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Neural Mechanisms of Pup-Affiliative Behavior in Adult Virgin California Mice (*Peromyscus californicus*)

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Dedication

I would like to dedicate this dissertation to my parents, Carlos and Graciela, who encouraged me to further my education and taught me to work hard for the things I aspire to achieve. Thank you for your constant support and love during the last six years. Los quiero mucho.

ABSTRACT OF THE DISSERTATION

Neural Mechanisms of Pup-Affiliative Behavior in Adult Virgin California Mice (*Peromyscus californicus*)

by

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Dr. Wendy Saltzman, Chairperson

In biparental species, in which both parents care for their offspring, the neural mechanisms underlying the initiation of paternal care are not well understood. The neural mediators of paternal behavior overlap substantially with those underlying maternal behavior; however, few studies have looked at the functional roles of classical neurotransmitter systems, such as norepinephrine (NE), in the initiation of paternal care. Moreover, the cellular and molecular mechanisms that govern neural plasticity during the transition to parenthood remain largely unexplored. To address this gap, studies in this dissertation examined the role of NE in the onset of paternal behavior, as well as the mechanisms underlying neural plasticity resulting from experience with pups in adult California mice (*Peromyscus californicus*), a biparental species. These experiments mainly focused on adult virgins, as I was interested in examining the neural mechanisms underlying the initiation of pup-affiliative behavior in this species. The first study investigated the effects of nepicastat, an inhibitor of NE synthesis, on pup-directed behavior in adult virgin California mice. I found that nepicastat significantly reduced the number of virgin males and females that approached an experimentally presented pup

and that displayed parental behavior. In the second study, I examined the effects of nepicastat on neural activation, as indicated by c-Fos expression, in brain regions associated with parental care and anxiety following exposure to pups. Virgin males injected with nepicastat exhibited significantly reduced neural activation in the medial amygdala following pup exposure compared to vehicle-injected controls. Finally, I examined the impact of exposure to pups on perineuronal net expression (PNN) in the brains of male and female California mice. PNNs are extracellular matrix structures that are known to influence synaptic plasticity. I used a pup sensitization paradigm that promotes the onset of paternal behavior in virgin California mice. I found that dynamic changes in PNN expression in the brain occur with repeated exposure to pups in male but not female California mice. These findings provide insights into the role of the noradrenergic system in the onset of parental behavior as well as the mechanisms regulating neuroplasticity of the paternal brain during the transition to parenthood.

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Chapter 1

Introduction

Fathers' involvement in parenting influences child development

Parents have profound effects on the development and later-life behavior of their offspring. Historically, research has focused mainly on the role of mother-infant interactions in offspring physical and psychological developmental outcomes, while the influence of father-infant interactions on these developmental outcomes is less understood. However, in contemporary industrialized societies increasing numbers of women have entered the workforce. Fathers have become more and more actively involved in the daily caregiving of their children, as the number of stay-at-home fathers and fathers raising their children as single parents has increased in the last few decades (Chesley, 2011; Fry, 2023; Livingston, 2013, 2014). Notably, growing evidence suggests that fathers, like mothers, play a critical role in the development of their offspring, as the quality of parenting by fathers contributes to a wide range of developmental outcomes in children (Barker et al., 2017). For example, high-quality paternal-child interactions are associated with improved child developmental, psychological, socioemotional, and cognitive outcomes (Bögels & Phares, 2008; Cabrera et al., 2018; Hofferth et al., 2002; St. George & Freeman, 2017; Tamis-LeMonda et al., 2004). Moreover, children of fathers who actively participate in childrearing, compared to children with absent fathers, are less likely to use drugs of abuse or become involved with the juvenile justice system (J. Rosenberg & Wilcox, 2006). Conversely, the incidence of certain affective disorders (e.g., depression or anxiety) in fathers during the postnatal period has been associated with adverse child outcomes, such as a greater risk of later behavioral and emotional

difficulties in offspring (Barker et al., 2017; Fredriksen et al., 2019; Ramchandani et al., 2008). Given the growing evidence of the critical impact of fathers on children's development, determining the neurobiological underpinnings of paternal care may have significant implications for society.

Parental care is essential for offspring survival in mammals

Parental care can be defined as any behavior that a parent engages in to ensure the survival and optimize the development of their offspring, thereby increasing the fitness of both the offspring and the parent. Parental care has been observed in various taxa throughout the animal kingdom, including invertebrates, fish, reptiles, amphibians, birds, and mammals. The extent to which each sex cares for their offspring varies widely across species. Females are the exclusive caregivers of young in many species, males are the sole care providers in others, and both parents provide care in some species (i.e., biparental species). Parental behavior can be observed before or after offspring are born and can include, but is not limited to, preparation of nests or burrows, guarding and brooding of eggs, nest defense, carrying offspring, resource provisioning, nursing, huddling, grooming, and even providing offspring with information to enhance survival (Clutton-Brock, 1991).

In mammals, females evolved the capacity to lactate, and young depend on the mother's milk to survive. Therefore, female care is obligatory for offspring survival, while male mammals are more likely to desert their offspring rather than share the responsibilities of rearing offspring (Dawkins & Carlisle, 1976; Trivers, 1972). Paternal care is rare in mammals, with males in only about 5 – 10% of mammalian species providing some form of direct parental care, and is seen most commonly in rodents,

carnivores, and primates (Kleiman & Malcolm, 1981; Rymer & Pillay, 2018). Male care may be rare in mammals because the benefits of paternal care often do not outweigh the costs, which can include increased predation risk (Schradin & Anzenberger, 2001), increased energy expenditure (Campbell et al., 2009), loss of mating opportunities (Houston et al., 2005), and reduced survival (Getz & McGuire, 1993). Most adult male mammals are infanticidal toward young, and infanticidal behavior is generally advantageous for males because lactating females in many species return to estrus more quickly following the loss of offspring, providing more mating opportunities for males (Hrdy, 1979). Biparental care is thought to occur when it provides some adaptive advantage and facilitates offspring survival and development (Clutton-Brock, 1991). While paternal care is rare among mammals, there is evidence that it can enhance offspring survival and strongly influence offspring's neural, cognitive, and behavioral development (Bales & Saltzman, 2016). Therefore, determining the factors that underlie the expression of paternal care in biparental species is important.

The neural basis of female mammalian parenting has been studied extensively, which makes sense given that female care is obligatory, and the role of specific neurotransmitters, hormones, and brain regions have been identified. While paternal behavior has begun to receive a significant amount of attention in recent years, the neural mechanisms underlying male parental care are not well understood. It is hypothesized that paternally behaving mammals use a similar mechanism as females to engage in pup-affiliative behavior and that similar regions in the male brain are activated in response to pups as in females (Wynne-Edwards & Reburn, 2000). However, it is worth noting that paternal care has evolved multiple times in mammals, likely leading to

variation in the mechanisms of paternal care across biparental mammalian species (Clutton-Brock, 1991).

The California mouse is a rodent model of biparental care

The California mouse (*Peromyscus californicus*) is a species of rodent that is found near the coast of California in chaparral and oak-laurel woodland regions, from the San Francisco Bay area to as far south as Baja California, Mexico (Merritt, 1974, 1978). The California mouse is monogamous and biparental and forms exclusive pair bonds in both the field and captivity; fathers nest with the female throughout gestation and remain with the female and their offspring in the nest following parturition (Dudley, 1974; Gubernick & Alberts, 1987; Gubernick & Teferi, 2000; Ribble & Salvioni, 1990). Fathers participate in all forms of parental behavior typical of mothers (e.g., huddling, grooming, and retrieving pups), except lactation (Gubernick & Alberts, 1987; Lee & Brown, 2002). Fathers have also been observed assisting in pup delivery during parturition, as well as ingesting placenta and amniotic fluid following parturition (Lee & Brown, 2002; Perea-Rodriguez et al., 2018). While virtually all fathers are attracted to pups, virgin males vary widely in their behavior toward unrelated pups, often avoiding, attacking, or even caring for foreign pups upon experimental exposure (Chauke et al., 2012; de Jong et al., 2009; Gubernick & Addington, 1994; Gubernick & Alberts, 1987; Horrell et al., 2017). However, behavioral sensitization to pups can occur in this species, as a previous study in our lab has indicated that repetitive pup exposure across a few days may facilitate the onset of pup-affiliative behavior in virgin males (Horrell et al., 2017). This difference in pup-directed behavior between fathers and virgins suggests that the neural control of pup-related behavior changes as males transition into fatherhood in this species.

Brain mechanisms regulating the expression of parental behavior

The current understanding of the neural substrates of parental care in mammals primarily comes from research in female rodents. Still, extensive evidence suggests that components of the underlying circuits controlling pup-affiliative and pup-aggressive behavior overlap substantially between the sexes, regardless of whether or not both sexes normally express that behavior (Dulac et al., 2014; Numan, 2020; Wu et al., 2014). However, accumulating evidence also suggests that within these shared networks, there are distinct neuronal populations in males and females that are anatomically and functionally dimorphic (Scott et al., 2015; Chen et al., 2019).

The initiation of parental behavior in many rodent species requires a transition in pup-directed behavior from initially avoiding or acting aggressively toward infant stimuli to shifting to approaching and being attracted to pup-related stimuli. The two components that underlie the initiation of parental behavior are mediated by distinct opposing, but interacting, neural systems, one that inhibits parental responsiveness in animals not rearing offspring (e.g., virgins) and one that promotes pup-affiliative behavior after the birth of offspring or repeated exposure to infants (Horrell et al., 2019; Numan, 2012). The circuit that controls avoidance and defensive behaviors in response to pup cues involves structures such as the accessory olfactory bulbs (AOB), medial amygdala (MeA), ventromedial hypothalamic nucleus (VMH), anterior hypothalamic nucleus (AHN), and periaqueductal grey. The circuit that regulates acceptance and attraction toward pup stimuli centers on the medial preoptic area (MPOA) of the hypothalamus and the bed nucleus of the stria terminalis (BNST). The MPOA and BNST receive inputs relaying information about infant cues and send projections to multiple target regions to coordinate parental behavior by 1) inhibiting circuits that promote avoidance, and 2)

innervating the mesolimbic dopamine (DA) reward system that promotes approach/attraction via activating ventral tegmental area (VTA) DA signaling to the nucleus accumbens (NAc) (Horrell et al., 2019; Numan & Stolzenberg, 2009; Fig. 1.1).

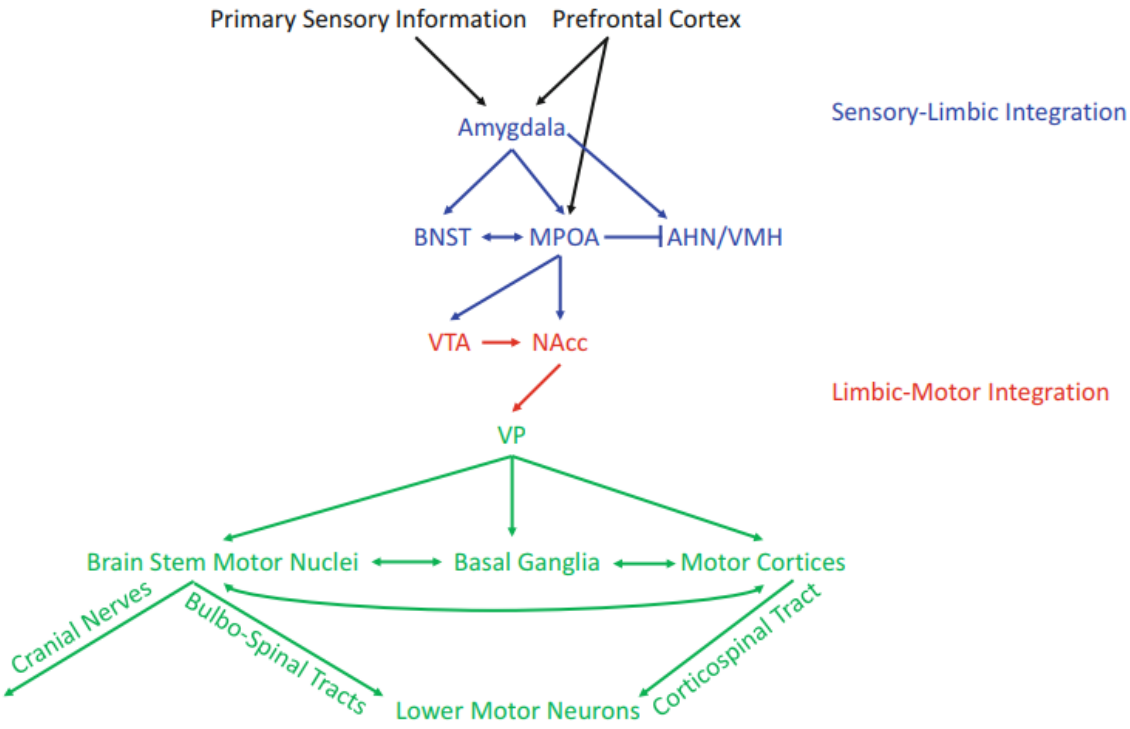

Figure 1.1. Circuit diagram of brain regions involved in parental care. The proposed circuit for the initiation of affiliative behavior involves the MPOA and BNST receiving inputs about offspring cues from several sensory modalities. MPOA and BNST projections activate the VTA DA input to the NAc to promote approach to reward-related stimuli. The NAc sends inhibitory (GABAergic) projections to the ventral pallidum (VP), a region that may be necessary for the execution of parental responses. In the presence of VTA-DA signaling to the NAc, inhibition of the VP by the NAc is released, which allows for approach/attraction of infants, while in the absence of VTA-DA signaling to the NAc, NAc inhibition onto the VP prevents approach and promotes avoidance (Horrell et al., 2019).

Researchers have generally studied the neural mechanisms of paternal care by using techniques such as measuring brain neurochemistry, quantifying immediately early gene expression, lesioning experiments, or using genetic targeting of neural circuits to influence paternal behavior. Exposure to infant-related sensory stimuli or direct exposure to infants increases brain activation in male rodents, as indicated by expression of

immediately-early genes, such as c-Fos, which are indirect markers of neural activity. Chemosensory pup stimuli acting through sensory neurons of the vomeronasal organ (VNO) and AOB are thought to promote infanticidal behavior in virgin male house mice (*Mus musculus*), as ablation of the VNO reduces pup-directed aggression and enhances paternal behavior (Tachikawa et al., 2013). Furthermore, pup exposure enhances Fos expression in downstream projection sites of the avoidance and aggression circuitry (e.g., the AHN and VMH) in virgin males. Fathers exhibit lower Fos expression in the VNO, AOB, and AHN compared to virgin males (Tachikawa et al., 2013). Therefore, the downregulation of the accessory olfactory system's response to pups may play a role in the suppression of infanticide and the onset of paternal care in house mice.

The MPOA and BNST receive information about pup-related sensory stimuli via several pathways. The MPOA is hypothesized to be a central integrator of infant stimuli and is critical for the induction of parental behavior in mammals (Numan, 2020). The MPOA and BNST are both activated in paternally behaving male rodents, and this activation often differs between fathers and virgin males. A study in California mice found that exposure to stimuli from an alien pup enhances Fos expression in California mouse fathers in the MPOA and BNST, and that this effect is attenuated in virgins (de Jong et al., 2009). Similarly, in both California mice and uniparental deer mice (*Peromyscus maniculatus*), fathers show increased Fos expression in the MPOA in response to pups in distress, compared to virgin males (Lambert et al., 2013). Finally, house mouse fathers exposed to an unrelated conspecific pup show increased Fos expression in the MPOA and BNST, compared to virgin males (Tachikawa et al., 2013; Wu et al., 2014). In particular, Wu et al. (2014) found that a population of MPOA neurons that express the neuropeptide galanin is activated in response to interactions with pups in fathers but not

in virgin males. Moreover, genetic ablation of these galanin-positive MPOA neurons causes deficits in parental behavior in fathers, while optogenetic activation of these neurons promotes paternal behavior and reduces aggression toward pups in virgins (Wu et al., 2014).

The amygdala, a subcortical region that integrates sensory information to encode and drive diverse affective behaviors, has been implicated in the expression of parental behavior in biparental rodents. In California mice, electrolytic lesions of the basolateral nucleus of the amygdala (BLA) impair paternal care in first-time fathers (Lee & Brown, 2007). In prairie voles (*Microtus ochrogaster*), virgin males exposed to pups show increased c-Fos immunoreactivity in the MeA compared to virgins exposed to a control object, while lesions to the MeA and corticomedial amygdala in this species decrease paternal care (Kirkpatrick, Carter, et al., 1994; Kirkpatrick, Kim, et al., 1994). More recently, Chen et al. (2019) found that GABAergic neurons in the MeA control pup-directed behavior in an activity-dependent manner, where optogenetic stimulation of these neurons at lower laser intensities induced pup-affiliative behavior, while stimulation at higher laser intensities induced infanticidal behavior.

In summary, the findings from the aforementioned studies suggest that the initiation of parental behavior in males is associated with changes in neural activation in many of the brain regions implicated in maternal care, including the MPOA, BNST, and amygdala (Feldman et al., 2019; Numan, 2020). However, although the primary neural circuits that mediate the initiation of paternal behavior have received increasing attention in recent years, the neurochemical systems that act within this paternal circuitry to promote the expression of pup-affiliative behavior remain largely unexplored. Most research has focused on the roles of neuropeptides and gonadal steroids, which have

been implicated strongly in the expression of paternal care (Horrell et al., 2019). In contrast, very little is known about the functional roles of classical neurotransmitter systems, such as catecholamines, in the initiation and maintenance of paternal care.

The noradrenergic system modulates arousal, attention, and behavioral flexibility

Norepinephrine is a catecholamine synthesized from dopamine (DA) by the enzyme dopamine β-hydroxylase (DBH). In the peripheral nervous system, NE is produced by postganglionic sympathetic neurons and underlies the flight-or-flight response. In the central nervous system (CNS), the noradrenergic system is comprised of two main ascending projections: (1) the dorsal noradrenergic bundle, which originates in the locus coeruleus (LC) and projects to the cerebellum, brainstem, hippocampus, cortical areas, and forebrain and (2) the ventral noradrenergic bundle, which arises in a number of nuclei of the pons and medulla and projects to the hypothalamus, midbrain, amygdala and BNST (Moore & Bloom, 1979). The widespread and distinct distribution of afferents from these neuroanatomical substrates places this system in a position to play a prominent regulatory role in the CNS. The noradrenergic system mediates a variety of sensory, affective, and cognitive functions, including arousal, facilitation of attention, stress reactivity, learning and memory, and motivation (e.g., Aston-Jones, 1985; Aston-Jones & Cohen, 2005; Berridge & Waterhouse, 2003; Bouret & Sara, 2005). Historically, activation of the locus coeruleus has been thought to result in widespread release of NE throughout the brain and to play an important role in determining the general level of behavioral activation and motivational state of an organism (Berridge & Waterhouse, 2003). Alternatively, recent evidence suggests that spatial modularity exists within the LC-NE neuronal cell groups and that distinct subpopulations of LC neurons have unique

efferent projections that can selectively mediate specific behaviors (Chandler et al., 2019; Poe et al., 2020).

Adrenergic receptors (ARs) are the target of NE and mediate both its peripheral and central actions. NE modulates synaptic transmission, plasticity, and cellular excitability via binding to three major G-protein-coupled AR subclasses (α_1, α_2, and β). The AR subclasses each elicit different cellular responses as they each couple to a distinct class of G proteins. Generally, G proteins either inhibit (via G_i) or stimulate (via G_s) the enzyme adenylyl cyclase or activate (via G_q) phospholipase C. NE has the highest affinity for the α_2 subclass (coupled to G_i), which has three known subtypes: $\alpha_{2A/D}$, α_{2B}, $\alpha_{2C.}$ NE has a relatively lower affinity for the α_1-AR subfamily (G_q-coupled receptors), which consists of three subtypes: α_{1A}, α_{1B}, and $\alpha_{1D.}$ Finally, NE has the weakest affinity for the β-AR subfamily (linked to G_s proteins), which also consists of three subtypes (β_1, β_2, and β_3). The effects of NE in each brain region are complex as they depend on the affinity of NE for the different receptor subtypes, as well as the expression patterns of adrenergic receptor subtypes in the target brain region.

As mentioned above, the noradrenergic system plays an essential role in modulating optimal behavioral responses, attention, and arousal across behavioral states (Aston-Jones & Cohen, 2005; Berridge & Waterhouse, 2003). NE release from the LC is thought to control cognitive flexibility and guide attention to novel, task-relevant, or emotionally salient stimuli in the environment, as salient/unexpected aspects of the environment activate the LC and result in brain-wide release of NE, irrespective of valence (Aston-Jones et al., 1994; Berridge & Waterhouse, 2003; Bouret & Sara, 2005; Joshi et al., 2016). NE seems to increase neural activation in response to relevant stimuli by enhancing signal-to-noise ratio in target systems, where NE suppresses

spontaneous impulse activity and enhances stimulus-evoked cellular responses (Berridge & Waterhouse, 2003; Servan-Schreiber et al., 1990).

The noradrenergic system regulates arousal, as the LC neuron discharge rate positively co-varies with arousal state, i.e., higher levels of LC activity are associated with greater arousal levels (Aston-Jones & Bloom, 1981). Many studies have provided evidence supporting an inverted U-shaped relationship between activation of the LC-NE system and behavioral performance (Aston-Jones & Cohen, 2005; Berridge & Waterhouse, 2003). Three patterns of LC activity corresponding to different behavioral states have been described: (1) a low tonic mode, (2) a phasic mode, and (3) a high-tonic mode. In the low tonic mode, LC neurons exhibit low-tonic LC activity (hypo-arousal) and individuals are inattentive, nonalert, minimally interactive with their environment, and disengaged from any current task, leading to poor behavioral performance. In the phasic mode, tonic LC activity increases to a moderate range. Synchronous phasic firing of electrotonically coupled LC neurons occurs in response to salient stimuli, and this activity pattern is associated with more focused attention and improved behavioral performance. As LC activity further increases, LC neurons exhibit a high level of ongoing tonic activity with elevated spontaneous discharge rates (hyper-arousal and stress), decreased synchrony, and diminished phasic response to specific stimuli. This mode is associated with a distractable attentional state characterized by less task engagement, labile attention, and increased behavioral flexibility (Aston-Jones et al., 1999; Aston-Jones & Cohen, 2005). Based on the proposed inverted-U relationship, the noradrenergic system seems to regulate the balance between selective attention and behavioral flexibility (Fig. 1.2). Notably, the three different modes of LC

activity described here may have distinct adaptive advantages under different environmental conditions.

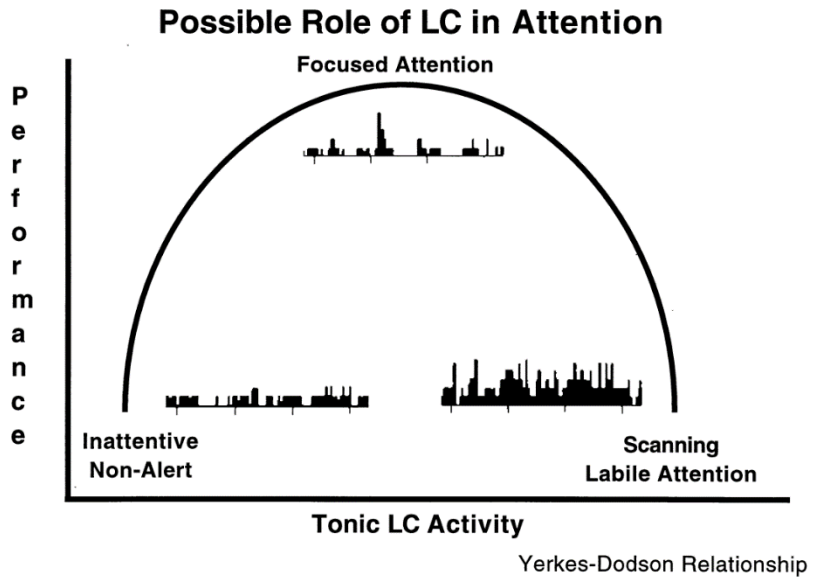

Figure 1.2. Role of the locus coeruleus in attention. Yerkes-Dodson relationship between LC activity and behavioral performance (Aston-Jones et al., 1999).

The noradrenergic system facilitates the initiation of parental behavior

Noradrenergic neurotransmission has been reported to facilitate the onset of maternal behavior in some mammalian species. Early studies in lab rats (*Rattus norvegicus*) reported that virgin rats induced to act maternally by pup exposure had higher NE metabolism in the hypothalamus compared to unresponsive virgins (P. Rosenberg et al., 1976). An additional study by this research group indicated that intracerebroventricular treatment of primiparous rats with the catecholaminergic neurotoxin 6-hydroxydopamine during late pregnancy depletes hypothalamic NE, but not DA, and impairs nursing, nest-building, and litter weight gain (P. Rosenberg et al., 1977). Moreover, a final study from this research group showed that lesioning the dorsal

noradrenergic bundle in expectant rat mothers, which leads to a depletion of cortical and hippocampal NE, causes a similar deficit in maternal behavior (Steele et al., 1979). Conversely, Bridges et al. (1982) found that lesioning midbrain NE pathways in peripartum rats had virtually no effect on postpartum maternal behavior, with the exception of a slightly inferior performance in lactation in lesioned subjects compared to controls (Bridges et al., 1982). Overall, these results suggest that NE plays a vital role in the initiation of maternal behavior in rats, but not the maintenance of that behavior once it has been established.

Genetic knockout studies have provided evidence that NE plays a vital role in the onset of parental behavior in house mice. House mouse mothers with a deletion of the DBH gene are unable to synthesize NE and exhibit severe deficits in maternal behavior; most of their pups die within several days of birth (Thomas & Palmiter, 1997). Moreover, treatment of DBH-knockout mothers with a synthetic precursor for NE production prior to parturition restores maternal behavior for the first litter and maintains maternal behavior in subsequent litters (Thomas & Palmiter, 1997). Furthermore, recent studies in house mice have found that pup retrieval correlates with LC firing in nulliparous surrogate female mice and lactating dams (Dvorkin & Shea, 2022). Additionally, a follow-up study from this research group found that connections between the LC and anterior cingulate cortex are critical for the expression of pup retrieval behavior in primiparous mouse dams (Corona et al., 2023). Taken together, these results suggest that NE signaling during the peri-partum period is responsible for long-lasting changes that promote maternal behavior in mice.

Central noradrenergic projections from the locus coeruleus to the olfactory bulb are involved in maternal recognition of offspring. For example, in sheep, destruction of

the noradrenergic inputs to the olfactory bulb prevents ewes from forming a selective attachment to their lambs (Pissonnier et al., 1985). Similarly, lesions of the noradrenergic projections to the olfactory bulb in female mice lead to primiparous mothers cannibalizing their pups on the first day postpartum, but the same lesions have no effect on cannibalism in multiparous mice (Dickinson & Keverne, 1988). The results from these studies suggest that noradrenergic signaling at the level of the olfactory bulbs is important for olfactory learning of offspring cues.

Prior to the research in my dissertation, only a single study, to my knowledge, had investigated the role of NE in the onset of pup-affiliative behavior in males. Virgin male DBH-knockout house mice exhibited low rates of pup retrieval when presented with unfamiliar pups, compared to heterozygous males (Thomas & Palmiter, 1997). It is important to note, however, that male house mice do not generally exhibit spontaneous paternal care in the wild (Gandelman et al., 1970; McCarthy & vom Saal, 1986); therefore, these findings may not be applicable to naturally biparental species. In the biparental prairie vole, exposure of adult virgin males and fathers to unfamiliar pups increases sympathetic excitation of the heart (Kenkel et al., 2013, 2014), but whether this increase in activity of the sympathetic nervous system and, presumably, increased release of NE into the periphery and possibly the brain contribute to the expression of paternal behavior has not been examined.

Perineuronal nets are dynamic extracellular structures that play an important role in experience-dependent plasticity

The chemical synapse has historically been considered to be composed of three main elements: the presynaptic element, the postsynaptic element, and surrounding glial

cells. Astrocytes, a type of glial cell, often enwrap neuronal synapses and play an important role in regulating neuronal function (Araque et al., 1999; Perea et al., 2009). The pre- and postsynaptic elements and associating astrocytes form an anatomical and functional unit classically known as the tripartite synapse (Araque et al., 1999). More recently, studies have shown that the extracellular matrix (ECM), the network of proteins that surrounds cells within the CNS, also plays an important role in modulating neural function and synaptic transmission (Dityatev & Rusakov, 2011; Kazantsev et al., 2012). Based on this recent evidence, the notion of the 'tetrapartite synapse' has emerged, comprised of the presynaptic and postsynaptic elements, astrocytic processes, and the perisynaptic ECM, as well as the interactions between the different components (Dityatev & Rusakov, 2011). Increasing evidence supports the hypothesis that the tetrapartite synapse as an integrated unit contributes to the regulation of synaptic plasticity and function, with an emphasis on perineuronal nets (PNNs) participating in synaptic plasticity.

PNNs are specialized pericellular aggregates of ECM that surround cell bodies and proximal dendrites of neurons in the CNS (Celio et al., 1998; Celio & Blumcke, 1994). PNNs are highly structured, lattice-like structures with holes for synaptic boutons from incoming neurons to synapse onto the underlying neurons. They are made up of four main structural components, including hyaluronan, chondroitin sulfate proteoglycans (CSPGs) of the lectican family, link proteins, and tenascin proteins (Wang & Fawcett, 2012). Hyaluronan forms the backbone of PNNs, CSPGs interact with the hyaluronan, link proteins stabilize the interactions between hyaluronan and CSPGs, and tenascin-R proteins bind to the opposite end of CSPGs not bound by link proteins. Together, these

molecules form the PNN structure that wraps around neurons (Fig. 1.3; Kwok et al., 2010).

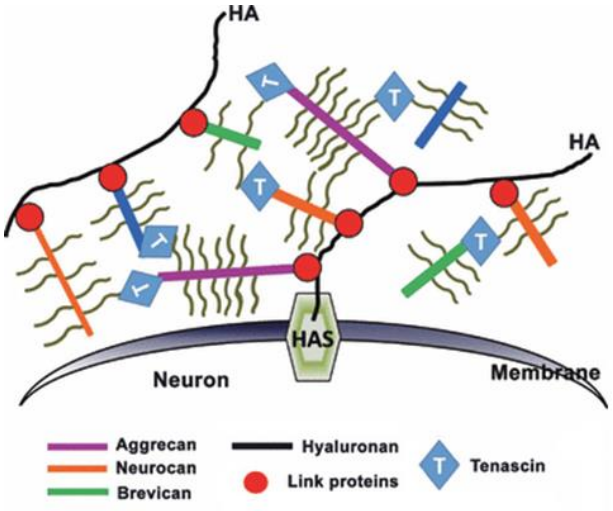

Figure 1.3 Schematic of perineuronal net organization. Cells expressing the hyaluronan synthase enzyme (HAS) secrete hyaluronan (HA), which forms the backbone of PNNs. CSPGS of the lectican family (e.g., aggrecan, brevican, neurocan) have a globular hyaluronan binding domain which allows them to interact with the hyaluronan backbone. Link proteins stabilize the interaction between the hyaluronan and CSPG, while tenascin proteins bind to the opposite end of the CSPGs (Kwok et al., 2010).

PNNs are prevalent throughout the mammalian CNS; however, cell-type-, region-, and species-specific differences in PNN expression exist. The majority of rodent cortical PNNs seem to preferentially envelop parvalbumin-positive (PV+) fast-spiking inhibitory interneurons, while few cortical PNNs are associated with excitatory pyramidal neurons (Alpár et al., 2006; Wegner et al., 2003). While the majority of rodent PNNs in the cortex surround PV+ inhibitory neurons, PNNs seem to surround both excitatory and inhibitory neurons in subcortical regions (Carstens et al., 2016; Horii-Hayashi et al., 2015; Mészár et al., 2012).

Regional differences in the expression of PNNs exist throughout the CNS of rodents. PNNs have been observed in multiple brain regions in rats; however, few studies have extensively examined the expression patterns of PNNs within brain regions implicated in motivation, reward, and reinforcement. While PNN expression seems to be sparse in the striatum of rats, high levels of PNN expression have been reported throughout the striatum and limbic system of mice, including the amygdala, hippocampus, hypothalamus, ventral pallidum, and prefrontal cortex (Bertolotto et al., 1996; Carstens et al., 2016; Horii-Hayashi et al., 2015; Mészár et al., 2012; Seeger et al., 1994; Uriarte et al., 2020). Given that some studies report high PNN expression across the circuitry mediating reward, reinforcement, and motivation, PNNs may influence brain circuits underlying motivated behaviors.

PNN formation commences during brain development and is finished by early adulthood in the rodent cortex, coinciding with the closure of the critical periods of neural development (Pizzorusso et al., 2002). Critical periods are windows of heightened plasticity and malleability of circuits during brain development which permit environmental factors to shape developing circuits via selective strengthening and refinement of different synapses (Hensch, 2005; Knudsen, 2004; Miao et al., 2016). The enhanced neuronal plasticity that occurs during critical periods no longer exists once mature circuit functions are established, as synaptic mechanisms exist to actively restrict plasticity in the adult brain (Kuhlman et al., 2013; Ribic et al., 2019). PNNs are one of the synaptic mechanisms that regulate plasticity during development, as PNN expression increases during development and this process restricts plasticity. Evidence from a large body of literature spanning a variety of species suggests that PNNs play a role in circuit stabilization.

PNNs serve multiple functions in the CNS, as they contribute to stabilization of synaptic connectivity, protection of neurons against firing-induced oxidative stress, buffering of the ionic microenvironment, and suppression of plasticity (Brückner et al., 1993; Carstens et al., 2016; Frischknecht et al., 2009; Wang & Fawcett, 2012). PNNs can physically block incoming axonal input to underlying neurons as they can form physical barriers for axonal growth cones and can act as chemical barriers by harboring chemorepulsive proteins such as semaphorins. Therefore, PNN formation in a given brain region is thought to be an indicator of a mature circuit element that no longer forms new synapses (Sorg et al., 2016).

In addition to playing a role in the neural plasticity associated with critical periods of development, PNNs can be dynamically regulated in adulthood. Normal environmental stimulation can alter PNN formation in the adult rodent CNS (Banerjee et al., 2017; Foscarin et al., 2011). Interestingly, numerous studies have shown that manipulations to remove PNNs in the adult CNS can reinstate juvenile-like states of plasticity, promote learning capabilities, and enhance cognitive flexibility (Carulli et al., 2010; Geissler et al., 2013; Gogolla et al., 2009; Happel et al., 2014; Hirono et al., 2018; Pizzorusso et al., 2002; Romberg et al., 2013). Regarding learning and memory, previous studies utilizing the enzyme chondroitinase ABC (ChABC), which can digest PNNs, in the cortex or amygdala have shown that PNNs play a role in the maintenance of both auditory and visual fear memories, as well as addiction-related memories (Banerjee et al., 2017; Thompson et al., 2018; Xue et al., 2014), suggesting PNNs may provide stability to CNS networks, which may be essential for the formation and recall of long-term memories.

Few studies have examined PNNs in the context of reproduction; however, recent studies in rodents have found dynamic changes in PNN expression in response to reproductive events. In a mouse model of Rett syndrome, an autism spectrum disorder, female mice with the mutation for Rett syndrome show precocious formation of PNNs, which leads to early onset and closure of the critical period, and these mutant virgin mice have impairments in pup-gathering behavior (Krishnan et al., 2017). However, the degradation of PNNs with ChABC corrects this impairment (Krishnan et al., 2017). Additionally, PNNs have been examined across the reproductive cycle in female rats. While virgin females do not exhibit any changes in PNN expression in the MPOA across the different phases of the estrous cycle, PNN expression in this region increases during gestation in rat dams, increasing progressively starting at about day 10 of gestation and peaking right before parturition at day 21 (Uriarte et al., 2020). However, PNN expression in the MPOA fades following the birth of offspring, during the lactation period. Furthermore, Uriarte and colleagues (2020) found that simulating the hormonal changes of pregnancy, via systemically administering estrogen and progesterone to ovariectomized virgin female rats, could induce PNN expression in the MPOA, while ovariectomized rats that received a vehicle solution did not exhibit any expression of PNNs in the same region. Interestingly, the cells surrounded by PNNs in the MPOA also expressed estrogen and progesterone receptors (Uriarte et al., 2020). Finally, Lau et al. (2020) found that PNN expression was altered in the primary somatosensory cortex following experience with pups in virgin female mice, in a region-specific and hemisphere-specific manner. Together, these findings suggest that hormonal changes experienced by new mothers or interactions with pups may alter some components of neural plasticity through modulation of PNNs. However, whether PNNs contribute to the

neuroplasticity of the parental brain during the transition to parenthood in a biparental species has not been explored (Horrell et al., 2021).

Examining the neural mechanisms underlying the initiation of parental behavior in the California mouse

The aims of the studies in this dissertation are to examine the effects of reduced noradrenergic neurotransmission on parental behavior, to determine the locations within the brain in which noradrenergic signaling might be important for promoting parental behavior, and to determine if dynamic changes in PNN expression occur with pup experience in adult California mice. Because I am interested in the neural mechanisms underlying the initiation of pup-affiliative behavior in both sexes, I mainly focused on virgin male and female California mice for these experiments.

Chapter 2 was written and published in 2022, titled *Acute inhibition of dopamine β-hydroxylase attenuates behavioral responses to pups in adult virgin California mice (Peromyscus californicus)* (Acosta et al., 2022). I administered the dopamine β-hydroxylase inhibitor nepicastat prior to exposing adult virgin male and female California mice to an unrelated pup. This study showed how reduced NE synthesis altered behavioral responses to pups in adult virgin California mice. I found that nepicastat-treated mice exhibited less pup-affiliative behavior compared to vehicle-treated controls. For Chapter 3, I examined the effects of reduced noradrenergic neurotransmission on pup-induced neural activation, as indicated by c-Fos expression, in nuclei associated with parental care and anxiety. I administered nepicastat or vehicle prior to exposing adult virgin male mice to a pup for one hour and then collected brains for c-Fos immunohistochemistry.

Chapter 4 looks at the mechanisms associated with neural plasticity resulting from experience with infants in adult California mice. Horrell et al. (2017) found that repeated exposure to pups results in the induction of parental behavior in virgin male California mice. I tested the hypothesis that this plasticity in responses to pups in virgin males is mediated by PNNs. The experiments in Chapter 4 characterize the effects of repeated pup exposure on PNN expression in virgin male and female California mice. I measured PNN density in brain regions implicated in the regulation of parental care in rodents. This allowed us to examine the dynamic changes in PNN expression that may occur with pup experience in this species.

Together, the results of these studies provide new insights into neural mechanisms underlying the onset of pup-directed affiliative behavior in both males and females of a biparental species. Specifically, they indicate that NE might play a critical role in this process and that the neural plasticity associated with the onset of pup-affiliative behavior is associated with dynamic changes in PNNs. Overall, data from this work provides the foundation for future studies investigating the neural substrates of paternal behavior.

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Chapter 2

Acute inhibition of dopamine β-hydroxylase attenuates behavioral responses to pups in adult virgin California mice (*Peromyscus californicus*)

Abstract

In biparental species, in which both parents care for their offspring, the neural and endocrine mediators of paternal behavior appear to overlap substantially with those underlying maternal behavior. Little is known, however, about the roles of classical neurotransmitters, such as norepinephrine (NE), in paternal care and whether they resemble those in maternal care. We tested the hypothesis that NE facilitates the initiation of nurturant behavior toward pups in virgin male and female California mice (*Peromyscus californicus*), a biparental rodent. Virtually all parents in this species are attracted to familiar and unfamiliar pups, while virgins either attack, avoid, or nurture pups, suggesting that the neurochemical control of pup-related behavior changes as mice transition into parenthood. We injected virgin males and females with nepicastat, a selective dopamine β-hydroxylase inhibitor that blocks NE synthesis (75 mg/kg, i.p.), or vehicle 2 hours before exposing them to a novel pup, estrous female (males only), or pup-sized novel object for 60 min. Nepicastat significantly reduced the number of males and females that approached the pup and that displayed parental behavior. In contrast, nepicastat did not alter virgins' interactions with an estrous female or a novel object, suggesting that nepicastat-induced inhibition of interactions with pups was not mediated by changes in generalized neophobia, arousal, or activity. Nepicastat also significantly reduced NE levels in the amygdala and prefrontal cortex and increased the ratio of

dopamine to NE in the hypothalamus. Our results suggest that NE may facilitate the initiation of parental behavior in male and female California mice.

Introduction

In all mammals, with the possible exception of humans, care by mothers is essential for offspring survival. Males, too, provide parental care in approximately 5-10% of mammalian species (Kleiman & Malcolm, 1981; Woodroffe & Vincent, 1994). The primary neural circuits that mediate the expression of paternal behavior have received increasing attention in recent years and appear to overlap substantially with those underlying maternal behavior (Feldman et al., 2019; Numan, 2020). On the other hand, the neurochemical systems that act within this paternal circuitry to promote the expression of pup-affiliative behavior remain largely unexplored. Most research has focused on the roles of neuropeptides and gonadal steroids, which have been implicated strongly in the expression of paternal care (Horrell et al., 2019). In contrast, very little is known about the functional roles of classical neurotransmitter systems, such as monoamines, in the initiation and maintenance of paternal care. The catecholamine norepinephrine (NE) has been implicated in the activation of maternal behavior, but a potential role of NE in the control of paternal care has received very little attention.

Norepinephrine is synthesized from dopamine (DA) by the enzyme dopamine β-hydroxylase (DBH). In the peripheral nervous system, NE is produced by postganglionic sympathetic neurons and underlies the flight-or-flight response. In the central nervous system, NE is synthesized primarily in the locus coeruleus, which sends noradrenergic projections throughout the brain to mediate a variety of sensory, affective, and cognitive functions, including arousal, attention, mood, learning, and memory (e.g., Aston-Jones,

1985; Aston-Jones & Cohen, 2005; Berridge & Waterhouse, 2003; Bouret & Sara, 2005). Historically, activation of the locus coeruleus has been thought to result in widespread release of NE throughout the brain and to play an important role in determining the general level of behavioral activation and motivational state of an organism (Berridge & Waterhouse, 2003). Alternatively, recent evidence suggests that spatial modularity exists within the LC-NE neuronal cell groups and that distinct subpopulations of LC neurons have unique efferent projections that can selectively mediate specific behaviors (Chandler et al., 2019; Poe et al., 2020). Given the role of NE in arousal and attention, this neurochemical system might facilitate the onset of pup-affiliative behavior in adult caregivers, possibly through an increase in alertness and attention to infant-related stimuli.

Noradrenergic neurotransmission has been reported to facilitate the onset of maternal behavior in several mammalian species. House mouse (*Mus musculus)* mothers with a deletion of the DBH gene are unable to synthesize NE and exhibit severe deficits in maternal behavior; most of their pups die within several days of birth (Thomas & Palmiter, 1997). Moreover, treatment of DBH-knockout mothers with a synthetic precursor for NE production prior to parturition restores maternal behavior (Thomas & Palmiter, 1997). Similarly, intracerebroventricular (ICV) treatment of primiparous rats with the catecholaminergic neurotoxin 6-hydroxydopamine during late pregnancy depletes hypothalamic NE, but not DA, and impairs nursing, nest-building, and litter weight gain (Rosenberg et al., 1977). Lesioning the dorsal noradrenergic bundle in expectant rat mothers, which leads to a depletion of cortical and hippocampal NE, causes a similar deficit in maternal behavior (Steele et al., 1979). Additionally, central noradrenergic projections from the locus coeruleus to the olfactory bulb are involved in maternal

recognition of offspring. For example, in sheep, destruction of the noradrenergic inputs to the olfactory bulb prevents ewes from forming a selective attachment to their lambs (Pissonnier et al., 1985), and similar lesions in house mice lead to primiparous mothers cannibalizing their pups on the first day postpartum (Dickinson & Keverne, 1988).

Only a single study has investigated the role of NE in the onset of pup-affiliative behavior in males. Virgin male DBH-knockout house mice exhibited low rates of pup retrieval when presented with unfamiliar pups, compared to heterozygous males (Thomas & Palmiter, 1997). It is important to note, however, that male house mice do not generally exhibit spontaneous paternal care in the wild (Gandelman et al., 1970; McCarthy & vom Saal, 1986); therefore, these findings may not be applicable to naturally biparental species. In the biparental prairie vole (*Microtus ochrogaster*), exposure of adult virgin males and fathers to unfamiliar pups increases sympathetic excitation of the heart (Kenkel et al., 2013, 2014), but whether this increase in activity of the sympathetic nervous system and, presumably, increased release of NE into the periphery and possibly the brain contribute to the expression of paternal behavior has not been examined.

The California mouse (*Peromyscus californicus*) is socially and genetically monogamous and biparental in both the field (Gubernick & Teferi, 2000; Ribble & Salvioni, 1990) and the lab (Gubernick & Alberts, 1987). Fathers participate in all forms of parental care typical of mothers (e.g., huddling, grooming, and retrieving pups) with the exception of lactation (Gubernick & Alberts, 1987; Lee & Brown, 2002). While virtually all fathers are attracted to experimentally presented, familiar or unfamiliar pups, virgin males vary widely in their behavior toward unrelated pups, either avoiding, attacking, or caring for pups upon exposure (de Jong et al., 2009; Chauke et al., 2012;

Gubernick & Addington, 1994; Gubernick & Alberts, 1987; Horrell et al., 2017). This difference in pup-directed behavior between fathers and virgins suggests that the neurochemical control of pup-related behavior changes as males transition into fatherhood.

In this study, we tested the hypothesis that NE signaling facilitates the onset of pup-affiliative behavior in adult virgin male and female California mice. While the role of NE in the onset of maternal behavior has been examined in uniparental mammalian species, the role of NE in the onset of pup-affiliative behavior in biparental species has not been addressed; therefore, both males and females were used in this study. To manipulate the noradrenergic system, we injected mice systemically with nepicastat, a highly selective and potent inhibitor of DBH that crosses the blood-brain barrier and reduces NE content in peripheral and central nervous tissues (Stanley et al., 1997). We first examined the effects of acute nepicastat treatment on behavioral responses to unrelated pups in virgin males and females. To determine whether effects of nepicastat on pup-directed behavior were associated with generalized changes in neophobia and/or behavioral activation, we next examined the effects of acute nepicastat treatment on virgins' behavioral response to a novel pup-sized object (both sexes) or an estrous female (males only). Finally, we examined the effects of acute nepicastat treatment on catecholamine levels in the hypothalamus and amygdala, subcortical regions implicated in the onset of pup-affiliative behavior, as well as in the prefrontal cortex (PFC), an area that receives similar dopaminergic and noradrenergic innervation, to confirm that nepicastat inhibited central activity of DBH.

Methods

Animals

Subjects were 110 male and 44 female adult virgin California mice aged 151-212 days (i.e., approximately 5-7 months). Mice were bred at the University of California, Riverside (UCR) and were descendants of mice from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). They were housed in 44 × 24 × 20 cm polycarbonate cages with aspen shavings and cotton wool for nesting material and with Purina Rodent Chow 5001 (LabDiet, Richmond, IN, USA) and water available *ad libitum*. Humidity was approximately 60–70%, temperature was maintained at 21 ± 1 °C, and lights were on a 14:10 light: dark cycle (lights on from 0500 h to 1900 h).

Juveniles were weaned from their parents at 27-32 days of age and housed in same-sex groups of 3-4 age-matched individuals in a colony room containing only virgin mice, to prevent exposure to sensory stimuli from pups. Two weeks prior to testing, each mouse was pair-housed with a related or unrelated cage mate from its virgin group and randomly assigned to an experimental condition (Table 2.1).

All experimental procedures were approved by UCR's Institutional Animal Care and Use Committee and conform to *the Guide for the Care and Use of Laboratory Animals*. UCR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Experimental design

This study consisted of five experiments. In experiments 1-4, we administered nepicastat or vehicle solution to determine the effects of NE inhibition on behavioral

responses of virgin males and females to a pup (Experiment 1), behavioral responses of virgin males and females to a novel, pup-sized object (Experiment 2), locomotor or anxiety-related behavior of virgin males in the open-field test (Experiment 3), and appetitive behavior of virgin males toward an estrous female (Experiment 4). In Experiment 5 we used high-performance liquid chromatography (HPLC) to examine whether nepicastat administration altered catecholamine content in the hypothalamus, amygdala, and prefrontal cortex. Sample sizes for each experiment are shown in Table 2.1.

Drug administration

Nepicastat (Adooq Bioscience, Irvine, CA, USA) was dissolved in a solution of 5% Tween 80, 30% polyethylene glycol (PEG), and 65% ddH20 and injected as a suspension at a volume of 1 mL/kg body mass (75 mg/kg). The vehicle solution was prepared in an identical manner but without any drug. The dose, route of administration, and latency from drug treatment to behavioral testing or brain collection for HPLC were based on previous findings in rats (Schroeder et al., 2010, 2013). Nepicastat administered at a dose of 50 mg/kg, i.p. 2 h prior to testing reduces content of NE in the cerebral cortex by 40%, increases the DA/NE ratio in the cortex, and alters drug-, cue-, and stress-primed reinstatement of cocaine seeking in rats (Schroeder et al., 2010, 2013). Nepicastat doses ranging from 5 to 50 mg/kg do not alter exploratory behavior in rats, while drug administration at a dose of 100 mg/kg has been shown to suppress exploratory behavior (Schroeder et al., 2013). In pilot studies, we found that i.p. injection of 50 mg/kg nepicastat did not alter pup-directed behavior of California mice 2 h later. Therefore, nepicastat was administered at a dose of 75 mg/kg (i.p.) 2 h prior to behavioral testing in experiments 1, 2, and 4 and 2 h prior to euthanasia and brain

collection in experiment 5. Mice in experiment 3 were tested in an open field immediately after undergoing a novel-object test (see below); consequently, they were tested at 180 min after nepicastat or vehicle injection. All behavioral tests were conducted within the time frame of the known maximal brain concentrations of nepicastat (120 - 240 minutes) following systemic administration (Loureiro et al., 2015).

Experiment 1: effects of nepicastat on parental behavior in male and female California mice

Adult virgin male and female mice received an injection of nepicastat (75 mg/kg, i.p.) or vehicle between 8:00 and 11:00 h and were immediately placed alone in a clean cage. After 2 h, an unrelated, unfamiliar, 2- to 5-day-old stimulus pup was placed in the cage, in the opposite corner from the adult subject, and the subject was videotaped for 1 h. Testing was terminated immediately if the subject attacked the pup. Some stimulus pups were used for multiple tests, but in no more than one test per day. The following behaviors of the subjects were scored from videotapes: latency to approach the pup, latency to initiate parental behavior (grooming or huddling the pup), total time spent sniffing the pup, total time spent in parental behavior, total time spent in general exploratory activity without pup contact, total time spent autogrooming, total number of backward flips (a common stereotyped behavior in captive California mice: Minie et al., 2021), and total time spent resting without pup contact. Pup retrieval was not scored or analyzed because pup-carrying behavior is not common in this species (Harris et al., 2011). All videos were scored by a single observer.

Experiment 2: Effects of nepicastat on neophobia in male and female California mice

We evaluated behavioral responses of adult virgin male and female mice to a novel object, 2 h following treatment with either nepicastat or vehicle, to determine the effects of nepicastat on neophobia. These tests were identical to parental-behavior tests except that the stimulus was a pup-sized pebble, similar in shape and size to a 2- to 5-day-old pup. Behaviors scored from videotapes were latency to approach the object, total time spent sniffing the object, total time spent in general activity without object contact, total time spent autogrooming, total number of backward flips, and total time spent resting without object contact. All videos were scored by a single observer.

Experiment 3: effects of nepicastat on anxiety-related behavior in male California mice

Open-field tests were performed as previously described (Perea-Rodriguez et al., 2018) between 11:00 and 14:00 h. A subset of male mice from Experiment 2 were tested in the open-field apparatus immediately following the novel-object test (180 min after injection with nepicastat or vehicle). For each test, the mouse was placed for 10 min in the center of a square arena (1 x 1 x 0.5 m) made of non-reflective dark, opaque plastic walls, with a piece of clean white butcher paper placed on the arena floor to enhance contrast between the floor and the dark-furred mice. The arena was located in a sound-attenuating chamber maintained at 1400 lx with two overhead white lights. Behavior was recorded by a video camera suspended above the center of the arena. After each test, the arena was cleaned with 75% alcohol solution and the butcher paper was replaced. Exploratory behavior was analyzed using TopScanLite v.2 tracking software (Clever Sys Inc., Reston, VA, USA), which allowed for automatic measurement of several parameters of mouse movement. The software was used to divide the arena into two

concentric zones: an inner square in the center of the arena (0.5 × 0.5 m) and an outer zone that extended 0.5 m from the wall to the perimeter of the inner square. Parameters scored were total distance traveled, duration of time spent in the inner square of the arena, and duration of time spent in the outer zone of the arena (Perea-Rodriguez et al., 2018). All videos were scored by a single observer.

Experiment 4: effects of nepicastat on sociosexual behavior in male California mice

Sociosexual-behavior tests were identical to parental-behavior tests, except that instead of a pup, the stimulus animal was an ovariectomized, estrogen/progesterone-treated, adult virgin female (see below) that was unrelated and unfamiliar to the male subject. Females were restrained with a custom-made harness made of smooth paracord. The harness measured approximately 30 cm, with a sewn secure hand loop and adjustable toggle to fit the mouse; full leg movement was possible with no throat pressure. The end of the paracord harness was attached to a 5 cm metal necklace extender with a lobster clasp for attaching the harness to the cage. Females were allowed to habituate to the harness for 15 min the day prior to testing.

On the day of testing, males were injected with nepicastat or vehicle and placed alone in a clean cage. After 2 hours, the restrained female was introduced into the opposite side of the test cage from the male for 1 h. Following the test, the harness was wiped down with 70% isopropyl alcohol, rinsed with water, and allowed to dry overnight prior to the next test. The following behaviors of the male were scored: latency to approach the female, total time spent huddling the female, total time spent sniffing the female, total time spent sniffing the female's genitals, total time spent in general activity

without female contact, total time spent autogrooming, and total time spent resting without female contact. All videos were scored by a single observer.

Ovariectomies and hormone treatment

Stimulus females were ovariectomized under isoflurane anesthesia using sterile conditions and standard surgical procedures as previously described (Zhao et al., 2018). A ventral midline incision (~ 1 cm) was made above the genital area, and each ovary was severed from the oviduct and removed. The incision was closed with absorbable sutures (Monocryl Suture 4-0 FS-2, Ethicon, San Angelo, TX, USA), and the skin was sealed with tissue glue (Vetbond Tissue Adhesive 1469SB, St. Paul, MN, USA). After surgery, females were housed individually for one week and then paired with another virgin female. Two days before testing, ovariectomized stimulus females were injected with estradiol benzoate (Sigma-Aldrich, St. Louis, MO USA; 0.072 mg, s.c.) dissolved in sesame oil. Forty-eight hours later, the stimulus females were injected with progesterone (Sigma-Aldrich; 0.48 mg, s.c.) dissolved in sesame oil and then tested with a virgin male 4 h later. This steroid administration protocol has been shown to induce sexual receptivity in ovariectomized California mice (Zhao et al., 2018).

Experiment 5: effects of nepicastat on brain catecholamine levels in male California mice

Adult virgin male mice were injected with nepicastat or vehicle as described above at 8:00 - 11:00 h and placed alone in a clean cage. Two hours after injection, each mouse was sacrificed by CO_2 administration and decapitated, and the brain was rapidly removed. Tissue blocks of the whole hypothalamus and amygdala were dissected using the Palkovits and Brownstein microdissection technique (Palkovits &

Brownstein, 1988) and a standard mouse brain atlas for reference (Paxinos & Franklin, 2008), while the PFC was dissected according to Spijker (Spijker 2011). Brain segments were flash-frozen on dry-ice and stored at -80° C until they were shipped to Emory University for HPLC analysis.

The tissue was thawed on ice and sonicated in 0.1 N perchloric acid (10 μl/mg tissue) for 12 s with 0.5 s pulses. Sonicated samples were centrifuged (16100 rcf) for 30 min at 4°C, and the supernatant was then centrifuged through 0.45 μm filters at 4000 rcf for 10 min at 4°C. For HPLC, an ESA 5600A CoulArray detection system equipped with an ESA Model 584 pump and an ESA 542 refrigerated autosampler was used. Separations were performed at 28ºC using an MD-150 × 3.2 mm C18 column. The mobile phase consisted of 1.6 mM 1-octanesulfonic acid sodium, 75 mM NaH_2PO_4, 0.025% triethylamine, and 8% acetonitrile at pH 2.98. Twenty μl of sample was injected. The samples were eluted isocratically at 0.4 mL/min and detected using a 6210 electrochemical cell (ESA, Bedford, MA) equipped with 5020 guard cell. Guard cell potential was set at 475 mV, while analytical cell potentials were −175, 150, 350 and 425 mV. NE, DA, and their primary metabolites (MHPG, DOPAC, HVA) were measured with electrochemical detection. The analytes were identified by the matching criteria of retention time to known standards (Sigma Chemical Co., St. Louis MO). Compounds were quantified by comparing peak areas to those of standards on the dominant sensor.

Statistical analyses

Data were tested for assumptions of normality using the Shapiro-Wilk test and homogeneity of variance using Levene's test. Behavioral data tended to violate these assumptions; therefore, we used nonparametric Mann-Whitney U tests to compare

behavior of nepicastat- and vehicle-treated mice within each sex and to compare males and females within each treatment group. Catecholamine data were normally distributed and were analyzed by between-subjects Student's t-tests. Data that were analyzed parametrically are presented as mean ± SE, and data that were analyzed non-parametrically are presented as median + 1st and 3rd quartiles. Effect size estimates were assessed by Cohen's d or Eta squared (η2) where appropriate (https://www.psychometrica.de/effect_size.html). All data were analyzed using GraphPad Prism version 8.0.0 for Windows (San Diego, CA, USA) with alpha set at 0.05 (2-tailed).

Results

Experiment 1: nepicastat treatment reduced interactions with pups in male and female California mice

Nepicastat-treated and vehicle-treated mice showed marked differences in their behavioral responses toward an unfamiliar pup (Fig. 2.1; Table 2.2). All of the virgin males and females treated with vehicle approached and smelled the pup, while the majority of nepicastat-treated mice did not. No statistically significant sex differences were found in any measure.

Males

Significantly fewer nepicastat-treated male mice approached the pup during the hour-long test compared to males treated with vehicle (5/16 vs 16/16, respectively; $p < 0.0001$, Fisher's Exact test). Additionally, nepicastat-treated males were significantly more likely to avoid the pup (i.e., to spend less than 60 s investigating the pup) than vehicle-treated males (14/16 vs 3/16, respectively, $p = 0.002$, Fisher's exact test; Fig. 2.1A).

For analyses of latency to approach the pup, adult mice that did not approach the pup were assigned a latency of 3600 s (the length of the test). Males in the nepicastat-treated group had a longer approach latency than vehicle-treated mice ($U = 27.50$, $p < 0.0001$, $d = 1.80$, Mann-Whitney U; Fig. 2.2A). However, when males that did not approach the pup were excluded from the analysis, the latency to approach did not differ between treatment groups ($p > 0.05$; Table 2.2). Nepicastat-treated males also spent significantly less time sniffing the pup in comparison to vehicle-treated males ($U = 11.50$, $p < 0.0001$, $d = 2.46$, Mann-Whitney U; Fig. 2.2C, Table 2.2).

Nepicastat also influenced parental behavior (i.e., grooming or huddling). Only one nepicastat-treated male engaged in parental behavior, while 9 of the 16 vehicle-treated males did so ($p = 0.0059$, Fisher's Exact test; Fig. 2.1A). For latency to initiate parental behavior, subjects that did not groom or huddle the pup were assigned a latency of 3600 s. Nepicastat-treated males had a longer latency to initiate parental behavior ($U = 60.50$, $p = 0.0014$, $d = 1.01$, Mann-Whitney U; Fig. 2.2B, Table 2.2) and spent less time engaging in parental behavior compared to vehicle-treated males ($U = 62.50$, $p = 0.0025$, $d = 0.97$, Mann-Whitney U; Fig. 2.2D, Table 2.2). Group sizes were not sufficient for statistical analysis of latency to initiate parental behavior when males that did not display this behavior were excluded. No difference in aggressive behavior towards pups was observed between drug-treated and vehicle-treated males (Fig. 1.1A); only 1 of 16 nepicastat-treated males attacked the pup, whereas 4 of 16 controls did so ($p > 0.05$). Finally, time spent engaging in general, non-pup-directed activity (i.e., autogrooming or exploratory behavior) and total number of flips did not differ significantly between groups (all $p > 0.05$, Mann-Whitney U; Table 2.2).

Females

Significantly fewer nepicastat-treated females approached the pup during the hour-long pup exposure compared to females treated with vehicle (4/11 vs 11/11; respectively; $p = 0.0039$; Fisher's Exact test). Similar to males, the overall proportion of females that avoided the pup was significantly higher in females that received nepicastat than vehicle-treated females (9/11 vs 2/11, respectively, $p = 0.0089$, Fisher's exact test; Fig. 2.1B).

The latency to approach the pup was significantly longer for drug-treated females than for vehicle-treated controls ($U = 4$, $p < 0.0001$, $d = 2.59$, Mann-Whitney U; Fig. 2.2A). When females that did not display approach behavior were excluded, nepicastat treatment still resulted in a significantly longer latency to approach the pup compared to vehicle treatment (Table 2.2). Nepicastat-treated females also spent significantly less time sniffing the pup than did vehicle-treated females ($U = 2.5$, $p < 0.0001$, $d = 2.78$, Mann-Whitney U; Fig. 2.2C, Table 2.2).

In females, as in males, nepicastat treatment reduced the likelihood of displaying parental behavior (Fig. 2.1B). Five of the 11 females in the vehicle group behaved parentally towards the pup, while none of the nepicastat-treated females did so ($p = 0.0351$, Fisher's Exact test). Therefore, the latency to initiate parental behavior was significantly greater for drug-treated females compared to vehicle-treated controls ($U = 33$, $p = 0.0351$, $d = 0.83$, Mann-Whitney U; Fig. 2.2B), and drug-treated females spent less (or no) time engaging in parental behavior in comparison to vehicle-treated females ($U = 33$, $p = 0.0351$, $d = 0.83$, Mann-Whitney U; Fig. 2.2D; Table 2.2). Similar to the males, aggression towards pups did not differ between treatment conditions (Fig. 2.1B),

as only 2 of 11 nepicastat-treated females and 4 of 11 controls attacked the pup ($p > 0.05$). Additionally, too few females exhibited flipping behavior during the pup tests (1/11 nepicastat vs 2/11 vehicle; respectively) to permit statistical analysis. Finally, time spent engaging in autogrooming or exploratory behavior did not differ significantly between groups (both $p > 0.05$, Mann-Whitney U; Table 2.2).

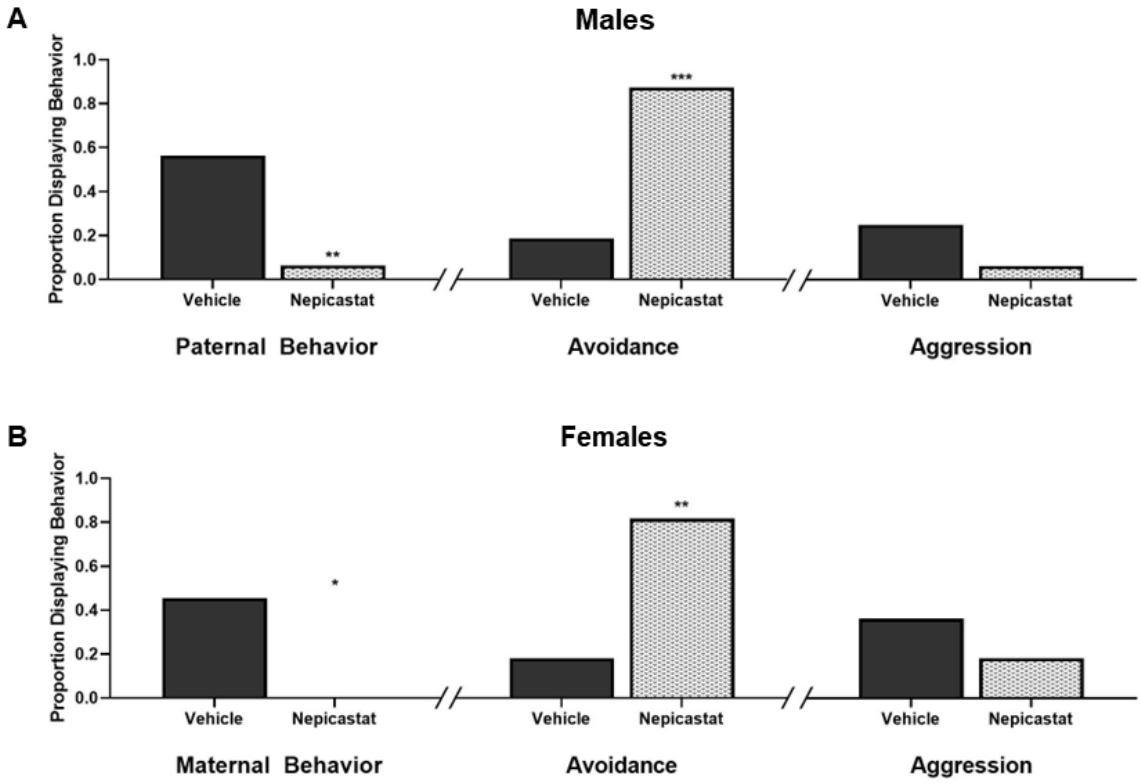

Figure 2.1. Effects of nepicastat (75 mg/kg, i.p.) on the proportion of adult virgin male (A) and female (B) California mice that displayed parental behavior (huddle/groom), avoidance, or aggression towards infants during the parental-behavior test. (*), (**), and (***) indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared with the vehicle-treated control group, respectively.

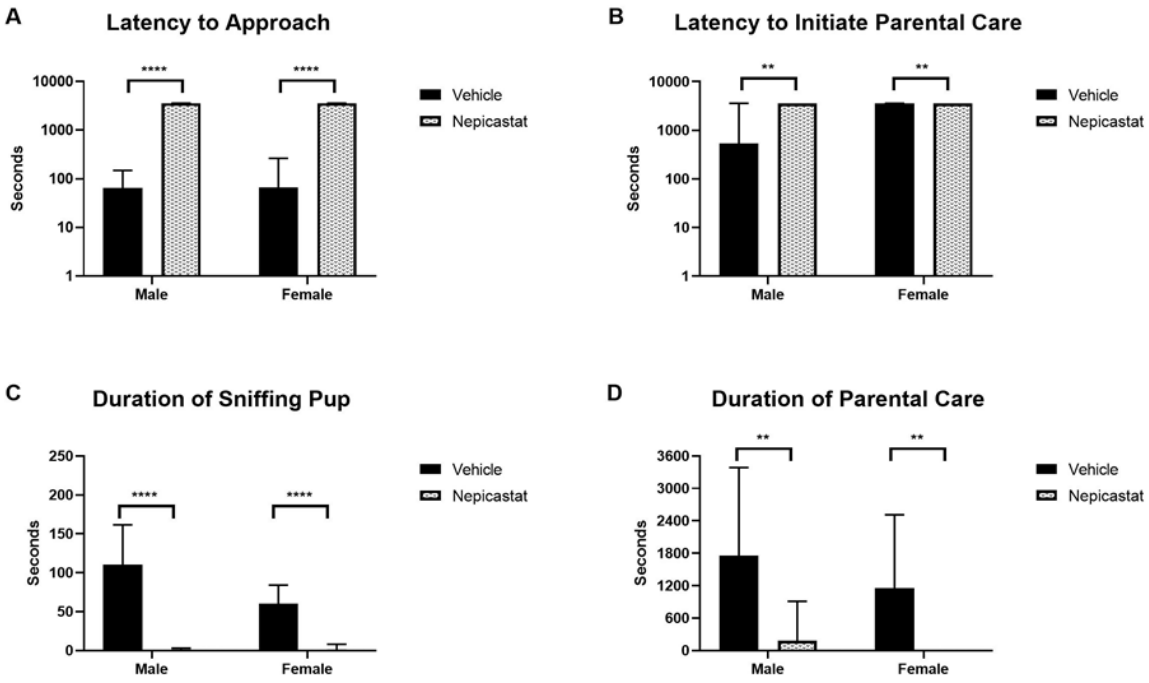

Figure 2.2. Effects of nepicastat on pup-directed behaviors (median ± 1st and 3rd quartiles) in adult virgin male (N = 16 per group) and female (N = 11 per group) California mice. A. Latency to approach a pup was longer in drug-treated virgin males and females compared to vehicle-treated mice. B. Latency to initiate parental care was longer in subjects that received nepicastat compared to virgins that received vehicle. C. Duration of time spent sniffing the pup was lower in nepicastat-treated males and females than in vehicle-treated controls. D. Duration of time spent engaging in parental behavior (huddle/groom) was lower in drug-treated males and females than vehicle-treated controls. (**) and (****) indicate $P < 0.01$ and $P < 0.0001$ compared with the control group, respectively

Experiment 2: nepicastat did not affect neophobia in male or female California mice

Nepicastat had no effect on the behavioral response to a novel, pup-sized pebble (Table 2.2). No significant differences were found between nepicastat- and vehicle-treated animals within either sex or between males and females within each treatment group in terms of number of mice that approached the object, latency to approach the object, or duration of sniffing the object (all $p > 0.05$) during the hour-long exposure. Additionally, there were no differences in the total number of flips between nepicastat- and vehicle- treated males ($p > 0.05$), while too few females exhibited flipping behavior

during the novel-object test to permit statistical analysis (4/10 nepicastat vs 1/10 vehicle, respectively). We also found no differences between treatment groups or between the sexes in the duration of general activity without object contact or duration of autogrooming during the novel-object test (both $p > 0.05$; Table 2.2).

To address the possibility that nepicastat might have affected only the initial response to the novel object, we also examined males' and females' behavior during the first 5 minutes of the test. No significant differences were found between nepicastat- and vehicle-treated mice within either sex (all $p > 0.08$; data not shown).

Experiment 3: nepicastat did not alter locomotor or anxiety-related behavior in male California mice

Locomotor and anxiety-related behavior of virgin males were assessed in the open-field test beginning 180 min following nepicastat or vehicle administration. Both nepicastat- and vehicle-treated males spent the majority of the 10-min open-field test in the peripheral zone of the arena compared to the central zone ($p > 0.05$). The total distance travelled in the open field did not differ between the two treatment groups ($p > 0.05$; Table 2.2).

Experiment 4: nepicastat did not alter sociosexual interactions with an estrous female in male California mice

Nepicastat-treated and control males spent a similar amount of time interacting with an estrous female during the hour-long test (Table 2.2). The majority of virgin males investigated the hormone-primed female; however, copulation was not attempted by the majority of males, regardless of treatment condition. Only one vehicle-treated male attempted to mount the female while none of the nepicastat-treated males did so ($p >$

0.05). The number of mice that exhibited appetitive behavior (i.e., active pursuit) toward the female did not differ between nepicastat- and vehicle-treated mice (7/11 vs 6/11, respectively, p = 1.0, Fisher's Exact test). Similarly, no significant differences were found between groups in latency to approach the female or in duration of non-genital sniffing of the female, sniffing the female's genitals, or huddling with the female (all p > 0.05; Table 2.2). Finally, time spent engaging in autogrooming or exploratory behavior did not differ significantly between groups (both p > 0.05).

Experiment 5: nepicastat decreased brain norepinephrine levels in male California mice

Norepinephrine, dopamine, and the DA/NE ratio were examined in brain tissue collected 2 h following injection of nepicastat or vehicle to confirm that systemic nepicastat administration inhibited DBH activity. Nepicastat significantly reduced NE in the prefrontal cortex (t_{14} = 5.547, p < 0.0001, d = 2.77) and amygdala (t_{14} = 3.836, p = 0.0018, d = 1.92), but not in the hypothalamus (Fig. 2.3; Table 2.3). Dopamine levels were not significantly altered in any of these regions following nepicastat treatment (all p > 0.05; Table 2.3). Finally, the DA/NE ratio was increased in the hypothalamus of nepicastat-treated mice compared to vehicle-treated controls (t_{14} = 3.74, p < 0.0022, d = 1.87), but did not differ between groups in the prefrontal cortex or amygdala (both p > 0.05).

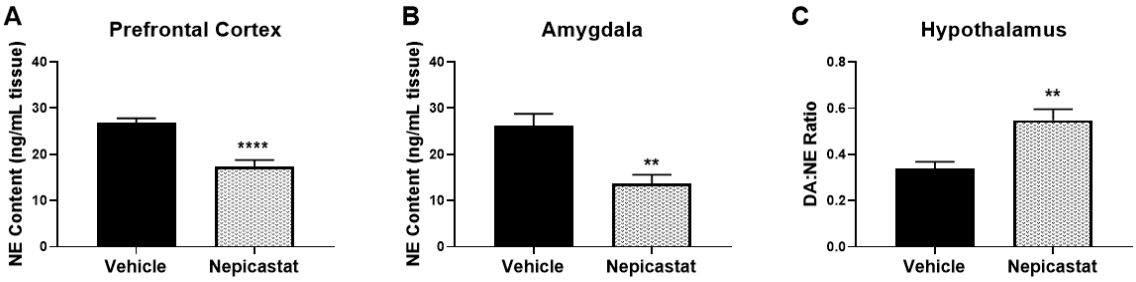

Figure 2.3. Effects of nepicastat on brain catecholamine levels (mean ± SEM). A. NE levels in the prefrontal cortex, B. NE levels in the amygdala, and C. the DA/NE ratio in the hypothalamus after treatment with vehicle or nepicastat (75 mg/kg, i.p.; N = 8 per group). (**) and (****) indicate $P < 0.01$ and $P < 0.0001$ compared with the vehicle group, respectively.

Discussion

Fathers in biparental species undergo neuroendocrine changes during the transition to fatherhood, which might facilitate the onset of parental behavior; however, the neurochemical mechanisms that regulate paternal care are not well understood. The noradrenergic system facilitates the onset of maternal behavior in several mammalian species, including house mice, rats, and sheep, but a possible role for this neuromodulatory system in the proximate regulation of paternal care has received very little attention. In this study, we investigated the role of NE in the onset of parental care in reproductively naïve male and female California mice by treating animals with the dopamine β-hydroxylase inhibitor nepicastat. Our main finding was that nepicastat treatment inhibited interactions with pups in both sexes, indicating that NE may facilitate the onset of pup-affiliative behavior in both male and female California mice. In contrast, we found no effects of nepicastat on neophobia, anxiety-related behavior, or sociosexual behavior. Acute nepicastat administration caused a reduction in NE content in the brains of virgin male California mice, confirming that DBH inhibition occurred following drug administration.

Systemic administration of nepicastat profoundly reduced interactions with an experimentally presented pup in both male and female virgins. In both sexes, nepicastat-treated California mice were less likely to approach the pup, had longer latencies to approach the pup, and spent less time engaging in parental behavior, compared to vehicle-treated controls. These results are consistent with findings on effects of depleting NE on the initiation of pup-affiliative behavior in virgin or primiparous female rats and house mice (Rosenberg et al., 1977; Steele et al., 1979; Thomas & Palmiter, 1997; but see Bridges et al., 1982) however, the role of NE signaling in the maintenance of maternal behavior after its initial onset is not clear. Smith and colleagues (2012) found that enhancing NE neurotransmission within the ventral bed nucleus of the stria terminals (BNSTv) and medial preoptic area (MPOA) inhibited certain aspects of maternal behavior in multiparous rat dams. Dams that received yohimbine, an α2 adrenoreceptor antagonist that enhances NE release, exhibited severe deficits in pup retrieval compared to vehicle-treated controls. Conversely, the authors reported that nulliparous virgin female rats that received bilateral noradrenergic lesions to the MPOA and BNSTv had non-significant increases in the number of days it took to exhibit maternal behavior during repeated exposure to pups (Smith et al., 2012). Therefore, while α2 adrenoreceptor antagonism within the maternal-care circuitry may disrupt certain aspects of ongoing maternal caregiving behavior in dams, the results suggest that inhibition of NE neurotransmission in the MPOA and BNST does not facilitate the initiation of maternal behavior in sexually inexperienced rats. Taken together with the results from the current study, these findings suggest that NE may play a faciliatory role in the initiation of parental care behavior.

We found no differences in infant-directed aggression between nepicastat- and vehicle-treated control mice in our study, suggesting that NE may not be involved in the expression of infant-directed aggression. However, a previous study showed that NE may be associated with aggression, as DBH -/- knockout mice display reduced resident-intruder aggression towards adult conspecifics (Marino et al., 2005). Therefore, our failure to find a difference in aggression between groups may be due to a floor effect, as we observed low aggression levels in the vehicle-treated mice. Alternatively, the differences between or findings and those of the earlier study might reflect differences in the neurochemical control of aggression in different contexts.

Effects of nepicastat on interactions with pups in our study could not be attributed to generalized effects on activity levels or investigatory behavior; nepicastat did not alter locomotor activity when mice were tested with a pup, novel object, or estrous female, or in open-field tests, compared to treatment with vehicle. Furthermore, we found no effect of nepicastat treatment on anxiety-related behavior, as the time spent in the central or peripheral zones of the open-field arena did not differ between treatment groups. Thus, we conclude that nepicastat at a dose of 75 mg/kg did not have a sedative effect on California mice, and effects of the drug on pup-directed behavior were not due to drug-induced alterations of general activity or anxiety levels.

Consistent with our findings, Zaru et al., (2013) found that a high dose of nepicastat (100 mg/kg) did not affect locomotor activity in a novel test cage in rats. In another study of rats, however, Schroeder et al. (2013) found that locomotor activity in a novel cage was inhibited by 100 mg/kg but not by lower doses (25-50 mg/kg) of nepicastat. The disparity in findings of these two studies might be attributable to a difference in the latency to test locomotor activity following drug administration, as Zaru

et al. (2013) examined locomotion 3 h following nepicastat administration while Schroeder et al. (2013) characterized locomotion 2 h after drug administration.

Inhibition of pup-directed behavior by nepicastat in male California mice did not reflect generalized effects on responses to social stimuli, as nepicastat-treated and control males did not differ in their responses to an ovariectomized, hormone-primed female. Only one control male and none of the drug-treated males attempted to mount the female; however, this may have been due to the harness that we used to restrict movement of the females, which might have affected the males' ability to mount. Additionally, or alternatively, the low level of sexual behavior might have resulted from our use of sexually inexperienced males; in rats, substantial numbers of sexually naïve males fail to mate with sexually receptive females upon first exposure (Clark et al., 1984; Whalen et al., 1961; reviewed in Ågmo, 1999).

Studies of sexually experienced male rodents have generally found that copulatory behavior is enhanced by noradrenergic signaling, especially via α2-adrenergic receptors (Clark et al., 1984; Clark et al., 1985; McIntosh & Barfield, 1984; Smith et al., 1987; Thomas & Palmiter, 1997). On the other hand, few studies in rodents have directly measured female-directed pursuit, such as approach and follow, to determine if NE is essential for appetitive sexual behavior. Moreover, the majority of studies examining the effects of NE on male sexual behavior have used non-monogamous rodent species. In the monogamous, biparental zebra finch (*Taeniopygia guttata*), however, ICV treatment of virgin males with the neurotoxin DSP-4, which depletes telencephalic NE, increased the latency to sing in response to a female but did not alter pursuit behaviors such as approaching or following the female (Barclay et al., 1996). In sum, our finding that nepicastat did not affect males' responses to sexually

receptive females, in contrast to findings in other rodents, might reflect methodological differences among studies or differences among species, potentially related to differences in mating systems.

In our study, systemic (i.p.) nepicastat administration reduced NE content in the prefrontal cortex of virgin male California mice, confirming that nepicastat successfully inhibited DBH. This result is in line with previous studies in which nepicastat administration caused a significant reduction of NE tissue levels within the rat PFC (Devoto et al., 2014; Schroeder et al., 2010). However, nepicastat did not substantially increase tissue DA content in the PFC in our study, while 50 mg/kg of nepicastat has been shown to enhance tissue levels of DA in the rat PFC (Devoto et al., 2014; Schroeder et al., 2010). The reason for this disparity between species is not clear.

We found that in the amygdala, as in the PFC, nepicastat reduced tissue levels of NE but did not affect tissue levels of DA; however, it is not clear whether or how decreased NE signaling in the amygdala might alter pup-directed behavior. The amygdala is a heterogeneous structure composed of structurally and functionally distinct subnuclei, which contribute to emotional arousal, learning, memory, motivation, and reward processing (Adolphs, 2010; McGaugh, 2004; Murray, 2007). It contains high densities of the major subtypes of adrenergic receptors and receives dense noradrenergic innervation from noradrenergic nuclei in the brain stem (Alexander et al., 1975; Asan, 1998; Byrum & Guyenet, 1987; Unnerstall et al., 1984; Woulfe et al., 1990). Previous studies have implicated specific subnuclei within the amygdala in paternal responsiveness, as lesions to the medial amygdala reduced alloparental behavior in virgin male prairie voles, while lesions to the basolateral amygdala decreased pup-affiliative behavior in California mouse fathers (Kirkpatrick et al., 1994; Lee & Brown,

2007). Therefore, the noradrenergic system might influence amygdala function to promote negative or positive affective behavioral responses to pups. Future studies should examine the effects of NE signaling within the amygdala on pup-directed behavior and whether the initiation of parental behavior may be mediated by NE's effects on arousal within this region.

In contrast to the PFC and amygdala, nepicastat did not significantly alter NE levels in the hypothalamus but increased the DA/NE ratio. The disparity in results may be due to differences in NE innervation at the different sites. The PFC receives dense NE innervation exclusively from the LC, while the major NA inputs to the hypothalamus originate from the medullary nuclei of the brain stem, with less innervation from the LC (Cunningham & Sawchenko, 1988; Fritschy & Grzanna, 1989; Morrison et al., 1978); therefore, our findings could potentially result from differences in sensitivity to DBH inhibition by nepicastat among different noradrenergic nuclei within the brain. It is also important to note that NE directly regulates excitatory tone of dopaminergic cells, as noradrenergic neurons originating in the LC project to the mesolimbic dopaminergic system, and NE normally promotes DA transmission. Therefore, pharmacological treatment with DBH inhibitors might reduce the facilitatory role of NE on dopaminergic cell activity and DA release in target regions (Gaval-Cruz & Weinshenker, 2009; Weinshenker & Schroeder, 2007). Thus, while the DA/NE tissue ratio was increased in the hypothalamus by nepicastat administration in our study, it is possible that DBH inhibition by nepicastat had the opposite effect on DA release.

The noradrenergic system plays an essential role in modulating optimal behavioral responses, attention, and arousal across behavioral states (Aston-Jones & Cohen, 2005; Berridge & Waterhouse, 2003). Previous studies have established a

critical role for NE signaling in many aspects of social behavior, including social recognition memory (Dluzen et al., 1998; Griffin & Taylor, 1995; Marino et al., 2005), maternal offspring recognition (Dickinson & Keverne, 1988; Pissonnier et al., 1985), and maternal behavior (Rosenberg et al., 1977; Steele et al., 1979; Thomas & Palmiter, 1997). Overall, our results are consistent with previous findings in female rodents that suggest enhanced NE activity may facilitate the onset of pup-affiliative behavior. Based on the results of the current study, we conclude that DBH inhibition selectively altered pup-directed behavior in both virgin male and female California mice, as nepicastat administration selectively reduced engagement of adult mice in any sort of interaction with pups. Therefore, DBH inhibition might not alter the valence of pup stimuli to adult mice but might affect their attentiveness to and/or interest in pups. Future studies should examine the neural mechanisms, including the subtypes and locations of receptors, by which NE may influence the onset of parental behavior in a monogamous and biparental species.

Experiment	Procedure	Sex	Treatment	N
1	Parental-behavior test	Male	Vehicle	16
		Male	Nepicastat	16
		Female	Vehicle	11
		Female	Nepicastat	11
2	Novel-object test	Male	Vehicle	16
		Male	Nepicastat	16
		Female	Vehicle	10
		Female	Nepicastat	10
3	[a]Open-field test	Male	Vehicle	10
		Male	Nepicastat	10
4	Sociosexual behavior test	Male	Vehicle	12
		Male	Nepicastat	12
5	HPLC	Male	Vehicle	8
		Male	Nepicastat	8

Table 2.1. Sample sizes for each experiment.
[a] Mice tested in the open field were tested with a novel object immediately prior. These animals are included in the sample sizes shown for both the novel-object test and the open-field test.

Test	Measure	Nepicastat Males	Vehicle Males	Nepicastat Females	Vehicle Females	Mann-Whitney (Nepicastat vs Vehicle Males) U, p	Mann-Whitney (Nepicastat vs Vehicle Females) U, p
Experiment 1: Parental Behavior	Latency to approach pup (s)	3600.00 (644.80, 3600.00)	64.50 (38.75, 149.00)	3600.00 (1046.00, 3600.00)	65.00 (15.00, 265.00)	27.5, **<0.001**	4.0, **<0.001**
	Latency to approach pup (s) (excluding nondisplayers)	81.00 (38.00, 149.00)	64.50 (38.75, 149.00)	700.50 (225.30, 1058.00)	65.00 (15.00, 265.00)	27.5, 0.320	4.0, **0.018**
	Latency to initiate parental behavior (s)	3600.00 (3600.00, 3600.00)	532.50 (148.00, 3600.00)	3600.00 (3600.00, 3600.00)	3600.00 (122.00, 3600.00)	60.5, **0.001**	33.0, **0.035**
	Duration of sniffing pup (s)	0.00 (0.00, 3.00)	110.50 (19.50, 161.50)	0.00 (0.00, 8.00)	60.00 (34.00, 84.00)	11.5, **<0.001**	2.5, **<0.001**
	Duration of parental behavior (s)	0.00 (0.00, 0.00)	2458.00 (0.00, 3285.00)	0.00 (0.00, 0.00)	0.00 (0.00, 2486.00)	62.5, **0.003**	33.0, **0.035**
	Duration of autogrooming during pup test (s)	87.00 (8.75, 163.50)	16.50 (4.00, 194.50)	0.00 (0.00, 264.00)	0.00 (0.00, 145.00)	106.0, 0.417	46.5, 0.364
	Duration of exploratory behavior (s)	62.00 (19.00, 133.30)	132.00 (28.00, 480.50)	47.00 (5.00, 189.00)	23.00 (9.00, 129.00)	93.5, 0.120	57.5, 0.858
Experiment 2: Novel Object	Latency to approach object (s)	186.00 (64.75, 3600.00)	503.50 (245.00, 3600.00)	2626.00 (60.75, 3600.00)	3600.00 (82.50, 3600.00)	88.5, 0.129	42.0, 0.538
	Duration of sniffing object (s)	8.50 (0.00, 31.25)	2.00 (0.00, 60.00)	2.00 (0.00, 112.50)	0.00 (0.00, 67.75)	110.0, 0.497	47.0, 0.845
	Duration of autogrooming (s)	27.00 (0.00, 90.75)	88.50 (6.25, 212.50)	263.50 (38.00, 500.30)	193.50 (70.00, 344.80)	92.5, 0.177	46.0, 0.796
	Duration of exploratory behavior (s)	84.50 (10.50, 184.30)	336.50 (12.50, 1071.00)	110.00 (32.75, 981.80)	59.00 (10.00, 248.30)	90.0, 0.153	37.0, 0.353
Experiment 3: Open Field	Total distance travelled (m)	37.50 (22.50, 69.57)	27.38 (6.84, 70.10)	-	-	38.0, 0.393	-
	Duration in center (s)	24.72 (18.63, 75.23)	14.27 (1.00, 53.97)	-	-	34.0, 0.240	-
	Duration in periphery (s)	576.00 (527.80, 582.70)	586.20 (547.80, 599.10)	-	-	35.0, 0.280	-
Experiment 4: Sociosexual Behavior	Latency to approach female (s)	445.00 (240.00, 730.00)	151.00 (62.00, 287.00)	-	-	37.0, 0.133	-
	Duration of sniffing female (s)	278.00 (3.00, 519.00)	401.00 (133.00, 537.00)	-	-	51.0, 0.549	-

Duration of huddling female (s)	0.00 (0.00, 1024.00)	68.00 (0.00, 1650.00)	-	-	53.0, 0.625	-
Duration of autogrooming (s)	245.00 (0.00, 527.00)	183.00 (49.00, 305.00)	-	-	59.5, 0.960	-
Duration of exploratory behavior (s)	230.00 (16.00, 668.00)	338.00 (213.00, 650.00)	-	-	42.0, 0.236	-

Table 2.2. **Behavior of nepicastat- and vehicle-treated virgin males and females during the parental behavior, neophobia, open-field, and sociosexual behavior tests**. Behavior scores (median (first and third quartiles)) and results of Mann-Whitney tests comparing vehicle and drug-treated mice are shown. For all latencies, animals that did not interact with the stimulus were assigned the maximum duration of the test (3600 s). P-values < 0.05 are in bold.

Brain Region	Norepinephrine		Dopamine	
	Nepicastat	Vehicle	Nepicastat	Vehicle
Prefrontal Cortex	17.39 ± 1.39****	26.84 ± 0.99	12.49 ± 2.55	10.80 ± 3.52
Amygdala	13.71 ± 1.87**	26.13 ± 2.64	115.0 ± 43.10	131.70 ± 29.25
Hypothalamus	85.85 ± 10.10	100.70 ± 12.08	46.45 ± 5.65	33.72 ± 3.95

Table 2.3. **Tissue catecholamine levels in the prefrontal cortex, amygdala, and hypothalamus of virgin male California mice treated with nepicastat (75 mg/kg, i.p., 2 hours before mice were sacrificed) or vehicle**. Values are expressed as ng/mL tissue. The mean ± SEM and results of Student's t-tests tests comparing vehicle and drug-treated mice are shown. (**) and (****) indicate $P < 0.01$ and $P < 0.0001$ compared with vehicle.

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Chapter 3

Acute inhibition of dopamine β-hydroxylase alters neural responses to pups in adult virgin male California mice (*Peromyscus californicus*)

Abstract

The neural mechanisms underlying paternal care in biparental mammals are not well understood. The California mouse (*Peromyscus californicus*) is a biparental rodent in which virtually all fathers are attracted to pups, while virgin males vary widely in their behavior toward unrelated infants, ranging from attacking to avoiding to huddling and grooming pups. We previously showed that pharmacologically inhibiting the synthesis of the neurotransmitter norepinephrine (NE) with the dopamine β-hydroxylase inhibitor nepicastat reduced the propensity of virgin California mice to interact with pups. The current study tested the hypothesis that nepicastat would reduce pup-induced c-Fos immunoreactivity, a cellular marker of neural activity, in the medial preoptic area (MPOA), medial amygdala (MeA), basolateral amygdala (BLA), and bed nucleus of the stria terminalis (BNST), brain regions implicated in the control of parental behavior and/or anxiety. Virgin males were injected with nepicastat (75 mg/kg, i.p.) or vehicle 2 hours prior to exposure to either an unrelated pup or novel object for 60 minutes. Immediately following the 60-minute stimulus exposure, mice were euthanized and their brains were collected for c-Fos immunohistochemistry. Nepicastat significantly reduced c-Fos expression in the MeA and MPOA of pup-exposed virgin males compared to vehicle-injected controls. In contrast, nepicastat did not alter c-Fos expression in any of the above brain regions following exposure to a novel object. Overall, these results

suggest that the noradrenergic system might influence MeA and MPOA function to promote behavioral interactions with pups in virgin males.

Introduction

In mammals, maternal care is obligatory for offspring survival, while paternal care is rare, with fathers in only about 5-10% of species providing some form of direct care for their offspring (Kleiman & Malcolm, 1981; Rymer & Pillay, 2018). Fathers in biparental species undergo neurochemical changes during the transition to fatherhood, which might facilitate the onset of parental behavior (Horrell et al., 2019); however, the neural mechanisms underlying male parental care are not well understood. Monoamine neurochemical systems have been strongly implicated in the expression of maternal behavior (Numan & Stolzenberg, 2009; Pawluski et al., 2019), but very little is known about the functional roles of monoamine neurotransmitter systems in the initiation and maintenance of paternal care.

The catecholamine norepinephrine (NE) has been reported to facilitate the onset of maternal behavior in several mammalian species. House mouse (*Mus musculus*) mothers with a deletion of the gene for dopamine-β-hydroxylase (DBH), the enzyme that synthesizes NE from dopamine, exhibit severe impairments in maternal behavior (Thomas & Palmiter, 1997). Importantly, restoring NE synthesis shortly before parturition rescues maternal behavior in DBH-knockout mothers (Thomas & Palmiter, 1997). In lactating dams and “surrogate” mice (nulliparous females housed with primiparous dams and their offspring), activity in the locus coeruleus (LC), the primary source of NE in the brain, correlates with pup retrieval (Dvorkin & Shea, 2022). Similarly, LC projections to the prefrontal cortex are involved in pup-retrieval behavior in mouse dams (Corona et al.,

2023). In the olfactory system, destruction of projections from the LC to the olfactory bulbs results in the majority of primiparous house mouse mothers cannibalizing their offspring and inhibits maternal recognition of offspring in sheep (*Ovis aries*; Dickinson & Keverne, 1988; Pissonnier et al., 1985).

Very few studies have examined the role of the noradrenergic system in the onset of paternal behavior. In house mice, DBH-knockout virgin males exhibit low rates of pup retrieval when presented with unfamiliar pups, compared to heterozygous males (Thomas & Palmiter, 1997). However, male house mice do not typically provide parental care under naturalistic conditions; hence, these findings may not be applicable to biparental species (McCarthy & vom Saal, 1986). A recent study in our lab investigated the role of NE in the onset of parental care in the California mouse (*Peromyscus californicus*), a socially monogamous, biparental rodent in which males provide extensive care for their offspring (Gubernick & Alberts, 1987). Pharmacological inhibition of NE synthesis with nepicastat, a highly selective and potent inhibitor of DBH, blocked the onset of pup-affiliative behavior in reproductively naïve adult male and female California mice (Acosta et al., 2022). On the other hand, nepicastat did not influence neophobia (behavioral responses to a novel object) in virgin males or females, anxiety-related behavior (in an open field) in virgin males, or sociosexual behavior (responses to a sexually receptive female) in virgin males, suggesting that the effects of the drug may be specific to pup-directed behavior. In addition, acute systemic nepicastat administration reduced NE levels in the prefrontal cortex and amygdala of virgin male California mice (Acosta et al., 2022). Overall, these results suggest that NE signaling may facilitate the onset of pup-affiliative behavior in adult males in this species.

The neural mechanisms underlying the effect of nepicastat on pup-directed behavior are not yet known. In the current study, therefore, we examined the effects of nepicastat administration on pup-induced c-Fos immunoreactivity, a cellular marker of neural activity, in brain regions implicated in parental responsiveness, anxiety, and avoidance. We administered nepicastat or vehicle to adult virgin males and characterized c-Fos expression following exposure to either an unfamiliar pup or a control object (pup-sized pebble). Brain regions examined included the medial preoptic area (MPOA), dorsal bed nucleus of the stria terminalis (dBNST), ventral bed nucleus of the stria terminalis (vBNST), medial amygdala (MeA), and basolateral amygdala (BLA). The MPOA, vBNST, MeA, and BLA have been implicated in the regulation of parental care in virgin rodents, while the BLA and dBNST have known associations with avoidance and anxiety-related behaviors, which are associated with inhibition of parental behavior in virgin rodents (Davis et al., 2010; Horrell et al., 2017; Kirkpatrick, Kim, et al., 1994; Numan, 2020; Saltzman et al., 2017; Tye et al., 2011). Importantly, all the proposed regions receive moderate to dense noradrenergic innervation from noradrenergic nuclei in the brainstem (Byrum & Guyenet, 1987; España & Berridge, 2006; Woulfe et al., 1990). Given NE's role in modulating attention and arousal across behavioral states (Aston-Jones & Cohen, 2005; Berridge & Waterhouse, 2003), we predicted that mice treated with the NE synthesis inhibitor would show reduced c-Fos expression in the brain regions of interest following exposure to a pup, compared to vehicle-treated controls.

Methods

Animals

Animals were maintained as described previously (Acosta et al., 2022). Briefly, they were housed in standard polycarbonate cages (44 × 24 × 20 cm) with aspen shavings as bedding and cotton wool for nesting, and were fed Purina Rodent Chow 5001 (LabDiet, Richmond, IN, USA) and water *ad libitum*. Colony rooms were kept under a 14:10-hour light/dark cycle with lights on at 05.00 h. Room humidity was approximately 60–70%, and temperature was maintained at 21 ± 1 ◦C.

We used 22 adult virgin male California mice aged 150–212 days (i.e., approximately 5–7 months) that were born and reared in our breeding colony at the University of California, Riverside (UCR) and descended from animals purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). Mice were weaned from their parents at 27-32 days of age and housed in same-sex groups consisting of 3-4 age-matched individuals.

All experimental procedures were approved by UCR's Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. UCR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Experimental Design

At least two weeks before testing, each mouse was pair-housed with a related or unrelated cage mate from its original group of virgin males. Within each pair, the two virgin males were assigned to different treatment conditions (nepicastat or vehicle) and

tested with the same type of stimulus (pup or novel object [pup-sized pebble]; see below). Beginning two hours after nepicastat or vehicle injection, each subject was exposed to either an unrelated pup or a novel object for 60 minutes. It was then immediately sedated and perfused and its brain collected for Fos-immunohistochemistry. See Fig. 3.1 for the experimental timeline.

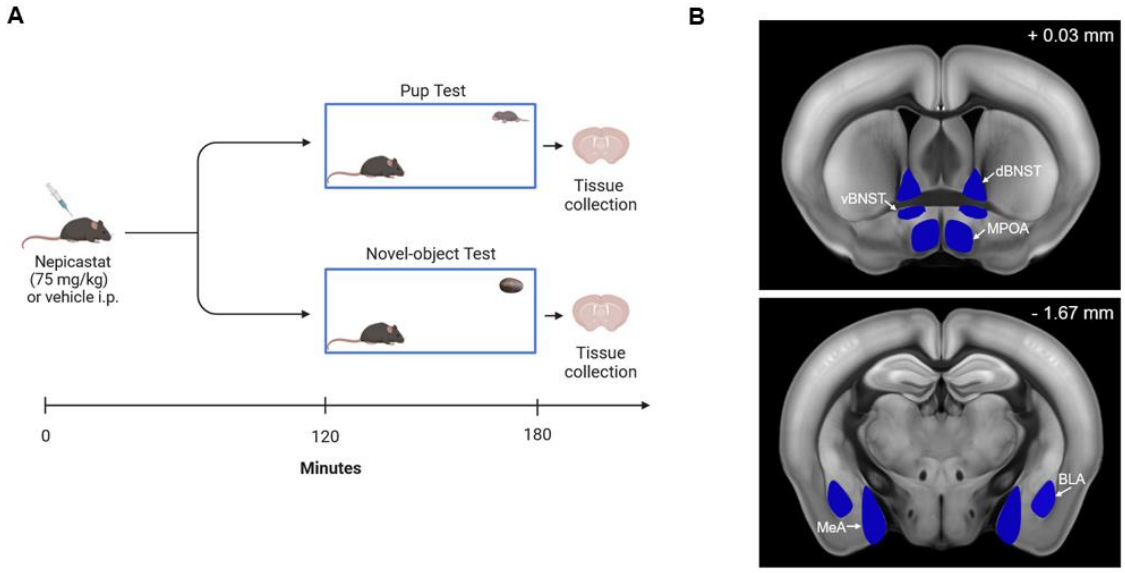

Figure 3.1. Schematic of the experimental design. **A.** Virgin males were injected i.p. with nepicastat (75 mg/kg) or vehicle 2 hours prior to exposure to either an unrelated pup or novel object for 60 minutes. Immediately following the stimulus exposure, mice were sedated and perfused and their brains collected for Fos-immunohistochemistry. **B.** Schematic representation of the brain regions (blue shaded areas) in which c-Fos-immunoreactive cells were quantified. Brain schematics were drawn with the aid of the Scalable Brain Atlas (Bakker et al., 2015), and regions were manually outlined according to Paxinos and Franklin (2012). The coordinates represent the distance relative to Bregma of the rostral face of each section. MPOA = medial preoptic area, MeA = medial amygdala, BLA = basolateral amygdala, vBNST = ventral bed nucleus of the stria terminalis, dBNST = dorsal bed nucleus of the stria terminalis.

Drug Administration

Nepicastat (Adooq Bioscience, Irvine, CA, USA) was dissolved in a solution of 5% Tween 80, 30% polyethylene glycol (PEG), and 65% ddH20 and injected as a suspension at a volume of 1 ml/kg body mass (75 mg/kg i.p.) 2 hours prior to behavioral

testing. The vehicle solution was prepared in an identical manner but without any drug, and a comparable volume was injected i.p. The dose, route of administration, and latency from drug treatment to behavioral testing were based on previous findings in our lab (Acosta et al., 2022). The 2-hour time window from drug treatment to behavior testing is consistent with findings from other studies, in which nepicastat levels in the brain peaked 120-240 minutes following systemic administration (Loureiro et al., 2015).

Behavioral Testing

Pup and novel-object tests were conducted as previously described (Acosta et al., 2022). Briefly, adult virgin male mice received an injection of nepicastat or vehicle between 8:00 h and 11:00 h and were immediately placed alone in a clean cage containing bedding, food, and water. After 2 h, an unfamiliar, unrelated, 2- to 5-day-old stimulus pup or novel object was introduced into the corner of the cage farthest from the adult subject for 1 h, followed immediately by sedation, perfusion, and collection of the brain for Fos-immunohistochemistry (see below). If a pup was attacked by the subject, it was removed immediately and the subject remained alone in the test cage for the remainder of the 60-min test, after which it was sedated and perfused. Some stimulus pups were used for multiple tests, but in no more than one test per day.

To examine the relationship between the density of c-Fos+ cells and behavior following nepicastat and vehicle administration, all 1-h tests were video-recorded. Videos from pup tests were later scored for pup-directed behavior [latency to approach the pup, latency to initiate parental behavior (grooming or huddling the pup), total time spent sniffing the pup, and total time spent in parental behavior]; videos from novel-object tests were scored for latency to approach the object and total time spent sniffing the object. All

videos were scored by a single observer blind to the treatment condition using The Observer software v. 11.5 (Noldus, Wageningen, Netherlands).

Immunohistochemistry, Imaging, and Cell Quantification.

Immediately following the test with a pup or novel object, mice were deeply anesthetized with pentobarbital and perfused intracardially with cold phosphate-buffered saline (PBS, 0.1 M, pH 7.4) and 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were removed and stored overnight in 4% PFA at 4°C and then immersed in a 30% sucrose solution for 24 to 48 h. The brains were stored in a freezing solution (30% ethylene glycol, 1% polyvinylpyrrolidone-40, 30% sucrose in 0.1M PBS) at -20°C until further processing. Brains were sectioned coronally at 40 µm thickness using a cryostat (Leica Biosystems, Deer Park, IL, USA; CM 1860). For each brain, an average of 8-10 slices of each region of interest were obtained for immunohistochemistry and stored at 4°C in 0.1M PBS with 0.05% sodium azide. We focused on regions of the hypothalamus and extended amygdala that are implicated in the control of parental behaviors, avoidance, and/or anxiety, including the MPOA (+0.03 mm from bregma), vBNST and dBNST (+0.18 mm from bregma), and BLA and MeA (−1.7 mm from bregma; Fig. 3.1B). Brain regions selected for quantification were identified by cross-referencing a standard mouse brain atlas (Paxinos & Franklin, 2012) and images of Nissl-stained California mouse sections (http://brainmaps.org). Each of the 4 treatment (nepicastat, vehicle) x stimulus (pup, novel object) groups was represented in every batch of histology.

Briefly, free-floating sections were washed three times in 0.1M PBS, incubated in 2% bovine serum albumin (BSA) in 0.1M PBS for 30 min, rinsed once in 0.1M PBS, and then incubated overnight at room temperature with the primary antibody rabbit anti-c-Fos

(1:2500; Synaptic Systems, Göttingen, Germany, Cat# 226 003) in 0.1M PBS containing 0.1% Triton X-100 with 2% BSA. The following day, sections were washed three times in 0.1M PBS, incubated for 90 min at room temperature with the secondary antibody donkey anti-rabbit Alexa Fluor 555 (1:500, Invitrogen, Waltham, MA, USA, Cat# 31572) in 0.1M PBS containing 2% BSA, washed three times in 0.1M PBS, mounted onto slides, and coverslipped using EMS Shield Mount with DABCO mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA).

Imaging for c-Fos immunohistochemistry was performed on an inverted Zeiss LSM 880 confocal microscope (10x objective; Carl Zeiss Microscopy, White Plains, NY, USA) equipped with Airyscan Fast. Images were collected in z-stacks covering 30 µm (1 µm step size). All images were acquired under identical conditions. Each z-stack was imported into ImageJ software (NIH), collapsed into a single image by projection, and then converted to a TIFF file.

Pipsqueak Pro™ (https://rewire.ai/pipsqueak-pro/) was used to identify and quantify the number of c-Fos-positive cells. A consistent background threshold was set and applied to all images for comparison. Pipsqueak was run in semi-automatic mode to select ROIs to identify c-Fos+ cells, which were then verified by a trained scorer who was blind to the experimental conditions during cell quantification. All areas were analyzed bilaterally in each section. Data for each region for each animal were averaged from 3-4 sections, and these averages were compared across groups.

Statistical Analyses

Data were tested for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. c-Fos density data were normally distributed; thus, they

were analyzed by between-subjects Student's t-tests within each stimulus condition and are presented as mean ± SE. The behavioral data from the subjects in the current study have been published previously (Acosta et al., 2022); in this paper, therefore, we limit behavioral analyses to correlations with c-Fos expression. Correlational analyses were performed separately within each stimulus condition, using both nepicastat- and vehicle-treated animals. Using the Shapiro-Wilk test, the behavioral data were determined to be non-normal, so Spearman's correlation was used. All data were analyzed using GraphPad Prism version 10.0.0 for Windows (San Diego, CA, USA) with alpha set at 0.05 (2-tailed). A p value of < 0.05 was considered statistically significant.

Only one mouse, which was treated with vehicle, attacked a pup during the 60-min test. The attack occurred 17 min after the pup was introduced into the male's cage, and the pup was removed immediately; therefore, this mouse was exposed to a pup for only 17 min. Consequently, we did not included data from this animal in any analyses.

Results

Nepicastat treatment reduced neural activation in the medial amygdala and medial preoptic area following pup exposure

Virgin males in the nepicastat-treated group had significantly reduced c-Fos expression in the MeA and MPOA following pup exposure, compared to males treated with vehicle (MeA: $t [7] = 2.37$, $p < 0.049$; Table 3.1; Fig. 3.2; MPOA: ($t [7] = 2.82$, $p = 0.026$; Fig. 3.3), suggesting that NE deficiency impairs MeA and MPOA activation in response to pups. c-Fos expression in the vBNST, dBNST, and BLA following exposure to pups was not influenced by nepicastat treatment (Table 3.1; Fig. 3.2 and 3.4).

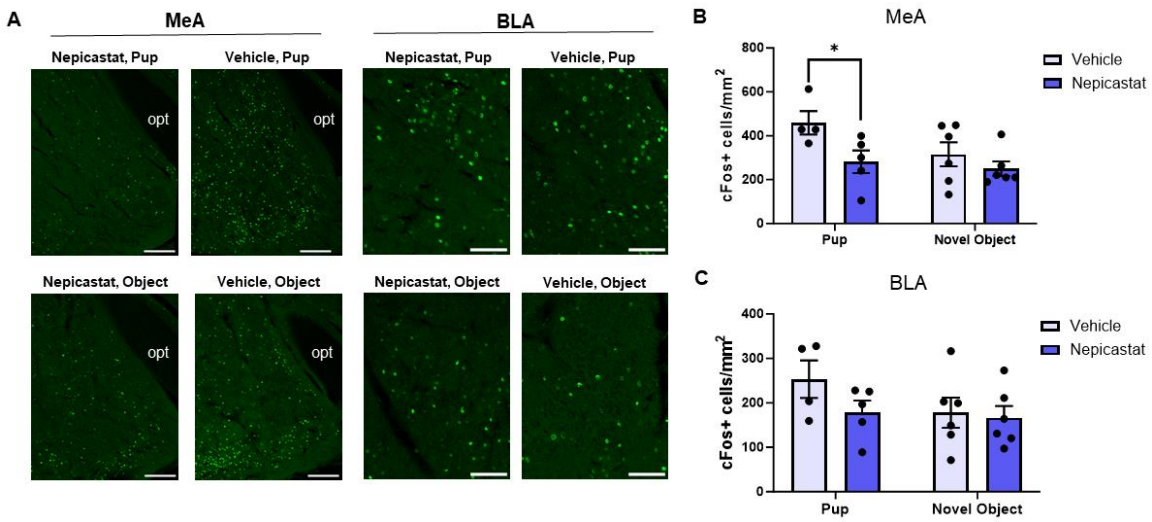

Figure 3.2. Effects of nepicastat on c-Fos expression in the amygdala. A. Representative photomicrographs of c-Fos immunoreactivity in the medial amygdala (MeA, left) and basolateral amygdala (BLA, right) of nepicastat- (75 mg/kg, i.p.) and vehicle-treated mice following exposure to a pup or novel object (pup-sized pebble). MeA scale bar, 200 µm; BLA scale bar, 100 µm. **B.** In comparison to vehicle treatment, nepicastat produced a significant decrease in c-Fos expression (mean ± SE) in the MeA following exposure to a pup (n = 4 vehicle, 5 nepicastat). Treatment groups did not differ significantly in c-Fos expression in the MeA following exposure to the novel object (n = 6 per group). **C.** c-Fos density in the BLA following exposure to either a pup (n = 4 vehicle, 5 nepicastat) or a novel object (n = 6 per group) was not influenced significantly by treatment. * indicates $p < 0.05$ compared with the vehicle group. opt = optic tract.

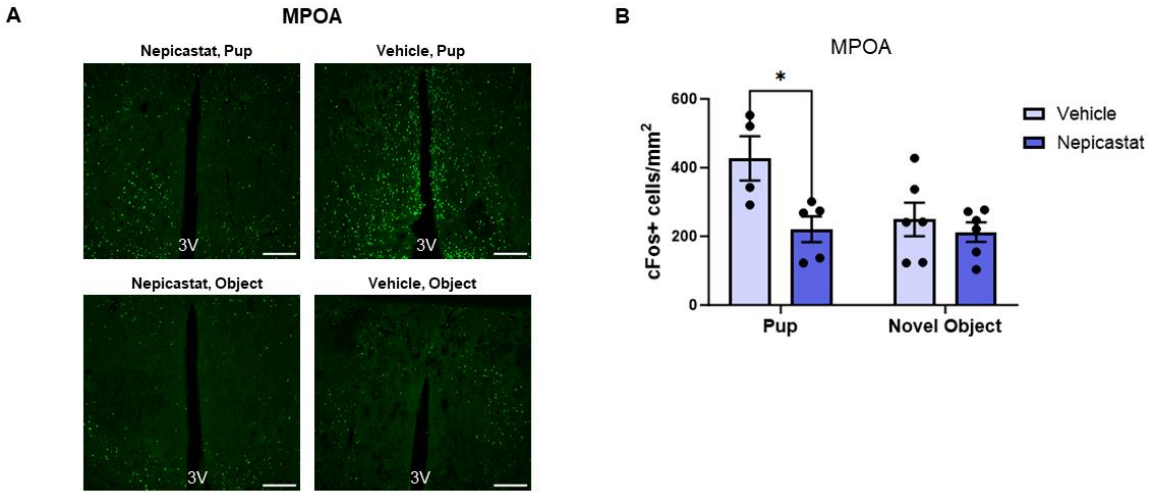

Figure 3.3. **Effects of nepicastat on c-Fos expression in the medial preoptic area (MPOA). A.** Representative photomicrographs of c-Fos immunoreactivity in the MPOA of nepicastat- (75 mg/kg, i.p.) and vehicle-treated mice following exposure to a pup or novel object. Scale bar, 200 µm. **B.** In comparison to vehicle, nepicastat administration significantly decreased c-Fos density (mean ± SE) in the MPOA following exposure to a pup (n = 4 vehicle, 5 nepicastat). Treatment groups did not differ significantly in c-Fos expression in the MPOA following exposure to the novel object (n = 6 per group). * indicates $p < 0.05$ compared with the vehicle group. 3V = third ventricle.

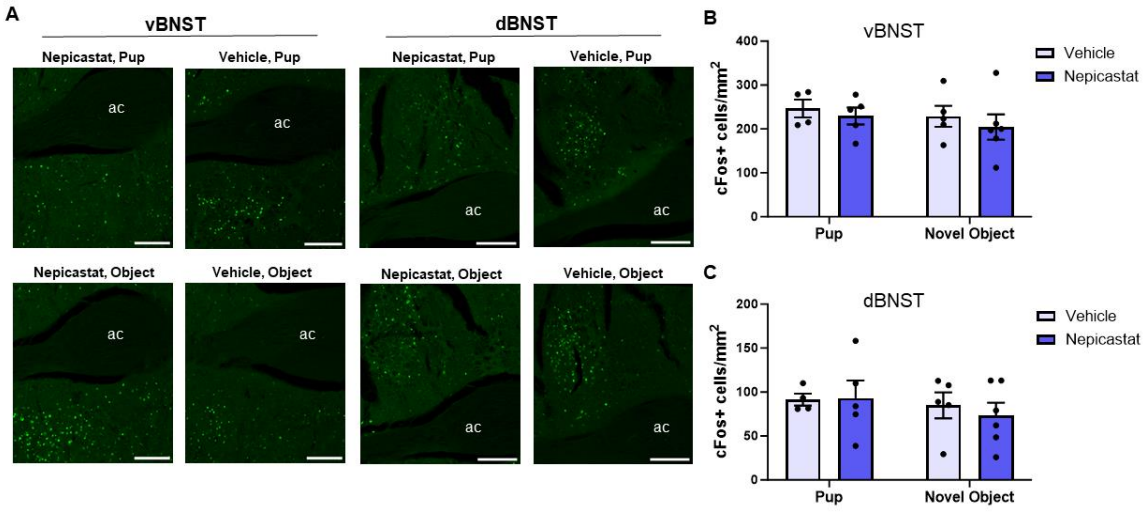

Figure 3.4. Effects of nepicastat on c-Fos expression in the bed nucleus of the stria terminalis (BNST). A. Representative photomicrographs of c-Fos immunoreactivity in the ventral BNST (vBNST, left) and dorsal BNST (dBNST, right) of nepicastat- and vehicle-treated mice following exposure to a pup or novel object. Scale bar, 200 µm. **B, C.** c-Fos density (mean ± SE) in the vBNST (B) and dBNST (C) following exposure to either a pup (n = 4 vehicle, 5 nepicastat) or a novel object (n = 5 vehicle, 6 nepicastat) was not influenced significantly by treatment. ac = anterior commissure.

Nepicastat treatment did not alter neural activation following novel-object exposure

No significant differences in c-Fos expression were found between nepicastat- and vehicle-treated males in any brain region examined (all $p > 0.3$) following the hour-long exposure to a novel, pup-sized pebble (Table 3.1).

Pup-directed behavior correlated with c-Fos immunoreactivity in the MPOA and amygdala

We examined whether pup-directed behavior correlated with c-Fos density in brain regions implicated in parental behavior and/or anxiety, combining data from nepicastat- and vehicle-treated mice. In the MPOA, c-Fos density correlated positively with total duration of parental behavior ($r [7] = 0.78$, $p = 0.020$; Table 3.2, Fig. 3.5A) and negatively with latency to initiate parental behavior ($r [7] = -0.78$, $p = 0.020$; Fig. 3.5B). In the BLA, we found a significant positive correlation between time spent sniffing the pup and c-Fos density ($r [7] = 0.71$, $p = 0.040$; Fig. 3.5C). In the MeA, c-Fos density correlated positively, but not significantly, with time spent sniffing the pup ($r [7] = 0.64$, $p = 0.071$; Fig. 3.5E). No significant correlations were found between c-Fos expression and latency to approach the pup, time spent sniffing the pup, latency to initiate parental behavior, or duration of parental behavior in the vBNST or dBNST of virgin males (Table 3.2).

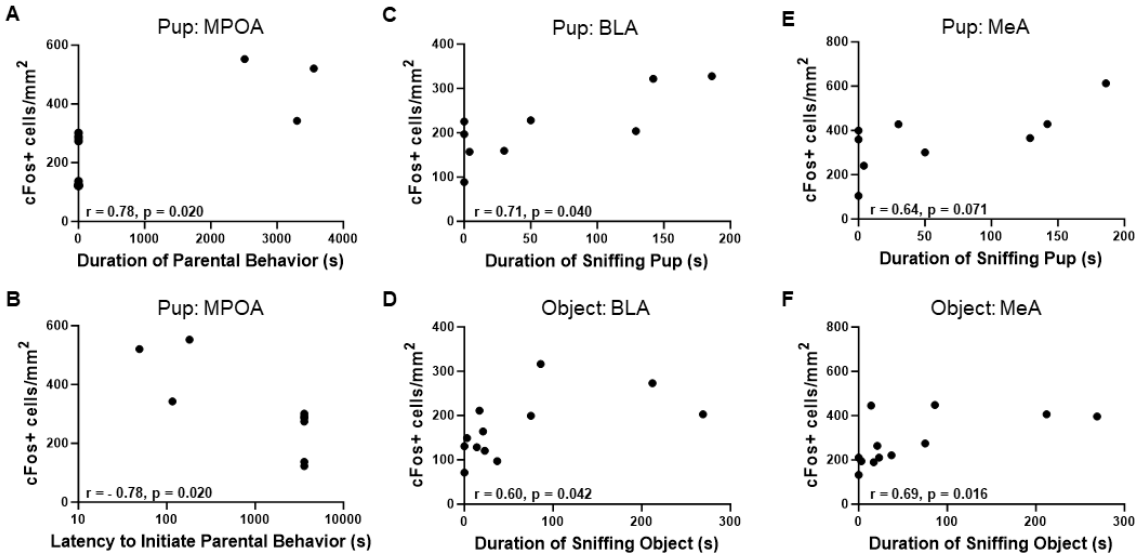

Figure. 3.5. Spearman correlations of c-Fos density and stimulus-directed behavior in adult virgin male California mice. **A, B**. Relationship between c-Fos density in the medial preoptic area (MPOA) and pup-directed behavior. **C, D**. Relationship between c-Fos density and pup- (C) or object- (D) directed behavior in the basolateral amygdala (BLA). **E**, **F**. Relationship between pup- (E) or object- (F) directed behavior and c-Fos density in the medial amygdala (MeA). No behavioral measures correlated significantly with c-Fos density in the bed nucleus of the stria terminalis.

Object-directed behavior correlated with c-Fos immunoreactivity in the amygdala

We evaluated relationships between behavioral and neural responses to the novel object, using data from nepicastat- and vehicle-treated mice combined. c-Fos density in both the BLA and the MeA showed significant positive correlations with time spent sniffing the object (BLA: r [10] = 0.60, p = 0.042; Table 3.2, Fig. 3.5D; MeA: (r [10] = 0.69, p = 0.016; Fig. 3.5F). No additional significant correlations between c-Fos expression and behavior were found in these regions or in the MPOA, vBNST, or dBNST of object-exposed virgin males (p > 0.1).

Discussion

The noradrenergic system facilitates the onset of maternal behavior in several mammalian species, but the role of norepinephrine in the initiation of paternal care is not well understood. A previous study in our lab found that systemic treatment with the dopamine β-hydroxylase inhibitor nepicastat inhibited interactions with experimentally presented, unrelated pups in adult virgin California mice of both sexes (Acosta et al., 2022). These findings prompted us to examine whether nepicastat administration influenced c-Fos immunoreactivity, a cellular marker of neural activity, in brain regions implicated in parental responsiveness and anxiety, as the initiation of parental behavior in rodents requires an increase in activation of brain regions that promote affiliative behavior toward infants as well as a concomitant reduction in activation of regions associated with stress responses and anxiety-like behavior (Numan, 2020). We found that virgin male mice treated with nepicastat exhibited significantly lower levels of neural activation in response to pups in the MeA and MPOA, compared to controls. In contrast, nepicastat did not alter neural activation in response to pups in the BLA, vBNST, or dBNST.

Previous lesion experiments and immunohistochemical studies characterizing c-Fos expression have implicated the amygdala in paternal responsiveness. In virgin male prairie voles (*Microtus ochrogaster)*, another monogamous, biparental rodent, MeA lesions reduce parental behavior, while interactions with pups increase c-Fos expression in the postero-dorsal division of the MeA (Kirkpatrick, Carter, et al., 1994; Kirkpatrick, Kim, et al., 1994; Northcutt & Lonstein, 2009). Moreover, the MeA has been implicated in the control of aggression in California mouse fathers, as fathers exhibit enhanced c-Fos expression following a resident-intruder test compared to virgin males (Trainor et al.,

2008). In the present study, systemic administration of nepicastat significantly decreased c-Fos expression in response to experimentally presented pups in the MeA of virgin male California mice, suggesting that NE facilitates neuronal response to pups in this region. Furthermore, the density of c-Fos-immunoreactive cells in the MeA was positively correlated with total time spent sniffing the pup. In house mice, the MeA influences both prosocial and aggressive responses to social stimuli in an activity-dependent manner, where low-intensity activation of GABAergic neurons promotes prosocial behaviors toward adult or infant stimuli while high-intensity activation of the same neurons promotes aggressive responses (P. B. Chen et al., 2019; Hong et al., 2014). The MeA receives substantial innervation by noradrenergic terminals from the brainstem (Moore & Bloom, 1979; Roder & Ciriello, 1993); therefore, it is possible that the noradrenergic system modulates MeA activity to promote positive or negative affective behavioral responses to pups in an activity-dependent manner.

In addition to the MeA, the MPOA and BNST have been implicated in the onset of parental behavior, as these regions regulate attraction toward pup stimuli (reviewed in Horrell et al., 2019; Numan, 2020). Previous findings have shown higher expression of c-Fos in the MPOA of parentally responsive virgin male house mice compared to unresponsive virgin males (Tsuneoka et al., 2015). Consistent with these findings, we found that c-Fos density in the MPOA was positively correlated with the total duration of parental behavior and negatively correlated with latency to initiate parental behavior in this study. Furthermore, nepicastat administration reduced neural activation following pup exposure in the MPOA, but not BNST, of virgin male California mice, suggesting that NE facilitates neuronal response to pups in the MPOA. However, the mechanisms by which NE signaling within the MPOA might promote the onset of pup-affiliative

behavior are not clear, as few studies have directly manipulated noradrenergic signaling within the core MPOA and vBNST parental-care circuitry to examine if NE can influence parental behaviors. In rats, certain components (retrieval and nursing) of postpartum maternal behavior are disrupted by pharmacologically blocking noradrenergic α2 receptor activity, thereby enhancing NE release in the MPOA and vBNST; however, pharmacological depletion of NE in the MPOA does not influence the initiation of maternal responsiveness in virgin females (Smith et al., 2012).

Limited evidence suggests that NE signaling in the MPOA and BNST changes during the transition to parenthood in female mammals, corresponding to the onset of parental behavior. NE release into the MPOA and BNST in sheep increases at parturition compared to NE levels 10 – 0 hours before birth (Kendrick et al., 1992). In rats, tissue content of NE in the MPOA is reduced in early postpartum dams (postpartum days 7 - 8) compared to virgins (Winokur et al., 2019). In contrast, house mouse mothers (postpartum day 16) exhibit elevated tissue levels of NE in the hypothalamus compared to virgins (Avraham et al., 2017). Notably, neither of these rodent studies examined NE content in the MPOA on the day of parturition, nor did they directly assess NE release within the MPOA; therefore, whether enhanced NE signaling in the MPOA occurs at the time of parturition and promotes the onset of pup-affiliative behavior in rodents has not been established. Furthermore, the spatiotemporal dynamics of NE release in the MPOA of male rodents during pup interactions and whether they might contribute to the onset of paternal behavior have not been examined. Given that noradrenergic signaling within the MPOA has been shown to enhance arousal (reviewed in España et al., 2016), NE signaling in the MPOA might affect parental behavior through effects on arousal and alertness in response to infant-related sensory cues. Moderate levels of arousal may

enhance attentiveness to these cues and promote caregiving behavior, whereas high arousal and anxiety might disrupt the onset of parental care.

The present study found that nepicastat treatment did not influence neural responses to a novel object in any brain region examined. These results are consistent with our previous finding that nepicastat did not alter the behavioral response to a novel object in virgin male California mice (Acosta et al., 2022). Noradrenergic signaling in the BLA has been implicated in object recognition memory (Barsegyan et al., 2014; Y.-F. Chen et al., 2022; Roozendaal et al., 2008); however, few studies have examined the role of NE signaling in the BLA in neophobia. Lustberg and colleagues (2020) found that noradrenergic signaling in the amygdala modulates the initial response to novelty, as the BLA exhibits reduced neural activation after exposure to a novel environment in DBH-knockout mice compared to control mice (Lustberg et al., 2020). In contrast, we tested mice with a pup-sized novel object in an environment similar to the animals' home environment; therefore, it is possible that the novel stimulus in our experiment was not as emotionally arousing as the novel environment in Lustberg et al.'s (2020) study. Notably, we found that the densities of c-Fos-expressing cells in the BLA and MeA were positively correlated with time spent investigating the object, suggesting that these regions may be involved in attending to novel stimuli. Thus, while noradrenergic signaling in the amygdala might not be involved in the regulation of neophobia in California mice, it is possible that increased activation of the amygdala may promote memory consolidation for different types of novel cues in this species.

In summary, our findings demonstrate that NE acting at the level of the MeA and MPOA may facilitate attention toward pup-related stimuli, as nepicastat administration reduced neural activation in these regions in response to pups. Future research

investigating the role of specific subtypes of NE receptors and the locations of the receptors along the parental-care circuit might reveal the mechanisms by which NE acts to promote the onset of pup-affiliative behavior in California mice.

Brain region	Pup			Object		
	Nepicastat (n=5)	Vehicle (n=4)	T-test results (t, p)	Nepicastat (n=6)	Vehicle (n=6)	T-test results (t, p)
MPOA	224.44 ± 38.82	427.04 ± 64.59	2.822, **0.026**	211.95 ± 28.04	245.93 ± 48.97	0.602, 0.561
MeA	281.24 ± 51.55	458.95 ± 53.44	2.371, **0.049**	250.24 ± 32.78	315.30 ± 55.13	1.014, 0.334
BLA	179.29 ± 26.01	253.18 ± 42.28	1.558, 0.163	166.16 ± 26.80	177.85 ± 34.14	0.270, 0.793
vBNST	229.59 ± 19.33	246.70 ± 20.10	0.608, 0.562	204.63 ± 28.63	229.05 ± 23.78	0.639, 0.539
dBNST	93.14 ± 19.88	91.39 ± 6.85	0.075, 0.942	73.60 ± 14.35	84.84 ± 14.81	0.542, 0.601

Table 3.1. Density (number per mm2; mean ± SE) of c-Fos-immunoreactive cells in all quantified brain areas of virgin male California mice after being tested with an unfamiliar pup or novel object (pup-sized pebble), following treatment with nepicastat (75 mg/kg, i.p.) or vehicle. p-values < 0.05 are in bold. MPOA = medial preoptic area, MeA = medial amygdala, BLA = basolateral amygdala, vBNST = ventral bed nucleus of the stria terminalis, dBNST = dorsal bed nucleus of the stria terminalis.

Region	Condition	N	Latency to approach stimulus	Time spent sniffing stimulus	Latency to initiate parental behavior	Time spent in parental behavior
MPOA	Pup	9	r = -0.593 p = 0.102	r = 0.559 p = 0.127	**r = -0.782** **p = 0.020**	**r = 0.782** **p = 0.020**
	Object	12	r = 0.476 p = 0.119	r = -0.252 p = 0.425	-	-
MeA	Pup	9	r = -0.458 p = 0.221	r = 0.644 p = 0.071	r = -0.426 p = 0.254	r = 0.426 p = 0.254
	Object	12	r = -0.298 p = 0.345	**r = 0.690** **p = 0.016**	-	-
BLA	Pup	9	r = -0.390 p = 0.302	**r = 0.712** **p = 0.040**	r = 0.009 p > 0.999	r = -0.009 p > 0.999
	Object	12	r = -0.136 p = 0.694	**r = 0.603** **p = 0.042**	-	-
vBNST	Pup	9	r = 0.187 p = 0.638	r = 0.322 p = 0.400	r = -0.069 p = 0.861)	r = 0.069 p = 0.861)
	Object	12	r = 0.455 p = 0.191	r = -0.164 p = 0.634	-	-
dBNST	Pup	9	r = 0.000 p > 0.999	r = 0.017 p = 0.982	r = 0.119 p = 0.766	r = -0.119 p = 0.766
	Object	12	r = 0.014 p = 0.973	r = 0.346 p = 0.294	-	-

Table 3.2. Spearman correlations between behavioral responses to a pup or novel object and c-Fos density in the brain regions of interest. p-values < 0.05 and the associated correlation coefficients are in bold; p-values between 0.05 and 0.1 and the corresponding correlation coefficients are underlined. MPOA = medial preoptic area, MeA = medial amygdala, BLA = basolateral amygdala, vBNST = ventral bed nucleus of the stria terminalis, dBNST = dorsal bed nucleus of the stria terminalis.

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Chapter 4

Sex-specific effects of experience with pups on perineuronal net expression in the biparental California mouse (*Peromyscus californicus*)

Abstract

In biparental species, in which both parents care for their offspring, repeated exposure to infants can induce parental behavior in virgin males and females. Little is known, however, about the mechanisms underlying neural plasticity resulting from experience with infants. Extracellular matrix assemblies called perineuronal nets (PNNs) are key structures that regulate neuroplasticity in adult rodents. Here, we examined the impact of pup exposure on PNN expression in the medial prefrontal cortex (mPFC), medial amygdala (MeA), and medial preoptic area (MPOA), brain regions implicated in the control of parental caregiving behavior, in male and female California mice (*Peromyscus californicus*), a biparental rodent. We stained brain sections with the PNN marker *Wisteria floribunda agglutinin* to examine PNN expression in adult virgin California mice exposed to pups 1, 2, or 3 times, as well as new parents. Control groups were similarly tested with a novel pup-sized object. We found that pup exposure altered PNN density in the mPFC and MeA, but not MPOA, of male mice. Virgin males exposed to pups twice exhibited reduced PNN density in the mPFC compared to virgin males exposed to pups only once. Moreover, pup-exposed fathers demonstrated a reduction in PNN density in the MeA compared to virgin males exposed to pups for the first time. In contrast, pup exposure did not alter PNN density in the mPFC, MeA, or MPOA of females. Additionally, PNN density in the mPFC was higher in males than in females. Finally, PNNs preferentially associated with GABAergic cells in the mPFC of male mice,

whereas in the MeA, PNNs associated with a similar percentage of GABAergic and CAMKII+ neurons. Our results suggest that dynamic changes in PNN expression in the mPFC and MeA occur with repeated exposure to pup stimuli in male California mice and might contribute to the onset of paternal behavior.

Introduction

In mammals, maternal care is obligatory for offspring survival, and the onset of female parenting is associated with structural and functional changes in the brain (Kinsley & Lambert, 2008; Leuner & Sabihi, 2016; Medina & Workman, 2020). Paternal care is rare in mammals, with fathers providing some form of care for their offspring in only ~5–10% of species (Kleiman & Malcolm, 1981). Males in these biparental species often exhibit marked changes in their behavioral response to infants as they transition into fatherhood: whereas reproductively naïve males may exhibit avoidance, aggression, or affiliative behavior toward experimentally presented infants, most fathers are attracted to and nurturant toward their own offspring as well as unfamiliar infants (de Jong et al., 2009; Duclot et al., 2022; Saltzman et al., 2017; Song et al., 2010). This change in behavioral responses to pups is accompanied by plasticity in neural circuits that mediate paternal behavior (reviewed in Horrell et al., 2021; Wilson et al., 2023). However, the molecular and cellular mechanisms underlying neuroplasticity in the onset of parental behavior in males have not been elucidated.

The California mouse (*Peromyscus californicus*) is a rodent species that is monogamous and biparental in both the field and captivity (Dudley, 1974; Gubernick & Alberts, 1987; Gubernick & Teferi, 2000; Ribble & Salvioni, 1990). Fathers spend as much time as mothers caring for offspring (e.g., huddling, grooming, and retrieving pups)

(Gubernick & Alberts, 1987; A. W. Lee & Brown, 2002). Under experimental conditions, fathers are typically attracted to and will care for both their own offspring and unrelated pups, while adult virgin males may either avoid, attack, or care for foreign pups (Chauke et al., 2012; de Jong et al., 2009; Gubernick & Addington, 1994; Gubernick & Alberts, 1987; Horrell et al., 2017). Behavioral sensitization to pups can occur in male California mice, as repeated exposure to pups across several days facilitates the onset of pup-affiliative behavior in virgin males (Horrell et al., 2017). However, the mechanisms that contribute to behavioral sensitization to pups in adult California mice are not known.

Increasing evidence supports the hypothesis that specialized assemblies of the extracellular matrix, perineuronal nets (PNNs), regulate neural plasticity. PNNs are pericellular aggregates of extracellular matrix that surround cell bodies and proximal dendrites of neurons in the central nervous system (Celio & Blumcke, 1994; Celio et al., 1998). PNNs are highly organized, lattice-like structures with holes allowing axonal boutons to synapse onto the underlying neurons (Wang & Fawcett, 2012). They are prevalent throughout the mammalian central nervous system; however, the majority of studies have focused on cortical PNNs, which preferentially envelop parvalbumin-positive (PV+), fast-spiking inhibitory interneurons (Härtig et al., 1992). In contrast, PNNs in subcortical regions seem to surround both excitatory and inhibitory neurons (Carstens et al., 2016; Horii-Hayashi et al., 2015; Mészár et al., 2012). PNNs have been observed in multiple brain regions in rodents; however, the expression patterns and plasticity of PNNs within brain regions implicated in motivation, reward, and reinforcement are only beginning to be understood. High levels of PNN expression are present throughout the striatum and limbic system, including the amygdala, hippocampus, hypothalamus, ventral pallidum, and prefrontal cortex (Bertolotto et al., 1996; Carstens et al., 2016;

Horii-Hayashi et al., 2015; Mészár et al., 2012; Seeger et al., 1994; Uriarte et al., 2020). Such high PNN expression across the circuitry mediating reward, reinforcement, and motivation suggests that PNNs may influence neuroplasticity in brain circuits underlying social behaviors, including parental behavior.

PNNs play an important role in the neural plasticity associated with critical periods of development (Carulli et al., 2010; Pizzorusso et al., 2002). In addition, PNNs can be dynamically regulated in adulthood. Manipulations to remove PNNs in the adult central nervous system can reinstate juvenile-like states of plasticity, promote learning capabilities, and enhance cognitive flexibility (Geissler et al., 2013; Gogolla et al., 2009; Happel et al., 2014; Hirono et al., 2018; Pizzorusso et al., 2002; Romberg et al., 2013). Additionally, studies that have depleted PNNs in the cortex or amygdala have shown that they play a role in the maintenance of both auditory and visual fear memories, as well as addiction-related memories (Banerjee et al., 2017; Thompson et al., 2018; Xue et al., 2014), suggesting PNNs may provide stability to brain networks, which may be essential for the formation and recall of long-term memories.

Recent studies in rodents have found dynamic changes in PNN expression associated with reproductive behavior. In a house mouse (*Mus musculus*) model of Rett syndrome, an autism spectrum disorder, female mice with the mutation for Rett syndrome show precocious formation of PNNs, which leads to early onset and closure of the critical period, and these mutant virgin mice have impairments in maternal pup-gathering behavior (Krishnan et al., 2017). However, degradation of PNNs with the enzyme chondroitinase ABC (ChABC) corrects this impairment (Krishnan et al., 2017). Additionally, PNN density in female rats (*Rattus norvegicus*) changes across pregnancy in the medial preoptic area (MPOA), a key brain region implicated in parental behavior.

While virgin females do not exhibit any changes in PNN expression in the MPOA across the estrous cycle, PNN expression in this region increases during gestation in rat dams and peaks shortly before parturition (Uriarte et al., 2020). PNN expression in the MPOA subsequently fades during the lactation period. Furthermore, simulating the hormonal changes of pregnancy in ovariectomized virgin female rats via systemically administering estrogen and progesterone induces PNN expression in the MPOA (Uriarte et al., 2020). Finally, Lau et al. (2020) found that PNN density was altered in primary somatosensory cortex following experience with pups in virgin female mice, in a region- and hemisphere-specific manner. Together, these findings suggest that hormonal changes experienced by new mothers and/or interactions with pups may facilitate neural plasticity through modulation of PNNs. Whether or not PNNs contribute to neuroplasticity of the parental brain during the transition to parenthood in a biparental species, or in males of any species, has not been explored (Horrell et al., 2021).

Given the role of PNNs in plasticity related to experience-dependent learning, it is possible that PNNs may regulate plasticity resulting from experience with pups in males of a biparental species. In the present study, therefore, we investigated the effects of pup experience on PNN expression in virgin male and female California mice. We predicted that PNN density in brain regions that play a key role in parental behavior (MPOA, medial amygdala [MeA], and medial prefrontal cortex [mPFC]; reviewed in Horrell et al., 2019; Numan, 2020) would decrease during initial exposure to pups, when pup-directed behavior is highly variable, thereby facilitating synaptic reorganization and behavioral modifications. We also examined whether PNNs preferentially associate with GABAergic or glutamatergic cells in the cortex (mPFC) and subcortically (MeA) in the

California mouse to determine if cell-type-specific associations differ from those previously described in rats and house mice.

Methods

Animals

California mice aged 161-226 days (i.e., approximately 5.5 - 7.5 months) were used in this study (n = 48 males and 48 females). Mice were bred at the University of California, Riverside (UCR) and housed and maintained as described previously (Acosta et al., 2022). The breeders were descendants of mice from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). Mice were housed in 44 × 24 × 20 cm polycarbonate cages with aspen shavings for bedding and cotton wool for nesting material and with Purina Rodent Chow 5001 (LabDiet, Richmond, IN, USA) and water available *ad libitum*. Humidity was approximately 60–70%, temperature was maintained at 21 ± 1 °C, and lights were on a 14:10 light: dark cycle (lights on from 2300 h to 1300 h).

Juveniles were weaned from their parents at 27-32 days of age and housed in same-sex groups of 3-4 age-matched individuals in a colony room containing only virgin mice, to prevent premature exposure to stimuli from pups. Two weeks before testing, virgin mice were pair-housed with an unrelated, age-matched same-sex mouse from their original same-sex group, and pairs were randomly assigned to experimental groups.

All experimental procedures were approved by UCR's Institutional Animal Care and Use Committee and conform to the *Guide for the Care and Use of Laboratory*

Animals. UCR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Experimental Design

Each virgin mouse was assigned to one of six experimental groups and underwent data collection over a 1- to 5-day period using a design modified from Horrell et al. (2017) (Fig. 4.1A). Groups differed in the stimulus with which they were tested and the number of exposures to the stimulus. Half of the virgin males and half of the virgin females underwent a 60-minute test with an unrelated, 2- to 5-day-old pup; the other half were tested identically with a novel object (pup-sized pebble). Two hours after the beginning of the 60-minute test with a pup or novel object, the mouse was perfused and its brain collected (see below).

Mice in the Pup-1 and Object-1 groups underwent a single 60-minute test with the pup or object, respectively (Fig. 4.1A). Mice in the Pup-2 and Object-2 groups underwent one 20-minute exposure to the pup or object, respectively, 2 days before their 60-minute test with the same stimulus and subsequent brain collection. Mice in the Pup-3 and Object-3 groups underwent 20-minute exposures to the pup or pebble, respectively, both 2 days and 4 days before their 60-minute test with the same stimulus. Within each pair of same-sex virgins, the two mice underwent the same number of stimulus exposures, but one was tested with a pup and the other with a novel object. We used first-time fathers and mothers, housed as breeding pairs with their first litter of pups, as positive controls. Five to 7 days after the birth of their first litter, breeders were tested for 60 min with an unrelated pup (Breeder-Pup) or novel object (Breeder-Object), followed by perfusion and brain collection.

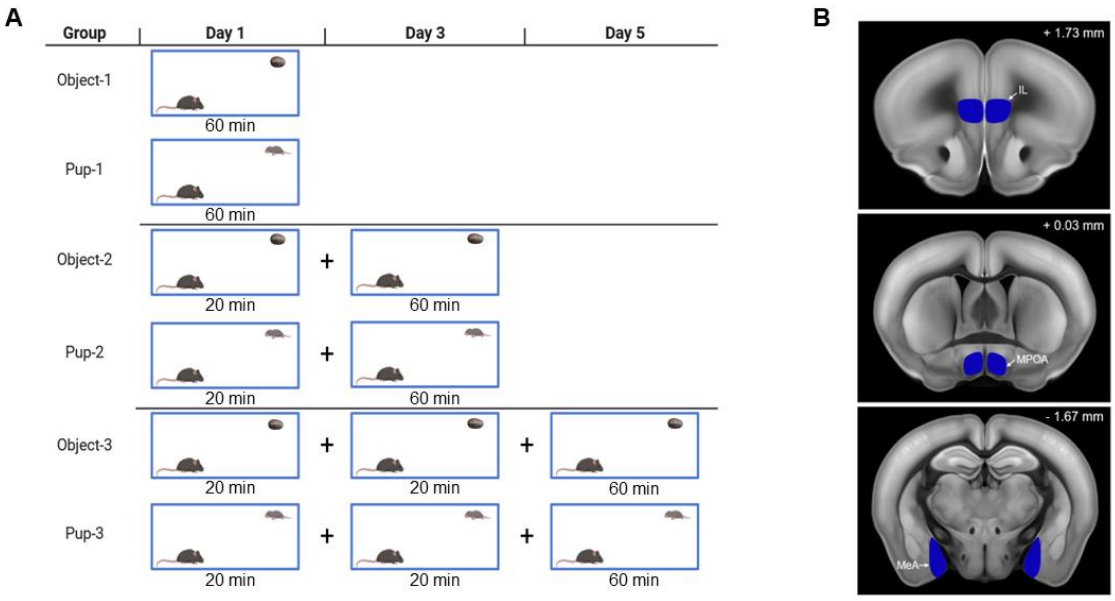

Figure 4.1. Schematic of the experimental design. A. Virgin mice were exposed 1, 2, or 3 times to either an unrelated pup or a novel pup-sized pebble in a clean cage. Two hours after the start of the final stimulus presentation, mice were euthanized and perfused transcardially, and their brains were removed. Not pictured is a group of first-time parents that were tested once with a novel object or unrelated pup as a positive control. N = 6 males and 6 females per group. **B.** Schematic representation of the brain regions (blue shaded areas) in which the density of WFA+ cells was measured. Brain schematics were drawn with the aid of the Scalable Brain Atlas (Bakker et al., 2015), and regions were manually outlined according to Paxinos and Franklin (2012). The coordinates represent the distance relative to Bregma of the rostral face of each section. IL = infralimbic medial prefrontal cortex, MPOA = medial preoptic area, MeA = medial amygdala.

Pup and Novel-object Exposures and Tests

For each 20-min stimulus exposure or 60-min test, virgin same-sex cage mates or first-time parents were removed from their home cage between 8:00 h and 11:00 h and placed individually in clean cages, identical to the home cages, containing aspen shavings, food, and water. Animals were tested in new cages to allow testing of both cage mates around the same time under identical conditions. The cage was placed on an isolated shelf in a colony room housing only virgin mice. After a 10-min habituation

period, an unfamiliar, unrelated, 2- to 5-day-old pup or a pup-sized pebble was introduced into the corner of the cage farthest from the subject for 20 (stimulus exposure) or 60 (stimulus test) minutes. If a subject attacked a pup, the exposure or test was immediately concluded and the pup was euthanized with pentobarbital (Fatal-Plus solution, Vortech Pharmaceuticals, Dearborn, MI, USA). These pup-aggressive mice then remained alone in the test cage and were either returned to their home cage 20 min after the start of the pup presentation (pup exposures) or perfused 2 h after the start of pup presentation (pup tests). Some stimulus pups were used for multiple tests but were used in no more than one test per day; subjects were never tested with the same pup more than once.

To examine the relationship between density of PNNs and pup-directed behavior, all pup tests were videotaped and subsequently scored by a single observer blind to the experimental condition using Behavioral Observation Research Interactive Software (BORIS, Friard & Gamba, 2016). The parameters scored were latency to approach the pup, latency to initiate parental behavior (grooming and/or huddling the pup), total time spent sniffing the pup, and total time spent in parental behavior. For novel-object tests, behaviors scored from videotapes were latency to approach the object and total time spent sniffing the object (Acosta et al., 2022; Nguyen et al., 2020).

Histology

Two hours after the beginning of the 60-minute test with a pup or novel object, mice were anesthetized with sodium pentobarbital (Fatal-Plus solution, Vortech Pharmaceuticals, Dearborn, MI, USA) and perfused intracardially with cold phosphate-buffered saline (PBS, 0.1 M, pH 7.4) and 4% paraformaldehyde (PFA). Brains were

removed and stored overnight in 4% PFA at 4°C and then immersed in a 30% sucrose solution for 24-48 h. The brains were stored in a freezing solution (30% ethylene glycol, 1% polyvinylpyrrolidone-40, 30% sucrose in 0.1M PBS) at -20°C until further processing. Brains were sectioned coronally at 40 µm thickness using a cryostat (Leica Biosystems, Deer Park, IL, USA; CM 1860). For each brain, an average of 8-10 slices of each region of interest was obtained for histochemistry and stored at 4°C in 0.1M PBS with 0.05% sodium azide. For each animal, 3–4 slices containing the infralimbic mPFC (+1.73 mm from bregma), MPOA (+0.03 mm from bregma) and MeA (−1.67 mm from bregma), respectively, were processed for each batch of immunohistochemistry (Fig. 4.1B). Brain regions selected for quantification were identified by cross-referencing a standard mouse brain atlas (Franklin and Paxinos, 2012) and images of Nissl-stained California mouse sections (http://brainmaps.org). Within each sex, each of the 8 exposure x stimulus treatment groups (Pup-1, Pup-2, Pup 3, Object-1, Object-2, Object-3, Breeder-Pup, and Breeder-Object) was represented in every batch of histology.

To visualize PNNs, we used fluorescent lectin staining procedures with fluorescein-labeled *Wisteria floribunda* agglutinin (WFA; 1:500; Vector Laboratories, Burlingame, CA, USA, Cat# FL135). WFA binds to the N-acetylgalactosamine residues of the PNN hyaluronan backbone and is a widely established marker for PNNs (Härtig et al., 1992). In this study, therefore, the term ‘PNNs’ refers specifically to WFA-positive (WFA+) PNNs. Free-floating sections were washed in 0.1M PBS (3 × 5 min), quenched in 50mM ammonium chloride in 0.1M PBS for 15 min, washed in 0.1M PBS (3 × 5 min), incubated with WFA overnight at 4°C, washed in 0.1M PBS (3 × 5 min), mounted onto slides, and coverslipped using EMS Shield Mount with DABCO mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA).

To determine whether PNNs preferentially associate with GABAergic or glutamatergic cells in the cortex (mPFC) and subcortically (MeA), we performed co-localization studies with the GABAergic neuron marker glutamic acid decarboxylase 67 (GAD67) and the glutamatergic neuron marker Ca^{2+}/calmodulin kinase II alpha (CaMKII-*α*). We did not perform co-localization studies in the MPOA because we did not observe group differences in PNN expression in this region (see Results) and because PNN density was low in the MPOA. For co-localization of WFA and CaMKII-*α*, free-floating 40 µm sections were washed in 0.1M PBS (3 × 10 min), quenched in 50mM ammonium chloride for 15 min, and washed again in 0.1M PBS (3 × 10 min). The slices were incubated in a blocking solution with 3% bovine serum albumin (BSA, Fisher Scientific, Waltham, MA, USA) and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 6 h at room temperature and then incubated overnight at 4°C with the primary antibody against CaMKII-*α* (1:1500; Enzo Life Sciences, Farmingdale, NY, USA, Cat# ADI-KAM-CA002) in the blocking solution. The day following incubation with primary antibody, sections were washed in 0.1M PBS (4 × 10 min) and incubated with goat anti-mouse Alexa Fluor 555 secondary antibody (1:500; Invitrogen, Waltham, MA, USA, Cat# A28180) in blocking solution for 2 h at room temperature. Sections were then washed in 0.1M PBS (3 × 10 min), incubated with WFA (1:500) overnight at 4°C, washed in 0.1M PBS (3 × 10 min), mounted onto slides, and coverslipped.

We processed another subset of mPFC and MeA slices for WFA staining and GAD67 immunoreactivity. Free-floating sections were washed in 0.1M PBS (3 × 10 min), quenched in 50mM ammonium chloride for 15 min, washed in PBS (3 × 10 min), and incubated in a blocking solution (3% BSA, 5% normal goat serum, and 0.1% Triton X-100) overnight at 4°C. The next day, tissue samples were rinsed with 0.1M PBS

(1 × 5 min) and then incubated with mouse anti-GAD67 primary antibody (1:750; MillaporeSigma, Burlington, MA, USA, Cat# MAB5406) in blocking solution for 48 h at 4°C. After primary incubation, sections were washed in 0.1M PBS (4 × 10 min) and then allowed to incubate with goat anti-mouse Alexa Fluor 555 secondary antibody (1:500; Invitrogen, Waltham, MA, USA, Cat# A28180) in blocking solution for 4 h at room temperature. Next, sections were washed with 0.1M PBS (4 × 10 min) and incubated with WFA (1:500) overnight at 4°C. On the final day, sections were washed in 0.1M PBS (3 × 10 min), incubated with the nuclear stain NucBlue (Invitrogen, Carlsbad, CA, USA, Cat# R37606) for 5 min at room temperature, washed three times in 0.1M PBS, mounted onto slides, and coverslipped.

Imaging and Quantification

An inverted Zeiss LSM 880 confocal microscope (Carl Zeiss Microscopy, White Plains, NY, USA) equipped with Airyscan Fast was used for imaging. Imaging of WFA+ PNNs in the mPFC, MeA, and MPOA was performed at 10×, and co-expression of WFA around GAD67+ or CaMKII-*α*+ neurons in the mPFC and MeA was performed at 20×. Imaged z-stacks covering 30 µm (1 µm step size) were acquired. All images were captured under identical conditions. Each z-stack was imported into ImageJ software (NIH), collapsed into a single image by projection, and then converted to a TIFF file.

Pipsqueak Pro™ (https://rewire.ai/pipsqueak-pro/) software from Rewire AI (Portland, OR, USA) was used to identify and quantify the number of PNN cells, PNN/GAD67 colocalization, and PNN/CAMKII-*α* colocalization. A consistent background threshold was set and applied to all images for comparison. Pipsqueak was run in semi-automatic mode to select ROIs to identify PNNs and individual GAD67+ or CAMKII-*α* +

cells, which were then verified by a trained scorer who was blinded to the condition during cell quantification. Stained cells were counted bilaterally for each brain region. Data for each region for each animal were averaged from 3-4 sections, and these averages were compared across groups.

Statistical Analyses

Data were tested for assumptions of normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. WFA density data were normally distributed; thus, they were compared among groups within each sex and stimulus treatment (pup, novel object) by one-way ANOVAs, with Bonferroni multiple-comparison tests performed following significant ($p < 0.05$) ANOVAs. Two-way ANOVAs were performed to examine sex differences in PNN density in pup- and object-exposed mice, with sex and stimulus or sex and exposure as between-subject factors. ANOVA data are presented as mean ± SE.

Correlations between WFA expression and behavior across stimulus exposure conditions were analyzed separately within each stimulus condition. Using the Shapiro-Wilk test, the behavioral data were determined to be non-normal, so Spearman's correlation coefficient was used. All data were analyzed using GraphPad Prism version 10.0.0 for Windows (San Diego, CA, USA) with alpha set at 0.05 (2-tailed).

Results

Pup exposure altered PNN density in the mPFC and MeA of male, but not female, California mice.

To investigate the influence of pup exposure on PNN expression, we analyzed WFA+ PNN density in the mPFC, MeA, and MPOA of male and female mice with different amounts of pup experience (Pup-1, Pup-2, Pup-3, and Parents).

Males

In the infralimbic (IL) mPFC of males, a one-way ANOVA revealed a statistically significant difference in PNN expression between pup-exposure groups ($F [3, 20] = 3.12$, $p = 0.049$, $\eta 2 = 0.32$). Post hoc analyses indicated that WFA+ PNN density was significantly lower in the mPFC of virgin males that were exposed to pups twice (Pup-2) than virgin males exposed to pups only once (Pup-1; Fig. 4.2A and 4.2B). PNN expression in the mPFC did not differ significantly between any other groups of males ($p > 0.05$).

In the MeA, we found a statistically significant difference in WFA expression between pup-exposure groups in males ($F [3,19] = 4.30$, $p = 0.018$, $\eta 2 = 0.40$). Post hoc analyses indicated that WFA+ PNN density in the MeA was significantly lower in first-time fathers than in virgin males exposed to pups only once (Fig. 4.3A and 4.3B). No other pairwise differences were significant ($p > 0.05$).

Overall, we found a low amount of WFA staining in the MPOA of pup-exposed virgin male California mice. No significant differences in PNN expression were found between the different pup-exposure groups in this brain region (all $p > 0.05$; Fig. 4.4A and 4.4B).

Females

In females, one-way ANOVAs found no differences between pup-exposure groups for WFA+ PNN density in the IL mPFC, MeA, or MPOA (all $p > 0.11$; Fig. 4.2A and 4.2C; Fig. 4.3A and 4.3C; Fig. 4.4A and 4.4C). Thus, pup exposure changes PNN density in the IL mPFC and MeA of male but not female California mice.

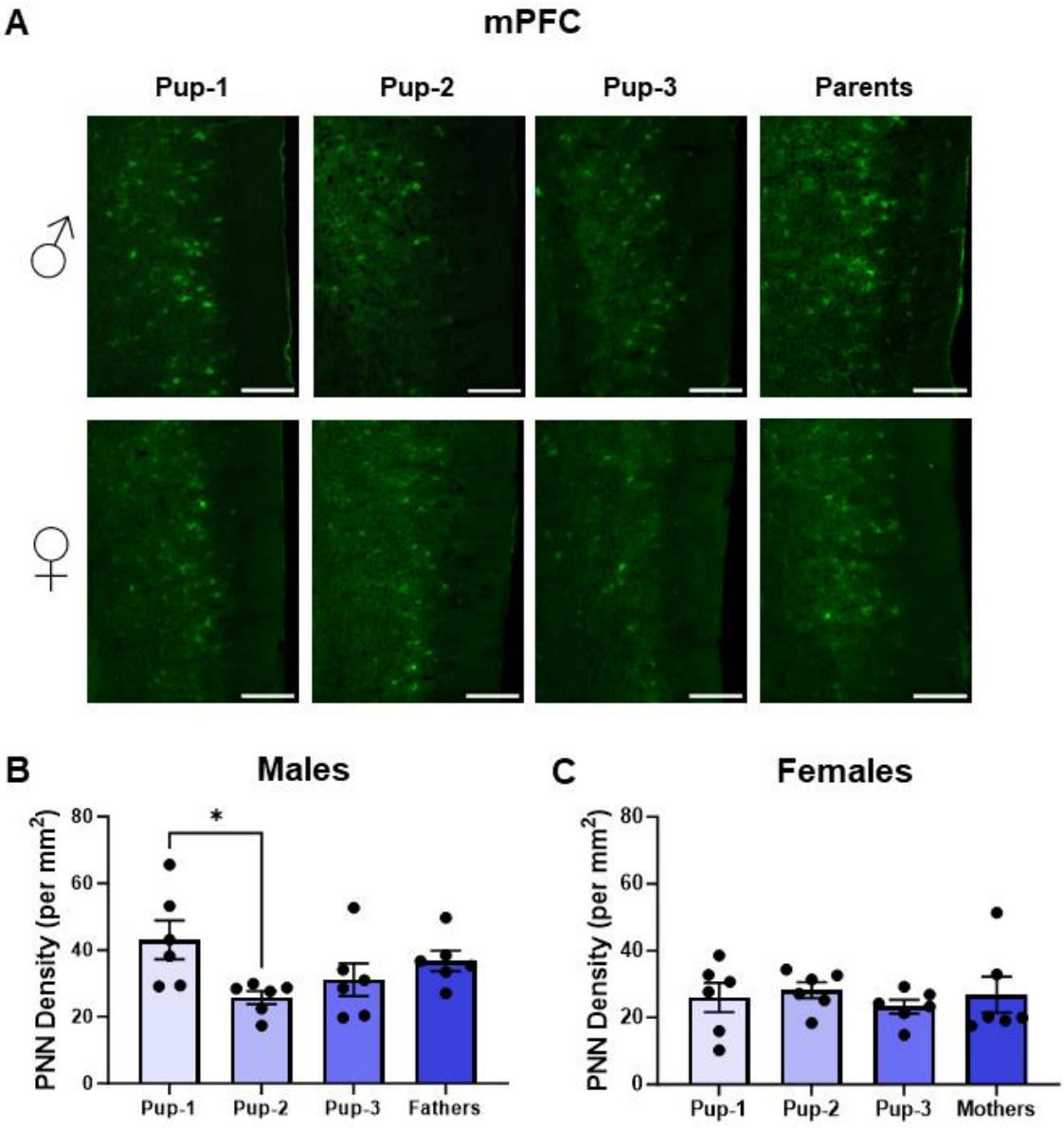

Figure 4.2. WFA expression (mean ± SEM) in the medial prefrontal cortex (mPFC) of pup-exposed adult male and female California mice. **A**. Representative photomicrographs of WFA histochemistry showing WFA+ PNNs in the infralimbic mPFC of male (top) and female (bottom) mice following 60-minute pup tests. Scale bar = 200 µm. **B.** In comparison to virgin males exposed to pups once, virgin males exposed to pups twice had significantly reduced WFA+ PNN density in the mPFC (n = 6 per group). **C.** WFA expression in the mPFC did not differ significantly between pup exposure groups in females (n = 6 per group). * indicates $p < 0.05$.

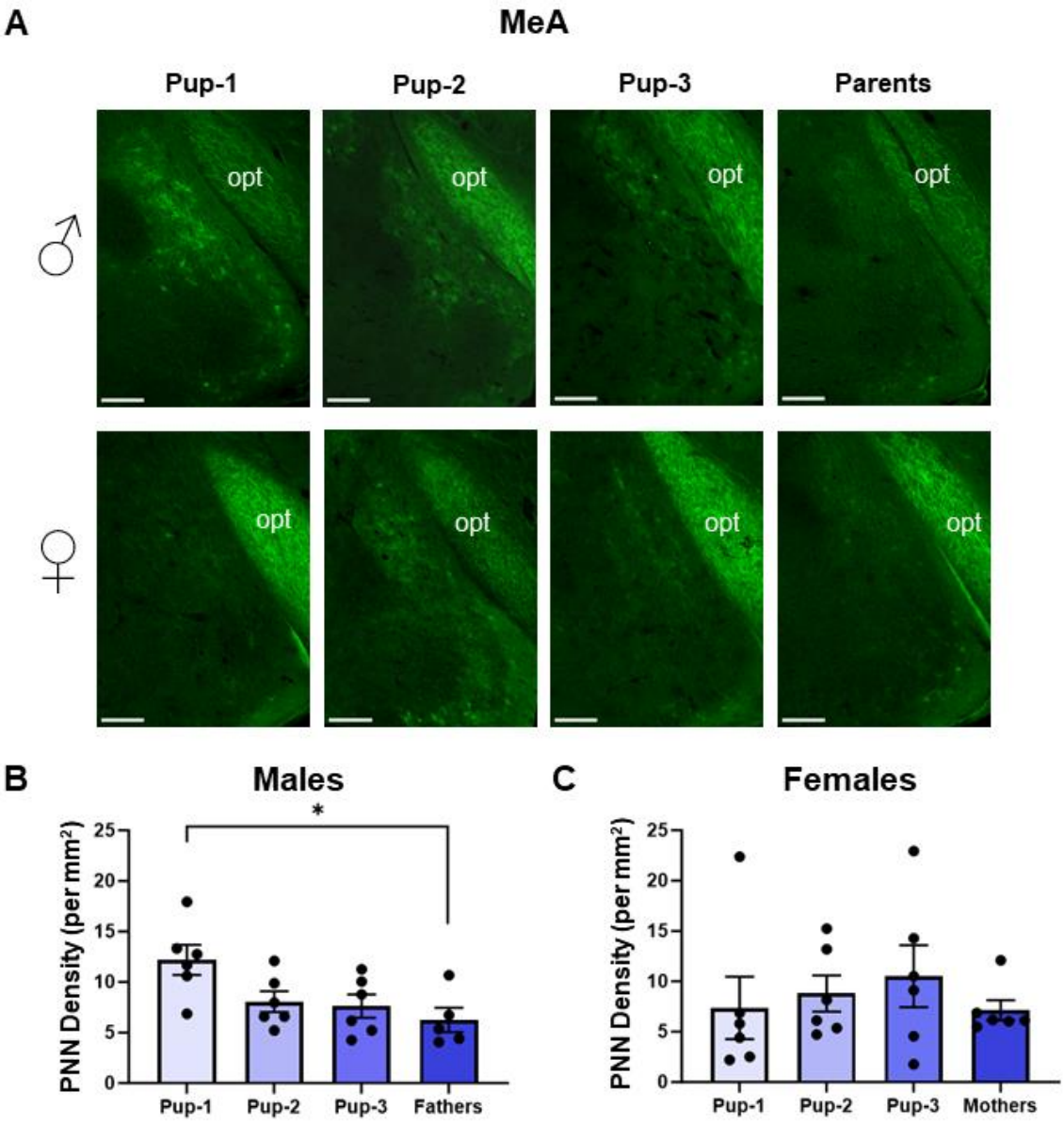

Figure 4.3. WFA expression (mean ± SEM) in the medial amygdala (MeA) of pup-exposed adult male and female California mice. A. Representative photomicrographs of WFA histochemistry showing WFA+ PNNs in the MeA of male (top) and female (bottom) mice following a 60-minute pup test. Scale bar = 200 µm. **B.** In comparison to virgin males exposed to pups once, fathers had significantly reduced WFA+ PNN density in the MeA (n = 6 per group). **C.** WFA expression in the MeA did not differ significantly between pup-exposure groups in females (n = 6 per group). * indicates $p < 0.05$. opt = optic tract.

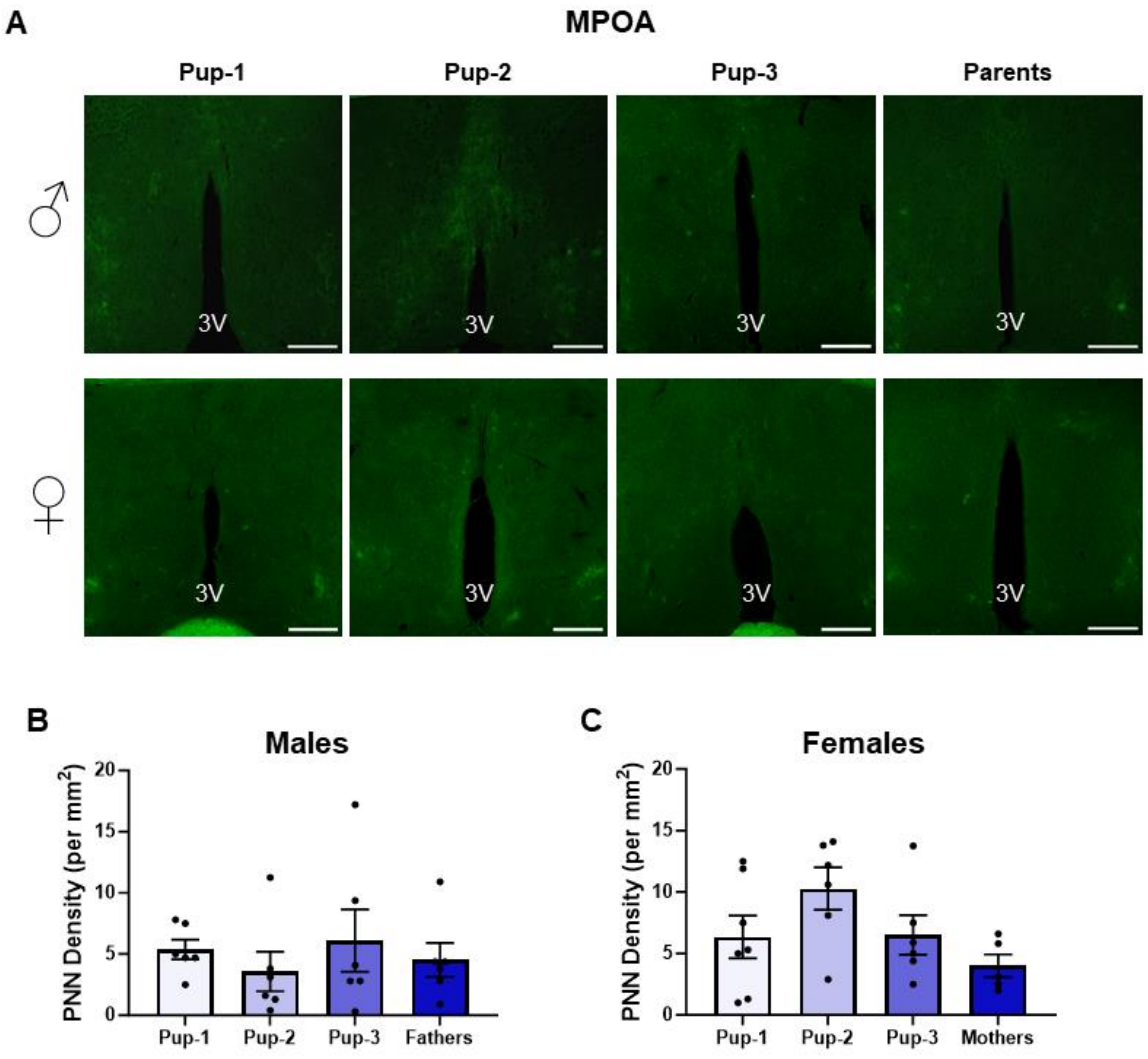

Figure 4.4. WFA expression (mean ± SEM) in the medial preoptic area (MPOA) of pup-exposed adult male and female California mice. **A.** Representative photomicrographs of WFA histochemistry showing WFA+ PNNs in the MPOA of male (top) and female (bottom) mice following a 60-minute pup test. Scale bar = 250 µm. **B, C.** WFA expression in the MPOA did not differ significantly between pup-exposure groups in males (B) or females (C; n = 6 per group). 3V = third ventricle.

Novel-object exposure did not alter PNN density in the mPFC, MeA, or MPOA of male or female mice.

To control for novelty, we also examined the effects of repeated exposure to a novel object (pup-sized pebble) on WFA+ PNN density in the mPFC, MeA, and MPOA of male and female California mice (Object-1, Object-2, Object-3, and Parents). Neither males nor females, showed any significant differences in WFA+ PNN density between object-exposure groups in the mPFC, MeA, and MPOA (all $p > 0.38$; Fig. 4.5).

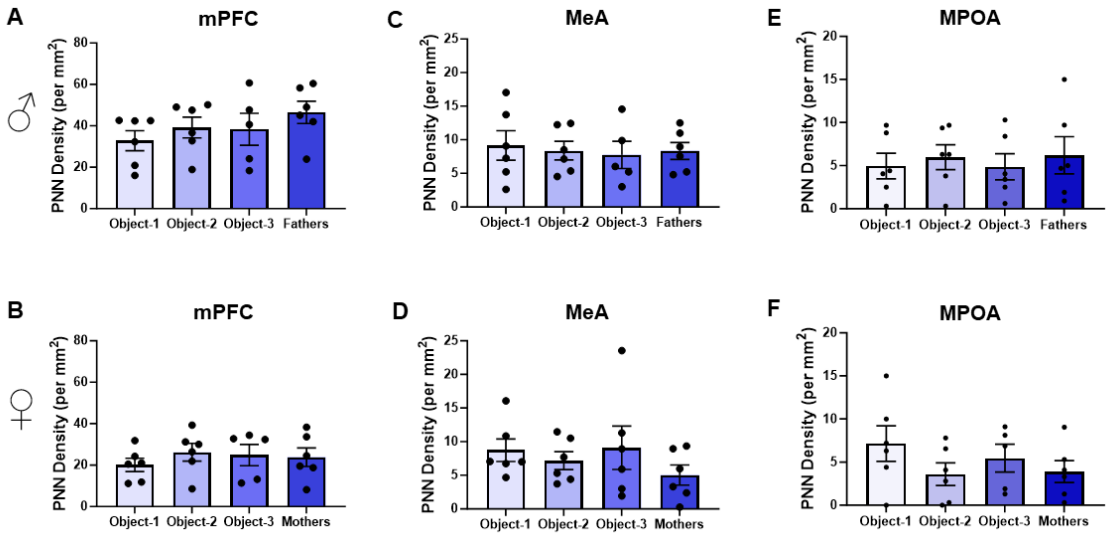

Figure 4.5. WFA expression (mean ± SEM) in the medial prefrontal cortex (mPFC), medial amygdala (MeA), and medial preoptic area (MPOA) of object-exposed adult male and female California mice. **A, B**. WFA expression in the mPFC did not differ significantly between object-exposure groups in males (A) or females (B). **C, D**. WFA expression in the MeA did not differ significantly between object exposure groups in males (C) or females (D; n = 6 per group). **E, F.** WFA expression in the MPOA did not differ significantly between object exposure groups in males (E) males or females (F; n = 6 per group).

Sex differences in PNN density were observed in the mPFC, but not MeA or MPOA, of pup- and object-exposed mice.

Next, we examined possible sex differences in WFA expression using two-way (sex x stimulus) ANOVAs. In the IL mPFC, a two-way ANOVA found a significant main effect of sex ($F [1,90] = 26.42$, $p = < 0.0001$, $\eta 2 = 0.22$), where males had significantly higher WFA expression than females ($p < 0.05$; Fig. 4.6B). WFA in the IL mPFC did not show a significant main effect of stimulus or a sex x stimulus interaction. WFA expression in the MeA and MPOA did not show any significant main effects of sex or stimulus or sex x stimulus interactions (Fig. 4.6C and 4.6D).

To determine whether sex differences in the IL mPFC were consistent across exposure conditions, two-way ANOVAs were conducted with sex and exposure as between-subject factors. Both pup- and object-exposed mice showed a significant main effect of sex in the IL mPFC ($F [1,40] = 8.07$, $p = 0.007$, $\eta 2 = 0.14$ for pup; $F [1,38] = 18.84$, $p = 0.001$, $\eta 2 = 0.31$ for object), with males having higher WFA+ PNN density than females. WFA in the IL mPFC did not show a significant main effect of exposure or a sex x exposure interaction. Overall, the data suggests male mice exhibit higher WFA+ PNN density in the IL mPFC compared to female mice.

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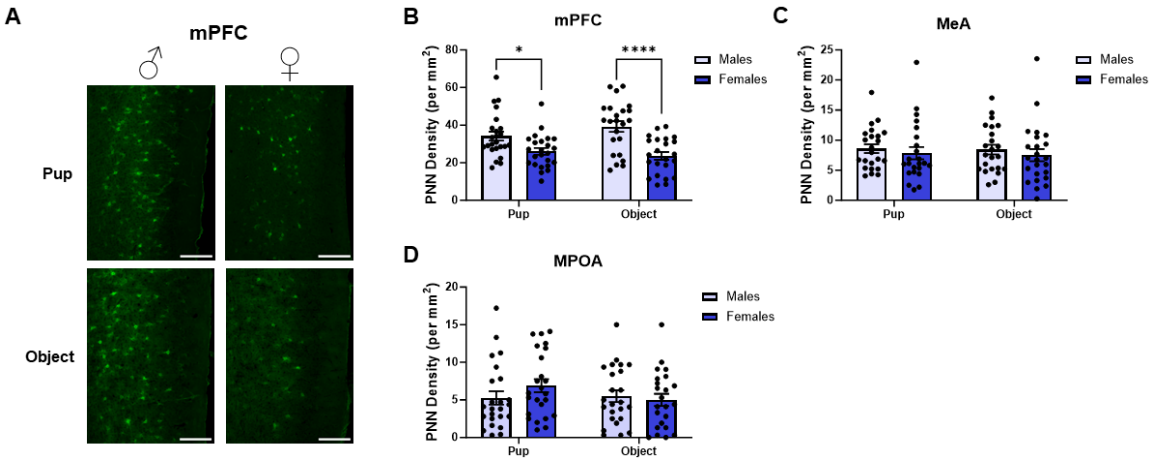

Figure 4.6. Sex differences in WFA expression (mean ± SEM). A. Representative photomicrographs showing WFA+ PNNs in the infralimbic medial prefrontal cortex (mPFC) of male (left) and female (right) mice following a 60-minute test with a pup (top) or object (bottom). Scale bar = 200 µm**.** B. WFA+ PNN density was significantly higher in the mPFC of males compared to females (n = 24 per group). **C, D**. Sex differences in PNN density were not observed in the medial amygdala (MeA; C) or medial preoptic area (MPOA; D) (n = 24 per group). (*) and (****) indicate $p < 0.05$, and $p < 0.0001$ compared with the opposite sex, respectively.

Pup-directed behavior correlated with WFA expression in the mPFC and MeA of virgin males.

Given that experience with pups influenced WFA expression in male mice, we examined whether WFA expression correlated with pup-directed behavior in males, analyzing mice from each exposure condition separately. In males exposed to a pup once, WFA+ PNN density in the mPFC correlated negatively with time spent sniffing the pup ($r [6] = -0.88$, $p = 0.044$; Fig.4.7A) and positively with latency to approach the pup ($r [6] = 0.84$, $p = 0.044$; Fig.4.7B). In the MeA, we found a significant negative correlation between WFA+ PNN density and latency to approach the pup in males exposed to a pup once ($r [6] = -0.87$, $p = 0.033$; Fig.4.7C). No significant correlations were found between WFA expression in the MPOA and behavior of pup-exposed males ($p > 0.05$). We also evaluated relationships between object-directed behavior and WFA expression in males.

No significant correlations between WFA expression and behavior were found in the mPFC, MeA, or MPOA of object-exposed males ($p > 0.05$).

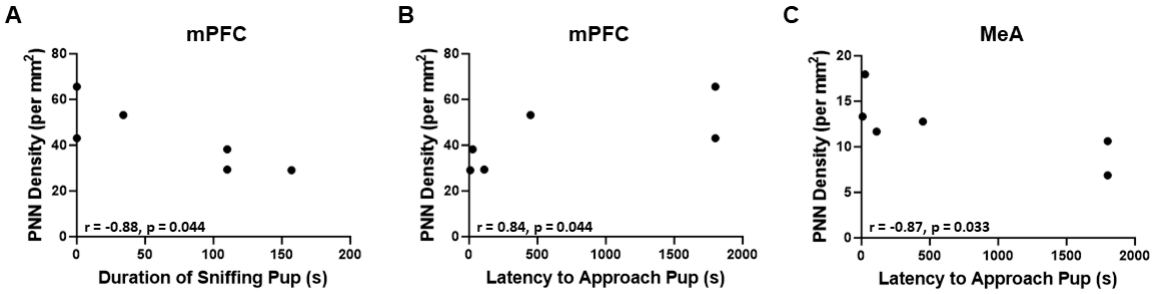

Figure 4.7. Spearman correlations of PNN densities and pup-directed behavior in adult virgin male California mice exposed to pups for the first time. **A, B.** Relationship between PNN density in the medial prefrontal cortex (mPFC) and time spent sniffing the pup (A) or latency to approach the pup (B). **C.** Relationship between PNN density in the medial amygdala (MeA) and latency to approach the pup.

PNNs in the mPFC, but not amygdala, primarily co-localize with GAD67 in California mice.

To gain insight into how circuit function may be modified by PNN plasticity following pup exposure, we determined the cell-type specificity of PNNs in the mPFC and MeA of adult male California mice. We measured co-localization of WFA+ PNNs with the GABAergic neuronal marker GAD67 or the glutamatergic neuronal marker CAMKII-α in these regions.

In the IL subregion of the mPFC, about 38% of GAD67+ neurons were enveloped by WFA+ PNNs, while fewer than 1% of CAMKII-α+ neurons co-localized with WFA+ PNNs (Fig. 4.8A and 4.8B). Moreover, approximately 69% of WFA+ PNNs co-localized with GAD67, whereas only around 6% WFA+ PNNs covered CAMKII-α+ glutamatergic neurons (Fig. 4.8C).

In the MeA, about 9% of GAD67+ cells were surrounded by WFA+ PNNs, while approximately 6% of CAMKII-α+ cells co-localized with WFA+ PNNs (Fig. 4.8A and 4.8B). On the other hand, the percentage of WFA+ PNNs containing GAD67 was about 23%, while ~28% of WFA+ PNNs covered CAMKII-α+ cells (Fig. 4.8C). Together, these data indicate that the majority of neurons surrounded by PNNs in the mPFC are GABAergic neurons, while WFA+ PNNs in the MeA do not preferentially co-localize with GABAergic or glutamatergic cell markers.

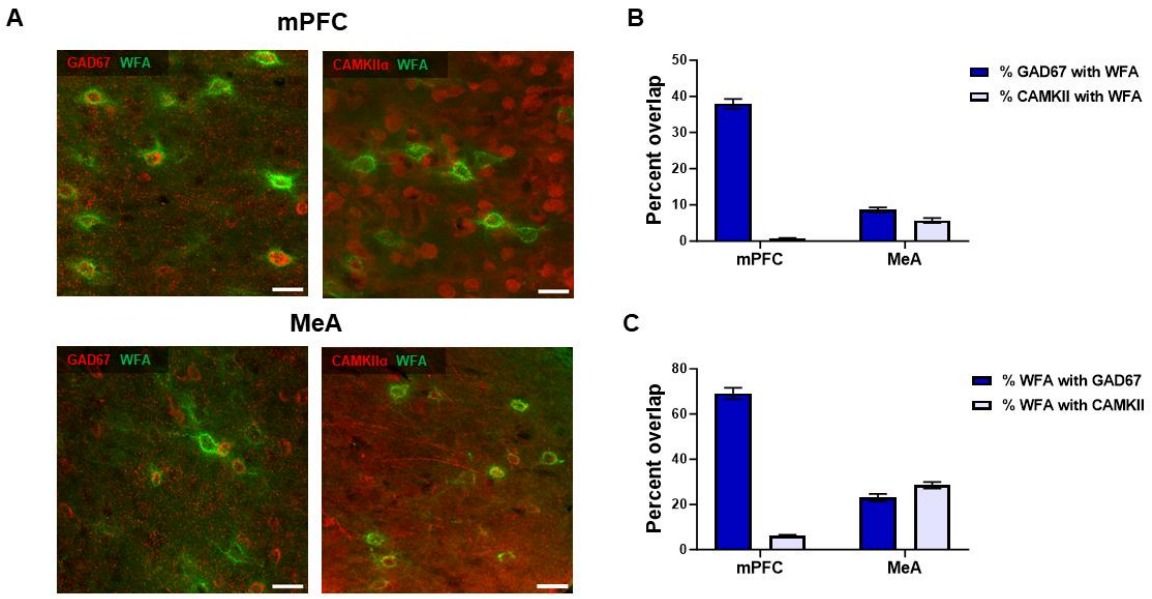

Figure 4.8. Co-localization of WFA with GABAergic (GAD67) and glutamatergic (CAMKIIα) cell markers in male California mice. A. Close-up images (20x) showing the co-localization of WFA+ and GAD67+ (left) or CAMKIIα+ (right) cells in the infralimbic mPFC (top) and MeA (bottom). Scale bar = 20 µm. **B.** Percentage (mean ± SEM) of GAD67+ and CAMKIIα+ neurons enveloped by WFA+ PNNs. **C**. Percentage (mean ± SEM) of WFA+ PNNs that contain GAD67 or CAMKIIα in the mPFC and MeA.

Discussion

Previous research in our lab has shown that repeated exposure to pups across several days facilitates the onset of pup-affiliative behavior in virgin male California mice

(Horrell et al., 2017). However, the mechanisms underlying the neural plasticity resulting from experience with infants are not well understood. Here, we examined the effects of experience with pups on expression of PNNs, extracellular structures that regulate synaptic plasticity, in male and female California mice. We found that the effects differed depending on brain region and sex. Compared to virgin males that were exposed to pups only once, virgin males exposed to pups twice had lower WFA+ PNN density in the mPFC, whereas first-time fathers had lower WFA+ PNN density in the MeA. Experience with pups did not alter WFA expression in the MPOA of males, nor did it influence WFA expression in the mPFC, MeA, or MPOA of adult female California mice. Repeated exposure to a pup-sized object did not alter WFA expression in the mPFC, MeA, or MPOA of males or females, suggesting the effects are not due to novelty. Finally, we found that PNNs in the mPFC are associated mostly with GABAergic neurons, as observed in other species, whereas PNNs in the MeA are associated with both GAD+ and CAMKIIα + cells.

The present study is the first to show that PNN density in the mPFC is dynamically altered with pup experience in virgin males. The mPFC plays a vital role in higher executive functioning and contributes to working memory, behavioral flexibility, response inhibition, attentional processes, emotional regulation, and the modulation of social behavior (Dalley et al., 2004; Etkin et al., 2011; Kim et al., 2015; Kingsbury et al., 2019; E. Lee et al., 2016; Wirt & Hyman, 2017). The mPFC has three primary subregions – the anterior cingulate cortex, the prelimbic cortex (PL), and the infralimbic cortex (IL) – and increasing evidence suggests the different subregions have specialized roles in social behavior. Notably, the PL and IL target the amygdala to differentially influence social behavior, as activation of PL projections to the basolateral amygdala

(BLA) impaired social interaction, while inhibition of IL-BLA projections also impaired social interaction (Huang et al., 2020). Moreover, Huang and colleagues (2020) found that IL neurons were preferentially activated following social interaction compared to PL neurons, indicating that the IL mPFC, through its connections to other brain regions involved in social interaction and emotional response, may regulate social behavior.

Some previously published studies support a role for the IL mPFC in rodent parental motivation (Pereira & Morrell, 2011, 2020); however, the neuroplastic changes that may occur in this region during the transition from pup-avoidant to pup-affiliative behavior have not yet been examined. In the current study, we found that virgin male California mice that were exposed to pups twice exhibited reduced PNN density in the IL mPFC compared to virgin males exposed to pups only once. Less dense PNNs in these males may have promoted plasticity in the mPFC, facilitating the transition from pup-avoidant to pup-affiliative behavior. Furthermore, PNN density in the mPFC was positively correlated with the latency to approach the pup and negatively correlated with the total time spent sniffing the pup in virgin males exposed to pups for the first time. It is possible that PNNs in this region initially stabilize social motivation circuits related to pup avoidance and then are dynamically reorganized with pup experience to stabilize circuits influencing pup approach behavior. Future studies utilizing chondroitinase ABC would allow us to degrade PNNs in the mPFC during the sensitization process to test this hypothesis.

PNNs preferentially surround fast-spiking parvalbumin (PV)-containing GABAergic interneurons in primary sensory areas (McRae et al., 2007). PV-positive (+) interneurons play an essential role in sensory learning in development and adulthood (Lupori et al., 2023; Rupert & Shea, 2022). Chemogenetic reduction of PV+ interneuron

activity reinstated heightened plasticity in adult sensory cortices, and activity of PV+ interneurons can influence PNN formation (Cisneros-Franco & de Villers-Sidani, 2019; Devienne et al., 2021). Additionally, plasticity of PV+ interneurons in the hippocampus is important for learning and memory processes in adulthood, implicating PV+ interneurons in experience-dependent plasticity (Donato et al., 2013, 2015). Consistent with studies in house mice, we found that most WFA+ PNNs surrounded GABAergic cells in the mPFC of male California mice (Ueno et al., 2017). Ueno and colleagues (2017) found that the majority of WFA+ PNNs were located in layers 5/6 of the IL mPFC of adult male mice, and about 60% of WFA+ PNNs surrounded GABAergic neurons in layers 5/6 of the IL. Notably, approximately 40% of WFA+ PNNs in layers 5/6 of the IL mPFC enveloped PV+ neurons (Ueno et al., 2017). Given that PV+ interneurons are important for experience-dependent plasticity and that PNNs surround PV+ cells in the mPFC (Ueno et al., 2017), it is possible that reduction of PNN density after pup exposure results in reduced inhibitory neurotransmission (Balmer, 2016; Lensjø et al., 2017) and facilitates cortical disinhibition to influence learning and memory.

Results of the current study suggest that twice-exposed males may have had a reduction in inhibitory tone in the IL mPFC compared to males exposed to pups for the first time, which suggests a possible disinhibition of projection neurons in these males. In house mice, elevated activity of subsets of mPFC projection neurons occurs during social behavior (Kim et al., 2015; E. Lee et al., 2016; Murugan et al., 2017). Notably, distinct ensembles of excitatory neurons in the mPFC are tuned to social performance, as some excitatory populations increase their activity during social exploration (Liang et al., 2018). In contrast, other populations exhibited decreased activation during social exploration, indicating diverse responses of mPFC projection neurons during social

exploration (Liang et al., 2018). Therefore, dynamic reorganization of PNNs in the mPFC may facilitate pup sensitization by reducing GABAergic interneuron feed-forward inhibition of long-range excitatory projections that influence social behavior. An important area of future research would be to examine the contribution of different interneuron cell types to pup-experience-dependent plasticity.

The MeA is a subregion of the amygdala implicated in the regulation of parental behavior in male rodents (Chen et al., 2019; Kirkpatrick, Carter, et al., 1994; Kirkpatrick, Kim, et al., 1994). We found that first-time fathers displayed reduced density of PNNs in the MeA compared to virgin males exposed to pups for the first time. Interestingly, PNN density in the MeA was negatively correlated with latency to approach the pup in virgin males exposed to pups for the first time. While PNNs in the BLA appear to play a role in the maintenance of fear- and addiction-related memories in rodents (Gogolla et al., 2009; Xue et al., 2014), a role of amygdalar PNNs in the modulation of social interactions has not been established; however, a recent study found that PNN removal in the posterior dorsal division of the MeA (MeApd) did not significantly affect aggressive behavior in adult male mice (Ciccarelli et al., 2021). The MeApd influences pup-directed behavior in a complex manner, as affiliative and aggressive responses to pups are regulated by GABAergic cells in an activity-dependent manner in male house mice; low-intensity optogenetic activation of GABAergic neurons promoted pup-affiliative behavior in virgin males, while high-intensity activation of the same neurons promoted aggressive behavioral responses toward pups (Chen et al., 2019). In this study, we found that while PNNs did not preferentially surround GABAergic neurons in the MeA of male California mice, a subset of MeA neurons surrounded by PNNs were GABAergic. Therefore, it is possible that PNNs regulate the activity of GABAergic cells involved in pup-directed

behavior. Future immunohistochemical studies examining co-localization of PNN-surrounded GABAergic neurons with a marker of neural activation, such as c-Fos, within the MeA would allow us to determine if GABAergic MeA neurons surrounded by PNNs are preferentially activated following pup exposure to help explore this hypothesis.

We found that PNNs surrounded a subpopulation of glutamatergic cells in the MeA of male California mice. Previous studies in house mice found that glutamatergic neurons in the MeApd promote self-maintenance behavior (self-grooming) but not social behavior (e.g., aggressive, sexual, and parental) in adult male mice (Chen et al., 2019; Hong et al., 2014). Thus, the PNNs around the glutamatergic cells in the MeA might influence the plasticity of the asocial circuit for self-maintenance behavior. However, the functional role of distinct neuronal subpopulations in the MeA in the control of behavior has not been explored in California mice; therefore, it is possible that glutamatergic cells in the MeA may influence parental behavior in our species.

In contrast to the mPFC and MeA, we found no effect of pup experience on PNN density in the MPOA of male California mice. Overall, we found very low levels of PNN expression in the MPOA of pup-exposed males (as well as all other groups in our study). These results are consistent with studies that have detected sparse WFA staining in the MPOA of male house mice (Horii-Hayashi et al., 2015, 2017) and male rats (Uriarte et al., 2020).Taken together, these results indicate that there is very low expression of WFA+ PNNs in the MPOA of male rodents; therefore, PNNs might not be involved in regulating structural plasticity in response to experience with pups in the MPOA.

Previous studies have found changes in PNN expression in response to reproductive events (i.e., pregnancy) in rat dams and interactions with pups in virgin

female house mice (Lau et al., 2020; Uriarte et al., 2020). In contrast, we found no effect of pup exposure on PNN density in the IL mPFC, MeA, or MPOA of female California mice. Notably, the effect of pup exposure on parental behavior (i.e., sensitization) has not been examined in females of this species. Many strains of inbred female house mice exhibit spontaneous parental behavior upon experimental exposure to unrelated pups (Gandelman, 1973; Mann et al., 1983; Tsuneoka et al., 2013; Wu et al., 2014); however, most adult virgin female rodents do not exhibit parental behavior when first presented with foster pups of the same species. Unlike laboratory mice, wild adult virgin female house mice often kill pups (Jakubowski & Terkel, 1982; McCarthy & Vom Saal, 1985). Similarly, adult virgin female rats tend to avoid or act aggressively towards experimentally presented pups upon initial exposure, but can become sensitized to pups following 6-8 days of pup exposure (Rosenblatt, 1967). Furthermore, in the naturally biparental prairie vole (*Microtus ochrogaster),* most adult females exhibit avoidant or infanticidal reactions when tested with unrelated pups; in contrast, the majority of adult virgin male prairie voles show parental behavior when tested under the same conditions (Lonstein & De Vries, 1999, 2001). Moreover, a previous study in our lab found that adult virgin female California mice are less motivated to engage in parental behavior toward experimentally presented pups compared to adult virgin males (Nguyen et al., 2020). Therefore, it is possible that virgin female California mice are more resistant to pup-stimulated parental behavior and may require a greater number of pup exposures to become parentally responsive. Thus, dynamic reorganization of PNNs across the sensitization period may occur in females, but this might happen following a greater number of pup exposures than males.

In our study, the number of neurons surrounded by WFA+ PNNs in the IL mPFC was significantly higher in male California mice than females. In contrast, no sex differences were found in PNN density in the mPFC of adult house mice reared under standard housing conditions, suggesting species differences in PNN expression might exist (Gildawie et al., 2020, 2021; Page & Coutellier, 2018). Notably, in our study, object-exposed females also exhibited lower PNN density compared to object-exposed males, suggesting the sex difference was not specific to the pup sensitization paradigm. Future studies should examine baseline levels of PNN in male and female California mice in the mPFC. Interestingly, a study in lab rats reported that females exhibited lower PNN density in the mPFC at the onset of puberty compared to post-pubertal females, while similar changes were not seen in males, suggesting a relationship between estrogen signaling and PNN expression in the mPFC of female rats (Drzewiecki et al., 2020). Gonadal steroid receptors are expressed in the prefrontal cortex of rodents, and gonadal hormones can influence synaptic formation and transmission of neurons in this region (Feng et al., 2010; Hajszan et al., 2007; Mitra et al., 2003; Montague et al., 2008; Nuñez et al., 2003; Yousuf et al., 2019; M. Zhang et al., 2021). Therefore, the sex differences in PNN density in the mPFC might be related to differences in circulating hormones and central hormone signaling between males and females; however, in this study, we did not explore the relationship between gonadal steroid receptors in the brain and PNN expression across our behavioral sensitization paradigm. Further studies characterizing co-localization of WFA+ PNNs with gonadal steroid receptors in the mPFC would help elucidate whether hormonal signaling influences PNN development around mPFC neurons in California mice.

While we found no difference in WFA+ PNN expression between males and females in the MeA, studies in other rodents have found that males exhibit greater PNN expression in the amygdala compared to females. In a study in rats, early-life adversity influenced PNN expression in the amygdala in a sex- and hemisphere-dependent manner, where juvenile male rats exhibited increased PNN expression in the right BLA compared to females following early-life adversity (Guadagno et al., 2020). Moreover, in house mice, adult males exhibited greater PNN expression in the MeApd compared to females, and, interestingly, the majority of WFA+ neurons in the MeApd also expressed estrogen receptors (Ciccarelli et al., 2021). Additionally, research examining sex differences in PNN expression in the hippocampus and hypothalamus has provided further evidence suggesting that PNN formation is influenced by hormones (Hernández-Vivanco et al., 2022; Laham et al., 2022; Uriarte et al., 2020; N. Zhang et al., 2021). Overall, the literature suggests that fluctuations in gonadal steroids influence cortical and subcortical PNN development; therefore, an important avenue of future research should be to explore the relationship between PNNs, gonadal steroid signaling, and behavioral sensitization to pups in male and female California mice.

While some similarities exist between the neural circuits that govern parental care in male and female rodents (Dulac et al., 2014; Wu et al., 2014), accumulating evidence also suggests sexually dimorphic neuronal populations exist within these shared networks (Scott et al., 2015; Chen et al., 2019). Notably, in biparental rodents, many of the regions that mediate maternal behavior are also involved in paternal behavior (Kirkpatrick, Kim, et al., 1994; A. W. Lee & Brown, 2002, 2007); therefore, differences in pup sensitization might arise from the same brain regions being differently regulated by hormones and/or by differential fine-tuned activation of neurons that

regulate pup-directed behavior in a sex-dependent manner. Our findings suggest that one such fine-tuning mechanism may be dynamic reorganization of PNN expression with pup experience in adult California mice. These findings, together with other studies that found changes in PNN expression following interactions with pups and across gestation in rodents (Krishnan et al., 2017; Lau et al., 2020; Uriarte et al., 2020), provide further support that pup interaction and hormones may alter neuroplasticity in the parental-care circuit through modulation of PNNs.

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Chapter 5

Conclusions

The neurochemical systems that act within brain circuits underlying males' nurturing behavior toward infants remain largely unexplored. These studies aimed to elucidate the role of norepinephrine NE (NE) in mediating the onset of paternal behavior, as well as to examine the mechanisms associated with neural plasticity resulting from experience with infants.

These studies use the California mouse (*Peromyscus californicus*), in which fathers participate extensively in all aspects of parental care except nursing, while adult virgin males vary widely in their behavior toward unrelated pups upon experimental exposure. Therefore, the neural control of pup-related behavior appears to change as males transition into fatherhood. In Chapters 2 and 3, I examined the role of NE in the onset of pup-affiliative behavior in virgin California mice. The experiments characterized parental care following pharmacological manipulations of NE signaling. In Chapter 2, I examined the effects of pharmacologically inhibiting NE synthesis on pup-directed behavior. I found that mice treated with the NE synthesis inhibitor nepicastat expressed less pup-affiliative behavior compared to vehicle-treated controls. In contrast, I found no effects of nepicastat on neophobia, anxiety-related behavior, or sociosexual behavior. These findings align with the literature suggesting a role for the noradrenergic system in the onset of parental behavior in female rodents.

In Chapter 3, I examined the effects of pharmacologically inhibiting NE synthesis on pup-induced expression of the protein Fos, a cellular marker of neural activity, in the brain. I used immunohistochemistry to quantify Fos in the medial preoptic area, bed

nucleus of the stria terminals, medial amygdala, and basolateral amygdala, brain regions that mediate paternal behavior and anxiety. I found that mice treated with nepicastat showed reduced Fos expression in the medial amygdala (MeA) following pup exposure, compare to vehicle-treated controls, which suggests that NE acting at the level of the MeA might be important for the onset of pup-affiliative behavior. Results from these experiments provide a novel understanding of the role of the noradrenergic system in the regulation of paternal responsiveness. Notably, these results might extend to human father-infant interactions, particularly under clinical conditions associated with dysregulated NE signaling, such as depression and anxiety.

In Chapter 4, I examined the mechanisms that may contribute to neural plasticity resulting from experience with pups in adult California mice. Specifically, I determined whether pup exposure affects perineuronal net (PNN) expression in the brains of male and female California mice. Repeated exposure to pups induces the onset of paternal responsiveness to pups in virgin males, and therefore, I examined changes in PNN expression in adult virgin mice exposed to pups 1, 2, or 3 times, and new parents. I found that experience with pups altered PNN density in the brain of virgin male, but not female, California mice. In the medial prefrontal cortex, PNN density decreased in virgin males that were exposed to pups twice compared to virgin males exposed to pups only once. In the MeA, first-time fathers exhibited lower PNN density compared to virgin males exposed to pups only once. These findings demonstrate that dynamic reorganization of PNN expression occurs with pup experience in adult male California mice.

Altogether, my dissertation research provides evidence for a role of the noradrenergic system in the onset of paternal behavior in a biparental mammalian

species. Additionally, this research is the first to suggest that PNNs might contribute to the regulation of the neuroplasticity of the paternal brain during the transition to parenthood in a biparental species. Overall, these studies provide novel insights into the key brain regions that influence the initiation of mammalian paternal behavior and the mechanisms underlying this regulation.

Future studies

An important next step to take in examining noradrenergic modulation of parental behavior is to test the effect of acute administration of noradrenergic receptor agonists/antagonists in the brain to determine the subtypes of adrenergic receptors, as well as the specific brain regions, that NE may be acting on to influence the onset of pup-affiliative behavior in the California mouse.

To further examine the role of PNNs in the neuroplasticity resulting from pup experience, future studies utilizing chondroitinase ABC would allow us to degrade PNNs in the brain of adult male California mice during the pup sensitization process. In pup-inexperienced virgins, PNNs may stabilize neural circuits underlying pup-avoidant behavior. Therefore, degrading PNNs in inexperienced virgins may promote plasticity to facilitate the transition from pup-avoidant to pup-affiliative behavior.