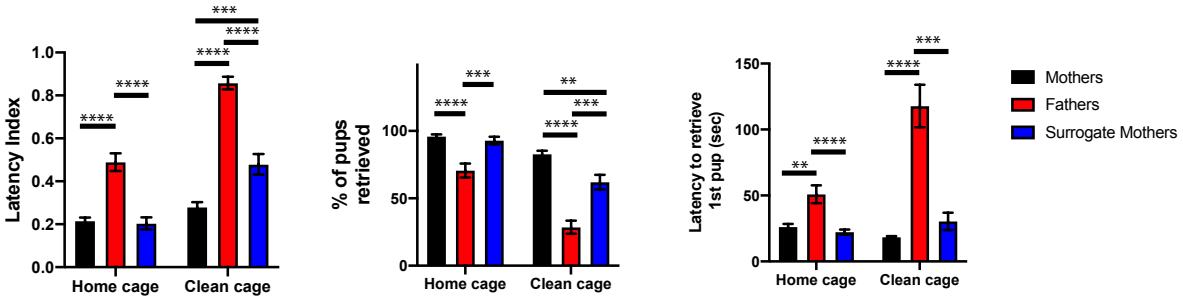


Figure 2.3. Fathers show higher latency to retrieve pups compared to females in their home cage and a novel cage.

A) Mean performance at PND 0 – 5 for mothers, fathers, and surrogates as measured by normalized latency. Lines represent mean ± s.e.m. (n=10 fathers, 20 mothers, and 10 surrogate mothers). Two-way ANOVA with Holm-Sidak's multiple comparisons test showed a significant interaction between sex and context: DF=2; ****p<0.0001; Mothers: home cage vs Mothers: novel cage p=0.1889; Fathers: home cage vs Fathers: novel cage ****p<0.0001; Surrogates: home cage vs Surrogates: novel cage ****p<0.0001; Mothers: home cage vs. Fathers: home cage ****p<0.0001; Mothers: home cage vs Surrogates: home cage p=0.9465; Fathers: home cage vs Surrogates: home cage ****p<0.0001; Mothers: novel cage vs Fathers: novel cage ****p<0.0001;Mothers: novel cage vs Surrogates: novel cage ****p<0.0001;Fathers: novel cage vs Surrogates: novel cage ****p<0.0001. B) Mothers: home cage vs Mothers: novel cage p=0.3891; Fathers: home cage vs Fathers: novel cage ****p<0.0001; Surrogates: home cage vs Surrogates: novel cage p=0.6560; Mothers: home cage vs Fathers: home cage ***p=0.0002; Mothers: home cage vs Surrogates: home cage p=0.8574; Fathers: home cage vs Surrogates: home cage ***p=0.0002; Mothers: novel cage vs Fathers: novel cage ****p<0.0001; Mothers: novel cage vs Surrogates: novel cage p=0.2634; Fathers: novel cage vs Surrogates: novel cage ****p<0.0001. C) Mothers: home cage vs Mothers: novel cage **p=0.0039; Fathers: home cage vs Fathers: novel cage ****p<0.0001; Surrogates: home cage vs Surrogates: novel cage ****p<0.0001; Mothers: home cage vs Fathers: home cage ****p<0.0001; Mothers: home cage vs Surrogates: home cage p=0.5241; Fathers: home cage vs Surrogates: home cage ***p=0.0004; Mothers: novel cage vs Fathers: novel cage ****p<0.0001; Mothers: novel cage vs Surrogates: novel cage ***p=0.0001; Fathers: novel cage vs Surrogates: novel cage ****p<0.0001).

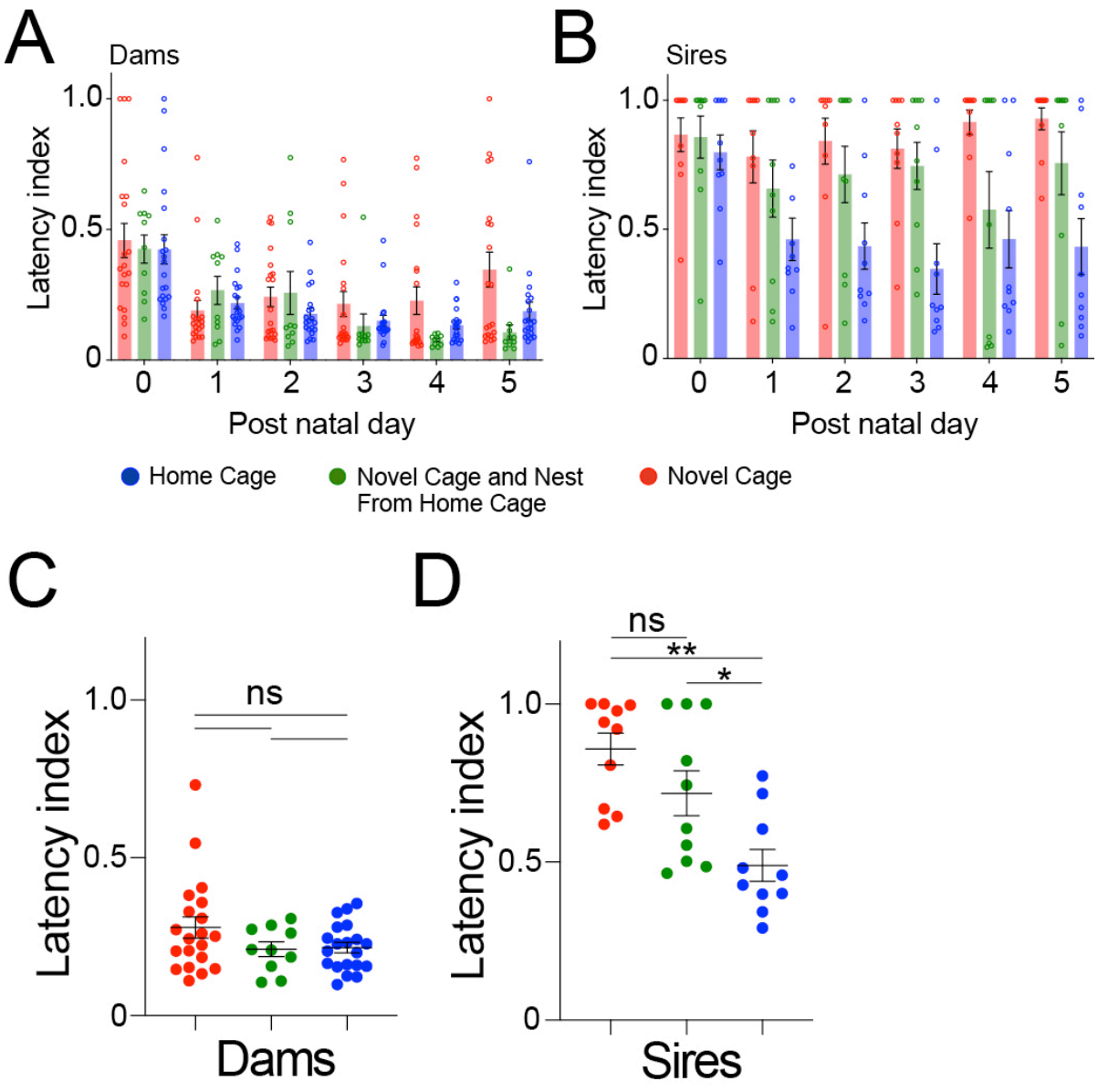


Figure 2.4. Paternal behavior shows contextual regulation.

A) Behavioral performance of dams across contexts from postnatal days 0 – 5. **B)** Behavioral performance of sires across contexts from postnatal days 0 – 5.**C)** The mean performance of normalized latency was averaged over all six sessions for three conditions. Dams' behavioral performance in the home cage, novel cage with nest from the home cage, and novel cage (n=20 in the home cage, n=20 in the novel cage, and n=10 in the novel cage plus nest from home cage). A Kruskal-Wallis with Dunn's multiple comparison test showed no significant differences in dams' performance across contexts p=0.4143. **D)** Sires' behavioral performance in the home cage, novel cage with nest from the home cage, and novel cage (n=10 per condition). A Kruskal-Wallis (**p=0.0015) with Dunn's multiple comparison test showed significant differences between the home cage vs novel cage comparison (**p=0.0013) and the home cage vs novel cage and nest from home cage comparison (*p=0.0436).

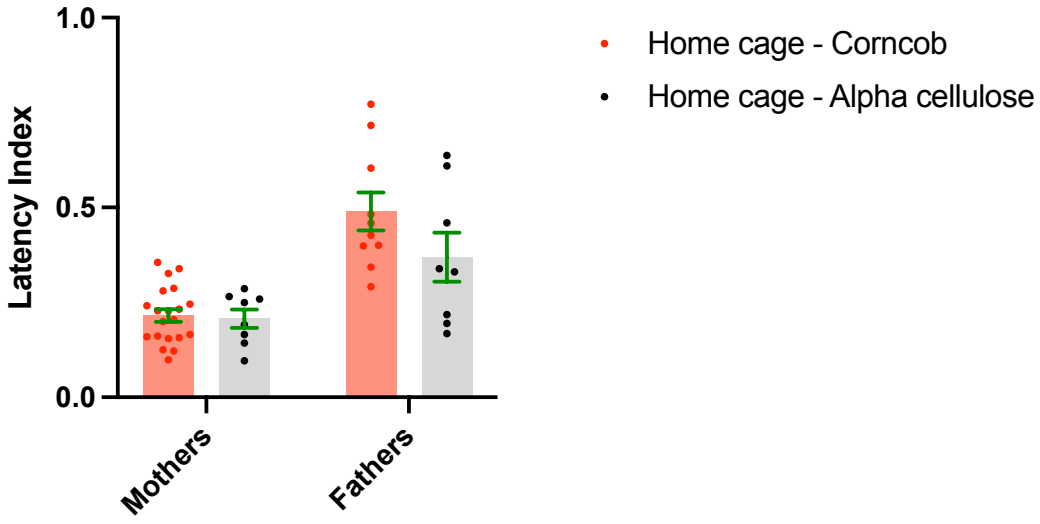


Figure 2.5. Housing mice with corncob bedding does not have an effect on parental behavior in males and females.

Mean pup retrieval behavioral performance of dams and sires in their home cage with corncob bedding (n=20 and n=10 respectively) and another group of dams and sires in their home cage with alpha cellulose bedding (n=10 and n=8, respectively). A Two-way ANOVA with Sidak's multiple comparisons test showed no significant differences between the two different bedding materials in both males and females.

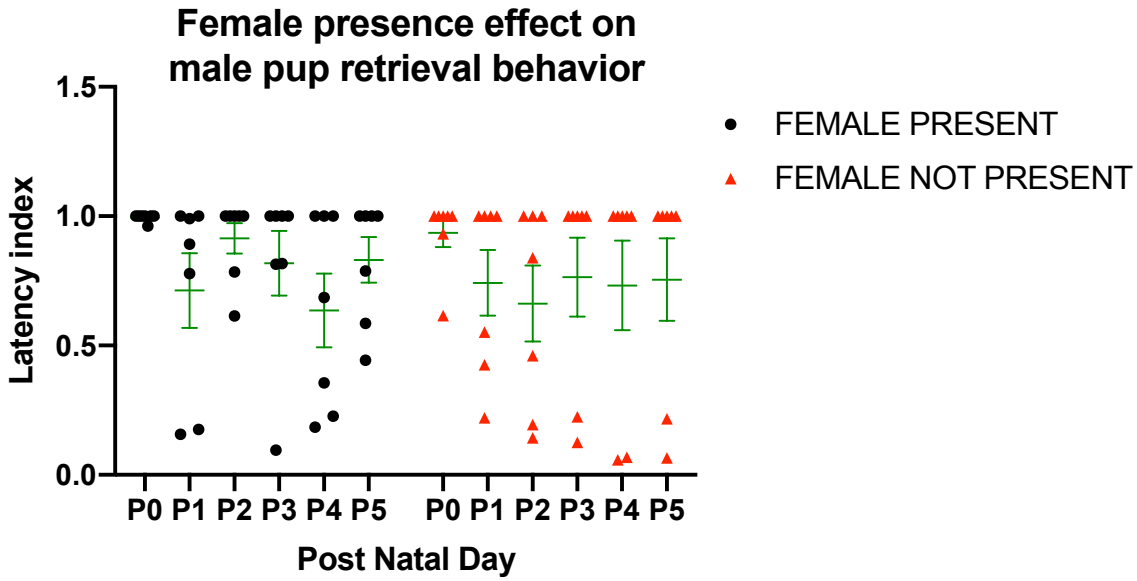


Figure 2.6. Pup retrieval behavioral performance of fathers in the presence of their female partners.

Fathers, behavioral performance in a novel cage, respectively. (N=7 mice per group; Lines represent mean ± s.e.m).

2.5 Effects of female mate presence in male retrieval behavior

Previous research suggests that females emit 38 KHz USVs that are sufficient to induce fast pup retrieval in males of the ICR strain (Liu et al. 2013). We designed a behavioral arena where animals can interact with each other through openings in a barrier that separates them. The arena has two chambers divided by a barrier with small openings that allow smell and sound to pass through but does not allow the animals to touch each other. We exposed CBA/CaJ fathers to pup retrieval behavioral assays in the presence or absence of their female partner in the other chamber and measured the latency to gather their pups. The pup retrieval assay was performed as follows: (1) The test subject was habituated with the pups in the nest for 10 minutes in the behavioral box, (2) the pups were then removed from the cage for 2 minutes, (3) the mother was placed in the small chamber in the behavioral arena, and (4) then the pups were scattered in the side of the cage with the father present. Each test subject had a maximum of 5 minutes to gather the pups to the nest. The same procedure was done at postnatal days 0 to 5. All assays were performed in the dark during the light cycle between 7:00am and 5:00pm and videos were recorded for further analysis. We did not observe any significant differences in male behavioral performance by the presence of the female (Figure 2.4).

2.6 Discussion

In this chapter, we described quantifiable differences in pup retrieval behavior between males and females of the CBA/CaJ strain and sex-dependent contextual regulation of retrieval behavior. Our results showed that sires display parental behavior towards their own pups. However, the sires' pup retrieval behavioral performance displayed greater variability compared to the dam's behavior. Interestingly, the variability in male behavior

was not explained by variability between individuals. Instead, it was explained by variability within individuals. We found that the sires' behavioral performance was inconsistent across post-natal days with males that retrieve some days but not others. However, we cannot explain the source of the behavioral variability with our experimental approach.

One possibility for the observed behavioral variability is that males are more sensitive to contextual changes compared to females. We therefore wanted to observe the animal's behavior in a novel environment to determine if there was sex-dependent contextual regulation of behavior in the CBA/CaJ strain. We found that paternal behavior is very sensitive to the change in context and showed that most males do not retrieve pups in a novel environment after a 20-minute assay. It is possible that a longer habituation period can facilitate better performance by the sires. Not surprisingly, the change in context did not perturbed the dam's behavior significantly. These results suggest that paternal behavior is subject to additional contextual regulation as compared to maternal behavior.

We wanted to determine if olfactory cues from the home cage were sufficient to improve the sire's behavioral performance in the novel environment. We transferred the home cage's scent by moving the nest from the home cage to the novel cage and repeated the assay. Although the manipulation did not reach statistical significance, there is a clear trend of the behavior moving toward home cage performance levels. These data suggest that paternal behavior is more "flexible" than maternal behavior which is typically very consistent and stable. An interesting possibility is that sires are more sensitive to stress compared to dams. It would be interesting to measure cortisol levels after interactions with pups in different contexts and compare male and female behavioral performance and the correlation with cortisol levels.

Nevertheless, we observed sex differences in parental behavior, so we sought to identify brain areas that may display sex-dependent activation patterns after interactions with pups. We used expression of the immediate early gene *c-fos* as a marker for neuronal activation and an automated pipeline to map brain-wide activity at cellular resolution after pup interactions in an unbiased way. I will discuss the results of the brain-wide activity screen in the next chapter.

Chapter 3: Brain-wide Expression Patterns of the Immediate Early Gene *c-fos* After Interactions with Pups

3.1 Introduction

Understanding the neural circuitry that drives parental interactions and how it differs in males and females is a fundamental issue in neuroscience. Previous studies have used unbiased mapping of immediate early genes (IEGs) expression as a measure for neuronal activation after parental interactions in mice (Renier et al., 2016; Kohl et al., 2018; Topilko et al., 2022). However, those studies did not focus on differences in pup retrieval behavior. Here, we aimed at identifying brain regions that modulate pup retrieval behavior differently in males and females. We compared the networks that underlie parenting behavior in male and female mice by using *c-fos* to map whole-brain activity at cellular resolution after interactions with pups. Due to the ubiquity of parental care in mammals, the understanding of parental circuits in male and female mice may have important implications in human parenting behaviors and neuropsychiatric disorders such as postpartum anxiety and depression.

3.2 Behavioral paradigm for *c-fos* induction and work-flow for brain-wide *c-fos* mapping

To identify regions of interest that drive and modulate parental interactions in mothers and fathers, we used induction of the immediate early gene *c-fos* and an automated pipeline to analyze its expression in the whole brain (Renier et al., 2016). Briefly, *c-fos* expression was induced by the behavior of interest and the mice were perfused 90 minutes after stimuli presentation, the brains were then stained for *c-fos* and cleared using iDISCO+, the tissue was automatically imaged using light-sheet microscopy, and *c-fos*$^{+}$ cells were

automatically detected and registered to the Allen Brain Atlas (Figure 3.1A). The pup exposure assay to induce *c-fos* expression was performed as illustrated in figure 3b. We examined whole brain *c-fos* expression patterns of dams and sires on post-natal day 3 under four conditions: Undisturbed: the test subject was kept in the home cage with the pups in the nest for a 3 – hour period. At 90 minutes, we opened the behavioral box and mocked the placement of the pups back in the cage by introducing the hand to the home cage to control for this disturbance in other groups. Isolated: the test subject was kept in the home cage without the pups for a 3 – hour period. At 90 minutes, the same control as the undisturbed group was performed. Reunion: the test subject was kept in the home cage without the pups for a 90 – minute period, and then all pups were returned in the nest for a second 90 – minute period. Retrieval: the test subject was kept in the home cage without the pups for 90 minutes, and then all pups were returned scattered in the cage for 90 minutes. Given the evidence of lateralization and preferential neural responses to pup USVs in the left AC (Ehret 1987; Marlin et al., 2015), we decided to image the left hemisphere for the *c-fos* screen. In dams, the undisturbed group showed significantly lower brain-wide *c-fos*$^+$ cell counts and less variability when compared to the isolated, reunion, and retrieval groups (Figure 3.1C). Sires showed greater variability and not significant differences across all of the groups in the total number of *c-fos*$^+$ cells in the entire left hemisphere (Figure 3.1D). Not surprisingly, the total number of *c-fos*$^+$ cells was generally positively correlated with the number of *c-fos*$^+$ cells in individual ROIs except in the baseline group in dams (Figure 3.2). Presumably, this is because the variability in the dam's baseline group is small (Figure 3.1C).

3.3 Brain-wide *c-fos* expression using ROI analysis

Statistical comparisons between different groups were run based on ROIs of the Allen Brain Atlas. A list of brain regions that were more active compared to baseline was generated for each condition in dams and sires and we also generated a list of brain regions that were upregulated in dams or sires by comparing the same condition across sex. We were interested in regions that were more active in the pup retrieval condition (Figure 3.3) and regions that were differentially activated in dams and sires (Figure 3.4). The variability in the baseline group in the sires was very high, so the statistical comparison with other conditions did not yield statistically significant ROIs in most comparisons (Figure 3.3). Among the regions that were more active in the retrieval group in dams were the medial hypothalamic zone (MEZ), which includes the MPOA, the bed nucleus of the stria terminalis (BNST), the basomedial amygdala (BMA), the central amygdala (CEA), the medial septal complex (MSC), and the anterior cingulate area (ACA). I explained the involvement of most of these regions in parenting behavior in chapter 1, but one region that retained our attention was the ACA (Figure 3.3C). This was because the involvement of the ACA in parenting behavior remains unclear and this region has been shown to be involved in sensitivity to social distress (Jeon et al., 2010; Smith et al., 2021). We then looked at *c-fos* expression in subdivisions of the ACA and found that the ventral part of the cingulate cortex (ACAv) shows stronger activation in the retrieval group in dams (Figure 3.5). Interestingly, the ACA and the infralimbic area (ILA) were among the most upregulated areas in dams of the retrieval condition compared to sires of the retrieval condition suggesting that pup retrieval behavior may evoked sex-dependent activation of the ACA (Figure 3.4D).

3.4 Behavioral observations during offspring interactions of *c-fos* induction mice

In order to understand differences in *c-fos* expression data, we were interested in identifying behavioral differences between groups and across sexes. We first quantified the amount of time the parents spent in the nest, and we found that mothers of the isolated group spent less time in the nest compared to the other groups (Figure 3.6A). We then correlated the amount of time spent in the nest with the number of *c-fos*$^+$ cells in 264 brain regions, and we found that all of the significant correlations were negative (Figure 3.6B). Notably, the amount of time the parents spent interacting with the pups is mostly time spent in the nest with them. We found that mothers of the undisturbed group spent less time nesting with the pups compared to the retrieval group (Figure 3.6C). Interestingly, when we correlated the time spent nesting with the pups by the mothers and the number of *c-fos*$^+$ cells in the same 264 brain regions, all significant correlations disappear. We noticed that some animals attempted to escape from the cage during the behavioral assay. We quantified the attempts to escape and found that, although not statistically significant, mothers of the isolated group showed the higher escape attempts compared to other groups (Figure 3.6D). All regions significantly correlated with the escape behavior were positively correlated with the number of *c-fos*$^+$ cells (Figure 3.6E). We quantified the amount of time the fathers spent in the nest, and found no significant differences across the different groups (Figure 3.6F). We then correlated the amount of time spent in the nest with the number of *c-fos*$^+$ cells in the same 264 brain regions, and we found that all of the significant correlations were negative (Figure 3.6G). We found that fathers of the undisturbed group spent less time nesting with the pups compared to the retrieval and the reunion groups (Figure 3.6H). In contrast with the mothers, we found that *c-fos*$^+$ cells in many regions were negatively correlated with the amount of time spent nesting with the pups (Figure 3.6I). We quantified

the attempts to escape and found that, fathers attempted to escape regardless of the presence or absence of the pups (Figure 3.6J). All regions significantly correlated with the escape behavior in fathers were positively correlated with the number of *c-fos*$^{+}$ cells (Figure 3.6K). Fathers of the retrieval group showed a greater latency to retrieve pups when compared to mothers (Figure 3.6L). We also recorded ultrasonic vocalizations (USVs) during the first 90 minutes of the assay and found that some mothers emit USVs in the absence of the pups (Figure 3.6M). We did not detect any USVs in the father experiments. Interestingly, the number of USVs was positively correlated with the number of escape attempts, suggesting that these behaviors may reflect pup separation anxiety in mothers (Figure 3.6N).

3.5 Discussion

A major goal in the field is to determine whether paternal behavior relies on distinct or identical neural circuits as compared to maternal behavior. In chapter 2, I showed that dams and sires display parental care toward their own pups. However, the behavior in males is more variable compared to female behavior, and it is inconsistent across post-natal days. Here, we examined brain-wide *c-fos* expression patterns as a proxy for neural activity in males and females. At a whole brain level, the variability of *c-fos*$^{+}$ cells is higher in sires compared to females, especially in the baseline condition (Figure 3.1C-D). These results suggest that the behavioral variability in males may be reflected in brain-wide *c-fos* expression variability as well.

One of the goals for the brain-wide *c-fos* screen was to identify brain regions activated in response to pup retrieval behavior. By making statistical comparisons between the different conditions, we were able to generate brain-wide activity maps for each

condition with respect to the baseline control. We found by comparing the baseline and retrieval groups in the dams that many brain regions previously implicated in maternal behavior were upregulated in the retrieval group. For example, the MEZ, which includes the MPOA – an essential regulator of parental behavior. We also identified the BMA which has recently been found to integrate the smell of pups and relay information to the auditory cortex and modulate responses to pup calls (Nowlan et al., 2022). The BNST and the CeA among many others. One ROI that captured our attention was the ACA.

The ACA was one of the most upregulated regions in the retrieval condition in dams (Figure 3.3C) and it was upregulated in sires that retrieve compared to sires from the reunion group (Figure 3.3H). Additionally, the ACA was previously implicated in maternal pup retrieval behavior in rats (Slotnick, 1966). However, the specific function and the timing of its involvement in pup retrieval behavior remains elusive. Interestingly, when we compared the retrieval groups between dams and sires, the ACA was one of the most upregulated regions in dams compared to sires suggesting that there is sex-dependent modulation of ACA during interactions with pups.

However, one of the limitations of using *c-fos* as a marker for neural activity is that it only provides a snapshot of activity at a given time point. Also, we did not obtain any cell type specificity information from the *c-fos* screen. Therefore, we decided to observe neural activity in ACA during pup retrieval behavior in freely moving dams and sires and in different cell populations. I will discuss the neural activity dynamics of ACA in males and females in the next chapter.

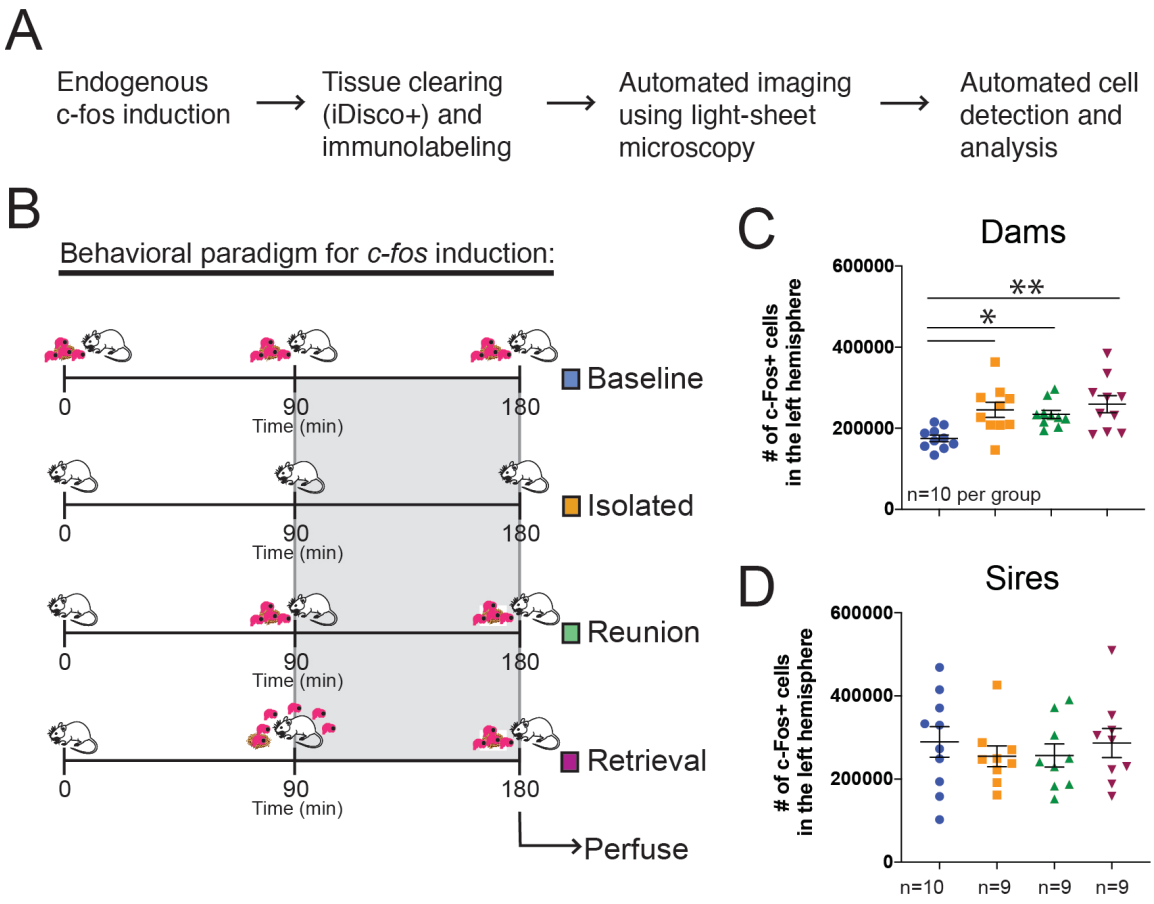


Figure 3.1. Automated pipeline for brain-wide *c-fos* mapping and behavioral paradigm for *c-fos* induction after interactions with pups.

A) Brain-wide *c-fos* expression mapping workflow. **B)** Schematic representation of behavioral paradigm to induce *c-fos* expression. There were four groups in the assay: undisturbed, isolated, reunion, and retrieval. The experiment was performed in mothers and fathers. All experiments were performed in the dark during the light cycle in a sound proof behavioral box. All mice were perfused immediately after the experiment was done. **C)** Quantification of *c-fos* positive cells in the left hemisphere; dams: n=10 per group (Kruskal-Wallis test; **p=0.0023; Dunn's multiple comparison test *p=0.0182; **p=0.0052). **D)** Quantification of *c-fos* positive cells in the left hemisphere; sires n= 10 undisturbed, 9 isolated, 8 reunion, and 9 retrieval (Kruskal-Wallis test not significant).

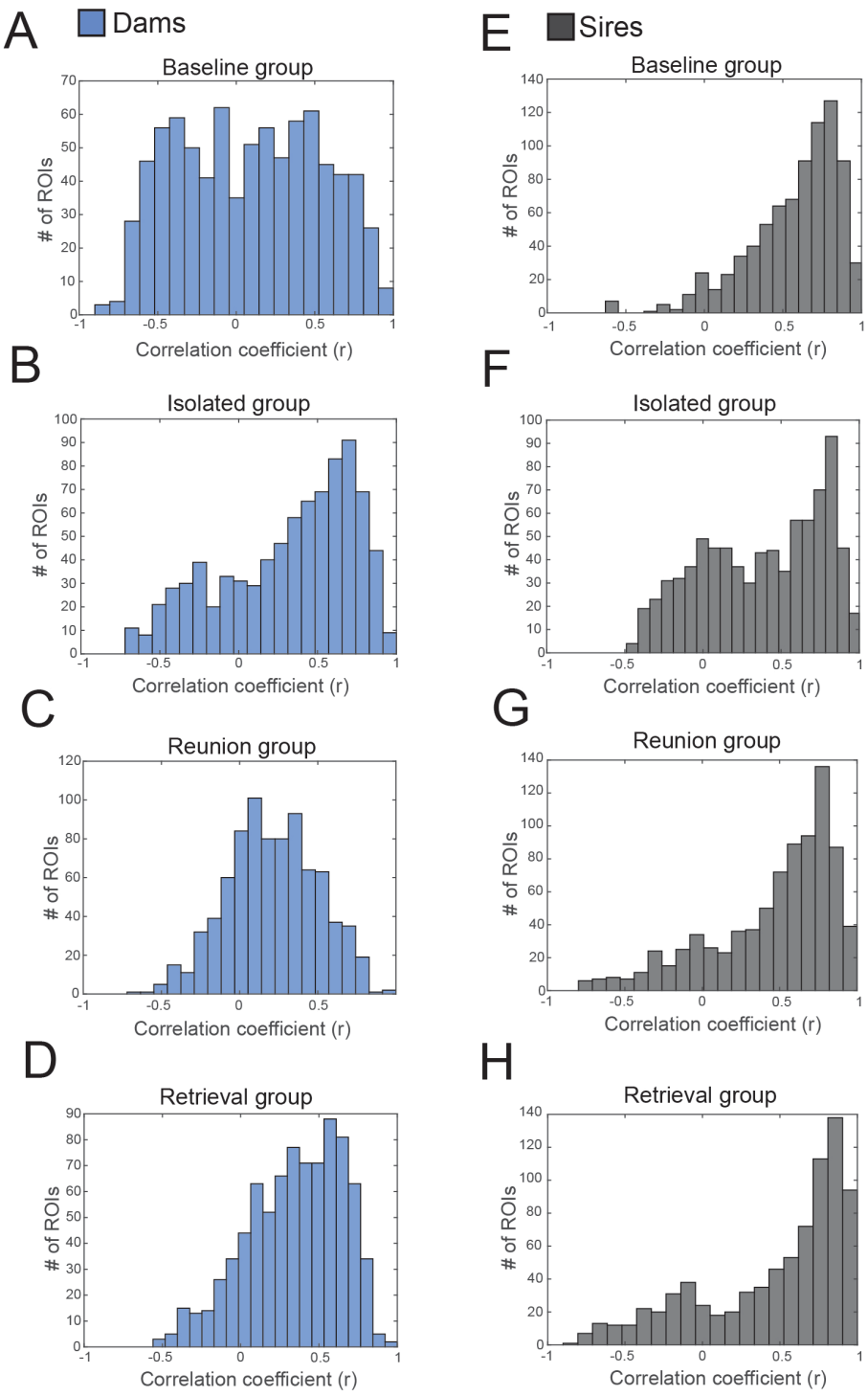


Figure 3.2. Total number of *c-fos*⁺ cells in the left hemisphere positively correlates with number of *c-fos*⁺ cells in individual ROIs.

A) Histograms of correlation coefficient (r) and the number of ROIs in the whole brain obtained by correlating the total number of *c-fos*⁺ cells in the left hemisphere with the number of *c-fos*⁺ in individual ROIs in dams. **B)** Same data analysis in sires.

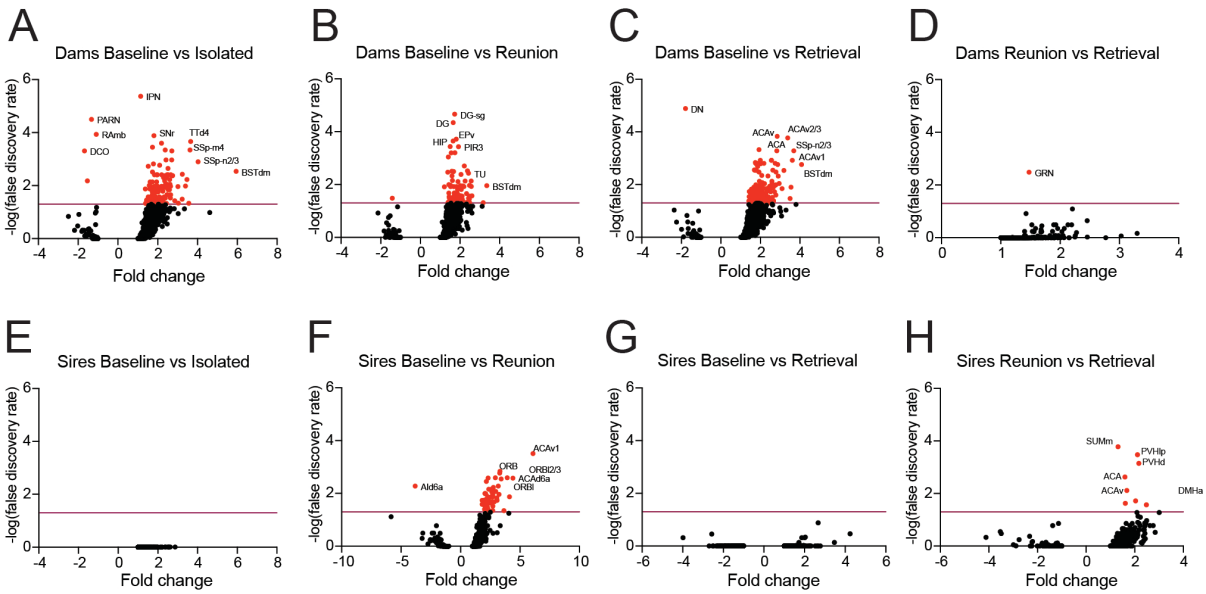


Figure 3.3. Volcano plots of regions induced by isolated, reunion and retrieval conditions in dams and sires as fold change in *c-fos*⁺ cells (baseline vs other conditions). Significant ROIs are depicted in red. Horizontal tan line depicts significant threshold with an FDR of 0.05. **A-C)** Statistical comparisons in dams between baseline and other conditions. A negative fold change represents an upregulation of *c-fos* expression in baseline condition and a positive fold change represents an upregulation of *c-fos* in the respective comparison. **D)** Statistical comparison between reunion and retrieval groups in dams. **E-G)** Statistical comparisons in sires between baseline and other conditions. A negative fold change represents an upregulation of *c-fos* expression in baseline condition and a positive fold change represents an upregulation of *c-fos* in the respective comparison. **H)** Statistical comparison between reunion and retrieval groups in sires.

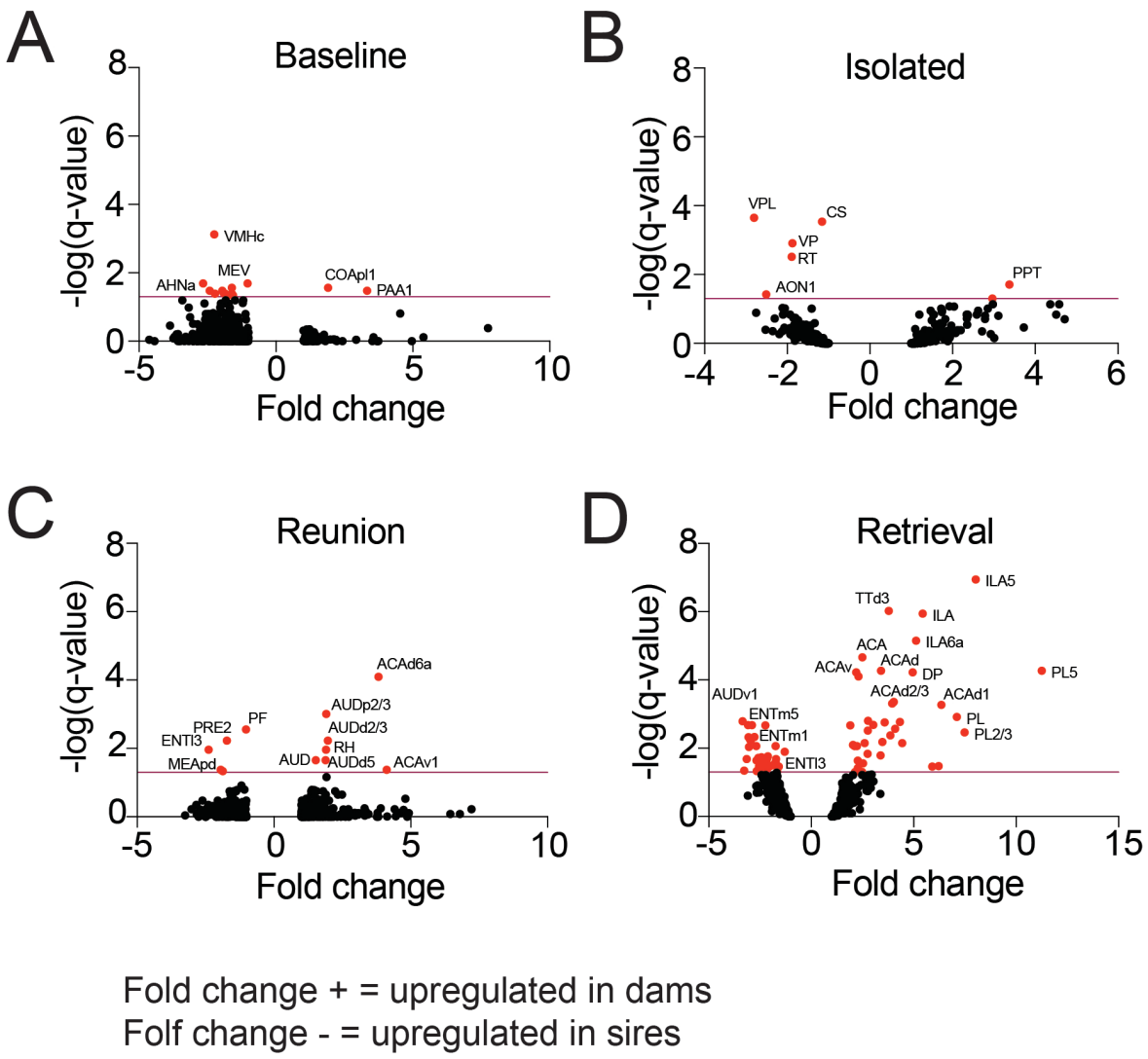


Figure 3.4. Volcano plots of regions induced by baseline, isolated, reunion and retrieval conditions in dams and sires as fold change in *c-fos*⁺ cells (dams vs sires within the same condition).

Significant ROIs are depicted in red. Horizontal tan line depicts significant threshold with an FDR of 0.05. **A)** Statistical comparisons of baseline groups between dams and sires. **B)** Statistical comparisons of isolated groups between dams and sires. **C)** Statistical comparisons of reunion groups between dams and sires. **D)** Statistical comparisons of retrieval groups between dams and sires. All positive fold changes indicate an upregulation in dams and negative fold changes indicate an upregulation in sires.

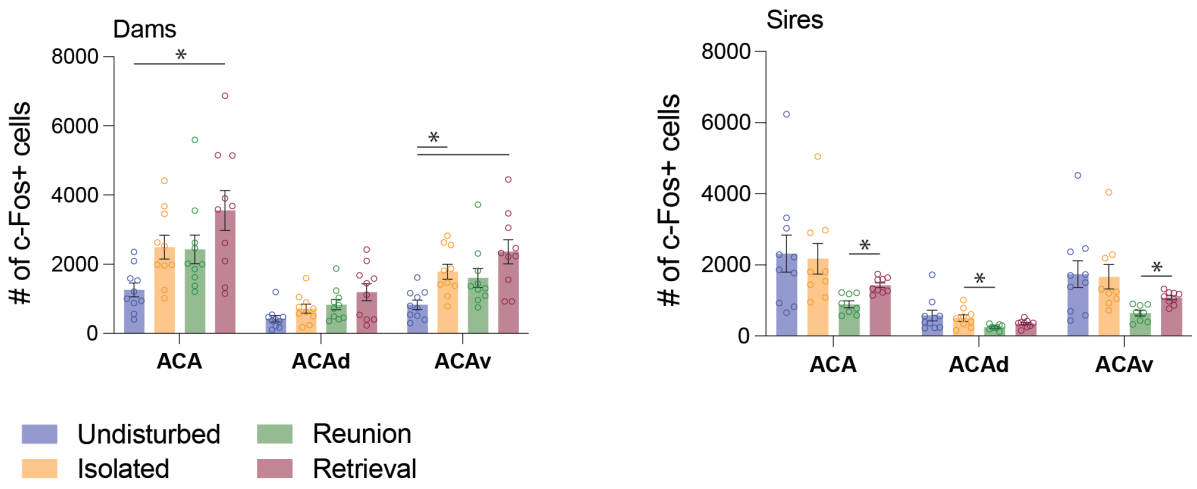


Figure 3.5. Anterior cingulate area *c-fos* expression is increased with pup retrieval behavior.

A) Quantification of *c-fos*+ cells in the anterior cingulate area of dams in response to pups (n=10 per group; Tukey's multiple comparison test *p<0.05). **B)** Quantification of *c-fos*+ cells in the anterior cingulate area of sires in response to pups (n=10 undisturbed, 9 isolated, 8 reunion, and 9 retrieval; Tukey's multiple comparison test *p<0.05).

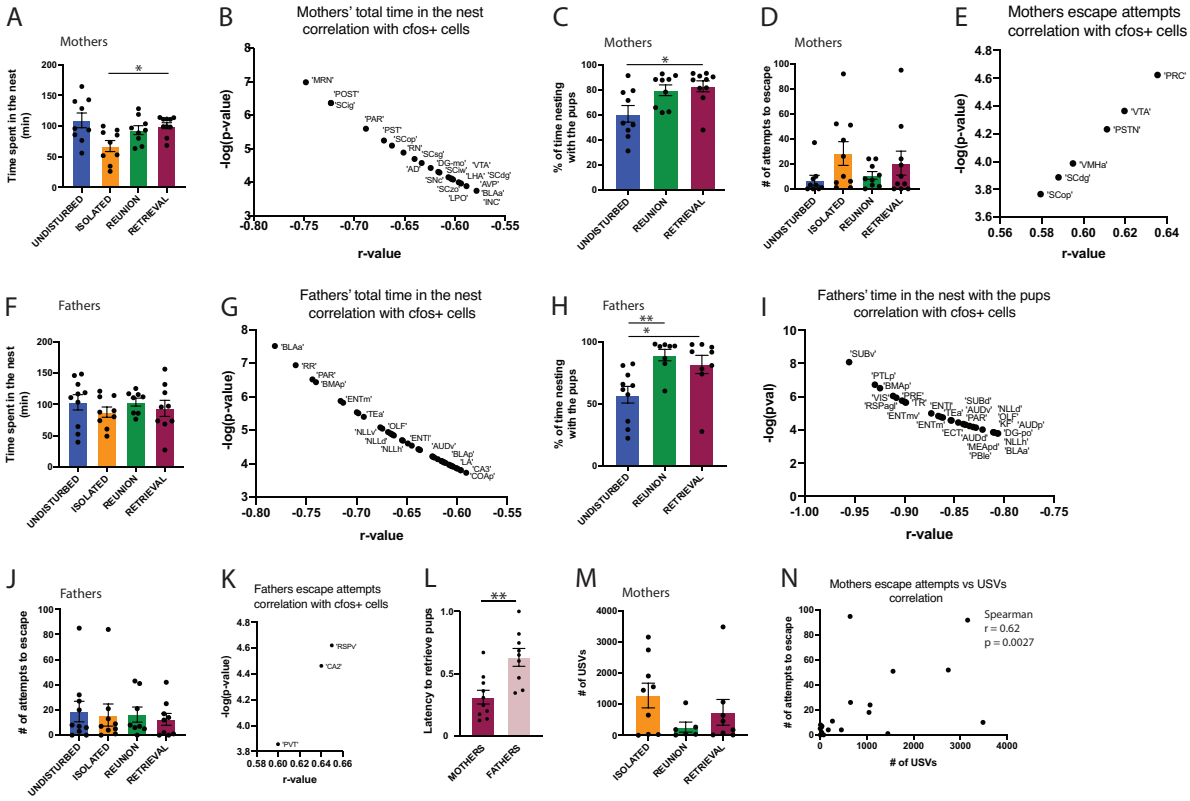

A
Mothers
Time spent in the nest (min)
UNDISTURBED
ISOLATED
REUNION
RETRIEVAL
B
Mothers' total time in the nest correlation with cfos+ cells
-log(p-value)
r-value
C
Mothers
% of time nesting with the pups
D
Mothers
# of attempts to escape
E
Mothers escape attempts correlation with cfos+ cells
F
Fathers
G
Fathers' total time in the nest correlation with cfos+ cells
H
Fathers
I
Fathers' time in the nest with the pups correlation with cfos+ cells
-log(pval)
J
Fathers
K
Fathers escape attempts correlation with cfos+ cells
L
Latency to retrieve pups
MOTHERS
FATHERS
M
Mothers
# of USVs
N
Mothers escape attempts vs USVs correlation
Spearman
r = 0.62
p = 0.0027

Figure 3.6. Behavioral paradigm for *c-fos* induction reveals differences in nesting behavior, ultrasonic vocalization output, and latency to retrieve pups between mothers and fathers and different correlation patterns with *c-fos* expression.

A) Quantification of total time spent in the nest for mothers during the 3-hour period; lines represent mean ± s.e.m (Kruskal-Wallis *p=0.0271; Dunn's multiple comparisons test: isolated vs retrieval *p=0.0452). **B)** Mother's data set correlation of time spent in the nest with *c-fos* positive cells across 264 ROIs; p<0.0001 for all ROIs shown. **C)** Quantification of time spent in the nest with the pups for the mothers' data set presented as a percentage; lines represent mean ± s.e.m (Kruskal-Wallis *p=0.0269; Dunn's multiple comparisons test: undisturbed vs retrieval *p=0.0298). **D)** Quantification of total number of escape attempts for the mother's data set. **E)** Mother data set correlation of number of escape attempts with *c-fos* positive cells across 264 ROIs; p<0.0001 for all ROIs shown. **F)** Quantification of total time spent in the nest for fathers during the 3-hour period; lines represent mean ± s.e.m. **G)** Fathers' data set correlation of time spent in the nest with *c-fos* positive cells across 264 ROIs; p<0.0001 for all ROIs shown. **H)** Quantification of time spent in the nest with the pups for the fathers' data set presented as a percentage; lines represent mean ± s.e.m (Kruskal-Wallis ***p=0.0005; Dunn's multiple comparisons test: undisturbed vs reunion **p=0.001; Undisturbed vs retrieval **p=0.009). **I)** Fathers' data set correlation of time spent in the nest with the pups and *c-fos* positive cells across 264 ROIs; p<0.0001 for all ROIs shown. **J)** Quantification of total number of escape attempts for the fathers' data set. **K)** Father data set correlation of number of escape attempts with *c-fos* positive cells across 264 ROIs; p<0.0001 for all ROIs shown. **L)** Latency index of mothers and fathers of the retrieval group; lines represent mean ± s.e.m (Mann Whitney test **p=0.0041). **M)** Quantification of USVs emitted by the mothers in the absence of the pups quantified using DeepSqueak; lines represent mean ± s.e.m. **N)** Correlation of number of USVs emitted by the mothers with the number of escape attempts (Spearman correlation r=0.62, p=0.0027).

Chapter 4: The Role of the Anterior Cingulate Area (ACA) in Parental Behavior

4.1 Introduction

In chapter 1, I briefly discussed the role of the mPFC in parental decision-making. The cerebral cortex is organized by defined layers that contain different inhibitory an excitatory cell types that can be categorized based on their anatomical projections, morphology or genetic profile (Harris and Mrsic-Flogel, 2013). Previous mPFC lesion experiments in parental behavior in rodents did not discriminate between different cell types and performed non-specific ablations. However, there is substantial evidence of different cortical interneurons types and functional heterogeneity during decision-making (Sohal et al., 2009; Veit et al., 2017). The functional heterogeneity of excitatory cortical neurons during decision-making is less well understood. Interestingly, in a recent report, Musall et al., identified cell-type-specific dynamics in pyramidal neurons in the frontal cortex during perceptual decisions (Musall et al., 2021). Moreover, using single cell calcium imaging it was shown that nonoverlapping subpopulations of ACA inhibitory neurons that express vasoactive intestinal peptide (VIP) respond to either social or non-social stimuli (Johnson et al., 2020). Parental decision-making research lacks a detailed description of the contribution of different cell types, so it will be interesting to investigate the contribution of excitatory and inhibitory populations in cortical regions during pup retrieval behavior.

4.2 Excitatory but not inhibitory neurons in the anterior cingulate cortex are differentially activated in dams and sires during pup retrieval behavior

Our results from the brain-wide *c-fos* screen identified the cingulate cortex as a candidate region to be likely associated with pup retrieval (Figure 3.3C). The ACA was one

of the most upregulated regions in dams compared to sires suggesting that there is sex-dependent modulation of ACA during interactions with pups. However, due to the slow temporal resolution of *c-fos*, we performed fiber photometry recordings in the cingulate cortex of freely moving mice during pup retrieval behavior to test this hypothesis (Figure 4.1). We expressed Gcamp6s in excitatory neurons in the cingulate cortex in males and females of the CBA/CaJ strain and recorded calcium activity from postnatal day 0 – 5 during free interactions with pups (Figure 4.1A-B). We confirmed the expression of the calcium indicator and the fiber placement using immunohistochemistry (Figure 4.1C-D and Figure 4.3). We aligned neural activity traces to the moment animals contacted a pup and retrieved it to the nest and also to the moment the animals dropped the pup back in the nest. We found that bursts of activity in excitatory cells are associated with pup retrieval events in dams and sires (Figure 4.1E-F). The pup retrieval evoked responses started approximately 1.5 seconds before pup contact and lasted about 2 seconds after pup contact (Figure 4.1G). Interestingly, the magnitude of these responses decreases over the course of postnatal day 0-5 (Figure 4.1I). To quantify pup retrieval evoked activity, we calculated the mean z-score of the df/f trace. We cut the traces from 4 seconds before the pup contact to 5 seconds after the contact and subtracted the first 2 seconds of baseline to determine the change of Ca^{2+} activity during pup retrieval. We measured the area under the curve for 2 seconds after the pup contact and observed a difference in the magnitude of these bursts when comparing dams to sires, with dams showing a stronger activation of the cingulate cortex during retrieval events (Figure 4.1G-H).

We were interested in whether different cell types in the ACA modulate different aspects of parental behavior in a sex-dependent manner. To test this idea, we injected cre-dependent Gcamp7s in VGAT-Cre male and female 8-week-old mice to restrict the

expression of the calcium indicator to inhibitory neurons in the cingulate cortex and recorded population calcium activity during pup retrieval behavior (Figure 4.2A-B). We confirmed the expression of the calcium indicator and the fiber placement using immunohistochemistry (Figure 4.2C-D and Figure 4.3). We aligned neural activity traces to the moment animals contacted a pup and retrieved it to the nest and also to the moment the animals dropped the pup back in the nest. We found that there was a dampening in neural activity when the mice contacted the pups and a peak in activity before the mice dropped the pups back in the nest (Figure 4.2E-F). The pup retrieval evoked dampening of activity in inhibitory cells lasted about 1.5 seconds after pup contact (Figure 4.2G). To quantify pup retrieval evoked activity, we calculated the mean z-score of the df/f from 4 seconds before the pup contact to 5 seconds after the contact and subtracted the first 2 seconds of baseline to determine the change of Ca^{2+} activity during pup retrieval. We measured the area under the curve for 2 seconds after the pup contact and observed no significant difference between dams and sires (Figure 4.2H-I).

4.3 Excitatory neuron activity patterns show a reciprocal relationship with inhibitory neurons in the cingulate cortex in dams

Inhibitory neurons in dams showed a reciprocal relationship when compared to excitatory neurons in the cingulate cortex during pup retrieval behavior (Figure 4.4 B-C). To quantify pup retrieval evoked activity, we calculated the mean z-score of the df/f from 4 seconds before the pup contact to 5 seconds after the contact and subtracted the first 2 seconds of baseline to determine the change of Ca^{2+} activity during pup retrieval. We measured the area under the curve for 2 seconds after the pup contact and observed a significant difference in the magnitude of responses between inhibitory and excitatory cells (Figure 4.4D). We also observed a peak of activity in the inhibitory population before the

dam dropped the pup in the nest that preceded a dampening in activity of the excitatory population. We measured the area under the curve from 1 second before the dam dropped the pup in the nest to 1 second after and we observe a significant difference between the inhibitory and excitatory populations (Figure 4.4E). Interestingly, we found that inhibitory neurons in the cingulate cortex decrease their activity when the animal enters the nest after all of the pups have been retrieved, and the activity increases when the animals exit the nest (Figure 3F-G). We found a stronger dampening of activity in inhibitory cells compared to excitatory cells when dams enter the nest by measuring the area under the curve for 4 seconds after entering the nest (Figure 4.4H-J). We did not observe a difference in activation between inhibitory and excitatory cells when dams exit the nest (Figure 4.4K).

Inhibitory neurons in sires did not show a reciprocal relationship when compared to excitatory neurons (Figure 4.5). We also found that inhibitory neurons in the cingulate cortex decrease their activity when the sires enter the nest after all of the pups have been retrieved, and the activity increases when the sires exit the nest (Figure 4.5F-G).

4.4 Pup distress jar paradigm

As I explained in section 1.5, the ACA is implicated in sensitivity to the distress of other conspecifics (Smith et al., 2021). For example, in mice, the typical freezing response as a result of painful stimulation can be learned by observation. Mice learn to freeze to a tone paired to another mouse receiving a foot shock. Bilateral lesions of ACA in mice impaired observational fear learning suggesting that neural activity in ACA is sensitive to the distress of others (Jeon et al., 2010). We were interested in the neural dynamics in the ACA during interactions with inaccessible pups in distress. We trapped the pups in a glass jar and put it in the home cage. The jar had a plastic lid with small holes on it, so the animals were able to

see, hear, and smell the pups, but were not able to touch them (Figure 4.6B). We recorded the animals' behavior for 5 minutes in their home cage with the pups trapped in a jar or 5 minutes in the home cage with an empty jar and the pups were taken out of the home cage. We found that trapping the pups in the jar elicited a strong response in the dams. Dams of the CBA/CaJ and VGAT-Cre strains spent significantly more time interacting with the jar with the pups compared to an empty jar (Figure 4.6E-F). Sires of the CBA/CaJ strain did not spend more time with the jar with pups compared to the empty jar (Figure 4.6H) To make sure that trapping the pups in the jar elicited a stressful reaction in the pups, we recorded USVs when the pups were in the jar and when they were in the nest. We found that they vocalize significantly more when they are trapped in the jar (Figure 4.6G). We also recorded calcium activity during these experiments, but the results are still under analysis. Example traces are shown in figure 4.6 C-D.

4.5 Silencing ACA excitatory neurons in dams increases the latency to retrieve pups and decreases maternal responsiveness to pups in distress

We showed that chemogenetic inactivation of excitatory neurons in ACA disrupts pup retrieval behavior in early postnatal days and decreases the time interacting with pups in distress. We expressed inhibitory Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) or GFP in excitatory neurons in the cingulate cortex of dams and recorded interactions with pups in freely moving mice 20 minutes after injecting the ligand clozapine or saline. We injected an AAV construct in the ACA expressing hM4D(Gi) under the control of the CaMKII promoter in CBA/CaJ mice to restrict expression to excitatory neurons (Figure 4.7A-B). Initially, it was reported that the designer ligand clozapine-N-oxide (CNO) directly activated these receptors (Nawaratne et al., 2008), but more recent evidence strongly suggests that they are in fact activated by the CNO metabolite clozapine

(Gomez et al., 2017). We performed pup retrieval assays from post-natal day 0 – 5 as illustrated in figure 4.7D. We alternated days of injection of the ligand clozapine and saline to have a control manipulation using the same subjects. We found that chemogenetic inactivation of excitatory neurons in ACA disrupts pup retrieval behavior and increases the latency to retrieve pups to the nest (Figure 4.7E). We then separated the data by post-natal days and found that the inhibition manipulation had a stronger effect on the first post-natal day compared to later days (Figure 4.7F). Additionally, we trapped the pups in a jar to make them inaccessible to the dams and measured the time they spent trying to access the pups as a measure of parental motivation. The lid of the jar had holes so that the test subject was able to hear and smell the pups, but it was not able to touch them. We compared the time GFP expressing dams and hM4D(Gi) expressing dams spent interacting with the pups in the jar after saline or clozapine injections. We found that hM4D(Gi) expressing dams spent significantly less time interacting with the jar compared to GFP controls injected with clozapine (Figure 4.8B). When we compared the same animals injected with saline, there was no significant difference in the time they spent interacting with the pups in the jar (Figure 4.8C). There was no difference in amount of time GFP and hM4D(Gi) expressing animals spent interacting with an empty jar with clozapine and saline injections (Figure 4.8D-E). In order to make sure that clozapine injection had an effect in our experiments, we compared hM4D(Gi) expressing dams injected with clozapine versus saline. We observed that the same animals injected with clozapine spent significantly less time interacting with the pups in the jar compared to when they were injected with saline (Figure 4F). We did not observe a significant difference in GFP expressing dams with clozapine or saline injection (Figure 4.8G). Additionally, GFP and hM4D(Gi) expressing dams spent significantly more

time interacting with the jar when the pups are present compared to the empty jar (Figure 4.8 H-I).

4.6 Silencing ACA excitatory neurons in sires may increase the latency to retrieve pups and decreases paternal responsiveness to pups in distress

We showed that, in sires, chemogenetic inactivation of excitatory neurons in ACA may disrupt pup retrieval behavior, and it may decrease the time interacting with pups in distress. However, we need to increase the sample size of our experiment to make a more accurate conclusion. We expressed inhibitory Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) or GFP in excitatory neurons in the cingulate cortex of sires and recorded interactions with pups in freely moving mice 20 minutes after injecting the ligand clozapine or saline. We injected an AAV construct in the ACA expressing hM4D(Gi) under the control of the CaMKII promoter in CBA/CaJ mice to restrict expression to excitatory neurons (Figure 4.9A-C). We performed pup retrieval assays from post-natal day 0-5 as illustrated in figure 4.7D. We alternated days of injection of the ligand clozapine and saline to have a control manipulation using the same subjects. We found that chemogenetic inactivation of excitatory cells in ACA showed an increase in the latency to retrieve pups in sires. However, we observed a similar increase in latency in hM4D(Gi) expressing sires even when we inject saline (Figure 4.9E). We then separated the sire's behavioral performance by post-natal days. However, the sire's behavior was variable across days, and none of the differences in individual post-natal days reached statistical significance (Figure 4.9F). We subjected the sires to the jar experiment as described in figure 4.10A. We observed that GFP and hM4D(Gi) expressing sires spent significantly more time interacting with the pups in the jar compared to the empty jar (Figure 4.10H-I). However, we did not see any significant differences when we compared GFP controls with hM4D(Gi) expressing sires (Figure 4.10B-

G). These results suggest that excitatory neurons in the ACA may be involved in processing distress from the pups in differentially in males and females.

4.7 Silencing ACA excitatory neurons does not affect anxiety-like behaviors

To determine whether the chemogenetic manipulation in ACA affected anxiety-like behaviors, we subjected the hM4D(Gi) dams and sires to the elevated-plus maze 20 minutes after injecting clozapine and control group of dams and sires that did not express the hM4D(Gi) construct (Figure 4.11). We found no difference between the two groups of dams and sires in the percent of time spent in the open arms and the number of entries to the open arms (Figure 4.11C-F). These results suggest that our manipulation did not have an effect on anxiety-like behaviors.

4.8 Activation of excitatory neurons in ACA

Our photometry results showed that excitatory neurons in ACA were more active in dams compared to sires during pup retrieval (Figure 4.1H). Therefore, we tested whether activating excitatory ACA neurons in males had a positive effect on pup retrieval behavior. To test this hypothesis, we injected an AAV construct in the ACA expressing hM3D(Gq) under the control of the CaMKII promoter in CBA/CaJ males to restrict expression to excitatory neurons (Figure 4.11A-C). We performed pup retrieval assays from post-natal day 0-5 as illustrated in figure 4.11D. We alternated days of injection of the ligand clozapine and saline to have a control manipulation using the same subjects. Surprisingly, we found that chemogenetic activation of excitatory neurons in ACA showed an increase in the latency to retrieve pups in sires, and in most cases, completely impaired pup retrieval

behavior (Figure 4.11E-F). There was a significant difference in pup retrieval behavior when we compared GFP controls injected with clozapine compared to hM3D(Gq) expressing sires injected with clozapine. However, we also observed a similar difference in the days we injected saline (Figure 4.11F).

4.9 Discussion

The neural substrates that modulate decision-making during parenting behaviors remain elusive. Starting from an unbiased brain-wide activity screen, we associated the anterior cingulate area with pup retrieval behavior. We also observed sex-dependent ACA activation patterns in different behavioral conditions in the whole-brain activity screen. However, we wanted to observe neural activity in freely moving mice to determine the temporal dynamics of ACA neurons during parenting behaviors. We were also interested in whether different cell types in ACA modulate different aspects of pup retrieval behavior. We assessed the activity dynamics of inhibitory and excitatory neurons in ACA during free interactions with pups, and we manipulated the activity of the excitatory population using chemogenetics.

First, we recorded calcium activity in CaMKII positive cells in ACA during free interactions with pups using fiber photometry. Our results showed stronger activation of CaMKII positive cells in dams compared to sires during pup retrieval behavior. Our data showed that the neural responses in ACA are stronger in early post-natal days when the pups are more vulnerable to environmental conditions. We observe that the responses during pup retrieval start before the animals contact a pup prior to retrieving it to the nest. These results suggest that the CaMKII neural responses are associated with the decision-making aspect of retrieving the pups. We do not believe these responses are sensory

responses as we did not see consistent activity during presentation of sensory cues alone (data not shown). The ACA is implicated in sensitivity to distress of conspecifics (Jeon, 2010; Smith et al., 2021). We believe that the ACA may be involved in processing pup distress to influence engagement with the pups. The differences in CaMKII neural activity between males and females may reflect differential sensitivity to pup distress.

The interplay between inhibitory and excitatory neural populations is essential for cortical processing during decision-making (Isaacson and Scanziani, 2011). We recorded the dynamics of inhibitory neurons in the ACA during pup retrieval behavior. Interestingly, we observed a reciprocal relationship with CaMKII positive cells in females but not in males. We also observe that inhibitory neurons in ACA dampen their activity when the mice are in the nest with pups, and it increases when the mice get out of the nest. One possibility is that the ACA modulates parental responsiveness and decision-making by putting the animals in a vigilant state during interactions with pups. It would be interesting to record the activity of different types of interneurons (e.g PV, SOM, VIP) in the cingulate cortex during free interactions with pups.

The cingulate cortex in humans responds to exposing mothers to infant cries and odors (Nishitani et al., 2014) These responses are not present in nonmothers suggesting that the ACA responds to infant distress depending on the female's state. We exposed dams and sires to inaccessible pups in distress. We trapped the pups in a glass jar, and we showed that pups vocalize significantly more in the jar compared to when they are in the nest. We observed that dams exhibit a strong behavioral response to the pups in the jar and spend a significant amount of time trying to access the pups. We do not observe the same response in males, suggesting that males and females exhibit differential sensitivity to pup distress.

These results also suggest that the jar behavioral paradigm can be used to assess parental responsiveness during pro-social behaviors.

Inactivation of the cingulate cortex impairs the acquisition of fear responses by observation (Jeon, 2010), and it also disrupts processing of pain in conspecifics (Smith et al., 2021). When pups get separated from the nest, they emit distress vocalizations that trigger immediate search by the dam. We hypothesized that the ACA is important in processing distress from the pups. Thus, inactivating excitatory neurons in ACA may disrupt pup retrieval behavior. We used inhibitory DREADDS to inactivate ACA during free interactions with pups. We found that the latency to retrieve the pups increases in early post-natal days and the animals' performance level goes back to baseline as the pups get older. These results are consistent with our photometry data that shows stronger ACA activation during pup retrieval behavior in early post-natal days. These activity patterns in ACA are also exhibited during a second pregnancy suggesting that the responses are dependent on the age of the pups. We speculate that when the pups are younger, mice are more vigilant as a result of the pups requiring more care. We do not see a consistent behavioral effect in males suggesting that pup distress is modulated in a sex-dependent manner in the ACA.

We trapped the pups in a jar and inactivated the ACA. We found that the inactivation decreases the amount of time animals spent trying to access the pups. These observations are dependent on the pups because we did not see any difference in the amount of time animals spent exploring an empty jar. Finally, we hypothesized that activating the ACA during pup retrieval behavior would improve sire's performance. We activated CaMKII positive cells using DREADDS. However, we found the opposite effect. As previously mentioned, the inhibitory/excitatory balance in the cortex is important for cortical processing during decision-making (Isaacson and Scanziani, 2011). Our activation

data suggest that sustained activation of the ACA perturbs the network in a way that is more detrimental to the animals' behavior. We propose that the cingulate cortex is involved in parental responsiveness and that it maintains differential sensitivity to pup distress in dams and sires.

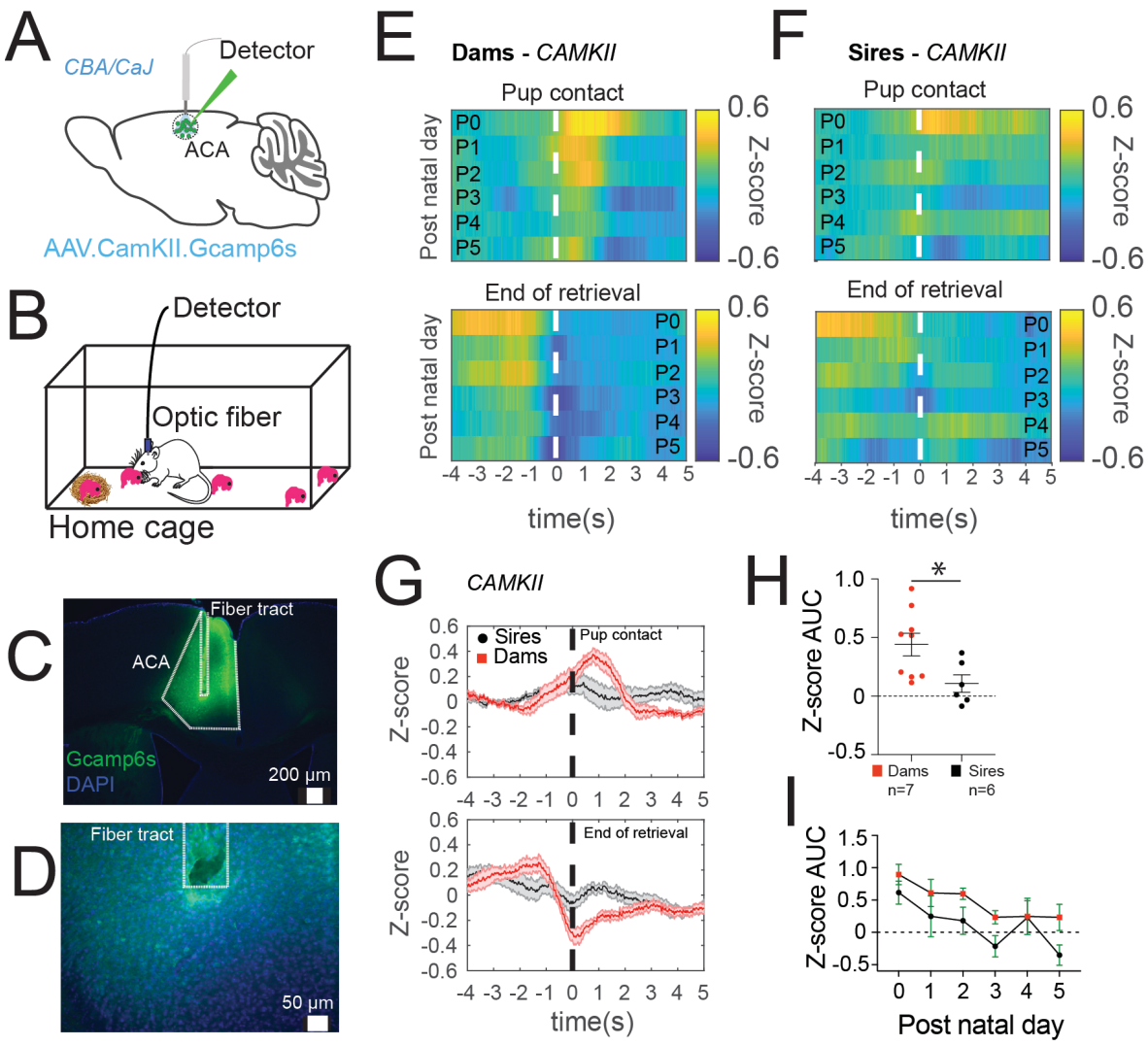


Figure 4.1. Cingulate cortex activity in excitatory cells increases during pup retrieval behavior.

A) Viral strategy to express GCaMP6s in excitatory neurons in the cingulate cortex. B) Schematic of behavioral paradigm. C-D) Representative images of fiber placement and GCaMP6s expression in the anterior cingulate area. E) These heatmaps depict fluorescent GCaMP6s signals detected from excitatory neurons in the cingulate cortex using fiber photometry during pup gathering events in mothers (n=7). The top panel is a heatmap aligned to the pup contact followed by retrieval events in which each row is the mean z-score for pup gathering event responses across all mice per day. The plot spans 6 days. The bottom panel shows the same signals aligned to the end of the retrieval events. F) These heatmaps depict fluorescent GCaMP6s signals detected from excitatory neurons in the cingulate cortex using fiber photometry during pup gathering events in fathers (n=6). G) Each panel shows the mean z-score for all of the data contrasting dams and sires. H) Quantification of the area under the curve from the pup contact to 2 seconds after; Mann Whitney test *p<0.05. I) Quantification of the area under the curve across post-natal days 0-5 shows a decline in the magnitude of activity.

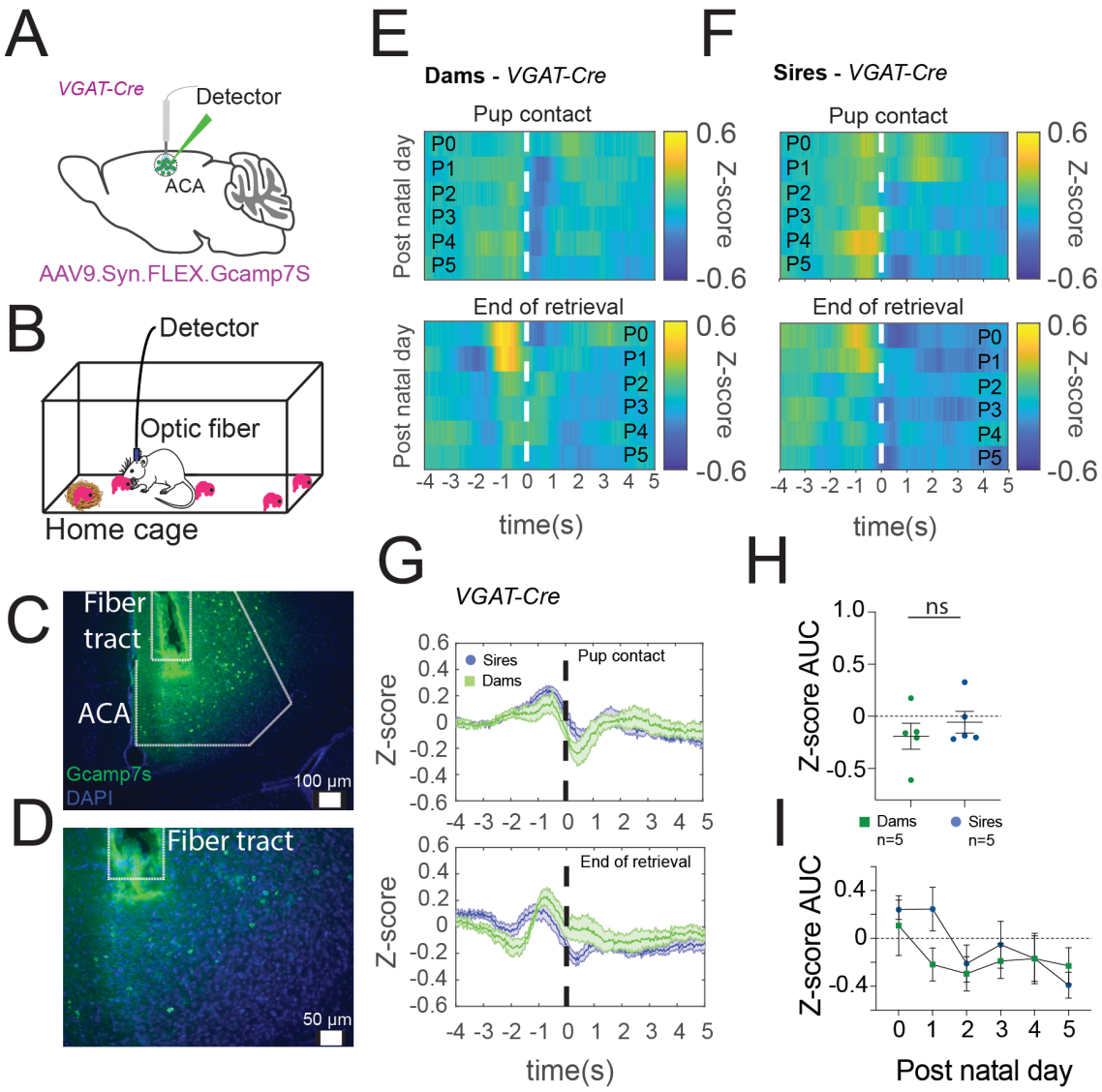


Figure 4.2. Cingulate cortex activity in inhibitory cells decreases during pup retrieval behavior.

A) Viral strategy to express GCaMP7s in inhibitory neurons in the cingulate cortex. B) Schematic of behavioral paradigm. **C-D)** Representative images of fiber placement and GCaMP7s expression in the cingulate cortex. **E)** These heatmaps depict fluorescent GCaMP7s signals detected from inhibitory neurons in the cingulate cortex using fiber photometry during pup gathering events in dams (n=5). The top panel is a heatmap aligned to the pup contact followed by retrieval events in which each row is the mean z-score for pup gathering event responses across all mice per day. The plot spans 6 days. The bottom panel shows the same signals aligned to the end of the retrieval events. **F)** These heatmaps depict fluorescent GCaMP7s signals detected from inhibitory neurons in the cingulate cortex using fiber photometry during pup gathering events in sires (n=5). **G)** Each panel shows the mean z-score for all of the data contrasting dams and sires. **H)** Quantification of the area under the curve from the pup contact to 2 seconds after; Mann Whitney test n.s. **I)** Quantification of the area under the curve across post-natal days 0-5 shows a decline in the magnitude of activity.

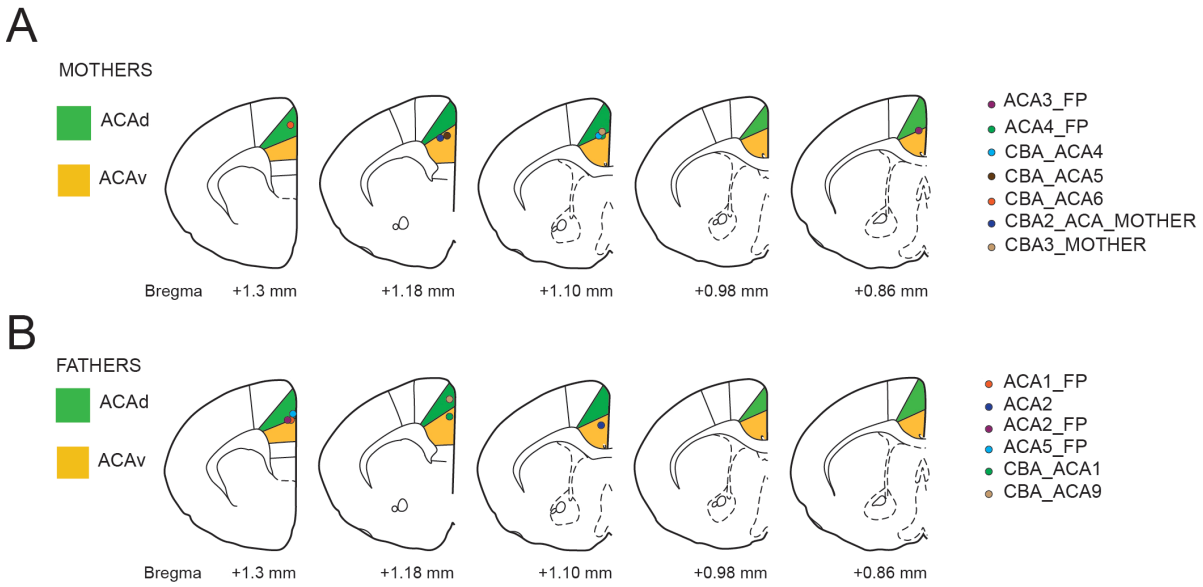
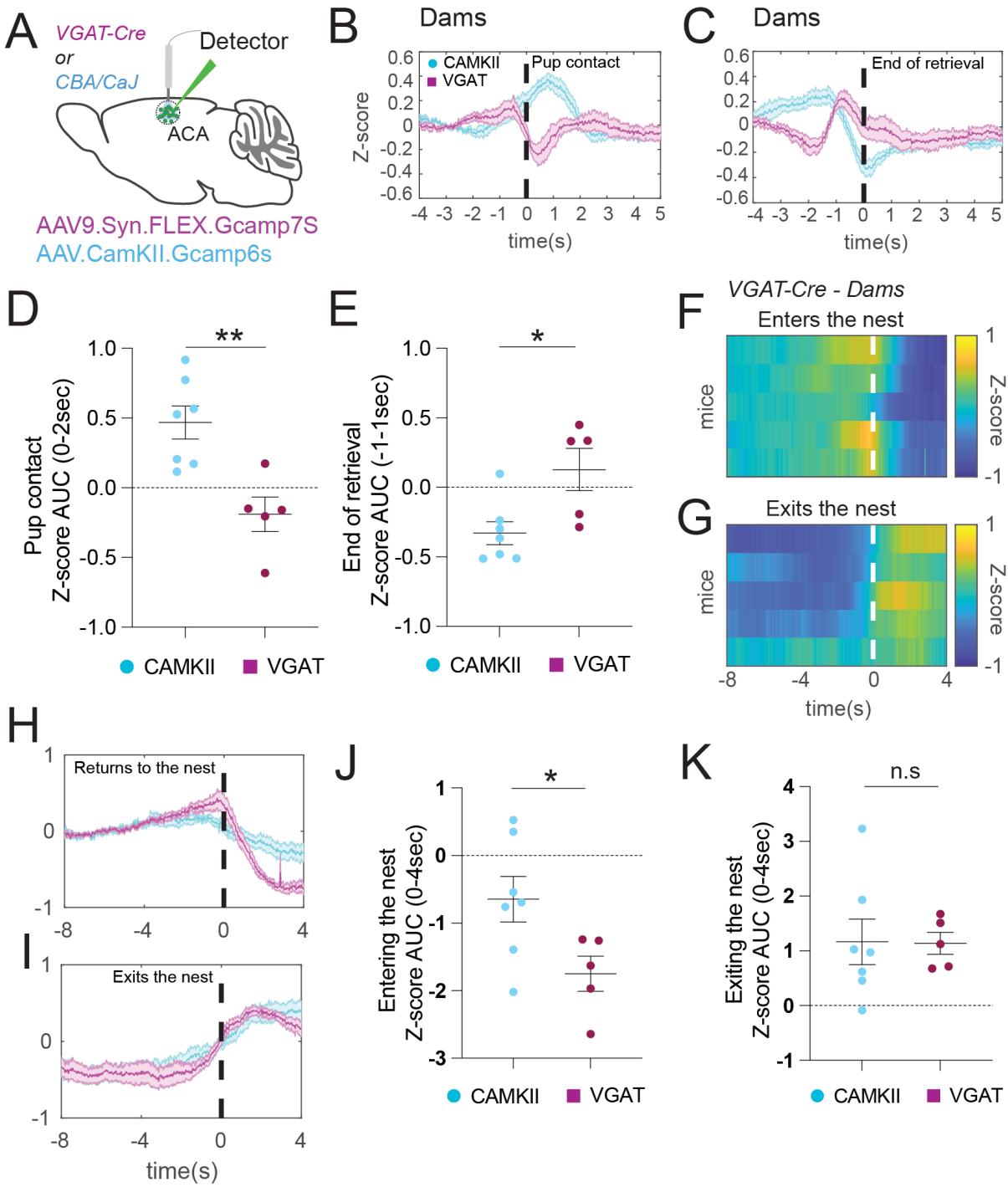


Figure 4.3. Fiber placement map of CAMKII fiber photometry recordings.

A) Schematic representations of the fiber locations for photometry recordings in excitatory cells in the anterior cingulate area of dams. **B)** Schematic representations of the fiber locations for photometry recordings in excitatory cells in the anterior cingulate area of sires. Green and yellow represent the dorsal and ventral parts of the anterior cingulate area, respectively.

Figure 4.4. Cingulate cortex activity in inhibitory cells decreases during pup retrieval behavior.

A) Viral strategy to express GCaMP7s in inhibitory neurons in the cingulate cortex. **B)** The panel shows the mean z-score for pup retrieval events aligned to the pup contact contrasting excitatory and inhibitory responses in dams (n=7 mice and n=5 mice respectively). **C)** The panel shows the mean z-score for pup retrieval events aligned to the end of the retrieval event contrasting excitatory and inhibitory responses in dams (n=7 mice and n=5 mice respectively). **D)** Quantification of the area under the curve from the pup contact to 2 seconds after; Unpaired t-test **p=0.0038 **E)** Quantification of the area under the curve from 1 second before the end of the retrieval to 1 second after; Unpaired t-test *p=0.0169 **F-G)** These heatmaps depict fluorescent GCaMP7s signals recorded from inhibitory neurons in the cingulate cortex using fiber photometry during free interactions with pups. The signals are aligned to the mother entering the nest after all the pups have been retrieved and to the mother leaving the nest. Each row represents the mean z-score from postnatal day 0-5 for each mouse (n=5 mice). **H-I)** Each panel shows the mean z-score for the data in F-G contrasting excitatory and inhibitory responses to entering and leaving the nest. **J)** Quantification of the area under the curve from entering the nest to 4 seconds after; Unpaired t-test *p=0.0378 **K)** Quantification of the area under the curve from exiting the nest to 4 seconds after; Unpaired t-test p=0.9613.


A
VGAT-Cre
or
CBA/CaJ
Detector
ACA
AAV9.Syn.FLEX.Gcamp7S
AAV.CamKII.Gcamp6s
B
Dams
CAMKII
VGAT
Pup contact
Z-score
time(s)
C
Dams
End of retrieval
time(s)
D
Pup contact
Z-score AUC (0-2sec)
**
CAMKII
VGAT
E
End of retrieval
Z-score AUC (-1-1sec)
*
CAMKII
VGAT
F
VGAT-Cre - Dams
Enters the nest
mice
Z-score
G
Exits the nest
mice
Z-score
time(s)
H
Returns to the nest
I
Exits the nest
time(s)
J
Entering the nest
Z-score AUC (0-4sec)
*
CAMKII
VGAT
K
Exiting the nest
Z-score AUC (0-4sec)
n.s
CAMKII
VGAT

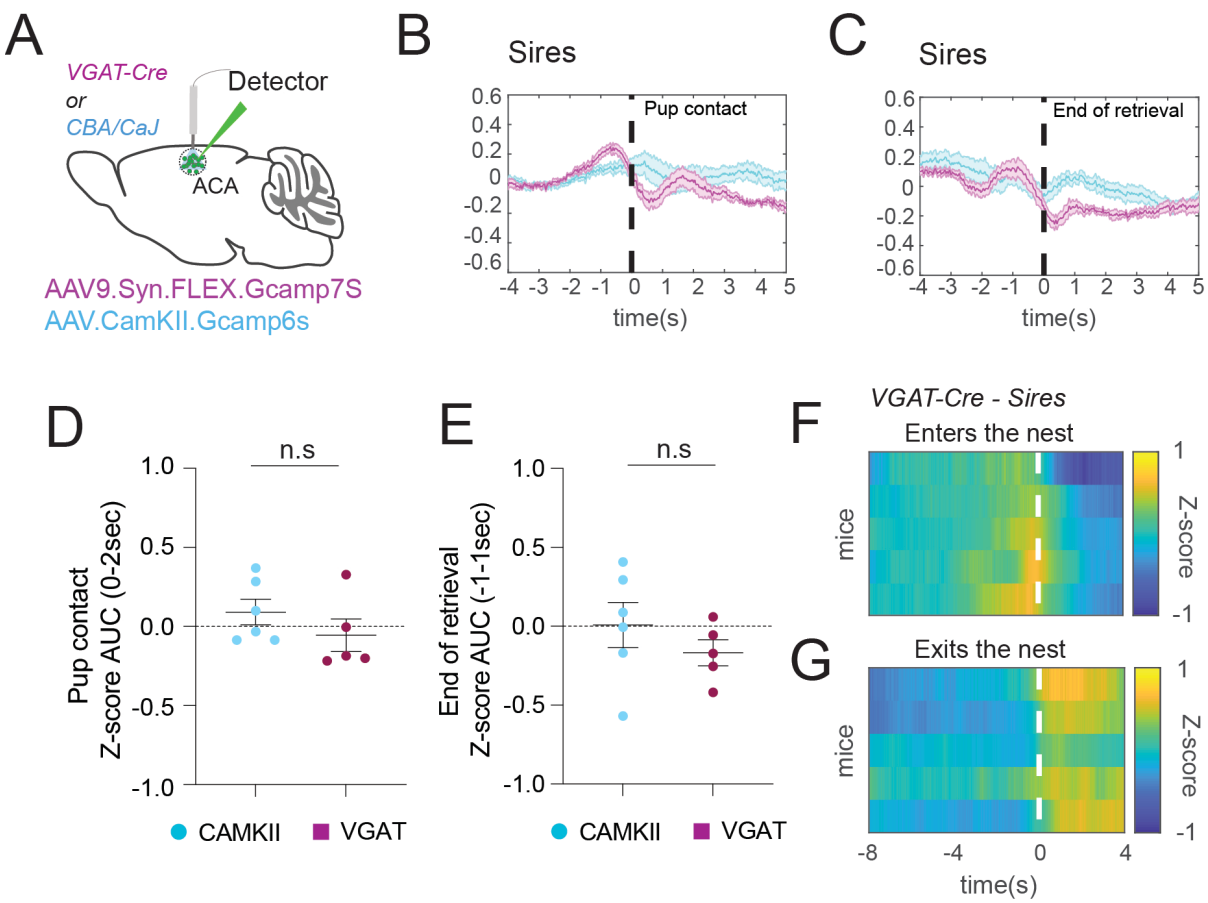
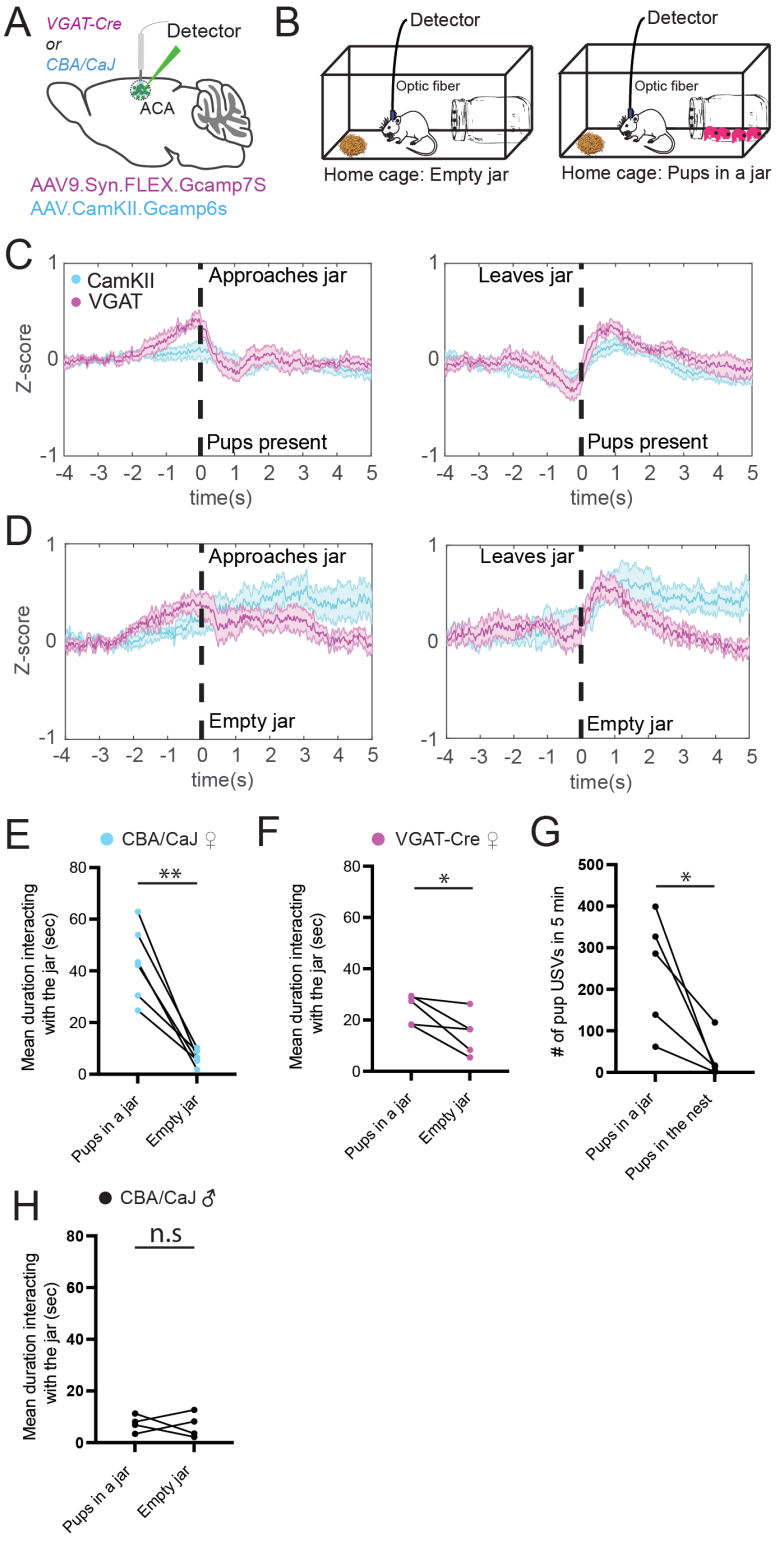


Figure 4.5. Cingulate cortex activity in inhibitory cells decreases during pup retrieval behavior.

A) Viral strategy to express GCaMP7s in inhibitory neurons in the cingulate cortex. **B)** The panel shows the mean z-score for pup retrieval events aligned to the pup contact contrasting excitatory and inhibitory responses in sires (n=6mice and n=5 mice respectively). **C)** The panel shows the mean z-score for pup retrieval events aligned to the end of the retrieval event contrasting excitatory and inhibitory responses in sires (n=6 mice and n=5 mice respectively). **D)** Quantification of the area under the curve from the pup contact to 2 seconds after; Unpaired t-test not significant **E)** Quantification of the area under the curve from 1 second before the end of the retrieval to 1 second after; Unpaired t-test not significant. **F-G)** These heatmaps depict fluorescent GCaMP7s signals recorded from inhibitory neurons in the cingulate cortex using fiber photometry during free interactions with pups. The signals are aligned to the sires entering the nest after all the pups have been retrieved and to the sires leaving the nest. Each row represents the mean z-score from postnatal day 0-5 for each mouse (n=5 mice).

Figure 4.6. Trapping pups in a jar induces pup USVs and elicits a behavioral response in dams.

A) Viral strategy to express GCaMP7s in inhibitory neurons in the cingulate cortex and GCaMP6s in excitatory neurons. **B)** Schematic of behavioral paradigm. **C)** Mean z-score traces of excitatory and inhibitory neuron responses when the pups are trapped in the jar aligned to when the dam approaches the lid of the jar and when the dam leaves the jar (VGAT-Cre n=5 inhibitory and CBA/CaJ n=6). **D)** Mean z-score traces of excitatory and inhibitory neuron responses when the jar is empty aligned to when the dam approaches the lid of the jar and when the dam leaves the jar (VGAT-Cre n=5 inhibitory and CBA/CaJ n=6). **E)** Mean duration of time interacting with the jar when it is empty and when the pups are trapped from P0-P5 (CBA/CaJ dams n=6). **F)** Mean duration of time interacting with the jar when it is empty and when the pups are trapped from P0-P5 (VGAT-Cre dams n=5). **G)** Number of USVs emitted by the pups in 5 minutes recordings when they are in the nest without the dam in the cage and when they are trapped in the jar (n=5 litters). **H)** Mean duration of time interacting with the jar when it is empty and when the pups are trapped from P0-P5 (CBA/CaJ sires n=5).


A
VGAT-Cre
or
CBA/CaJ
Detector
ACA
AAV9.Syn.FLEX.Gcamp7S
AAV.CamKII.Gcamp6s
B
Detector
Optic fiber
Home cage: Empty jar
Detector
Optic fiber
Home cage: Pups in a jar
C
CamKII
VGAT
Approaches jar
Pups present
Z-score
time(s)
Leaves jar
Pups present
time(s)
D
Approaches jar
Empty jar
Z-score
time(s)
Leaves jar
Empty jar
time(s)
E
CBA/CaJ ♀
**
Mean duration interacting with the jar (sec)
Pups in a jar
Empty jar
F
VGAT-Cre ♀
*
Mean duration interacting with the jar (sec)
Pups in a jar
Empty jar
G
*
# of pup USVs in 5 min
Pups in a jar
Pups in the nest
H
CBA/CaJ ♂
n.s
Mean duration interacting with the jar (sec)
Pups in a jar
Empty jar

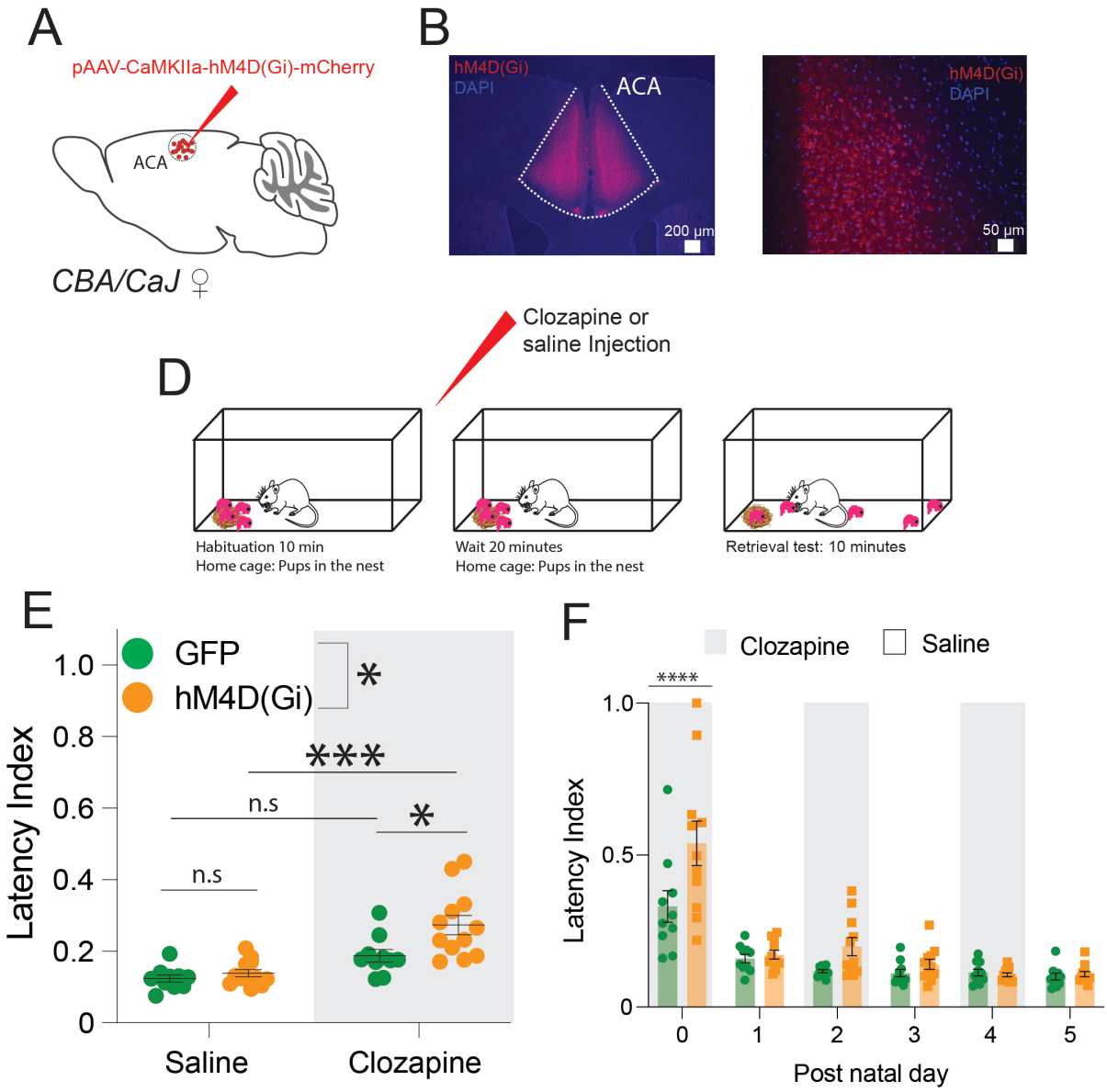
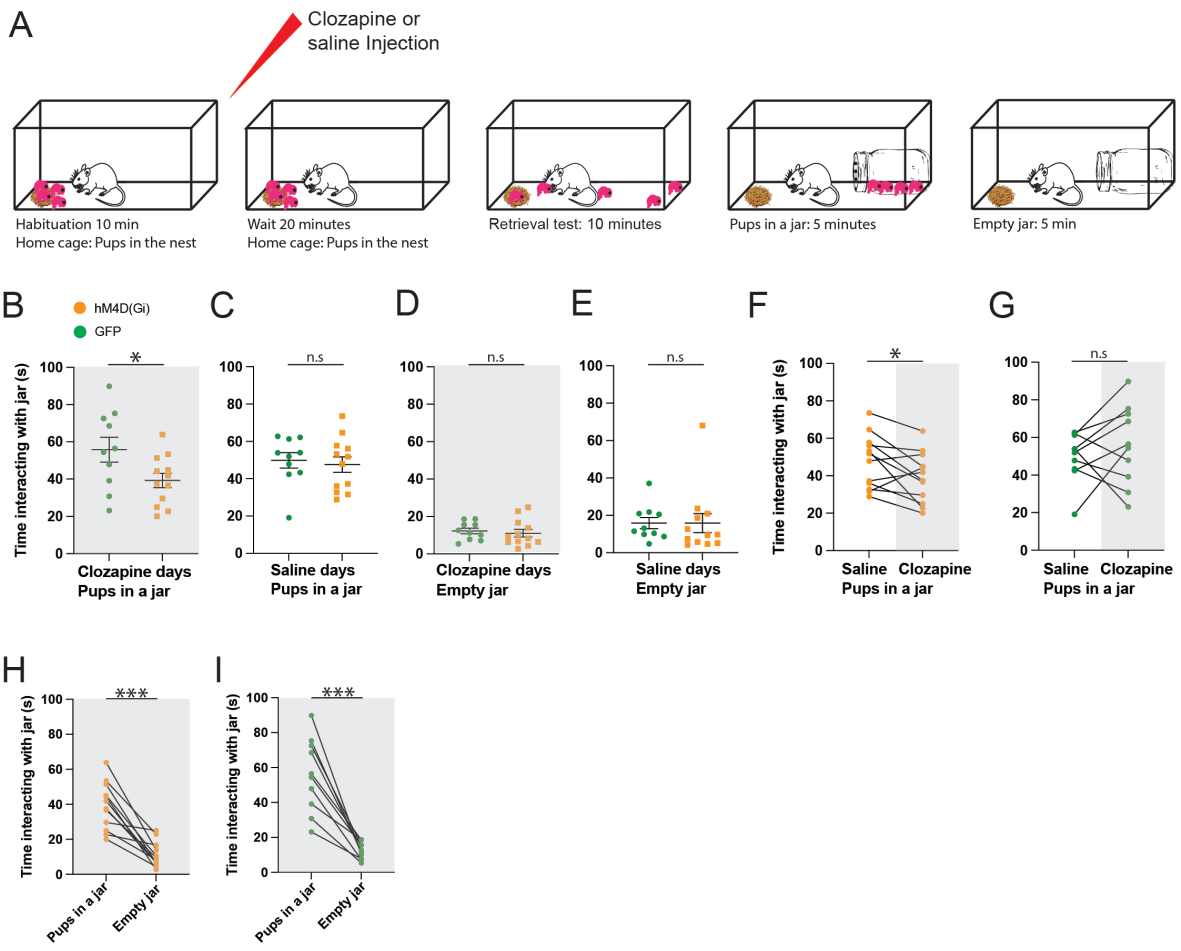


Figure 4.7. Chemogenetic inhibition of ACA disrupts pup retrieval behavior in dams
A) Viral strategy used to inhibit the cingulate cortex during interactions with pups. **B-C)** Representative images of the expression of the hM4D(Gi) construct in the ACA of dams. **D)** Schematic of behavioral paradigm used to measure pup retrieval performance. **E)** Mean latency index (± s.e.m.) in GFP expressing mothers injected with saline (n=10), GFP expressing mothers injected with clozapine (n= same 10 mice from saline experiment), hM4D(Gi) expressing mothers injected with saline (n=12), and hM4D(Gi) expressing mothers injected with clozapine (n= same 12 mice from saline experiment); Ordinary Two-way ANOVA GFP vs hM4D(Gi) ; **p=0.0081 ; F(1,40)=7.774; Saline vs clozapine ; ****p<0.0001 F(1,40)=29.64 ; Tukey's multiple comparison test, Clozapine: GFP vs. Clozapine: hM4D(Gi) **p=0.0094, Saline: hM4D(Gi) vs. clozapine: hM4D(Gi) ***p<0.0001. **F)** Same data as in E, but separated by different post-natal days. Mixed-effects model GFP vs hM4D(Gi); *p=0.0199; Sidak's multiple comparison test, p<0.0001 for P0; P1-P5 ns.

Figure 4.8. Chemogenetic inhibition of ACA decreases parental responsiveness in dams.

A) Schematic of behavioral paradigm used to measure pup retrieval performance and parental responsiveness to pups in distress. **B)** Time interacting with the jar when the pups are trapped by GFP expressing dams (n=10) and hM4D(Gi) expressing dams (n=12) injected with clozapine (Mann Whitney test *p=0.0426). **C)** Time interacting with the jar when the pups are trapped by GFP expressing dams (n=10) and hM4D(Gi) expressing dams (n=12) injected with saline (Mann Whitney test p=0.6277). **D)** Time interacting with the empty jar by GFP expressing dams (n=10) and hM4D(Gi) expressing dams (n=12) injected with clozapine (Mann Whitney test p=0.4176). **E)** Time interacting with the empty jar by GFP expressing dams (n=10) and hM4D(Gi) expressing dams (n=12) injected with saline (Mann Whitney test p=0.3463). **F)** Time interacting with the jar when the pups are trapped by hM4D(Gi) expressing dams injected with saline (n=12) and hM4D(Gi) expressing dams (n=12) injected with clozapine (Paired t test *p=0.0369; t=2.373; df=11). **G)** Time interacting with the empty jar by GFP expressing dams injected with saline (n=10) and GFP expressing dams (n=10) injected with clozapine (Paired t test p=0.4038; t=0.8759; df=9). **H)** Time interacting with the empty jar by hM4D(Gi) expressing dams injected with clozapine (n=12) and time interacting with the jar with the pups by hM4D(Gi) expressing dams (n=12) injected with clozapine (Paired t test ****p<0.0001; t=6.636; df=11). **I)** Time interacting with the empty jar by GFP expressing dams injected with clozapine (n=10) and time interacting with the jar with the pups by GFP expressing dams (n=10) injected with clozapine (Paired t test ****p<0.0001; t=6.717; df=9).


A
Clozapine or
saline Injection
Habituation 10 min
Home cage: Pups in the nest
Wait 20 minutes
Home cage: Pups in the nest
Retrieval test: 10 minutes
Pups in a jar: 5 minutes
Empty jar: 5 min
B
hM4D(Gi)
GFP
Time interacting with jar (s)
Clozapine days
Pups in a jar
C
n.s
Saline days
Pups in a jar
D
n.s
Clozapine days
Empty jar
E
n.s
Saline days
Empty jar
F
Saline
Clozapine
Pups in a jar
G
n.s
Saline
Clozapine
Pups in a jar
H
Pups in a jar
Empty jar
I
Pups in a jar
Empty jar

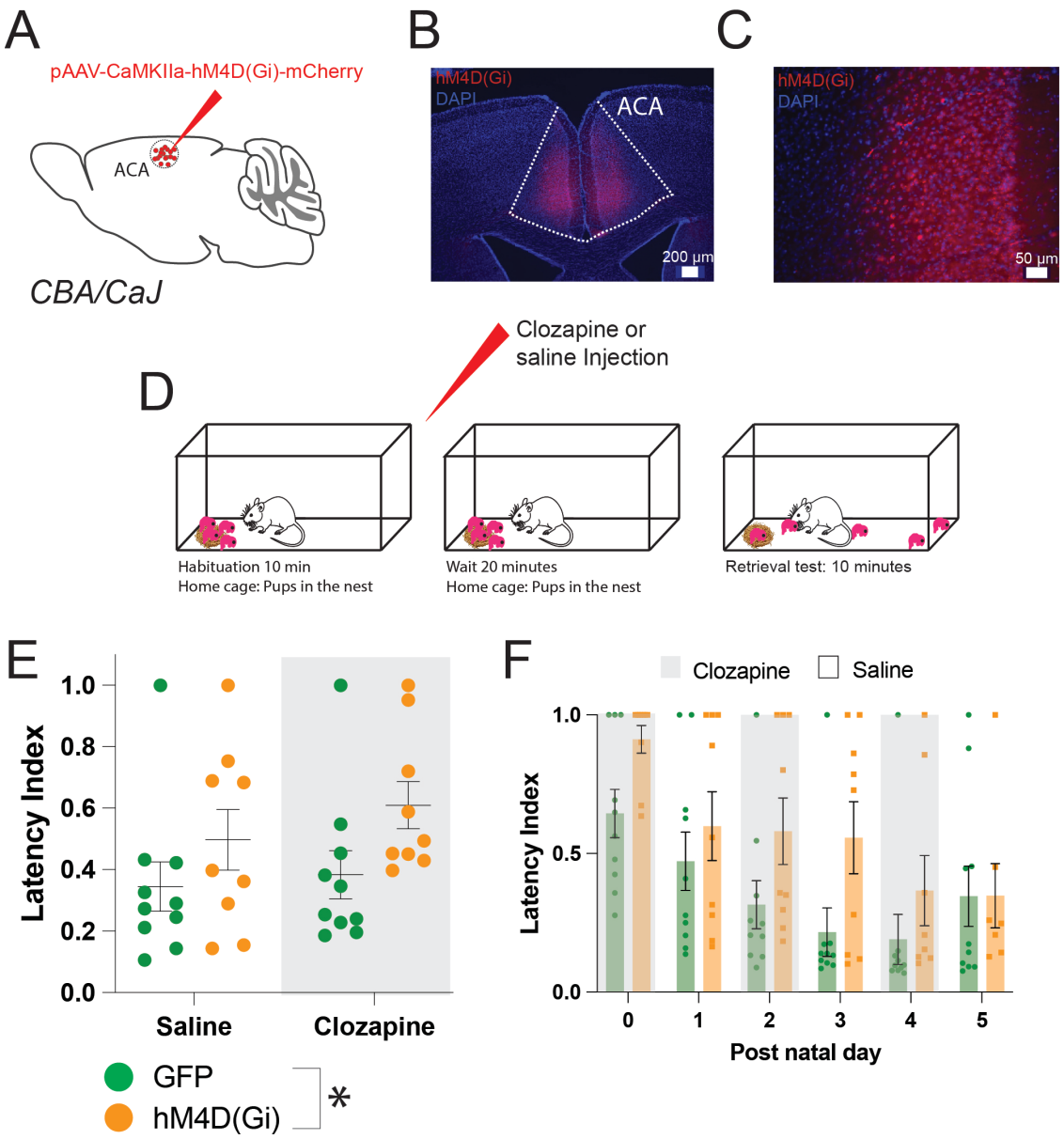


Figure 4.9. Chemogenetic inhibition of excitatory neurons in ACA does not disrupt pup retrieval behavior in sires.

A) Viral strategy used to inhibit the cingulate cortex during interactions with pups. **B-C)** Representative images of the expression of the hM4D(Gi) construct in the ACA of sires. **D)** Schematic of behavioral paradigm used to measure pup retrieval performance. **E)** Mean latency index (± s.e.m.) in GFP expressing sires injected with saline (n=10), GFP expressing sires injected with clozapine (n= same 10 mice from saline experiment), hM4D(Gi) expressing sires injected with saline (n=9), and hM4D(Gi) expressing sires injected with clozapine (n= same 9 mice from saline experiment); Ordinary Two-way ANOVA GFP vs hM4D(Gi) ; *p=0.0303 ; F(1,34)=5.110; Tukey's multiple comparison test, no significant comparisons. **F)** Same data as in E, but separated by different post-natal days. There were no statistically significant comparisons.

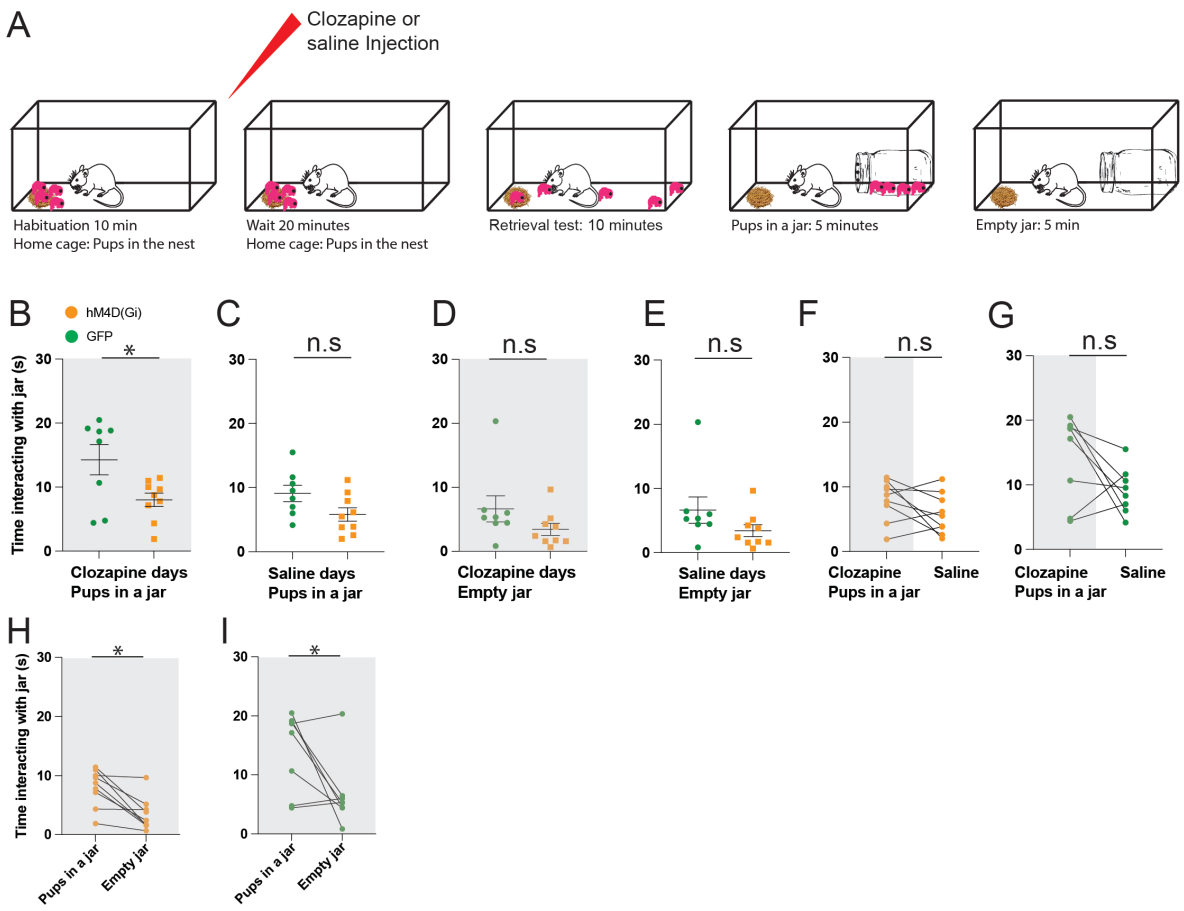


Figure 4.10. Chemogenetic inhibition of excitatory neurons in ACA does not decrease parental responsiveness in sires.

A) Schematic of behavioral paradigm used to measure pup retrieval performance and parental responsiveness to pups in distress. **B)** Time interacting with the jar when the pups are trapped by GFP expressing sires (n=8) and hM4D(Gi) expressing sires (n=9) injected with clozapine (Mann Whitney test p=0.0592). **C)** Time interacting with the jar when the pups are trapped by GFP expressing sires (n=8) and hM4D(Gi) expressing sires (n=9) injected with saline (Mann Whitney test p=0.0592). **D)** Time interacting with the empty jar by GFP expressing sires (n=8) and hM4D(Gi) expressing sires (n=9) injected with clozapine (Mann Whitney test p=0.0592). **E)** Time interacting with the empty jar by GFP expressing sires (n=8) and hM4D(Gi) expressing sires (n=9) injected with saline (Mann Whitney test p=0.2660). **F)** Time interacting with the jar when the pups are trapped by hM4D(Gi) expressing sires injected with saline (n=9) and hM4D(Gi) expressing sires (n=9) injected with clozapine (Paired t test p=0.1392; t=1.642; df=8). **G)** Time interacting with the empty jar by GFP expressing sires injected with saline (n=8) and GFP expressing sires (n=8) injected with clozapine (Paired t test p=0.1024; t=1.878 df=7). **H)** Time interacting with the empty jar by hM4D(Gi) expressing sires injected with clozapine (n=9) and time interacting with the jar with the pups by hM4D(Gi) expressing sires (n=9) injected with clozapine (Paired t test **p<0.0036; t=4.069; df=8). **I)** Time interacting with the empty jar by GFP expressing sires injected with clozapine (n=8) and time interacting with the jar with the pups by GFP expressing sires (n=8) injected with clozapine (Paired t test *p=0.0344; t=2.619; df=7).

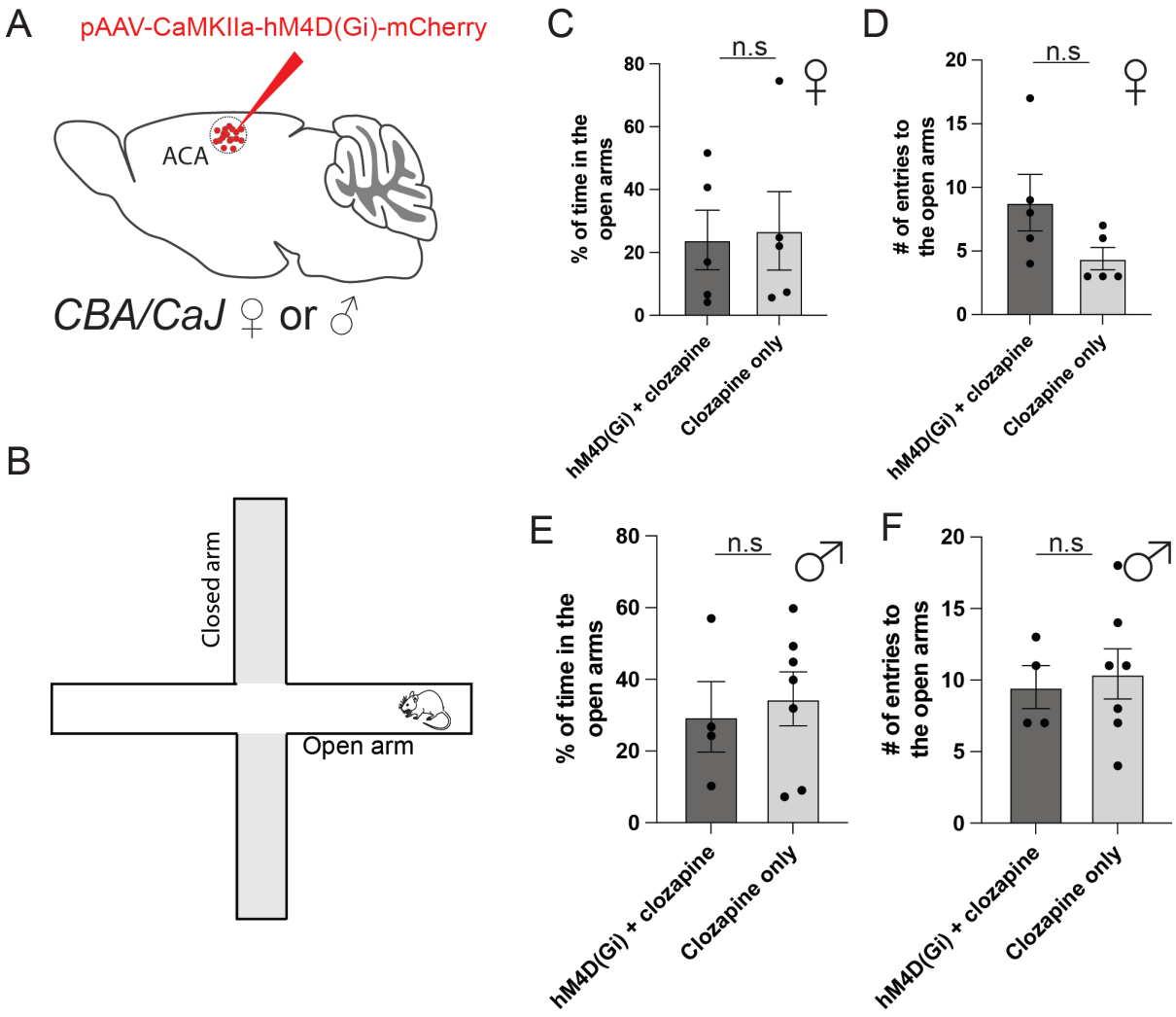


Figure 4.11. Inhibition of excitatory neurons in the anterior cingulate area does not alter anxiety-like behaviors in the elevated plus maze

A) Viral strategy to inhibit the ACA excitatory cell population using chemogenetics. **B)** Schematic of the elevated plus maze set up. **C)** Quantification of the percent of time spent in the open arms by the dams (hM4D(Gi) n=5; control n=5). **D)** Quantification of number of entries to the open arms by the dams (hM4D(Gi) n=5; control n=5). **E)** Quantification of the percent of time spent in the open arms by the sires (hM4D(Gi) n=4; control n=7). **F)** Quantification of number of entries to the open arms by the sires (hM4D(Gi) n=5; control n=7).

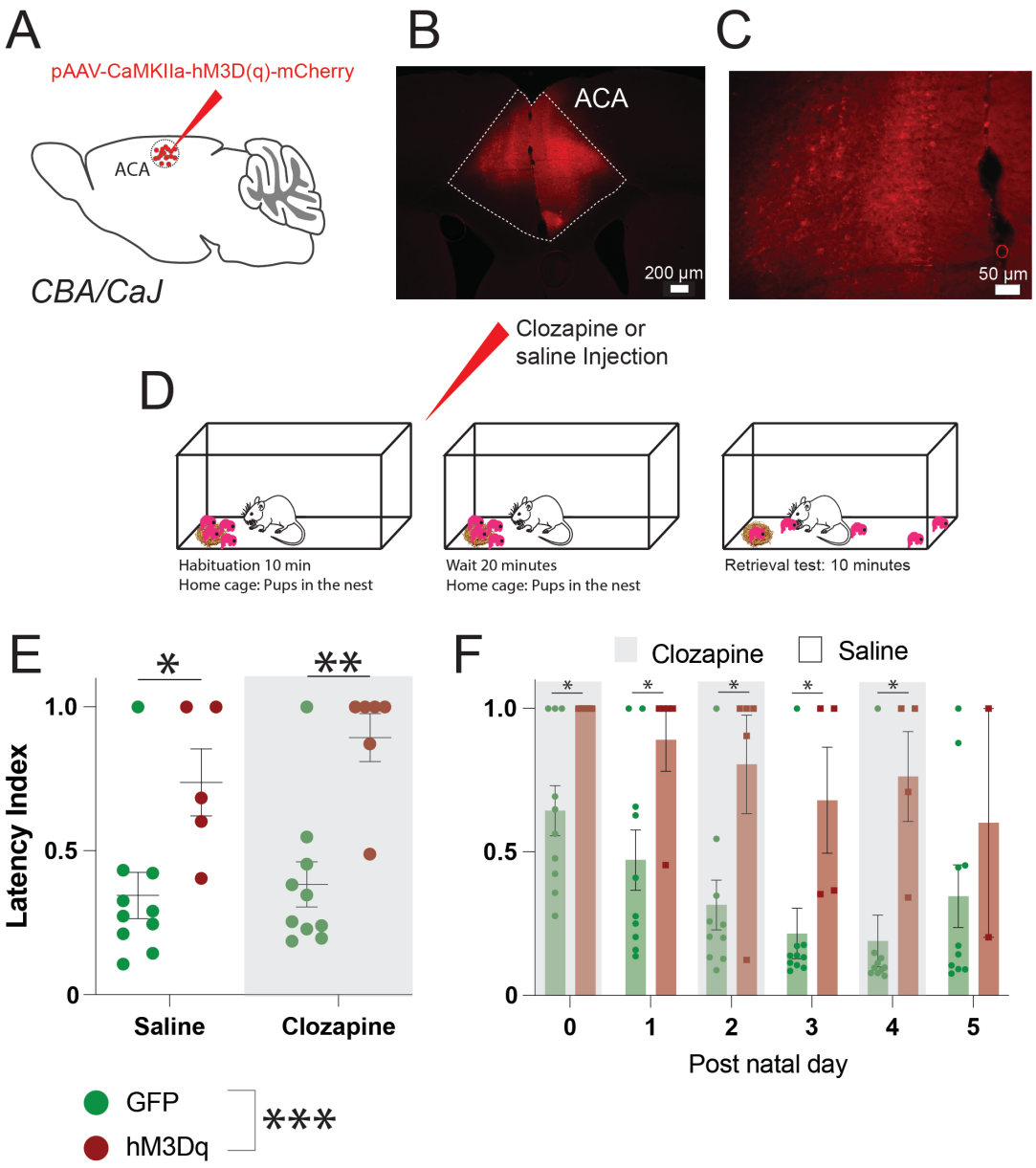


Figure 4. 12. Chemogenetic activation of excitatory neurons in ACA disrupts pup retrieval behavior.

A) Viral strategy used to activate the cingulate cortex during interactions with pups in sires. **B-C)** Representative images of the expression of the hM3D(Gq) construct in the ACA of sires. **D)** Schematic of behavioral paradigm used to measure pup retrieval performance. **E)** Mean latency index (± s.e.m.) in GFP expressing sires injected with saline (n=10), GFP expressing sires injected with clozapine (n= same 10 mice from saline experiment), hM3D(Gq) expressing sires injected with saline (n=5), and hM3D(Gq) expressing sires injected with clozapine (n= same 5 mice from saline experiment); Ordinary Two-way ANOVA GFP vs hM4D(Gi) ; ****p<0.0001 ; F(1,27)=24.17; Tukey's multiple comparison test, Clozapine: GFP vs. Clozapine: hM3D(Gq) **p=0.0021, Saline: hM3D(Gq) vs. clozapine: hM3D(Gq) not significant p=0.7199, Saline: GFP vs Saline: hM3D(Gq) p=0.0318. **F)** Same data as in E separated by postnatal days.

Chapter 5: A Noradrenergic Pathway from the Locus coeruleus to the Anterior Cingulate Cortex is Active During Pup Retrieval Behavior

5.1 Introduction

The anterior cingulate area is interconnected with the noradrenergic locus coeruleus, and activity patterns in ACA are coordinated with activity in LC. For example, in monkeys, the correlation of ACA activity with LC firing is reduced during tonic LC activity, but this correlation increases when external stimuli evoke phasic firing in LC (Joshi and Gold, 2022). The LC was proposed to contribute to goal-directed action selection during parental behavior with global release of noradrenaline (Dvorkin and Shea, 2022). However, the downstream targets of LC that modulate social behavior through NA signaling remain elusive. Here, we confirm a robust projection from LC to the ACA and observe LC activity patterns in males and females during parental behavior. We also measure NA release in ACA during goal-directed social behavior. Our data suggests that phasic firing events in LC evoked by pup retrieval behavior trigger NA release in ACA. Finally, we are currently working on chemogenetically inhibiting the cells that project from LC to ACA during parenting behavior.

5.2 The anterior cingulate area receives robust inputs from the noradrenergic locus coeruleus

Previous reports have shown an anatomical connection between the ACA and LC (Fillinger et al., 2017; Koga et al., 2020). For example, Koga et al., used an anterograde approach by injecting cre-dependent eYFP in the LC of DBH-cre mice and found that the projecting fibers densely innervated both hemispheres of the ACA (Koga et al., 2020). DBH-Cre mice express Cre recombinase in cells that express dopamine beta hydroxylase. This

enzyme converts dopamine into noradrenaline and therefore selectively labels noradrenergic LC neurons. On the other hand, Fillinger et al., used a retrograde approach to trace the afferents of the mouse ACA. They injected cholera toxin B (CTB) and FluoroGold (FG) and found robust labeling in the LC (Fillinger et al., 2017). To confirm those data, we injected a retrograde adeno- associated virus (AAV) driving expression of the fluorescent reporter tdTomato in CBA/CaJ mice. We sliced the LC and stained for tyrosine hydroxylase to define the boundaries of LC and identify retrogradely labeled cells within LC (Figure 5.1A). Furthermore, we injected cre-dependent tdTomato in the LC of DBH-Cre mice and used serial two-photon tomography to visualize the projection fibers in the ACA (Figure 5.1B). We confirmed robust projections from LC to ACA with both strategies. We did not quantify these data.

5.3 LC activity patterns in males and females during pup retrieval behavior

Previous data from our lab suggests that LC activity is associated with maternal behavior in virgin females that undergo surrogacy (data not shown). We noticed that activity in the ACA and the LC appear to be regulated at similar timing during pup retrieval behavior. To record LC activity during pup retrieval in dams and sires, we performed fiber photometry of GCaMP7s signal in LC of freely behaving mice. We injected a Cre-dependent adeno- associated virus (AAV) driving expression of the fluorescent activity reporter GCaMP7s into DBH-Cre mice that express Cre recombinase in cells that express dopamine beta hydroxylase (Figure 5.2 A-C). Our recordings showed time-locked responses to when dams and sires contact a pup prior to a retrieving event (Figure 5.2D-E). These responses are sustained for the entire pup retrieval event and get back to baseline activity levels when the mice drop the pup in the nest (Figure 5.2D-E). These LC responses are also very consistent

across post-natal days. We observed that the LC responses associated with pup retrieval are longer in sires compared to dams (Figure 5.2F). To quantify pup retrieval evoked activity, we calculated the mean z-score of the df/f trace. We cut the traces from 4 seconds before the pup contact to 5 seconds after the pup contact, and we calculated the area under the curve for 4 seconds after the pup contact preceding a retrieval event. We found that LC responses are weaker in dams compared to sires (Figure 5.2G).

We also quantified the duration of the retrieval events from postnatal days 0-5 and found that the sires retrieval events are significantly longer than the dams (Figure 5.3B) with sires averaging approximately 4 seconds and dams 3 seconds. We performed Spearman correlations of the area under the curve and the duration of the retrieval events. We found that there is a positive correlation between those two variables in all mice (Figure 5.3E, 5.4 females and 5.6 males). These data suggest that the temporal precision of the neural activity is related to the temporal precision of the behavior. We then quantified the duration of the intervals between retrieval events, and we observed that sires take longer than dams to engage in the next retrieval event (Figure 5.3C). On average, sires take approximately 18 seconds to engage in the next retrieval event and dams approximately 4.5 seconds. These data were collected from post-natal day 0-5 and each mouse retrieved its own litter of pups four times each day in 5-minute sessions. We correlated the duration of the intervals between retrieval events with the area under the curve of the subsequent trial and found a positive correlation in most mice (Figure 5.3D, Figure 5.5 females, and Figure 5.6 males). These results show that the longer the mice wait to engage in the next retrieval event can indicate how strong the response in the LC is in the next trial.

5.4 Noradrenaline release in ACA during parental behavior in males and females

Based on our previous data from LC and the hypothesis that LC shapes social goal-directed behavior by modulating motivational salience to social cues, we wanted to determine if there was noradrenaline (NA) release in the cingulate cortex during pup retrieval behavior. We injected a noradrenaline sensor (Feng et al., 2019) in the cingulate cortex and performed fiber photometry in freely behaving dams and sires (Figure 5.6A-B). We confirmed expression of the sensor and fiber placement (Figure 5.6C-D). We aligned the NA release traces to the moment animals contacted a pup and retrieved it to the nest and also to the moment the animals dropped the pup back in the nest. We found that bursts of NA release in ACA are associated with pup retrieval events in sires (Figure 5.6E-F). To quantify pup retrieval evoked NA release, we calculated the mean z-score of the df/f trace. We cut the traces from 4 seconds before the pup contact to 5 seconds after the contact. We measured the area under the curve for 5 seconds after the pup contact and observed a difference in the magnitude of these bursts when comparing dams to sires, with sires showing stronger NA release in the cingulate cortex during retrieval events (Figure 5.6G-H). These data suggest that there is a functional connection between the LC and the ACA associated with parental behavior.

5.5 Discussion

The LC plays an important role in arousal, formation and memory retrieval, modulation of the sleep/wake cycle, modulation of stress, attentional selection and goal-directed action selection among many other functions (Poe et al., 2020; Ross and Van Bockstaele, 2021). However, the neural mechanisms by which the noradrenergic system

regulates socially-motivated behavior remain poorly understood. Here, we confirmed a robust projection from the LC to the ACA. In chapter 4, I provided evidence that suggests that the ACA modulates the sensitivity to pup distress differentially in males and females. The LC is known to modulate stress responses through corticotropin releasing factor (CRF) which increases tonic firing in the LC (McCall et al., 2015). Interestingly, there is sex-dependent sensitivity to CRF in LC neurons, with female LC neurons being more sensitive to CRF compared to male LC neurons (Curtis et al., 2006). Thus, the LC is a putative candidate region to modulate sex-dependent stress responses through its projections to ACA and influence goal-directed social behavior.

We observed neural responses in the LC in dams and sires during pup retrieval behavior. These responses are longer in sires compared to dams. The magnitude of these responses is positively correlated with the duration of retrieval events and the duration of the intervals between retrieval events suggesting that sexually divergent activation of the LC leads to sex differences in parental behavior. We were interested in whether these sex-dependent differences in LC activity led to sex differences in NA release in downstream targets. We probed NA release in ACA during pup retrieval behavior using a genetically encoded fluorescent sensor (Feng et al., 2019). Consistent with the longer responses in LC activity in sires compared to dams, we found that pup retrieval evokes more transient NA release in sires compared to dams. However, when we look at sustained NA release in ACA for extended periods of times during parenting behavior (Figure 5.7), we see a sustained and tonic increase in NA release in dams and not in males. These results suggest that NA in ACA may be acting at different time scales in dams compared to sires. For example, one hypothesis is that the LC-NA system in males responds more to acute and salient

interactions with pups and females are more attentive and hypervigilant with the pups through sustained NA release in ACA.

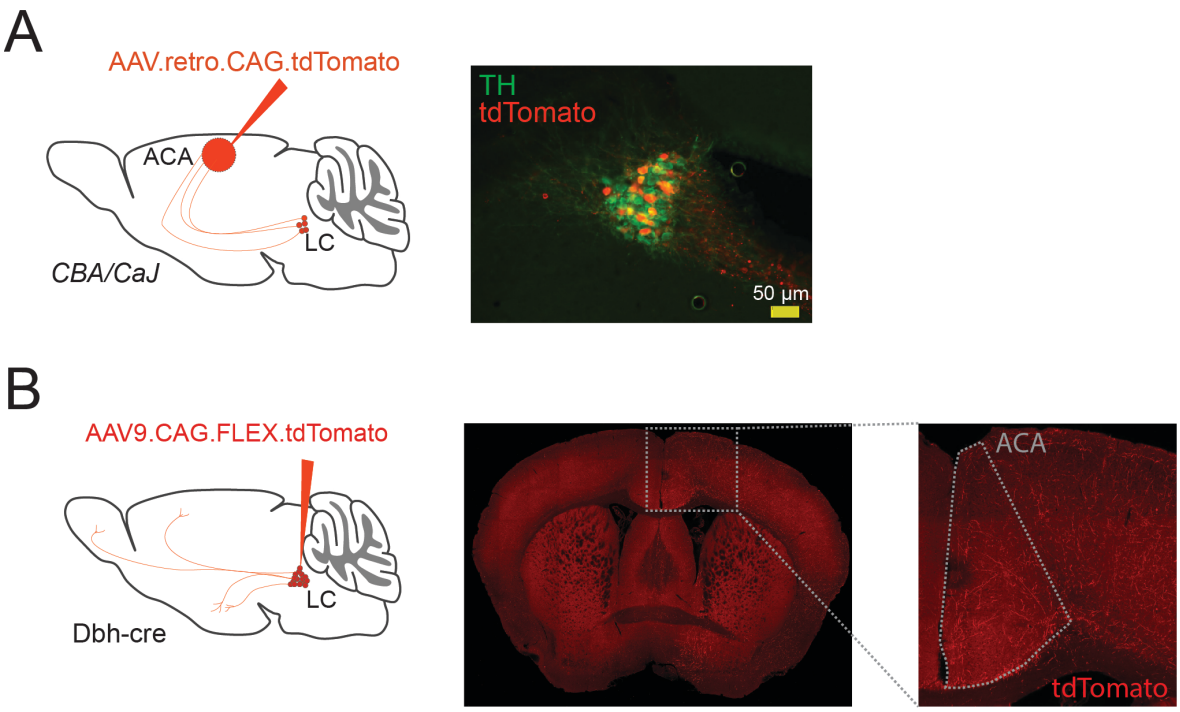
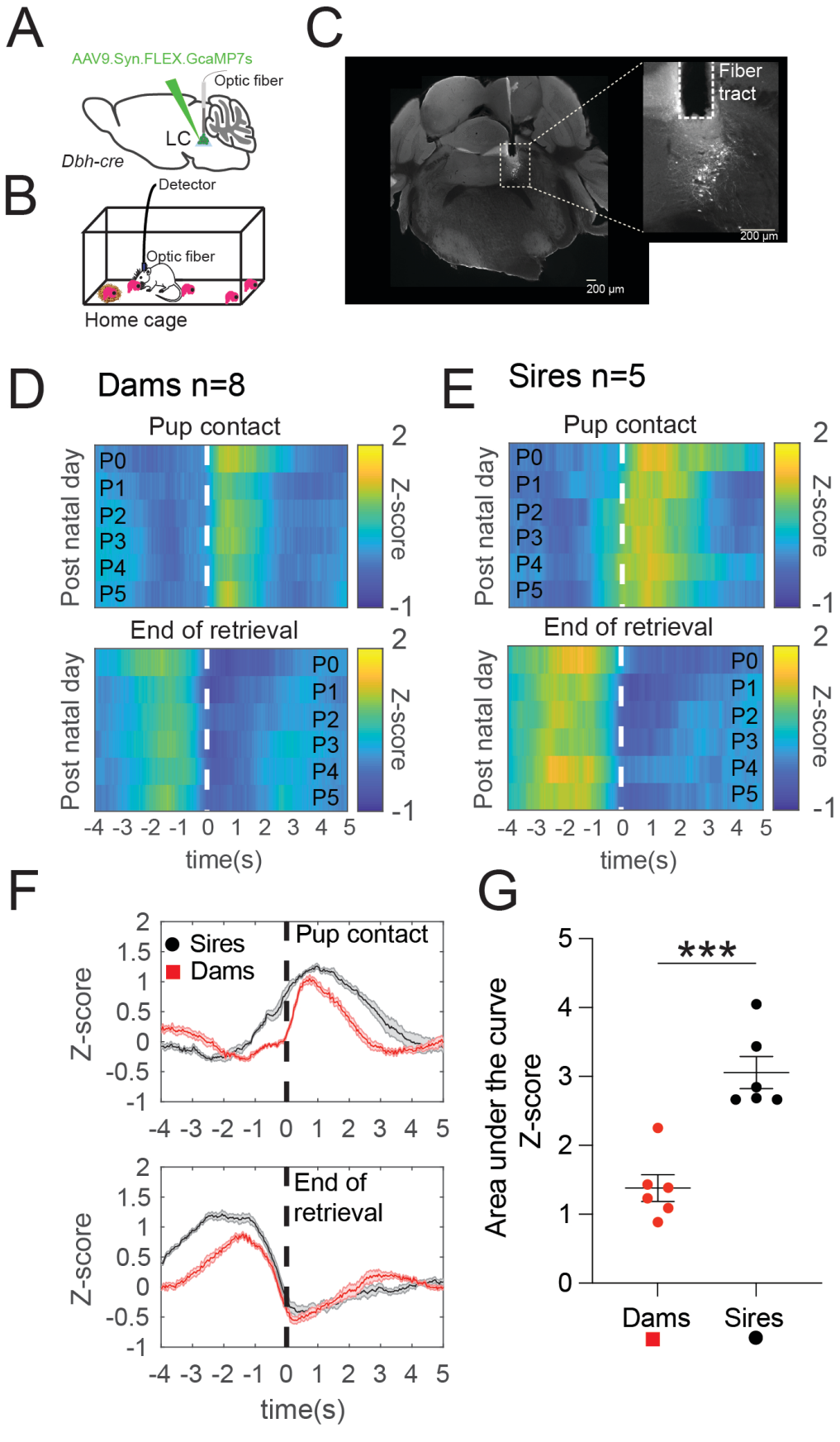


Figure 5.1. Noradrenergic projection from the locus coeruleus to the anterior cingulate area.
A) Representative image of retrograde labeling from ACA to LC. Green labeling is tyrosine hydroxylase (TH) antibody staining and red is tdTomato from the retrograde injection. **B)** Representative images of the ACA taken with serial two-photon tomography of a DBH-Cre mouse injected with cre-dependent tdTomato in LC.

Figure 5. 2. Timing and amplitude of LC activity associated with pup retrieval behavior is different in sires compared to dams.

A) Viral strategy to selectively express GCaMP7s in dbh+ neurons in the LC. **B)** Schematic of the pup retrieval behavioral paradigm. **C)** Representative image of the GCaMP7s expression in the LC in a coronal orientation. **D-E)** These heatmaps depict fluorescent GCaMP7s signals detected from LC using fiber photometry during pup gathering events in dams (n=8) and sires (n=5). The top panels are heatmaps aligned to the pup contact followed by a retrieval event in which each row is the mean z-score deltaf/f for pup gathering event responses per day across all mice. The plot spans 6 days. The bottom panels show the data aligned to the end of the retrieval events. **F)** The top panel shows the mean z-score deltaf/f for all of the data aligned to the pup contact followed by a retrieval event contrasting dams and sires. The bottom panel is aligned to the end of the retrieval events. **G)** Quantification of the area under the curve for the LC responses from the pup contact to 4 seconds after (Mann Whitney test ***$p<0.0001$).


A
AAV9.Syn.FLEX.GcaMP7s
Optic fiber
LC
Dbh-cre
B
Detector
Optic fiber
Home cage
C
Fiber tract
200 µm
200 µm
D
Dams n=8
Pup contact
Post natal day
P0
P1
P2
P3
P4
P5
Z-score
2
-1
End of retrieval
-4 -3 -2 -1 0 1 2 3 4 5
time(s)
E
Sires n=5
Pup contact
End of retrieval
F
Sires
Dams
Pup contact
End of retrieval
Z-score
2
1.5
1
0.5
0
-0.5
-1
-4 -3 -2 -1 0 1 2 3 4 5
time(s)
G
***
Area under the curve
Z-score
5
4
3
2
1
0
Dams
Sires

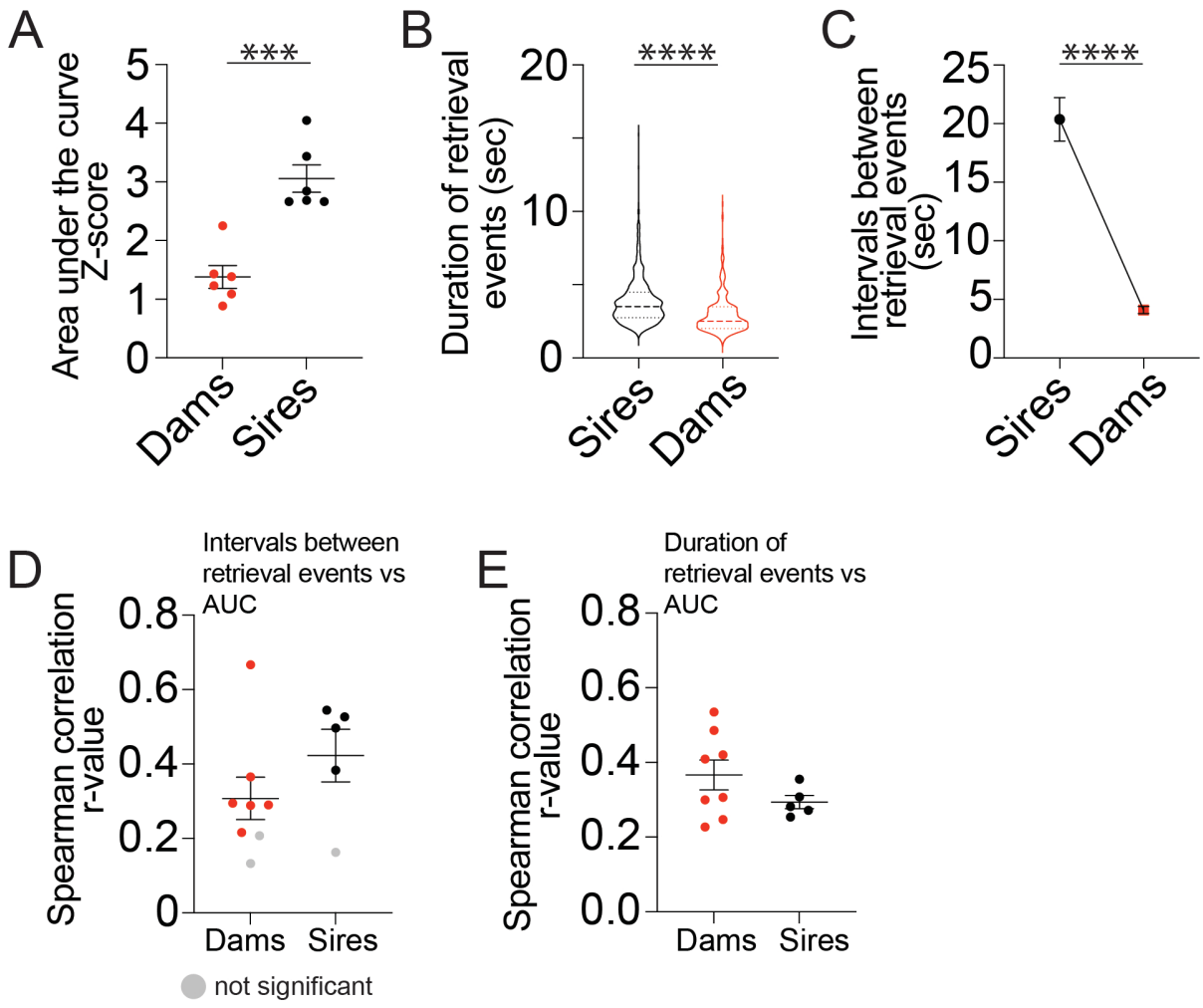


Figure 5.3. Amplitude of LC responses during pup retrieval correlate with the duration of the retrieval events and the duration of the intervals between events.
A) Quantification of the area under the curve for the LC responses from the pup contact to 4 seconds after (Mann Whitney test ***p<0.0001). **B)** Violin plots of the duration of retrieval events (sires n=5 mice, n= 618 events; dams n=8 mice, n=1238 events) Mann-Whitney test p<0.0001. **C)** Duration of intervals in between retrieval events (sires n=5 mice, n=508 events; dams n=8 mice, n=1072 events) Mann-Whitney test p<0.0001). **D)** Scatter plot of Spearman correlation r coefficients in dams and sires of the intervals between retrieval events correlated with magnitude of LC responses during pup retrieval. Gray dots represent not significant correlations. **E)** Scatter plot of Spearman correlation r coefficients in dams and sires of the duration of the retrieval events correlated with magnitude of LC responses during pup retrieval.

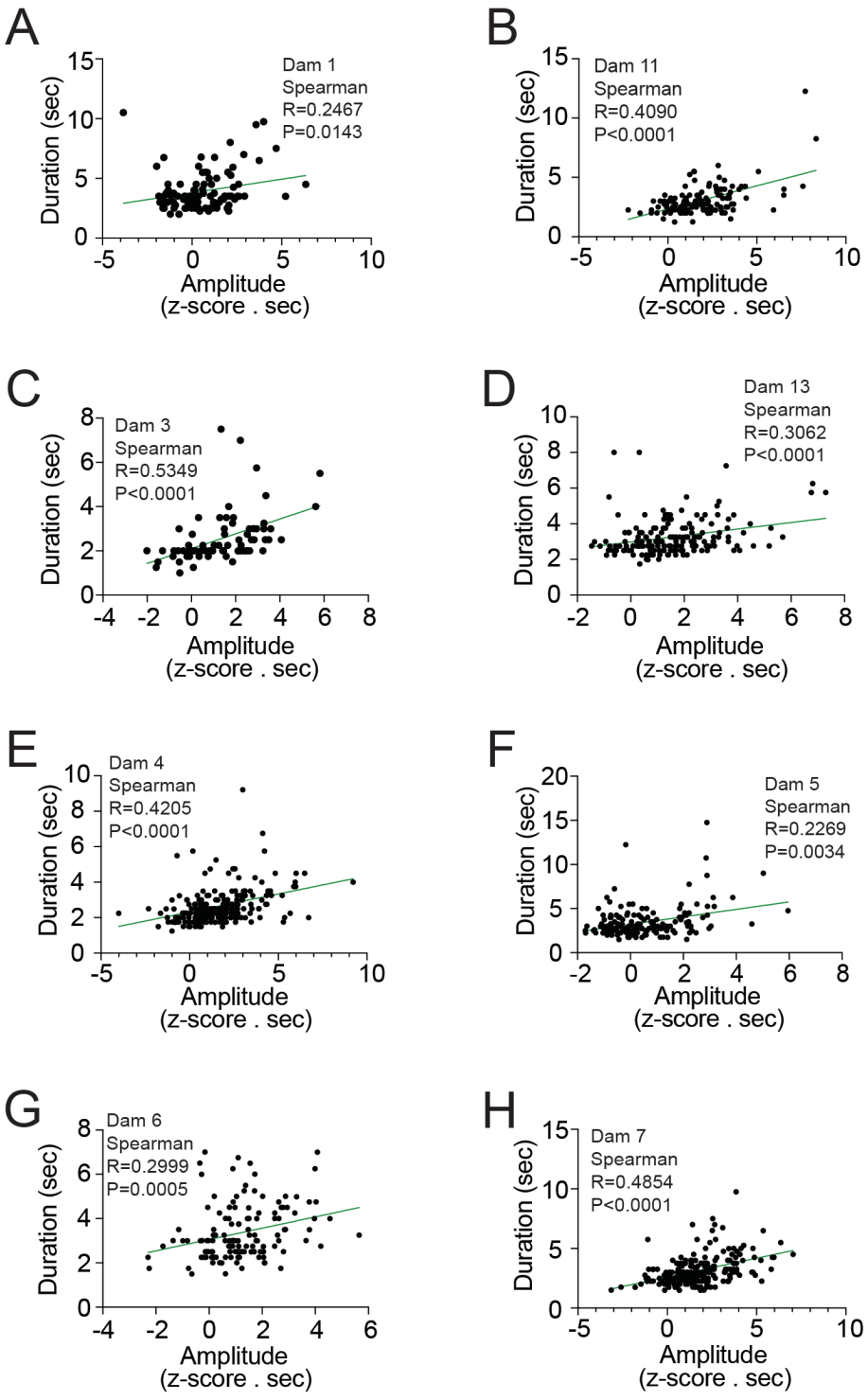


Figure 5. 4. Spearman correlations of the duration of retrieval events with the magnitude of LC responses in dams.

Each panel is a different mouse. p-values and r coefficients are indicated individually. Green line represents a linear regression.

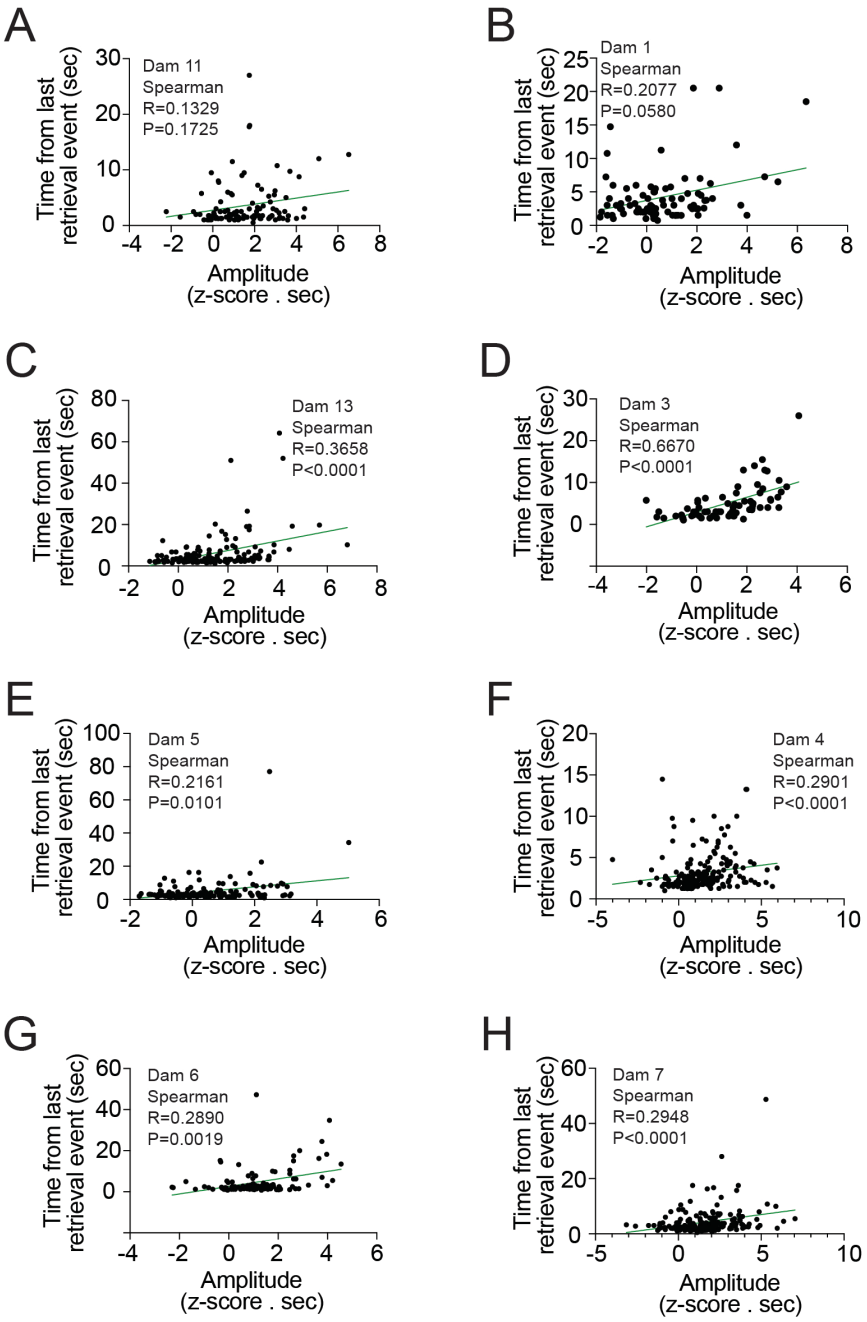


Figure 5.5. Spearman correlations of the duration of the intervals between retrieval events with the magnitude of LC responses in dams.

Each panel is a different mouse. p-values and r coefficients are indicated individually. Green line represents a linear regression.

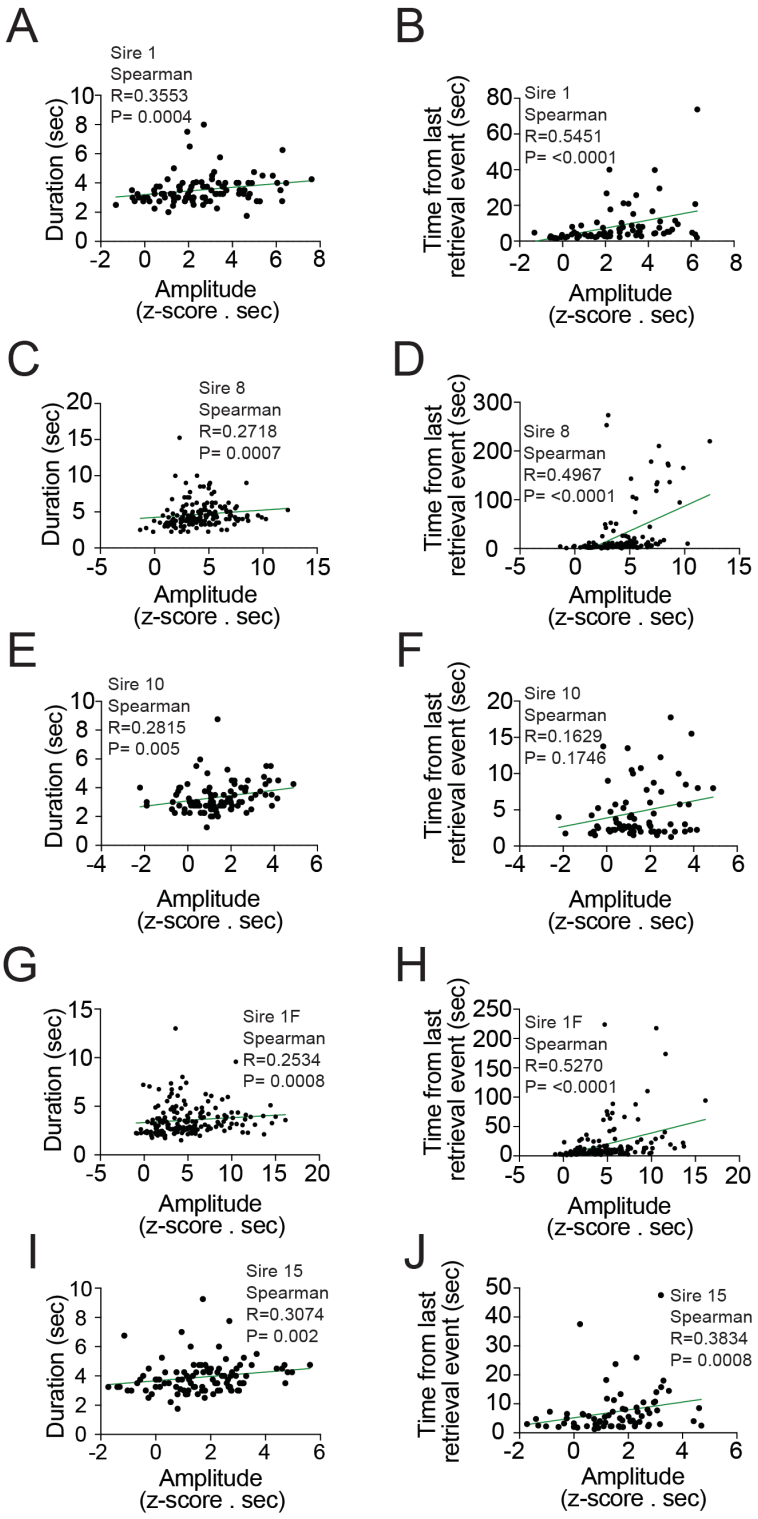


Figure 5. 6. Spearman correlations of the duration of the intervals between retrieval events and the duration of retrieval events with the magnitude of LC responses in sires.
Each panel is a different mouse. p-values and r coefficients are indicated individually. Green line represents a linear regression.

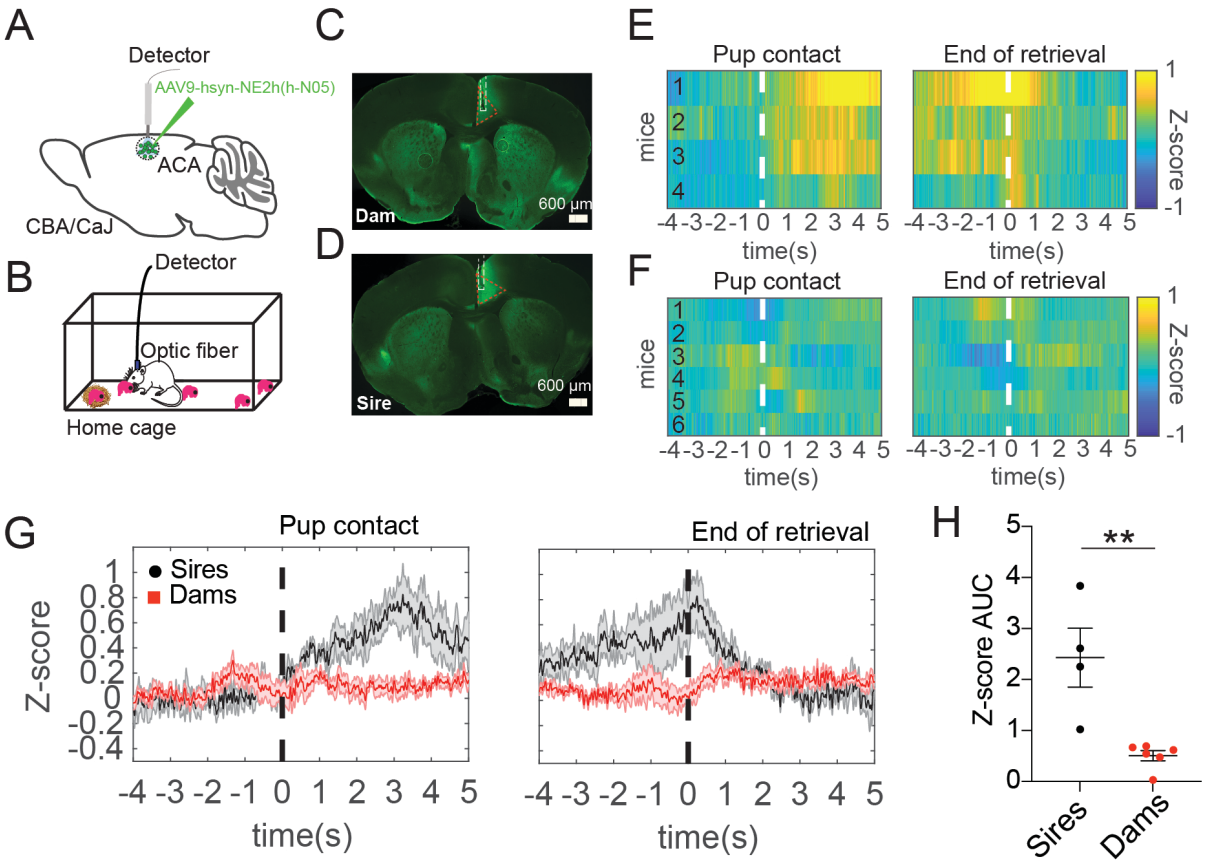


Figure 5. 7. Pup retrieval evokes noradrenaline release in ACA.

A) Viral strategy to express a noradrenaline sensor in ACA. **B)** Behavioral schematic of pup retrieval behavior. **C)** Representative image of expression of NA sensor and fiber placement in dams. **D)** Representative image of expression of NA sensor and fiber placement in sires. **E-F)** These heatmaps depict fluorescent NA sensor signals detected from ACA using fiber photometry during pup gathering events in dams (n=6) and sires (n=4). The left panels are heatmaps aligned to the pup contact followed by a retrieval event in which each row is the mean z-score deltaf/f for pup gathering event responses per mouse across post-natal days 0-5. The right panels show the data aligned to the end of the retrieval events. **G)** The left panel shows the mean z-score deltaf/f for all of the data aligned to the pup contact followed by a retrieval event contrasting dams and sires. The right panel is aligned to the end of the retrieval events. **H)** Quantification of the area under the curve for the NA release responses from the pup contact to 5 seconds after (Mann Whitney test **p<0.0095).

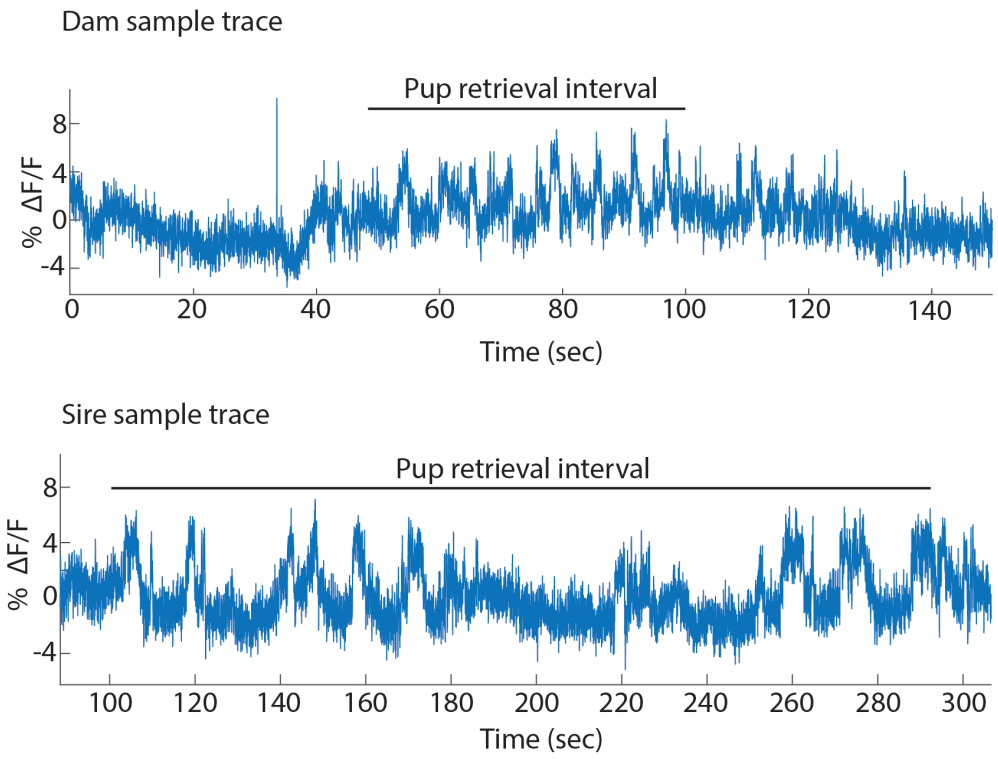


Figure 5. 8. Pup retrieval evokes noradrenaline release in the ACA in dams and sires.
Top panel: Representative deltaF/F trace of a dam interacting with pups. Black line above the trace indicates the time period when the dam was retrieving the pups to the nest. Bottom panel: Representative deltaF/F trace of a sire interacting with pups. Black line above the trace indicates the time period when the sire was retrieving the pups to the nest.

Chapter 6: Conclusions and Perspectives

The goal of this research was to identify whether sex-dependent differences in parental behavior arise from differences in neuronal circuitry or differences in neuronal activity within the same circuitry. Most evidence in the field supports the hypothesis of shared circuitry modulating parental displays in males and females. However, the extent to which parenting in males and females is modulated by identical regulatory mechanisms remains to be determined. The results described in this thesis provide insight into sex and context-dependent modulation of parenting in dams and sires. Additionally, starting from an unbiased whole-brain activity screen, we identified sex-dependent activation patterns in response to different behavioral conditions and interactions with pups. We chose to focus on one of the identified brain regions, the anterior cingulate cortex, because of its involvement in decision-making, sensitivity to social distress, and vicarious fear conditioning. To address the involvement of ACA in parental behavior and whether sex-dependent differences in neural activity lead to differences in behavior, we observed and manipulated activity in ACA in males and females. In this thesis work, we revealed the following key findings:

1) Pup retrieval behavior exhibits greater variability in males compared to females and male behavior is inconsistent across post-natal days. Additionally, sire's pup retrieval behavioral performance gets almost completely impaired by performing the assay in a novel environment. These results show that sire's behavior is subject to additional contextual regulation as compared to dam's behavior.
2) Brain-wide activity *c-fos* screen reveals brain regions likely associated with pup retrieval including the ACA. Comparing male and female *c-fos* expression levels, we revealed that the ACA is more active in dams compared to sires suggesting

that sex-dependent activation of ACA may lead to differences in behavior between males and females.

3) Excitatory neurons in ACA show stronger activation in dams compared to sires during pup retrieval behavior. Furthermore, inhibitory neurons in ACA show reciprocal activity patterns compared to excitatory neurons during pup retrieval behavior in dams but not in sires.
4) Chemogenetic inactivation of excitatory neurons in ACA increases the latency to retrieve pups and decreases parental responsiveness to interact with pups in distress in dams but not in sires. These results suggest that the ACA modulates sensitivity to pup distress differently in males and females.
5) The ACA receives robust inputs from the noradrenergic LC. DBH^+ neurons in LC display time-locked responses to pup retrieval events in dams and sires. These responses are longer and greater in magnitude in sires compared to dams.
6) Pup retrieval behavior evokes noradrenaline release in ACA in a sex-dependent manner.

1. Sex-dependent parental behavioral modulation

Parenting behavior in males and virgin females is not observed in wild mice of the *mus musculus* species. However, breeding selection allowed the expression of paternal behavior in laboratory mice, and virgin females show spontaneous maternal behavior when exposed to pups in the laboratory. The facilitation of paternal behavior in the lab is, in part, due to co-habituation with the female mate during the pregnancy period (Liu et al., 2013; Tachikawa et al., 2013). These observations in laboratory mice suggest that males and females share the neural mechanisms that give rise to parental care. However, our results in chapter 1 show that pup retrieval behavior can be modulated differently in male and female

laboratory mice. This sex-dependent modulation of behavior provides an opportunity to assess the extent to which the control of parenting in males and females is regulated by identical or distinct neural networks.

It is well known that stress related psychiatric disorders affect more females than males (Carter-Snell and Hegadoren, 2003; Curtis et al., 2006). Interestingly, lactating female mice show decreased levels of anxiety and stress responses (Numan, 2020). Moving mice to an unfamiliar environment increases their levels of anxiety-like behaviors. Our data in chapter 1 shows that lactating female mice retrieve their pups in a novel environment and males do not. One possibility for this difference in behavior is that the stress induced by the change in environment modulates the neural mechanisms that control parenting in a sex-dependent manner. For example, the presence of the pups could induce a hypervigilant state in lactating females to quickly respond to the needs of the pups. This hypervigilant state could suppress the stressful response to the change in environment allowing the female to exhibit maternal behavior. On the other hand, the presence of the pups may not reduce anxiety and stress responses in males. In fact, males may find the presence of the pups stressful. The change in environment is possibly more stressful for males compared to females, and as a consequence, it suppresses paternal behavior. It would be interesting to measure stress levels in male and female mice after interactions with pups. These sex-differences in parenting behavior and responses to stress could provide a useful model to study the modulation of anxiety and stress in male and female disease models.

2. Brain-wide activity patterns in response to pups in males and females

Male mice exhibit greater variability in parenting behavior compared to females. This variability in behavior is reflected in greater variability in *c-fos* expression levels as

shown in chapter 3. Therefore, it is challenging to find differences in *c-fos* expression levels comparing groups of males of different experimental conditions. However, we showed in females that an unbiased brain-wide approach is useful to identify regions of interest that are understudied in the regulation of parental behavior. For example, we identified the anterior cingulate area to be one of the most upregulated brain areas in response to pup retrieval in dams. Comparing male to female *c-fos* expression levels in animals that retrieved pups showed that the anterior cingulate area is only upregulated in dams and not in sires.

Other brain regions identified by the *c-fos* screen in dams that would be interesting to investigate are the medial septal complex (MSC) and the rostral part of the lateral septum (LSr). The lateral septum modulates stress responses in mice and increases coping with stress during the force swim test (Singewald et al., 2011). The increase in *c-fos* expression in these regions that we see in our data could be in response to coping with the distress of the pups to facilitate maternal behavior. However, the resolution of the *c-fos* screen does not provide temporal resolution to know when these regions are active. It would be informative to record neural activity in these regions during interactions with pups in distress in males and females.

3. Activity dynamics in the anterior cingulate area during parental care

Empathy can be defined as the adoption of another individual's emotional state. Rodents display empathy-like behaviors such as vicarious fear learning (Jeon, 2010), social transfer of pain and analgesia (Smith et al., 2021), and pro-social behaviors (Burkett et al., 2016).The anterior cingulate area processes information about the emotional state of others (Jeon, 2010; Smith et al., 2021). Parental care is essential for the well-being of the offspring, but an interesting aspect of it, is that there is no immediate benefit for the caregiver. It can

therefore be considered an "unselfish" pro-social behavior. During parental encounters, adults need to process offspring cues, usually distress cues, and then pick an appropriate behavioral response. An interesting candidate region to integrate distress signals from the offspring to facilitate a pro-social response is the anterior cingulate area.

In chapter 4, I discussed activity dynamics in excitatory and inhibitory neurons in the ACA. We showed that excitatory neurons are activated during pup retrieval behavior in a sex-dependent manner. Females show stronger activation than males. If the ACA processes distress from the pups, these results suggest that males and females process social distress from the pups in a sex-dependent manner. We also showed that chemogenetic inhibition of the ACA disrupts pup retrieval behavior and parental responsiveness in females, but not in males. However, the levels of parental responsiveness displayed by males at baseline conditions are already very low compared to females. It is possible that the ACA manipulation in males has no effect on behavior because the activity in ACA is already low. It would be interesting to determine if there is differential gene regulation in the ACA in sires compared to dams. I speculate that there will be differences in genes that are implicated in stress modulation.

4. Activity dynamics in the locus coeruleus and noradrenaline release in the anterior cingulate area during parental care

The noradrenergic LC has been implicated in the regulation of stress in a sex-dependent manner. For example, female LC neurons are more sensitive to CRF than those in males (Curtis et al., 2006). CRF signaling in LC neurons, induces an increase in tonic firing and reduces responses to salient stimuli (Curtis et al., 2006; Poe et al., 2020). In chapter 5, I described LC responses during pup retrieval behavior. We observe greater LC responses in sires compared to dams. These results suggest that males find interactions with pups more

stressful than females. We also confirmed an anatomical connection from LC to ACA. When we looked at noradrenaline release in the ACA, it appears that salient pup retrieval events evoke more noradrenaline release in sires compared to dams. Paradoxically, interactions with pups in females appear to increase the tonic release of noradrenaline in ACA making transient changes less evident. For example, females retrieve pups very fast and the levels of noradrenaline release remain high throughout the entire retrieval episode. Males on the other hand, take longer to retrieve the pups and the intervals in between retrieval events are longer. These longer intervals allow noradrenaline levels in ACA to return to baseline and make transient changes in NA release more evident than in females. Nevertheless, we observe sex-dependent noradrenaline release in ACA during interactions with pups. These results suggest that noradrenaline modulates activity in ACA differentially in males and females and these differences lead to sex differences in parenting behavior.

It has been shown that noradrenergic cells from LC project to excitatory cells in ACA and not to inhibitory cells (Koga et al., 2020). It would be interesting to pharmacologically block noradrenaline receptors in excitatory neurons in ACA and assess any effects on parental behavior. Furthermore, it would be interesting to analyze sex-dependent expression of NA receptors in ACA given that we observe differences in noradrenaline release in males and females. Finally, it would be interesting to disrupt the neural circuit from the LC to ACA and determine its contribution to social behavior.

Chapter 7: Materials and Methods

Animals

Adult mice (8–14 weeks old) were maintained on a 12h/12 h light-dark cycle (lights on 07:00 h) and received food ad libitum. Genotypes used were CBA/CaJ, VGAT-Cre (C57BL/6 background), and DBH-Cre (Tg(Dbh-cre)KH212Gsat/Mmucd, unfrozen stock, MMRRC). All procedures were conducted in accordance with the National Institutes of Health's Guide for the care and use of laboratory animals and approved by the Cold Spring Harbor Laboratory Institutional Animal Care and Use Committee.

Pup retrieval assay

The pup retrieval assay was performed as described in (Krishnan et al., 2017). Briefly, (1) The test subject was habituated with 5 pups in the nest of the home cage for 5 minutes in a soundproof behavioral box, (2) the pups were removed from the cage for 2 minutes, and (3) then the pups were scattered in the cage. The first pup was placed in the nest and then moving clockwise, a pup was placed in each corner and one in the center. Each test subject had a maximum of 5 minutes to gather the pups to the nest. The same procedure was repeated on postnatal days 0 to 5. All assays were performed in the dark during the light cycle and videos were recorded for further analysis.

For the behavioral analysis, we calculated the latency index for each mouse to gather all pups using the formula:

$$\text{latency index} = [(t1-t0) + (t2-t0) + \ldots + (tn-t0)] \,/\, (n \times L)$$

where: n = # of pups outside the nest, t0 = start of trial, tn= time of nth pup gathered, L = trial length.

The same experiment was conducted in a clean/novel cage with different mice.

Pup retrieval assay with nest from the home cage

The nest from the home cage was moved to a novel cage prior to the pup retrieval assay and the test subject was habituated with the pups in the nest for 5 minutes. The pups were then removed for 2 min, and then scattered in the novel cage. The test subject was allowed to gather the pups back to the nest for 5 min. The same procedure was repeated on postnatal days 0 to 5. All assays were performed in the dark during the light cycle and videos were recorded for further analysis.

Behavioral paradigm allowing male-female communication

We designed a behavioral arena where males and females can interact with each other through openings in a wall that separates them. The arena has two chambers divided by a wall with small openings that allow smell and sound to pass through. We exposed males to pup retrieval behavioral assays in the presence or absence of the female partner and measured the latency to gather the pups by the father.

Behavioral assay for brain-wide *c-fos* induction

Male and female wild-type CBA/CaJ mice breeding pairs were made at 8-10 weeks-old. The experiment included four behavioral groups, and it was performed on post-natal day 3. Undisturbed: the test subject was kept in the home cage with the pups in the nest for a 3-hour period. Isolated: the test subject was kept in the home cage without the pups for a

3-hour period. Reunion: the test subject was kept in the home cage without the pups for a 90-minute period, and then all pups were returned in the nest for a second 90-minute period. Retrieval: the test subject was kept in the home cage without the pups for 90 minutes, and then all pups were returned scattered in the cage for 90 minutes. The experiment was performed in mothers and fathers 12-14 weeks old. All experiments were performed in the dark during the light cycle in a sound proof behavioral box. All mice were perfused immediately after the experiment was done through the ascending aorta with 1% PBS, followed by 4% paraformaldehyde (PFA) in 0.1 M PBS (pH 7.4). The brains were removed and post-fixed in paraformaldehyde overnight before starting the clearing protocol.

Clearing protocol/*c-fos* staining

All brains were cleared using the iDisco+ protocol as described in (Renier et al., 2016).

Lightsheet imaging

Cleared samples were imaged in sagittal orientation (left hemisphere) on a light-sheet fluorescence microscope (Ultramicroscope II, LaVision Biotec) equipped with a sCMOS camera (Andor Neo) and a 4x/0.5 objective lens (MVPLAPO 4x) equipped with a 6-mm working distance dipping cap. Version v144 of the Imspector Microscope controller software was used. The samples were scanned with a step-size of 3 micrometers using the continuous light-sheet scanning method with the included contrast blending algorithm for the 640 nm and 595 nm channels (20 acquisitions per plane), and without horizontal scanning for the 480-nm channel.

Statistical analysis for *c-fos* mapping

Statistical comparisons between different groups were run based on either ROIs or evenly spaced voxels. Voxels are overlapping 3D spheres with 100 µm diameter each and spaced 20 µm apart from each other. The cell counts at a given location, Y, are assumed to follow a negative binomial distribution whose mean is linearly related to one or more experimental conditions, X: $E[Y]=\alpha+\beta X$. For example, when testing an experimental group versus a control group, the X is a single column showing the categorical classification of mouse sample to group id, i.e. 0 for the control group and 1 for the experimental group (Venables and Ripley, 2002; O'Hara and Kotze, 2010). We found the maximum likelihood coefficients α and β through iterative reweighted least squares, obtaining estimates for sample standard deviations in the process, from which we obtained the significance of the β coefficient. A significant β means the group status is related to the cell count intensity at the specified location. The z-values in our summary tables correspond to this β coefficient normalized by its sample standard deviation, which under the null hypothesis of no group effect, has an asymptotic standard normal distribution. The p-values give us the probability of obtaining a β coefficient as extreme as the one observed by chance assuming this null hypothesis is true. In the case of three (or more) groups, we utilize Tukey's Honest Significance test to adjust the p-values of the group factor coefficients to control for multiple comparisons: group1v2, group1v3 and group2v3. To account for multiple comparisons across all voxel/ROI locations, we thresholded the p-values and reported false discovery rates with the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). In contrast to correcting for type I error rates, this method controls the number of false positives among the tests that have been deemed significant.

Stereotaxic injections

All surgery was performed under aseptic conditions and body temperature was maintained with a heating pad. Standard surgical procedures were used for stereotaxic injection and implantation, as previously described (Dvorkin and Shea, 2022; Nowlan et al., 2022). Briefly, mice were anesthetized with isoflurane (2% in a mixture with oxygen, applied at 1.0 L/min), and head-fixed in a stereotaxic injection frame (Stereotax model). Ketamine was used as an anesthetic.

To prepare mice for the photometry experiments, we first made a small craniotomy in each mouse, unilaterally. We then lowered a glass micropipette (tip diameter, ~20 µm) containing viral solution to reach the ACA (coordinates: +0.55 mm posterior to Bregma, 0.3 mm lateral from midline, and -0.9 mm ventral from brain surface). The injection coordinates for the LC experiments were (+1.5mm posterior to lambda, 0.8mm lateral from the midline, and 2.8mm ventral from the brain surface). (About 0.2–0.3 µL of viral solution was delivered with pressure applications (5–20 psi, 5–20 ms at 1 Hz) controlled by a Picrospritzer and a pulse generator. The rate of injection was ~20 nl/min. The pipette was left in place for 5-10 minutes following the injection, and then slowly withdrawn. Infection of the ACA was performed in both hemispheres in mice dedicated to chemogenetic inhibition experiments and about 0.15 µL of viral solution was delivered to each hemisphere (coordinates: +0.55 mm posterior to Bregma, ± 0.3 mm lateral from midline, and -0.9 mm ventral from brain surface).

We then implanted optic fibers above injection locations (coordinates: +0.55 mm anterior to Bregma, 0.3 mm lateral from midline, and 0.9 mm vertical from brain surface). A

head-bar was also mounted for head-restraint. We waited one week for the mice to recover from surgery and pair them with a mate. We then waited for the pups to be born to start recording the photometry signals.

Fiber photometry recordings and behavior

To record the activity of CamkII+ cells in ACA *in vivo*, we used a custom-made fiber photometry system to measure GCaMP6s signals in these neurons through an optical fiber (Fiber core diameter, 200 μm; Fiber length, 2.0 mm; NA, 0.37; Inper, Hangzhou, China) unilaterally implanted in the ACA of 8-10 weeks-old male and female mice. Animals were habituated to the behavioral box in their home cage for 10 min starting at least 1 day before parturition. 4 retrieval sessions were recorded each day were all pups from the litter were scattered in the cage, and the test subject was allowed to retrieve all the pups. Each session was 5 minutes long and sessions were separated by 2 minutes. The same behavioral procedures were used to record neural activity from all of the populations described below. GCaMP signals were detected and measured as follows: briefly, activity-dependent GCaMP was delivered by AAV to **CamkII+** neurons under the expression of the CamkII promoter. An optical fiber cable was mated to the fiber implant in the ACA neurons before each optical recording session, and it was used to deliver 470 nm and 565 nm excitation light to the brain. The intensity of the light for excitation was adjusted to ~5-10 μW at the tip of the patch cord. The two wavelengths were sinusoidally modulated at 211 Hz and 180 degrees out of phase. Green and red emitted light signals were filtered and split to separate photodetectors and digitally sampled at 6100 Hz via a data acquisition board (National Instruments, Model # NI USB-6211). Peaks were extracted by custom Matlab software with an effective sampling rate of 211 Hz. Each signal was corrected for photobleaching by fitting

the decay with a double exponential, and then normalized to a Z score. After subtracting the activity-independent red signal to correct for movement artifacts, the green signal was transformed back to absolute fluorescence and DF/F was computed relative to the mean of the measured fluorescence minus the mean of the baseline fluorescence. The resulting traces from each recording session were converted to a Z score to compare between subjects and across days. All data analysis was performed using custom written code in Matlab.

To record activity of DBH+ cells in LC *in vivo*, we injected a cre-dependent AAV GCaMP7s into the LC of **DBH-Cre** mice and implanted a fiber unilaterally in the LC (Fiber core diameter, 200 μm; Fiber length, 5.5 mm; NA, 0.37; Inper, Hangzhou, China). The intensity of the light for excitation was adjusted to ~30 μW at the tip of the patch cord. To record the activity of inhibitory neurons in the ACA during parental behavior, we injected a cre-dependent AAV GCaMP7s into the cingulate cortex of **VGAT-Cre** mice and implanted a fiber unilaterally in the ACA (Fiber core diameter, 200 μm; Fiber length, 2.0 mm; NA, 0.37; Inper, Hangzhou, China). The intensity of the light for excitation was adjusted to ~5-10 μW at the tip of the patch cord. To record noradrenaline release in the cingulate cortex, we used fiber photometry and a noradrenaline sensor. We injected AAV-hsyn-NE2h into the cingulate cortex of **CBA/CaJ** mice and implanted a fiber unilaterally in the ACA (Fiber core diameter, 200 μm; Fiber length, 2.0 mm; NA, 0.37; Inper, Hangzhou, China). The intensity of the light for excitation was adjusted to ~5-10 μW at the tip of the patch cord.

Pups in a jar experiment

All pups from the litter were placed in a 4oz. glass jar with a plastic lid with holes in it (AOZITA; B07VHBX3ZC). The test subjects were able to hear and smell the pups in the jar, but were not able to touch them. The animal's behavior was recorded for 5 min with the

pups in the jar and 5 minutes with an empty jar in the home cage. Interactions with the lid of the jar were quantified as a proxy of motivation for the test subject to interact with the pups. All behaviors were scored with the software BORIS (Friard and Gamba, 2016). An interaction with the jar was quantified when the test subject was in close proximity to the lid of the jar, facing the jar, either touching, biting, of sniffing it. To confirm the jar was enough to put the pups in distress, we recorded USVs for 5 min when the pups were in the nest in the home cage and 5 minutes with the pups in a jar. All pups used to record USVs were 0-5 days old.

Chemogenetic Inhibition

Mice were habituated to the behavioral box for 10 minutes at least 24h before the experiment. Mice were maintained on a 12h/12 h light-dark cycle (lights on 10:00 h) and received food ad libitum. During the dark phase, test subjects were habituated in their home cage for 10 minutes. CBA/CaJ mice expressing hM4D(Gi) were injected intraperitoneally (i.p.) with either saline (0.9% NaCl) or clozapine (0.1 mg/kg) (HelloBio, Inc.) dissolved in saline. The injection of clozapine or saline was alternating in each mouse every other day; P0,P2, and P4 clozapine and P1,P3, and P5 saline. Twenty minutes after the injections, all pups were scattered in the home cage and the test subject's behavior was recorded for 10 minutes. Then, all pups were placed in a jar for 5 min and videos were recorded. Lastly, the test subjects were exposed to an empty jar and videos were recoded. All videos were manually scored using BORIS.

References

Afonso, V.M., Sison, M., Lovic, V., and Fleming, A.S. Medial Prefrontal Cortex Lesions in the Female Rat Affect Sexual and Maternal Behavior and Their Sequential Organization. 12.

Alsina-Llanes, M., and Olazábal, D.E. NMDA Lesions in the Prefrontal Cortex Delay the Onset of Maternal, but Not Infanticidal Behavior in Pup-Naïve Adult Mice (C57BL/6). 13.

Aston-Jones, G., and Cohen, J.D. 2005. AN INTEGRATIVE THEORY OF LOCUS COERULEUS-NOREPINEPHRINE FUNCTION: Adaptive Gain and Optimal Performance. *Annu. Rev. Neurosci.* 28: 403–450.

Bakowska, J.C., and Morrell, J.I. 1997. Atlas of the neurons that express mRNA for the long form of the prolactin receptor in the forebrain of the female rat. *J. Comp. Neurol.* 386: 161–177.

Bean, J.N., and Wysocki, C.J. 1989. Vomeronasal organ removal and female mouse aggression: The role of experience. *Physiol. Behav.* 45: 875–882.

Becker, J.B., McClellan, M.L., and Reed, B.G. 2017. Sex differences, gender and addiction: Sex, Gender, and Addiction. *J. Neurosci. Res.* 95: 136–147.

Benjamini, Y., and Hochberg, Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J. R. Stat. Soc. Ser. B Methodol.* 57: 289–300.

Berridge, C.W., and Waterhouse, B.D. 2003. The locus coeruleus–noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. *Brain Res. Rev.* 42: 33–84.

Bicks, L.K., and Morishita, H. 2015. Prefrontal Cortex and Social Cognition in Mouse and Man. *Front. Psychol.* 6: 15.

Blass, E.M., and Teicher, M.H. 1980. Suckling. *Science* 210: 15–22.

Bosch, O.J., and Neumann, I.D. 2008. Brain vasopressin is an important regulator of maternal behavior independent of dams' trait anxiety. *Proc. Natl. Acad. Sci.* 105: 17139–17144.

Bridges, R.S., Numan, M., Ronsheim, P.M., Mann, P.E., and Lupini, C.E. 1990. Central prolactin infusions stimulate maternal behavior in steroid-treated, nulliparous female rats. *Proc. Natl. Acad. Sci.* 87: 8003–8007.

Burkett, J.P., Andari, E., Johnson, Z.V., Curry, D.C., de Waal, F.B.M., and Young, L.J. 2016. Oxytocin-dependent consolation behavior in rodents. *Science* 351: 375–378.

Carter-Snell, C., and Hegadoren, K. 2003. Stress Disorders and Gender: Implications for Theory and Research. *CJNR* 35: 34–55.

Casada, J.H., and Dafny, N. 1993. Responses of neurons in bed nucleus of the stria terminalis to microiontophoretically applied morphine, norepinephrine and acetylcholine. *Neuropharmacology* 32: 279–284.

Chen, P.B., Hu, R.K., Wu, Y.E., Pan, L., Huang, S., Micevych, P.E., and Hong, W. 2019. Sexually Dimorphic Control of Parenting Behavior by the Medial Amygdala. *Cell* 176: 1206-1221.e18.

Choe, H.K., Reed, M.D., Benavidez, N., Montgomery, D., Soares, N., Yim, Y.S., and Choi, G.B. 2015. Oxytocin Mediates Entrainment of Sensory Stimuli to Social Cues of Opposing Valence. *Neuron* 87: 152–163.

Cohen, L., Rothschild, G., and Mizrahi, A. 2011. Multisensory Integration of Natural Odors and Sounds in the Auditory Cortex. *Neuron* 72: 357–369.

Curry, T., Egeto, P., Wang, H., Podnos, A., Wasserman, D., and Yeomans, J. 2013. Dopamine receptor D2 deficiency reduces mouse pup ultrasonic vocalizations and maternal responsiveness: DRD2-deficient mice: reduced pup USV and dam response. *Genes Brain Behav.* 12: 397–404.

Curtis, A.L., Bethea, T., and Valentino, R.J. 2006. Sexually Dimorphic Responses of the Brain Norepinephrine System to Stress and Corticotropin-Releasing Factor. *Neuropsychopharmacology* 31: 544–554.

Dudley, D. 1974. Paternal behavior in the California mouse, Peromyscus californicus. *Behav. Biol.* 11: 247–252.

Dulac, C., and Wagner, S. 2006. Genetic Analysis of Brain Circuits Underlying Pheromone Signaling. *Annu. Rev. Genet.* 40: 449–467.

Dvorkin, R., and Shea, S.D. 2022. Precise and pervasive phasic bursting in locus coeruleus during maternal behavior in mice. *J. Neurosci.* JN-RM-0938-21.

Ehret, G. 1987. Left hemisphere advantage in the mouse brain for recognizing ultrasonic communication calls. *Nature* 325: 249–251.

Ehret, G. 2005. Infant Rodent Ultrasounds ? A Gate to the Understanding of Sound Communication. *Behav. Genet.* 35: 19–29.

Ehret, G., and Bernecker, C. 1986. Low-frequency sound communication by mouse pups (Mus musculus): wriggling calls release maternal behaviour. *Anim. Behav.* 34: 821–830.

Fang, Y.-Y., Yamaguchi, T., Song, S.C., Tritsch, N.X., and Lin, D. 2018. A Hypothalamic Midbrain Pathway Essential for Driving Maternal Behaviors. *Neuron* 98: 192-207.e10.

Febo, M., Felix-Ortiz, A.C., and Johnson, T.R. Inactivation or inhibition of neuronal activity in the medial prefrontal cortex largely reduces pup retrieval and grouping in maternal rats. *BRAIN Res.* 12.

Fendt, M. 2005. Noradrenaline Transmission within the Ventral Bed Nucleus of the Stria Terminalis Is Critical for Fear Behavior Induced by Trimethylthiazoline, a Component of Fox Odor. *J. Neurosci.* 25: 5998–6004.

Feng, J., Zhang, C., Lischinsky, J.E., Jing, M., Zhou, J., Wang, H., Zhang, Y., Dong, A., Wu, Z., Wu, H., et al. 2019. A Genetically Encoded Fluorescent Sensor for Rapid and Specific In Vivo Detection of Norepinephrine. *Neuron* 102: 745-761.e8.

Fichtel, I., and Ehret, G. 1999. Perception and recognition discriminated in the mouse auditory cortex by c-Fos labeling: *NeuroReport* 10: 2341–2345.

Fillinger, C., Yalcin, I., Barrot, M., and Veinante, P. 2017. Afferents to anterior cingulate areas 24a and 24b and midcingulate areas 24a′ and 24b′ in the mouse. *Brain Struct. Funct.* 222: 1509–1532.

Fraser, E.J., and Shah, N.M. 2014. Complex Chemosensory Control of Female Reproductive Behaviors. *PLoS ONE* 9: e90368.

Freund-Mercier, M.J., Stoeckel, M.E., Palacios, J.M., Pazos, A., Reichhart, J.M., Porte, A., and Richard, Ph. 1987. Pharmacological characteristics and anatomical distribution of [3H]oxytocin-binding sites in the Wistar rat brain studied by autoradiography. *Neuroscience* 20: 599–614.

Friard, O., and Gamba, M. 2016. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods Ecol. Evol.* 7: 1325–1330.

Gandelman, R., Zarrow, M.X., Denenberg, V.H., and Myers, M. 1971. Olfactory Bulb Removal Eliminates Maternal Behavior in the Mouse. *Science* 171: 210–211.

Gomez, J.L., Bonaventura, J., Lesniak, W., Mathews, W.B., Sysa-Shah, P., Rodriguez, L.A., Ellis, R.J., Richie, C.T., Harvey, B.K., Dannals, R.F., et al. 2017. Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. *Science* 357: 503–507.

Halpern, M., and Martinez-Marcos, A. 2003. Structure and function of the vomeronasal system: an update. *Prog. Neurobiol.* 70: 245–318.

Harris, K.D., and Mrsic-Flogel, T.D. 2013. Cortical connectivity and sensory coding. *Nature* 503: 51–58.

Hauser, H. 1985. Lever pressing for pups: Evidence for hormonal influence upon maternal behavior of mice. *Horm. Behav.* 19: 454–468.

Henschen, C.W., Palmiter, R.D., and Darvas, M. 2013. Restoration of Dopamine Signaling to the Dorsal Striatum Is Sufficient for Aspects of Active Maternal Behavior in Female Mice. *Endocrinology* 154: 4316–4327.

Horner, B.E. 1947. Paternal Care of Young Mice of the Genus Peromyscus. *J. Mammal.* 28: 31.

Insel, T.R., and Harbaugh, C.R. 1989. Lesions of the hypothalamic paraventricular nucleus disrupt the initiation of maternal behavior. *Physiol. Behav.* 45: 1033–1041.

Isaacson, J.S., and Scanziani, M. 2011. How Inhibition Shapes Cortical Activity. *Neuron* 72: 231–243.

Isogai, Y., Wu, Z., Love, M.I., Ahn, M.H.-Y., Bambah-Mukku, D., Hua, V., Farrell, K., and Dulac, C. 2018. Multisensory Logic of Infant-Directed Aggression by Males. *Cell* 175: 1827-1841.e17.

Jeon, D. 2010. Observational fear learning involves affective pain system and Cav1.2 Ca2+ channels in ACC. *Nat. Neurosci.* 13: 9.

Johnson, C., Kretsge, L.N., Yen, W.W., Sriram, B., Jimenez, J.C., Jinadasa, T.J., O'Connor, A., Sky Liu, R., Nguyen, T.P.H., Cho, E.S., et al. 2020. Distinct VIP interneurons in the cingulate cortex encode anxiogenic and social stimuli (Neuroscience).

Joshi, S., and Gold, J.I. 2022. Context-dependent relationships between locus coeruleus firing patterns and coordinated neural activity in the anterior cingulate cortex. *ELife* 11: e63490.

Kebschull, J.M., Garcia da Silva, P., Reid, A.P., Peikon, I.D., Albeanu, D.F., and Zador, A.M. 2016. High-Throughput Mapping of Single-Neuron Projections by Sequencing of Barcoded RNA. *Neuron* 91: 975–987.

Kirckpatrick, B., Williams, J.R., Slotnick, B.M., and Carter, S.C. 1994. Olfactory bulbectomy decreases social behavior in male prairie voles (Microtus ochrogaster). *Physiol. Behav.* 55: 885–889.

Kirkpatrick, B., Kim, J.W., and Insel, T.R. 1994. Limbic system fos expression associated with paternal behavior. *Brain Res.* 658: 112–118.

Klampfl, S.M., Brunton, P.J., Bayerl, D.S., and Bosch, O.J. 2014. Hypoactivation of CRF Receptors, Predominantly Type 2, in the Medial-Posterior BNST Is Vital for Adequate Maternal Behavior in Lactating Rats. *J. Neurosci.* 34: 9665–9676.

Koga, K., Yamada, A., Song, Q., Li, X.-H., Chen, Q.-Y., Liu, R.-H., Ge, J., Zhan, C., Furue, H., Zhuo, M., et al. 2020. Ascending noradrenergic excitation from the locus coeruleus to the anterior cingulate cortex. *Mol. Brain* 13: 49.

Kohl, J., Autry, A.E., and Dulac, C. 2017. The neurobiology of parenting: A neural circuit perspective. *BioEssays* 39: e201600159.

Kohl, J., Babayan, B.M., Rubinstein, N.D., Autry, A.E., Marin-Rodriguez, B., Kapoor, V., Miyamishi, K., Zweifel, L.S., Luo, L., Uchida, N., et al. 2018. Functional circuit architecture underlying parental behaviour. *Nature* 556: 326–331.

Krach, S. 2010. The rewarding nature of social interactions. *Front. Behav. Neurosci.*

Krishnan, K., Lau, B.Y.B., Ewall, G., Huang, Z.J., and Shea, S.D. 2017. MECP2 regulates cortical plasticity underlying a learned behaviour in adult female mice. *Nat. Commun.* 8: 14077.

Kuroda, K.O., Meaney, M.J., Uetani, N., Fortin, Y., Ponton, A., and Kato, T. 2007. ERK-FosB signaling in dorsal MPOA neurons plays a major role in the initiation of parental behavior in mice. *Mol. Cell. Neurosci.* 36: 121–131.

Lambert, K.G., Franssen, C.L., Hampton, J.E., Rzucidlo, A.M., Hyer, M.M., True, M., Kaufman, C., and Bardi, M. 2013. Modeling paternal attentiveness: Distressed pups evoke differential neurobiological and behavioral responses in paternal and nonpaternal mice. *Neuroscience* 234: 1–12.

Landeros, R.V., Morisseau, C., Yoo, H.J., Fu, S.H., Hammock, B.D., and Trainor, B.C. 2012. Corncob Bedding Alters the Effects of Estrogens on Aggressive Behavior and Reduces Estrogen Receptor-α Expression in the Brain. *Endocrinology* 153: 949–953.

Lee, A., Clancy, S., and Fleming, A.S. 1999. Mother rats bar-press for pups: effects of lesions of the mpoa and limbic sites on maternal behavior and operant responding for pup-reinforcement. *Behav. Brain Res.* 100: 15–31.

Lee, A.W., and Brown, R.E. 2007. Comparison of medial preoptic, amygdala, and nucleus accumbens lesions on parental behavior in California mice (Peromyscus californicus). *Physiol. Behav.* 92: 617–628.

Lepri, J.J., Wysocki, C.J., and Vandenbergh, J.G. 1985. Mouse vomeronasal organ: Effects on chemosignal production and maternal behavior. 35: 809–814.

Li, R., Ma, X., Wang, G., Yang, J., and Wang, C. 2017. Why sex differences in schizophrenia? 9.

Liman, E.R., Corey, D.P., and Dulac, C. 1999. TRP2: A candidate transduction channel for mammalian pheromone sensory signaling. *Proc. Natl. Acad. Sci.* 96: 5791–5796.

Liu, H.-X., Lopatina, O., Higashida, C., Fujimoto, H., Akther, S., Inzhutova, A., Liang, M., Zhong, J., Tsuji, T., Yoshihara, T., et al. 2013. Displays of paternal mouse pup retrieval following communicative interaction with maternal mates. *Nat. Commun.* 4: 1346.

Liu, R.C., and Miller, K.D. 2003. Acoustic variability and distinguishability among mouse ultrasound vocalizations. *J. Acoust. Soc. Am.* 114:.

Lonstein, J.S., and De Vries, G.J. 1999. Comparison of the Parental Behavior of Pair-Bonded Female and Male Prairie Voles (Microtus ochrogaster). *Physiol. Behav.* 66: 33–40.

Mandiyan, V.S., Coats, J.K., and Shah, N.M. 2005. Deficits in sexual and aggressive behaviors in Cnga2 mutant mice. *Nat. Neurosci.* 8: 1660–1662.

Marlin, B.J., Mitre, M., D'amour, J.A., Chao, M.V., and Froemke, R.C. Oxytocin enables maternal behaviour by balancing cortical inhibition. 22.

Martin, J., Walters, R.K., Demontis, D., Mattheisen, M., Lee, S.H., Robinson, E., Brikell, I., Ghirardi, L., Larsson, H., Lichtenstein, P., et al. 2018. A Genetic Investigation of Sex Bias in the Prevalence of Attention-Deficit/Hyperactivity Disorder. *Biol. Psychiatry* 83: 1044–1053.

Matsushita, N., Muroi, Y., Kinoshita, K., and Ishii, T. 2015. Comparison of c-Fos expression in brain regions involved in maternal behavior of virgin and lactating female mice. *Neurosci. Lett.* 590: 166–171.

McCall, J.G., Al-Hasani, R., Siuda, E.R., Hong, D.Y., Norris, A.J., Ford, C.P., and Bruchas, M.R. 2015. CRH Engagement of the Locus Coeruleus Noradrenergic System Mediates Stress-Induced Anxiety. *Neuron* 87: 605–620.

Musall, S., Sun, X.R., Mohan, H., An, X., Gluf, S., Drewes, R., Osten, P., and Churchland, A.K. 2021. Pyramidal cell types drive functionally distinct cortical activity patterns during decision-making (Neuroscience).

Nawaratne, V., Leach, K., Suratman, N., Loiacono, R.E., Felder, C.C., Armbruster, B.N., Roth, B.L., Sexton, P.M., and Christopoulos, A. 2008. New Insights into the Function of M_4 Muscarinic Acetylcholine Receptors Gained Using a Novel Allosteric Modulator and a DREADD (Designer Receptor Exclusively Activated by a Designer Drug). *Mol. Pharmacol.* 74: 1119–1131.

Nishimori, K., Young, L.J., Guo, Q., Wang, Z., Insel, T.R., and Matzuk, M.M. 1996. Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. *Proc. Natl. Acad. Sci.* 93: 11699–11704.

Nishitani, S., Kuwamoto, S., Takahira, A., Miyamura, T., and Shinohara, K. 2014. Maternal Prefrontal Cortex Activation by Newborn Infant Odors. *Chem. Senses* 39: 195–202.

Noirot, E. 1972. Ultrasounds and maternal behavior in small rodents. *Dev. Psychobiol.* 5: 371–387.

Nowlan, A.C., Kelahan, C., and Shea, S.D. 2022. Multisensory integration of social signals by a pathway from the basal amygdala to the auditory cortex in maternal mice (Neuroscience).

Numan, M. 2020. The Parental Brain Mechanisms, Development an Evolution (Oxford University Press).

Numan, M., and Corodimas, K.P. 1985. The effects of paraventricular hypothalamic lesions on maternal behavior in rats. *Physiol. Behav.* 35: 417–425.

Numan, M., and Numan, M. 1996. A lesion and neuroanatomical tract-tracing analysis of the role of the bed nucleus of the stria terminalis in retrieval behavior and other aspects of maternal responsiveness in rats. *Dev. Psychobiol.* 29: 23–51.

Numan, M., and Numan, M.J. Expression of Fos-Like Immunoreactivity in the Preoptic Area of Maternally Behaving Virgin and Postpartum Rats. 16.

Numan, M., Numan, M.J., Pliakou, N., Stolzenberg, D.S., Mullins, O.J., Murphy, J.M., and Smith, C.D. 2005. The effects of D1 or D2 dopamine receptor antagonism in the medial preoptic area, ventral pallidum, or nucleus accumbens on the maternal retrieval response and other aspects of maternal behavior in rats. *Behav. Neurosci.* 119: 1588–1604.

Numan, M., Rosenblatt, J.S., and Komisaruk, B.R. 1977. Medial preoptic area and onset of maternal behavior in the rat. *J. Comp. Physiol. Psychol.* 91: 146–164.

Numan, M., and Stolzenberg, D.S. 2009. Medial preoptic area interactions with dopamine neural systems in the control of the onset and maintenance of maternal behavior in rats. *Front. Neuroendocrinol.* 30: 46–64.

Numan, M., Stolzenberg, D.S., Dellevigne, A.A., Correnti, C.M., and Numan, M.J. 2009. Temporary inactivation of ventral tegmental area neurons with either muscimol or baclofen reversibly disrupts maternal behavior in rats through different underlying mechanisms. *Behav. Neurosci.* 123: 740–751.

O'Brien, A.P., McNeil, K.A., Fletcher, R., Conrad, A., Wilson, A.J., Jones, D., and Chan, S.W. 2017. New Fathers' Perinatal Depression and Anxiety—Treatment Options: An Integrative Review. *Am. J. Mens Health* 11: 863–876.

O'Hara, R.B., and Kotze, D.J. 2010. Do not log-transform count data: *Do not log-transform count data. Methods Ecol. Evol.* 1: 118–122.

Oliveras, D., and Novak, M. 1986. A Comparison of Paternal Behavior in the Meadow Vole Microtus-Pennsylvanicus, the Pine Vole Microtus-Pinetorum and the Prairie Vole Microtus-Ochrogaster. *Anim. Behav.* 32: 519–526.

Pedersen, C.A., Caldwell, J.D., Walker, C., Ayers, G., and Mason, G.A. Oxytocin Activates the Postpartum Onset of Rat Maternal Behavior in the Ventral Tegmental and Medial Preoptic Areas. 9.

Peters, Y.M., O'Donnell, P., and Carelli, R.M. 2005. Prefrontal cortical cell firing during maintenance, extinction, and reinstatement of goal-directed behavior for natural reward. *Synapse* 56: 74–83.

Pfaff, D., and Keiner, M. 1973. Atlas of estradiol-concentrating cells in the central nervous system of the female rat. *J. Comp. Neurol.* 151: 121–157.

Poe, G.R., Foote, S., Eschenko, O., Johansen, J.P., Bouret, S., Aston-Jones, G., Harley, C.W., Manahan-Vaughan, D., Weinshenker, D., Valentino, R., et al. 2020. Locus coeruleus: a new look at the blue spot. *Nat. Rev. Neurosci.* 21: 644–659.

Poindron, P. 2005. Mechanisms of activation of maternal behaviour in mammals. *Reprod. Nutr. Dev.* 45: 341–351.

Renier, N., Adams, E.L., Kirst, C., Wu, Z., Azevedo, R., Kohl, J., Autry, A.E., Kadiri, L., Umadevi Venkataraju, K., Zhou, Y., et al. 2016. Mapping of Brain Activity by Automated Volume Analysis of Immediate Early Genes. *Cell* 165: 1789–1802.

Rogers, F.D., and Bales, K.L. 2019. Mothers, Fathers, and Others: Neural Substrates of Parental Care. *Trends Neurosci.* 42: 552–562.

Rosenblatt, J.S., and Ceus, K. 1998. Estrogen Implants in the Medial Preoptic Area Stimulate Maternal Behavior in Male Rats. *Horm. Behav.* 33: 23–30.

Rosenblatt, J.S., Hazelwood, S., and Poole, J. 1996. Maternal Behavior in Male Rats: Effects of Medial Preoptic Area Lesions and Presence of Maternal Aggression. *Horm. Behav.* 30: 201–215.

Rosenblatt, J.S., Olufowobi, A., and Siegel, H.I. 1998. Effects of Pregnancy Hormones on Maternal Responsiveness, Responsiveness to Estrogen Stimulation of Maternal Behavior, and the Lordosis Response to Estrogen Stimulation. *Horm. Behav.* 33: 104–114.

Rosenblatt, J.S., and Siegel, H.I. Hysterectomy-Induced Maternal Behavior During Pregnancy in the Rat. 16.

Ross, J.A., and Van Bockstaele, E.J. 2021. The Locus Coeruleus- Norepinephrine System in Stress and Arousal: Unraveling Historical, Current, and Future Perspectives. *Front. Psychiatry* 11: 601519.

Rudebeck, P.H., Walton, M.E., Smyth, A.N., Bannerman, D.M., and Rushworth, M.F.S. 2006. Separate neural pathways process different decision costs. *Nat. Neurosci.* 9: 8.

vom Saal, F.S., and Howard, L.S. 1982. The Regulation of Infanticide and Parental Behavior: Implications for Reproductive Success in Male Mice. *Science* 215: 1270–1272.

Schwarz, L.A., Miyamichi, K., Gao, X.J., Beier, K.T., Weissbourd, B., DeLoach, K.E., Ren, J., Ibanes, S., Malenka, R.C., Kremer, E.J., et al. 2015. Viral-genetic tracing of the input–output organization of a central noradrenaline circuit. *Nature* 524: 88–92.

Scott, N., Prigge, M., Yizhar, O., and Kimchi, T. 2015. A sexually dimorphic hypothalamic circuit controls maternal care and oxytocin secretion. *Nature* 525: 519–522.

Sewell, G.D. 1970. Ultrasonic Communication in Rodents. *Nature* 227: 410–410.

Siegel, H.I., and Rosenblatt, J.S. 1975. Hormonal basis of hysterectomy-induced maternal behavior during pregnancy in the rat. *Horm. Behav.* 6: 211–222.

Simerly, R.B., and Swanson, L.W. 1986. The organization of neural inputs to the medial preoptic nucleus of the rat. *J. Comp. Neurol.* 246: 312–342.

Singewald, G.M., Rjabokon, A., Singewald, N., and Ebner, K. 2011. The Modulatory Role of the Lateral Septum on Neuroendocrine and Behavioral Stress Responses. *Neuropsychopharmacology* 36: 793–804.

Slotnick, B.M. 1966. Disturbances of maternal behavior in the rat following lesions of the cingulate cortex. *Behaviour* 29: 204–236.

Smith, C.D., Holschbach, M.A., Olsewicz, J., and Lonstein, J.S. 2012. Effects of noradrenergic alpha-2 receptor antagonism or noradrenergic lesions in the ventral bed nucleus of the stria terminalis and medial preoptic area on maternal care in female rats. *Psychopharmacology (Berl.)* 224: 263–276.

Smith, M.L., Asada, N., and Malenka, R.C. 2021. Anterior cingulate inputs to nucleus accumbens control the social transfer of pain and analgesia. Science 371: 153-159.

Smotherman, W.P., Bell, R.W., Starzec, J., Elias, J., and Zachman, T.A. 1974. Maternal responses to infant vocalizations and olfactory cues in rats and mice. *Behav. Biol.* 12: 55–66.

Sohal, V.S., Zhang, F., Yizhar, O., and Deisseroth, K. 2009. Parvalbumin neurons and gamma rhythms enhance cortical circuit performance. *Nature* 459: 698–702.

Soroker, V., and Terkel, J. 1988. Changes in incidence of infanticidal and parental responses during the reproductive cycle in male and female wild mice Mus musculus. *1988* 36: 1275–1281.

Stagkourakis, S., Smiley, K.O., Williams, P., Kakadellis, S., Ziegler, K., Bakker, J., Brown, R.S.E., Harkany, T., Grattan, D.R., and Broberger, C. 2020. A Neuro-hormonal Circuit for Paternal Behavior Controlled by a Hypothalamic Network Oscillation. *Cell* 182: 960-975.e15.

Stolzenberg, D.S., and Numan, M. 2011. Hypothalamic interaction with the mesolimbic DA system in the control of the maternal and sexual behaviors in rats. *Neurosci. Biobehav. Rev.* 35: 826–847.

Stolzenberg, D.S., Zhang, K.Y., Luskin, K., Ranker, L., Bress, J., and Numan, M. 2010. Dopamine D1 receptor activation of adenylyl cyclase, not phospholipase C, in the nucleus accumbens promotes maternal behavior onset in rats. *Horm. Behav.* 57: 96–104.

Stoop, R. 2012. Neuromodulation by Oxytocin and Vasopressin. *Neuron* 76: 142–159.

Sturgis, J., and Bridges, R. 1997. N-Methyl-DL-Aspartic Acid Lesions of the Medial Preoptic Area Disrupt Ongoing Parental Behavior in Male Rats. *Physiol. Behav.* 62: 305–310.

Sullivan, R., Wilson, D., and Leon, M. 1989. Norepinephrine and learning-induced plasticity in infant rat olfactory system. *J. Neurosci.* 9: 3998–4006.

Sullivan, R.M., Brake, S.C., Hofer, M.A., and Williams, C.L. 1986. Huddling and independent feeding of neonatal rats can be facilitated by a conditioned change in behavioral state. *Dev. Psychobiol.* 19: 625–635.

Tachikawa, K.S., Yoshihara, Y., and Kuroda, K.O. 2013. Behavioral Transition from Attack to Parenting in Male Mice: A Crucial Role of the Vomeronasal System. *J. Neurosci.* 33: 5120–5126.

Takayanagi, Y., Yoshida, M., Bielsky, I.F., Ross, H.E., Kawamata, M., Onaka, T., Yanagisawa, T., Kimura, T., Matzuk, M.M., Young, L.J., et al. 2005. Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice. *Proc. Natl. Acad. Sci.* 102: 16096–16101.

Thomas, S.A., and Palmiter, R.D. 1997. Impaired Maternal Behavior in Mice Lacking Norepinephrine and Epinephrine. *Cell* 91: 583–592.

Topilko, T., Diaz, S.L., Pacheco, C.M., Verny, F., Rousseau, C.V., Kirst, C., Deleuze, C., Gaspar, P., and Renier, N. 2022. Edinger-Westphal peptidergic neurons enable maternal preparatory nesting. *Neuron* S0896627322000484.

Unger, E.K., Burke, K.J., Yang, C.F., Bender, K.J., Fuller, P.M., and Shah, N.M. 2015. Medial Amygdalar Aromatase Neurons Regulate Aggression in Both Sexes. *Cell Rep.* 10: 453–462.

Valtcheva, S., and Froemke, R.C. 2018. Neuromodulation of maternal circuits by oxytocin. *Cell Tissue Res.* 375: 57–68.

Vandenbergh, J.G. 1973. Effects of central and peripheral anosmia on reproduction of female mice. *Physiol. Behav.* 10: 257–261.

Veit, J., Hakim, R., Jadi, M.P., Sejnowski, T.J., and Adesnik, H. 2017. Cortical gamma band synchronization through somatostatin interneurons. *Nat. Neurosci.* 20: 951–959.

Venables, W.N., and Ripley, B.D. 2002. MASS: modern applied statistics with S (New York: Springer).

Wakerley, J.B., and Lincoln, D.W. 1973. The milk-ejection reflex of the rat: a 20- to 40-fold acceleration in the firing of paraventricular neurones during oxytocin release. *J. Neuroendocrinol.* 57: 477–493.

Wisner, K.L., Bogen, D.L., Sit, D., McShea, M., Hughes, C., Rizzo, D., Confer, A., Luther, J., Eng, H., and Wisniewski, S.W. 2013. Does Fetal Exposure to SSRIs or Maternal Depression Impact Infant Growth? *Am. J. Psychiatry* 170: 485–493.

Wu, Z., Autry, A.E., Bergan, J.F., Watabe-Uchida, M., and Dulac, C.G. 2014. Galanin neurons in the medial preoptic area govern parental behaviour. *Nature* 509: 325–330.

Xie, Y., Huang, L., Corona, A., Pagliaro, A.H., and Shea, S.D. A reinforcement learning algorithm shapes maternal care in mice. *Biorxiv* 23.

Yoshihara, C., Numan, M., and Kuroda, K.O. 2017. Oxytocin and Parental Behaviors. *Curr. Top. Behav. Neurosci.* 35.