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Acute Inhibition of Dopamine β-Hydroxylase Attenuates Behavioral Responses to Pups in Adult Virgin California Mice (*Peromyscus californicus*)

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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.

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1. 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 and Malcolm, 1981; Woodroffe and 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; Berridge and Waterhouse, 2003; Aston-Jones and Cohen, 2005; Bouret and 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 and 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 pupaffiliative 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 and Palmiter, 1997). Moreover, treatment of DBH-knockout mothers with a synthetic precursor for NE production prior to parturition restores maternal behavior (Thomas and

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 and 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 and 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 and 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 (Ribble and Salvioni, 1990; Gubernick and Teferi, 2000) and the lab (Gubernick and 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 and Alberts, 1987; Lee and 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 (Gubernick and Alberts, 1987; Gubernick and Addington, 1994; de Jong et al., 2009; Chauke et al., 2012; 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.

2. Materials and methods

2.1. 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 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).

2.2. 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 1.

2.3. 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., 2013, 2010). 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).

2.4. 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 laboratory-housed 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.

2.5. 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.

2.6. 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 × 1 × 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.

2.7. 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.

2.7.1. 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).

2.8. 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 and Brownstein, 1988) and a standard mouse brain atlas for reference (Paxinos and 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.

2.9. 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 nonparametrically 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).

3. Results

3.1. 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 (Figure 1; Table 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 hourlong 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. 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. 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). 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. 2C, Table 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. 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 2B, Table 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. 2D, Table 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. 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).

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. 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. 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). 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. 2C, Table 2).

In females, as in males, nepicastat treatment reduced the likelihood of displaying parental behavior (Fig. 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. 2B), and drug-treated females spent less (or zero) time engaging in parental behavior in comparison to vehicle-treated females (U = 33, p = 0.0351, d = 0.83, Mann-Whitney U; Fig. 2D; Table 2). Similar to the males, aggression towards pups did not differ between treatment conditions (Fig. 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).

3.2. 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). 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).

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).

3.3. 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).

3.4. 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). 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). Finally, time spent engaging in autogrooming or exploratory behavior did not differ significantly between groups (both $p > 0.05$).

3.5. 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 (Table 3). Dopamine levels were not significantly altered in any of these regions following nepicastat treatment (all $p > 0.05$; Table 3). Finally, the DA/NE ratio was increased in the hypothalamus of nepicastattreated 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 $p > 0.05$).

4. 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 and 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 residentintruder 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 druginduced 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 (Whalen et al., 1961; Clark et al., 1984; 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; McIntosh and Barfield, 1984; Clark et al., 1985; Smith et al., 1987; Thomas and 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 (Schroeder et al., 2010; Devoto et al., 2014). 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 (Schroeder et al., 2010; Devoto et al., 2014). 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 (McGaugh, 2004; Murray, 2007; Adolphs, 2010). 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; Unnerstall et al., 1984; Byrum and Guyenet, 1987; Woulfe et al., 1990; Asan, 1998). 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 and 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 (Morrison et al., 1978; Cunningham and Sawchenko, 1988; Fritschy and Grzanna, 1989); 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 (Weinshenker and Schroeder, 2007; Gaval-Cruz and Weinshenker, 2009). 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 (Berridge and Waterhouse, 2003; Aston-Jones and Cohen, 2005). 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 and Taylor, 1995; Marino et al., 2005), maternal offspring recognition (Pissonnier et al., 1985; Dickinson and Keverne, 1988), and maternal behavior (Rosenberg et al., 1977; Steele et al., 1979; Thomas and 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.

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Highlights

- We injected virgin male and female California mice with either nepicastat or vehicle.
- Nepicastat inhibited interactions with pups in pup-naïve virgin male and female mice.
- Nepicastat reduced norepinephrine (NE) levels in the brain.
- NE may facilitate the onset of pup-affiliative behavior in California mice.

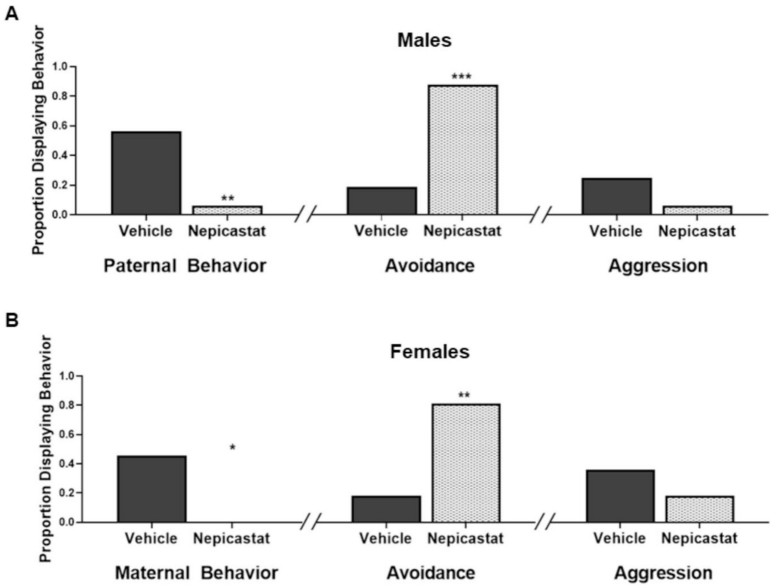

Fig. 1.

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.

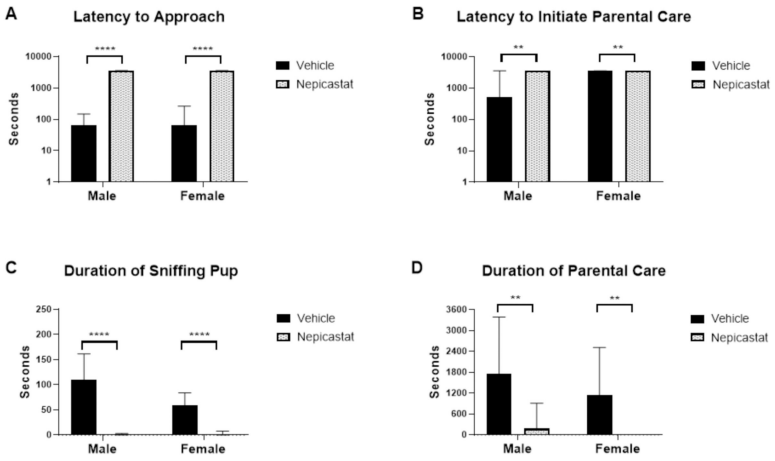

Fig. 2.

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

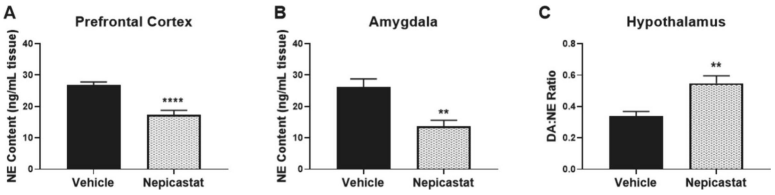

Fig. 3.

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.

Table 1.

Sample sizes for each experiment.

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

[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.

Table 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.

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	-

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
	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 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.

	Norepinephrine		Dopamine	
Brain Region	**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

** () and

**** () indicate P < 0.01 and P < 0.0001 compared with vehicle.