Genetic disruption of dopamine β-hydroxylase dysregulates innate responses to predator odor in mice

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Abstract

Rodents infected with the parasite *Toxoplasma gondii* show reduced aversion to predator odor and diminished neophobia, and infection in humans is associated with increased risk of neuropsychiatric disorders. Norepinephrine (NE) and dopamine (DA) modulate anxiety and predator odor responses, and *T. gondii* suppresses dopamine β-hydroxylase (*Dbh)* expression thereby reducing NE and increasing DA, which may contribute to the disruption of innate host behaviors. We examined the consequences of *Dbh* knockout (*Dbh -/-*) on responses to predator odor (bobcat urine) and compared them to Dbh-competent littermate controls. Over the first 10 min of predator odor exposure, controls exhibited robust defensive burying behavior, whereas *Dbh -/-* mice showed high levels of grooming. Defensive burying was potently suppressed in controls by drugs that reduce NE transmission, while excessive grooming in *Dbh -/-* mice was blocked by DA receptor antagonism. In response to a cotton square scented with a novel "neutral" odor (lavender), most control mice shredded the material, built a nest, and fell asleep within 90 min. *Dbh -/-* mice failed to shred the lavender-scented nestlet, but still fell asleep. In contrast, controls sustained high levels of arousal throughout the predator odor test and did not build nests, while *Dbh -/-* mice were asleep by the 90-min time point, often in shredded bobcat urine-soaked nesting material. Compared with controls exposed to predator odor, *Dbh -/-* mice demonstrated decreased c-fos induction in the anterior cingulate cortex (ACC), lateral septum (LS), periaqueductal gray (PAG), and bed nucleus of the stria terminalis, but increased c-fos in the LC and medial amygdala. These data indicate that relative ratios of central NE and DA signaling coordinate the type and valence of responses to predator odor, and suggest that *Dbh -/-* mice may represent a useful monogenetic model of neurobiological changes conferred by *T. gondii* infection.

Key words: *Toxoplasma gondii*, norepinephrine, dopamine, dopamine β-hydroxylase, predator

1. Introduction

1.1. Toxoplasma gondii infection disrupts innate fear responses

Responding to external stimuli with appropriate behaviors (e.g., approach or avoidance) is crucial for survival of most animals. In rodents, detection of cat odorants prompts adaptive defensive and escape behaviors that reduce risk of predation. The lifecycle of the parasite *Toxoplasma gondii* requires transmission through the feline gut before settling into chronic infection in larger mammals, including humans (Kannan et al 2010). *T. gondii* facilitates this process by first infecting rodents and suppressing neophobia and reducing aversion to predator odor, thus making it more likely the infected rodent will be eaten by a cat, and in turn, making it more likely the parasite will transmit to humans (Vyas et al 2007, Webster et al 1994). *T. gondii*-induced changes in behavioral responses to predator stress is not merely a rodent curiosity; approximately one-third of the human population carries a chronic *T. gondii* infection, and while most infected people are grossly asymptomatic, toxoplasmosis can have devastating effects on the fetal brain and is associated with greater risk for developing a host of neuropsychiatric disorders, and may even increase the risk of suicide and homicide (Cook et al 2015, Nayeri et al 2022). It is important to understand the neurobiological underpinnings of species-typical and maladaptive responses to threats because dysregulated behavioral responses to stressful stimuli represent many hallmark features of neuropsychiatric disorders, including posttraumatic stress disorder (PTSD), anxiety, and drug abuse (Dias et al 2013). The mechanisms by which *T. gondii* changes host behavior are not fully understood, although a recent study showed that *T. gondii* infection in rodents profoundly decreases expression of dopamine β-hydroxylase (DBH) (Alsaady et al 2019), the enzyme that converts dopamine (DA) into norepinephrine (NE) in noradrenergic cells. Thus, a reduction in DBH decreases NE while elevating DA in the brain.

1.2 Norepinephrine modulates anxiety-like traits

The catecholamine neuromodulators NE and DA govern emotional and motivated behavior. NE is critical for arousal, attention, orienting to salient stimuli, and organization of stress responses (Poe et al 2020, Sara 2009), while DA mediates reward and approach (Krach et al 2010). The locus coeruleus (LC) is the main source of NE throughout the brain and projects to regions implicated in responses to potential threats (Lustberg et al 2020a, Lustberg et al 2020b, Poe et al 2020, Schwarz & Luo 2015). Although NE is the dominant neurotransmitter produced by the LC, it can also release DA under certain conditions (Beas et al 2018, Kempadoo et al 2016, Petter et al 2023, Takeuchi et al 2016).

The LC is activated by novelty and predator odor, and NE transmission facilitates anxiety-like behavior, particularly in paradigms that involve stress and novelty (Curtis et al 2012, Day et al 2004, Hayley et al 2001, Lustberg et al 2020b). NE has also been previously implicated in predator odor responses as well. Exposure to trimethylthiazoline (TMT), a component of fox odor, promotes freezing behavior in rats, which is attenuated by pharmacological blockade of NE transmission in the bed nucleus of the stria terminalis (BNST) (Fendt et al 2005). Likewise, CRISPR-induced disruption of NE synthesis in the mouse LC blocks the ability of rat odor to trigger arousal and wakefulness (Yamaguchi et al 2018).

1.3 Norepinephrine and dopamine control distinct stress-induced behaviors

We have previously used DBH knockout (*Dbh -/-*) mice that lack NE and have excessive DA in LC and other noradrenergic cell groups to investigate the relative contribution of these catecholamines to stress responses. The anxiogenic effects of footshock and novelty stress are abolished in *Dbh -/-* mice, and they also fail to exhibit species-typical repetitive burying and nestlet shredding behaviors in novel environments (Lustberg et al 2020a, Lustberg et al 2020b, Tillage et al 2021). Pharmacological restoration of NE levels (without normalizing DA levels) rescues these phenotypes, while pharmacological blockade of NE transmission recapitulates

them in normal mice. These findings indicate that the lack of NE is primarily responsible for the aberrant responses.

However, we recently reported that both NE and DA contribute to novel odorant stress (Lustberg et al 2022). In response to non-threatening but novel olfactory stimuli (e.g. plant-derived essential oils presented in a novel environment), normal mice escalate digging, burrowing, and burying. In contrast, *Dbh -/-* mice do not engage in these behaviors, and instead groom excessively in novel environments regardless of the presence of various “neutral” odors. Importantly, acute suppression of NE transmission blocks novel odor-induced digging in control mice, while DA receptor antagonists attenuate grooming in *Dbh-/-* mice, indicating that the NE:DA ratio in noradrenergic neurons modulates the nature of these behavioral responses.

1.4 Study objectives and summary

Because *T.gondii* infection suppresses DBH expression, and NE and DA regulate stress-induces behaviors, we speculated that DBH inhibition may be the driving factor in the dramatic switch between adaptive (defend/avoid) and risky (approach) behavioral responses to predator odor. If this were true, we would expect *Dbh -/-* mice to lack species-typical defensive responses to predator odor and potentially display paradoxical appetitive/approach behaviors towards it. To test this, we exposed *Dbh -/-* and control mice to neutral odors or bobcat urine applied to cotton nestlets in a novel environment and measured digging, burying, nestlet shredding, and arousal. We then used a pharmacological approach to dissect the relative contributions of NE and DA. We also assessed the effects of DBH knockout on molecular signatures of neuronal activation in brain regions that receive innervation from the LC and are implicated in behavioral responses to predator odor. Our data suggest that *Dbh -/-* mice may represent a useful monogenetic model of neurobiological changes conferred by *T. gondii* infection.

2. Materials and methods

2.1 Animals

Dbh -/- mice were maintained on a mixed 129/SvEv and C57BL/6 J background, as previously described (Thomas et al 1998, Thomas et al 1995). *Dbh -/-* males were bred to *Dbh +/-* females, and pregnant *Dbh +/-* dams were administered drinking water containing the β-adrenergic receptor (AR) agonist isoproterenol and the α1AR agonist phenylephrine (20 μg/ml each; Sigma-Aldrich) with vitamin C (2 mg/ml) from E9.5–E14.5, and the synthetic NE precursor L-3,4-dihydroxyphenylserine (DOPS; 2 mg/ml; Lundbeck, Deerfield, IL) + vitamin C (2 mg/ml) from E14.5-parturition to prevent embryonic lethality resulting from complete *Dbh* deficiency. *Dbh -/-* mice are easily distinguished from their NE-competent littermates by their visible delayed growth and bilateral ptosis phenotypes. *Dbh* +/− littermates were used as controls because their behavior and catecholamine levels are indistinguishable from wild-type (*Dbh +/+*) mice (Bourdelat-Parks et al 2005, Szot et al 1999, Thomas et al 1998). Adult mice 4-10 months of age were used for all experiments.

Because no sex differences in stress-induced digging or grooming have been reported in past literature (Dixit et al 2020, Londei et al 1998, Smolinsky AN 2009) or were observed in published experiments from our lab (Lustberg et al 2022) and pilot experiments from this study, male and female mice from the same *Dbh* genotype were evenly distributed across drug treatment groups, and data were pooled between sexes. All animal procedures and protocols were approved by the Emory University Animal Care and Use Committee, in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Mice were maintained on a 12 h/12 h light/dark cycle (7:00/19:00 h) with access to food and water ad libitum, except during behavioral testing. Behavioral testing was conducted under standard lighting conditions during the light cycle (ZT5-ZT8) in the same room where the mice were housed to minimize the stress of cage transport on test days.

2.2 Behavioral analysis

Mice were group-housed in static cages in same-sex groups of 2–5. In the first set of experiments, individual mice were removed from their home cages and placed into new standard mouse cages (13″ x 7″ x 6″) containing only clean bedding substrate and a cotton nestlet square (5 cm × 5 cm, ~3 g) pre-soaked with either 1 ml of deionized water (odorless control) or predator odor (bobcat urine; Maine Outdoor Solutions, LLC, Hermon, Maine). Each mouse received both treatments in a counterbalanced order spaced 2 d apart. A clear plexiglass cover was pressed flush over the cages to prevent vaporization or dispersal of the odorants over the course of testing, while still allowing filming and behavioral scoring. The mice in the odorant-treated cages were filmed with a front-facing, mounted digital video camera for 10 min for assessment of defensive (digging/burying) and non-defensive (grooming) behaviors (De Boer & Koolhaas 2003, Kalueff & Tuohimaa 2005, Kemble & Bolwahnn 1997, Londei et al 1998, Lustberg et al 2020a, Pond et al 2021, Smolinsky AN 2009). Time spent in defensive burying and grooming was manually scored with digital stopwatches by a trained observer blind to genotype and treatment. Defensive burying involved vigorous displacement of the bedding toward the odorant stimulus, often resulting in the burying of the odorant-soaked nestlet. Defensive burying sometimes presented as intense burrowing away from the stimulus, which resembled “swimming” or tunneling through the bedding substrate (Bondi et al 2007, De Boer & Koolhaas 2003, Fucich & Morilak 2018, Lustberg et al 2020a, Treit et al 1981). Burying is considered aversion-related, because it resembles responses exhibited after exposure to the shock probe test or predator odor (Garbe CM 1993, Hwa et al 2019, McGregor et al 2002, Sluyter et al 1996). Grooming was defined as repetitive paw movements oriented around the whiskers and face, as well as licking or scratching of the body and tail (De Boer & Koolhaas 2003, Kalueff & Tuohimaa 2005, Smolinsky AN 2009). Some reports suggest that grooming may be a self-soothing behavior that increases under stressful conditions, while others propose

that it is a “rest behavior’ that indicates low levels of stress (Guild & Dunn 1982, Kemble & Bolwahnn 1997, Kemble ED 1995).

The second set of experiments was identical to the first set of experiments, except that mice were pretreated with saline, adrenergic, or dopaminergic drugs (see below) prior to predator odor exposure. All drugs were administered 30 min prior to testing except nepicastat, which was administered 2 h prior to testing. Each mouse received both a saline and drug pretreatment in a counterbalanced order spaced 2 d apart.

The third set of experiments were identical to the first set of experiments except that (1) the cotton nestlet square pre-soaked with either 1 ml of a novel “neutral” odor (lavender oil, 1:100 dilution in deionized water; Anjou, Fremont, CA) or bobcat urine, and (2) mice were left undisturbed for 90 min, after which a trained experimenter returned to record whether the mouse was asleep and whether the nestlet was fully (100%) shredded. Sleep was assessed behaviorally using EEG-validated scoring criteria, as we have described (Porter-Stransky et al 2019).

2.3 Drugs

The following drugs were intraperitoneally administered to dissect the relative contributions of NE and DA receptor signaling to behavior: nepicastat (100 mg/kg, DBH inhibitor) (Synosia Therapeutics, Basel, Switzerland); prazosin (0.5 mg/kg, α1AR antagonist) (Sigma-Aldrich, St. Louis, MO); propranolol (5 mg/kg, βAR antagonist) (Sigma-Aldrich); atipamezole (1 mg/kg, α2AR antagonist) (Sigma-Aldrich), flupenthixol (0.25 mg/kg, nonspecific DA receptor antagonist) (Sigma-Aldrich); SCH-23390 (0.003 mg/kg, D1 receptor antagonist) (Sigma-Aldrich); L-741,626 (10 mg/kg, D2 receptor antagonist) (Sigma-Aldrich). Doses selected were based on previous studies and pilot experiments to control for confounding effects such as sedation (Lustberg et al 2020a, Lustberg et al 2022, Mitchell et al 2008). All drugs were dissolved in bacteriostatic saline

except prazosin and L-741,626. Prazosin was first dissolved in 1.5% DMSO and 1.5% Cremophor EL before being added to saline, and L-741,626 was first dissolved in 10% ethanol and 1.5% Cremophor EL before being added to saline.

2.4 c-fos immunohistochemistry (IHC)

Dbh +/- and *Dbh -/-* mice were exposed to predator odor for 90 min as described above, then euthanized with an overdose of sodium pentobarbital (Fatal Plus, 150 mg/kg, i.p.; Med-Vet International, Mettawa, IL) for transcardial perfusion with cold 4% paraformaldehyde in 0.01 M PBS. After extraction, brains were post-fixed for 24 h in 4% paraformaldehyde at 4°C, and then transferred to cryoprotectant 30% sucrose/PBS solution for 72 h at 4°C. Brains were embedded in OCT medium (Tissue-Tek; Sakura, Torrance, CA) and serially sectioned by cryostat (Leica) into 20-μm coronal slices. Brain sections were stored in 0.01 M PBS (0.02% sodium azide) at 4°C before IHC.

For IHC, brain sections were blocked for 1 h at room temperature in 5% normal goat serum (NGS; Vector Laboratories, Burlingame, CA) diluted in 0.01 M PBS/0.1% Triton-X permeabilization buffer. Sections were then incubated for 48 h at 4°C in NGS blocking/permeabilization buffer, including primary antibodies raised against c-fos (rabbit anti-c-fos, Millipore, Danvers, MA, ABE457; 1:5000) and the NE transporter (NET; chicken anti-NET, #260006, Synaptic System, Goettingen, Germany; 1:3000). After washing in 0.01 M PBS, sections were incubated for 2 h in blocking/permeabilization buffer with goat anti-rabbit AlexaFluor 488 and goat anti-chicken AlexaFluor 568 (Invitrogen, Carlsbad, CA; 1:500). After washing, the sections were mounted onto Superfrost Plus slides and coverslipped with Fluoromount-G plus DAPI (Southern Biotech, Birmingham, AL).

2.5 Statistical analysis

The effects of the water vs predator odor on defensive burying and grooming in *Dbh +/-* and *Dbh -/-* mice were compared using repeated measures 2-way ANOVA (genotype x odorant), with post hoc Tukey tests for multiple comparisons where appropriate. The effects of drugs vs vehicle on time spent engaged in repetitive behaviors were assessed using paired sample t-tests. Contingency data of nestlet shredding and sleeping between genotypes were analyzed using Fisher's exact tests. Although the current study was not sufficiently powered to detect sex differences, preliminary analysis of the data revealed roughly equivalent distributions and means between males and females (data not shown), similar to what we previously reported for behavioral and neurochemical responses to novel odors (Lustberg et al 2022). Thus, male and female data were pooled within each genotype and treatment group.

3. Results

3.1 Dbh -/- mice exhibit decreased defensive burying and increased grooming in response to predator odor.

We first assessed the responses of *Dbh -/-* and NE-competent *Dbh +/-* control mice to predator odor and water by measuring defensive burying (**Fig. 1A, Supplemental movie 1**) and grooming (**Fig. 1B, Supplemental movie 1**). Control animals displayed modest levels of grooming during water exposure and no defensive burying. Predator odor dramatically increased defensive burying and total digging but had no impact on grooming in control mice. By contrast, *Dbh -/-* mice displayed high levels of grooming in response to water and were behaviorally indifferent to predator odor, with no emergence of defensive burying and no change in grooming.

For defensive burying, there were main effects of genotype [$F(1,24) = 15.96$, $p = 0.0005$], odorant [$F(1,24) = 15.96$, $p = 0.0005$], and odorant x genotype interaction [$F(1,24) = 15.96$, $p = 0.0005$]. Post hoc analysis revealed that *Dbh +/-* mice showed significantly increased defensive

burying ($p < 0.0001$) in the predator odor condition compared with water, and they also engaged in significantly more defensive burying ($p < 0.0001$) than the *Dbh -/-* mice in the predator odor condition (Fig. 1C). For grooming, there was a main effect of genotype [$F(1,24) = 18.82$, $p = 0.002$], but no main effect of odorant [$F(1,24) = 0.7293$, $p = 0.40$] or odorant x genotype interaction [$F(1,24) = 0.57$, $p = 0.46$].

3.2 Adrenergic antagonists suppress predator odor-induced digging behavior in control animals.

Our data from *Dbh -/-* mice suggested that NE is required for defensive burying in response to predator odor. To determine whether this is true in NE-competent mice as well as which receptors are involved, we next assessed the effects of specific AR antagonists on the predator odor responses of *Dbh +/-* mice. The compounds tested included prazosin (0.5 mg/kg, α1AR antagonist), atipamezole (1 mg/kg, α2AR antagonist), and propranolol (5 mg/kg, βAR antagonist). Compared with saline, each compound tested significantly reduced burying (prazosin: $t(7) = 2.46$, $p = 0.044$; atipamezole: $t(6) = 3.38$, $p = 0.015$); propranolol: $t(7) = 2.85$, $p = 0.025$) (**Fig. 2A-C**), indicating that α1AR, α2AR, and βAR activation all contribute to this predator odor-induced defensive behavior. Prazosin was the only compound that attenuated grooming in control mice (**Fig. 2D-F**) (prazosin: $t(7) = 3.03$, $p = 0.019$; atipamezole: $t(6) = 0.74$, $p = 0.49$; propranolol: $t(7) = 0.85$, $p = 0.43$). Overall, these results indicated that defensive burying was the predator odor-induced behavior most sensitive to blocking NE transmission.

3.3 Pharmacological DBH inhibition reduces defensive burying and increases grooming following exposure to predator odor in normal mice.

Because AR antagonists recapitulated the decrease in digging behaviors but not the increase in grooming observed in the *Dbh -/-* mice, we reasoned that increased grooming was mediated by ectopic DA produced by LC neurons in the knockouts. To test this idea, we used the selective

DBH inhibitor nepicastat, which unlike AR antagonists increases DA in addition to decreasing NE (Devoto et al 2015, Ohkubo et al 2019, Schroeder et al 2010, Stanley et al 1997). Nepicastat administration to *Dbh* +/- control mice phenocopied both aberrant responses of *Dbh* -/- mice to predator odor [defensive burying: $t(9)=3.29$, $p = 0.009$; grooming: $t(9)=2.60$, $p = 0.029$) (**Fig 3**), suggesting that increasing DA is necessary for abnormal *Dbh* -/- mouse predator odor-induced grooming.

3.4 D1, but not D2, antagonists attenuate excessive grooming in Dbh -/- mice in the presence of predator odor.

To directly test whether excessive DA transmission was required for predator odor-induced grooming in *Dbh* -/- mice, we assessed the effects of DA receptor antagonists on predator odor response in the knockouts. The drugs tested included flupenthixol (0.25 mg/kg, nonselective DA receptor antagonist), SCH-23390 (0.003 mg/kg, D1 receptor antagonist), and L-741,626 (10 mg/kg, D2 receptor antagonist). Flupenthixol [$t(12) = 2.76$, $p = 0.0037$] and SCH-23390 [$t(6) = 5.89$, $p = 0.0011$] significantly reduced grooming in *Dbh* -/- mice in the presence of predator odor, while L-741,626 had no effect [$t(7) = 0.75$, $p = 0.48$] (**Fig. 4**). Saline-treated *Dbh* -/- mice exhibited almost no defensive burying, and none of the drugs rescued this behavior (data not shown). It is worth noting that mice administered SCH-23390 ceased nearly all movement but responded to gentle prodding and regained normal mobility after being returned to their home cages. These results suggest that excessive grooming in *Dbh* -/- mice is mediated by D1, but not D2 transmission.

3.5 α1AR blockade has no effect on behavioral response to predator odor in Dbh -/- mice.

Our data thus far have indicated that NE transmission was responsible for defensive burying, while DA promoted grooming. However, because we observed a significant reduction in grooming in the *Dbh* +/- mice treated with prazosin, and DA can signal through α1ARs under

some conditions (Cilz et al 2014, Ozkan et al 2017, Paladini et al 2001), we speculated that some of the excessive grooming observed in *Dbh -/-* mice might be driven by DA-mediated α1AR transmission. To test this hypothesis, we administered prazosin (0.5 mg/kg) to *Dbh -/-* mice in the presence of predator odor, but failed to observe a drug effect on grooming (saline: 223.2 $\pm$ 49.85 sec; prazosin 216.3 $\pm$ 45.56 sec; $p = 0.8986$). Combined with the outcomes from the DA receptor antagonist experiments, these results suggest that while α1ARs modestly contribute to predator odor-induced grooming in normal mice, they are not required for the excessive grooming response of *Dbh -/-* mice, which appears to be mediated by excessive activation of D1 receptors.

3.6 D1 receptor blockade induces behavioral arrest in predator odor-exposed Dbh +/- control mice.

To determine whether SCH-23390-induced suppression of all behavior was unique to *Dbh -/-* mice or was a general effect of the drug in the presence of predator odor, we administered SCH-23390 (0.003 mg/kg) to bobcat urine-exposed *Dbh +/-* mice and found a significant decrease in defensive burying [$t(6) = 4.64$, $p = 0.0035$] and grooming [$t(6) = 3.47$, $p = 0.013$] (**Fig. 5**). While *Dbh +/-* mice treated with SCH-23390 were qualitatively more mobile than the *Dbh -/-* mice treated with SCH-23390, they remained mostly still until their return to their home cage, when they immediately regained normal activity. These results indicate that an interaction between predator odor and D1 blockade induces behavioral arrest in both normal and *Dbh -/-* mice.

3.7 Dbh -/- mice lack predator odor-induced arousal and aversion.

Predator odors typically elicit innate vigilance and aversion in prey that is resistant to habituation. We and others have shown that, when exposed to a novel environment and given access to a

cotton nestlet, normal mice rapidly shred the nestlet, build a nest, and fall asleep in it (Lustberg et al 2020a). To examine the effects of disrupting LC catecholamine homeostasis on these responses to predator odor, we measured nestlet shredding and arousal state in *Dbh* +/- and *Dbh* -/- mice after 90 min of exposure to a novel environment with a nestlet treated with bobcat urine. To control for the influence of a novel odor, a separate cohort of mice experienced the same paradigm except that a non-threatening but unfamiliar odor (lavender oil) was used instead of bobcat urine. In the presence of lavender, most (6/8) *Dbh* +/- mice shredded the nestlet and were asleep in the nest they built by the 90 min time point (**Fig. 6**). As we have shown previously with unscented nestlet (Lustberg et al 2020a), *Dbh* -/- mice failed to shred the nestlet (0/7) but still fell asleep (6/7) (**Fig. 6**). Fisher's exact tests revealed a significant difference between genotypes for shredding ($p=0.007$) but not sleeping ($p>0.99$).

In contrast to lavender odor, none (0/8) of the *Dbh* +/- mice shredded their nestlet or fell asleep in the presence of bobcat urine (**Fig. 6**). The behavior of *Dbh* -/- mice also changed when exposed to predator odor, but in unexpected directions. While *Dbh* -/- mice never engaged in shredding unscented or lavender-scented nestlets within 90 min, a majority (5/9) of them fully (100%) shredded the bobcat urine-soaked nestlet (**Fig. 6**). Those that did build a nest fell asleep in it, and altogether 8/9 of the *Dbh* -/- mice were asleep by the end of the test (**Fig. 6**). Fisher's exact tests revealed significant differences between genotypes for shredding ($p=0.0294$) and sleeping ($p=0.0004$). Pilot experiments using different source of predator odor (fox urine and tufts of domestic cat fur instead of bobcat urine) also revealed paradoxical appetitive responses in knockout mice; *Dbh* +/- animals engaged in typical defensive burying behavior, while most *Dbh* -/- mice instead sat on top of the cat fur and groomed themselves (**Supplemental movie 2**). These results demonstrate that predator odor elicits powerful arousal and aversion in normal mice, and that loss of DBH activity produces aberrant behavioral responses that include apparent affiliative properties.

3.8 c-fos immunohistochemistry reveals predator odor-responsive brain regions that differ between Dbh +/- and Dbh -/- mice.

To identify candidate brain regions that are activated by predator odor and facilitate differential responses in *Dbh* +/- and *Dbh* -/- mice, c-fos immunohistochemistry was conducted in animals exposed to a novel environment with a bobcat urine-treated nestlet. Analysis focused on the LC and other brain regions that receive innervation from noradrenergic neurons and have been implicated in predator odor responses, including the anterior cingulate cortex (ACC), dorsal BNST (dBNST), periaqueductal gray (PAG), periventricular nucleus of the hypothalamus (PVN), lateral septum (LS), and medial amygdala (MeA) (**Fig. 7**). Repeated measures 2-way ANOVA showed a main effect of brain region [$F(3,45) = 76.57$, $p < 0.0001$] and a genotype x brain region interaction [$F(6,90) = 44.84$, $p < 0.0001$]. Sidak's post hoc tests revealed that *Dbh* +/- mice had significantly more c-fos+ cells than *Dbh* -/- mice in the ACC ($t = 8.361$, $p < 0001$), dBNST ($t = 7.767$, $p < 0.0001$), PAG ($t = 9.897$, $p < 0.0001$), and LS ($t = 7.460$, $p < 0.001$), while *Dbh* -/- mice had significantly more c-fos+ cells in the LC ($t = 9.554$, $p < 0.0001$) and MeA ($t = 3.679$, $p = 0.0177$). No genotype differences were found in the PVN ($t = 0.956$, $p = 0.9542$). Integrated with the effects of genotype and drugs, these results imply that noradrenergic transmission in the ACC, dBNST, PAG, and/or the LS contribute to aversive/defensive effects of predator odor, while D1 signaling in the MeA promotes grooming and appetitive responses.

4. Discussion

4.1 Summary

Predator odors such as bobcat urine are ethologically relevant stressors that elicit intense, innate behavioral responses in mice (Ferrero et al 2011, Janitzky et al 2015). In this study, we investigated the effects of manipulating catecholamine transmission on predator odor-induced stress in mice by comparing *Dbh* -/- mice, which lack NE and instead produce DA in their

“noradrenergic” neurons, to their *Dbh* +/- littermates with normal catecholamine content. We also used pharmacological manipulation to target NE and DA signaling, allowing us to assess the effects of both chronic and acute catecholamine manipulation.

4.2 Adrenergic control of predator odor-induced defensive burying.

Defensive burying is considered an active coping strategy in response to stress, in which the mouse attempts to remove or avoid aversive stimuli. In the shock probe defensive burying test, mice will use bedding to bury electrified prods and remove the source of shock pain (Tillage et al 2020). The marble burying test has also demonstrated that burying is stress-sensitive, with stressed mice burying more marbles than unstressed mice (Kedia & Chattarji 2014), and we have shown that *Dbh* -/- mice bury fewer marbles than controls (Lustberg et al 2020a).

We found that both *Dbh* -/- and *Dbh* +/- mice engage in similarly low levels of defensive burying when exposed to a novel cage with a water-soaked cotton nestlet, indicating that this environment is not particularly stressful. *Dbh* +/- mice show increased defensive burying when exposed to predator odor as compared to water, suggesting that this behavior is an innate adaptive defensive response to threat. The combined results from our experiments with *Dbh* -/- mice, acute pharmacological DBH inhibition, and adrenergic receptor antagonists demonstrate that noradrenergic transmission is required for defensive burying during predator odor exposure. We previously showed that NE is critical for digging in the presence of novel odors but did not precisely identify the receptor subtypes responsible (Lustberg et al 2022).

The effects of βAR and α1AR activation are partially overlapping but can also be distinct. βAR activation is necessary for many types of anxiety-like and defensive behaviors, including responses to physical threats (e.g. shock) (Bondi et al 2007), psychological threats (e.g. novel environments) (Lustberg et al 2022), and anxiogenic drugs (e.g. cocaine) (Schank et al 2008),

while α1AR signaling promotes both exploratory and aversive behaviors in novel environments (Lustberg et al 2020a, Stone et al 2006, Stone et al 1999). α1AR blockade decreases defensive behavior in the presence of a traumatic cue in rats (Ketenci et al 2020), and antagonism of βARs attenuates cocaine withdrawal-induced defensive burying in rats (Harris and Aston-Jones, 1993). Interestingly, the βAR antagonist propranolol blocks the anxiogenic effects of optogenetic LC stimulation in the elevated zero maze, while only the α1AR antagonist prazosin prevents real-time place aversion (McCall et al 2015). We have shown that propranolol diminishes nestlet shredding, while prazosin attenuates marble burying in a novel environment (Lustberg et al 2020a). By contrast, antagonism of either receptor reduces defensive burying in the shock probe test. Thus, the current finding that both βARs and α1ARs contribute to defensive burying behavior during predator odor exposure is consistent with previous literature.

The ability of the α2AR antagonist atipamezole to reduce predator odor-induced defensive burying was more of a surprise. α2ARs are expressed widely throughout the brain but are enriched in LC neurons, where they function as inhibitory autoreceptors (Aghajanian & VanderMaelen 1982, Timmermans & van Zwieten 1982). Thus, α2AR agonists often decrease, while α2AR antagonists can increase NE transmission and anxiety-like behaviors (Lustberg et al 2020a, Lustberg et al 2020b). In the current study, atipamezole had effects similar to those of propranolol and prazosin, suggesting that it was not primarily acting on α2AR autoreceptors but rather by blocking α2AR heteroreceptors on LC target cells. α2ARs are expressed in stress-sensitive brain regions such as the prefrontal cortex (PFC, which includes the ACC) and the dBNST, where they can have paradoxical excitatory effects on neurons and networks (Harris et al 2018, Wang et al 2007). For example, stimulation of postsynaptic α2ARs in the dBNST enhances neuronal activity and promotes anxiety-like behavior in the elevated plus maze (Harris et al 2018). We speculate that atimpamezole-induced reduction of predator odor-induced defensive burying is mediated by similar mechanisms.

4.3 Dopaminergic control of grooming.

Grooming, unlike digging, is not typically considered a stress coping response, as mice will groom in situations of both comfort and distress (Smolinsky AN 2009). Indeed, cat odor often inhibits grooming in rodents (McGregor et al 2004). Repetitive, purposeless self-grooming is often observed in animal models of obsessive-compulsive disorder (OCD), Tourette's syndrome (TS), and autism spectrum disorder (ASD) (Lewis 2011, Nordstrom & Burton 2002, Welch et al 2007). Dopamine signaling has been previously implicated in facilitating grooming behavior. For example, D1, but not D2, receptor signaling contributes to stereotyped self-grooming in rats (Berridge & Aldridge 2000a, Berridge & Aldridge 2000b) and novelty-induced grooming in mice (Drago et al 1999).

As a precursor for NE, DA is produced in noradrenergic neurons. Due to incomplete conversion to NE by DBH, DA can also be released by these cells and mediates some LC functions including specific forms of learning and memory (Chowdhury et al 2022, Kempadoo et al 2016, Wagatsuma et al 2018). *Dbh -/-* mice lack NE and instead exclusively release DA from LC neurons under conditions that normally result in NE transmission. Converging lines of evidence from our study indicate that the excessive grooming displayed by *Dbh -/-* mice is mediated by DA. First, high levels of grooming in the presence of predator odor were observed in control mice treated with the DBH inhibitor nepicastat, but not in mice following AR blockade. Second, grooming behavior in *Dbh -/-* mice was attenuated by DA receptor antagonists.

Our findings that the non-selective DA receptor antagonist flupenthixol and the D1-selective antagonist SCH-23390, but not the selective D2 antagonist L-741,626 blocked grooming in *Dbh -/-* mice exposed to predator odor indicates this behavior is mediated by D1 receptors. One caveat to this interpretation is that even low dose SCH-23390 (0.003 mg/kg) eliminated most motor behavior in both *Dbh -/-* and *Dbh +/-* mice, not just grooming. Animals immediately resumed normal activity upon return to their home cage, indicating a specific

interaction between the drug and the environmental context. D1 blockade is well known to suppress many aspects of motor activity, including cocaine-induced hyperactivity (Cabib et al 1991) and rearing in a novel cage (Dreher & Jackson 1989). Another study using SCH-23390 also showed that it can induce catalepsy (Chinen & Frussa-Filho 1999), although at a much higher dose (0.1 mg/kg) than the one we used. Prazosin reduces grooming in *Dbh +/-* but not *Dbh -/-* mice, indicating that NE signaling through α1ARs promote this behavior exclusively in normal animals. This is consistent with our previous finding that prazosin reduces amphetamine-induced locomotion in *Dbh +/-* but not *Dbh -/-* mice (Weinshenker et al 2002), and further implicates D1 receptor mediation in knockouts.

We previously showed that *Dbh -/-* mice groom more than *Dbh +/-* mice in the presence of a novel but non-threatening odorant, and this excessive grooming was reduced by administration of flupentixol (Lustberg et al 2022). Therefore, the results from our genetic and pharmacological experiments suggest that the neurochemistry and circuity that drive predator odor-induced grooming resemble those controlling grooming responses to novel odors and/or environments.

4.4 Dbh -/- mice have impaired arousal and aversion responses to predator odor

Both NE and DA promote arousal. Activation of noradrenergic neurons in the LC and dopaminergic neurons in the ventral tegmental area (VTA) and ventral PAG (vPAG) induce wakefulness, while their suppression reduces arousal (Carter et al 2010, Cho et al 2017, Eban-Rothschild et al 2016, Lu et al 2006, Poe et al 2020, Porter-Stransky et al 2019). Many environmental challenges such as low dose amphetamine, white noise, and human handling that increase wakefulness in normal mice fail to do so in *Dbh -/-* mice (Hunsley & Palmiter 2003, Hunsley & Palmiter 2004), CRISPR-mediated knockout of DBH in the mouse LC attenuates arousal triggered by rat bedding (Yamaguchi et al 2018), and AR antagonists can also reduce wakefulness in control mice (Mitchell & Weinshenker 2010, Porter-Stransky et al 2019).

Sustained arousal is an adaptive response to threats such as predator odor. Normal mice exposed to a new cage with an unscented cotton nestlet will initially explore the novel environment, then shred the material and fall asleep in the nest within 60-90 min (Lustberg et al 2020a). In the current study, we showed that while most control mice shredded a cotton square containing square containing a novel but non-threatening odor (lavender) and fell asleep in the nesting material, none shredded a bobcat urine-soaked nestlet and all remained vigilant for at least 90 min. *Dbh -/-* mice had a paradoxical response to novel and threatening odors. The knockouts fail to shred odorless (Lustberg et al 2020a) or lavender-scented (this study) material, but remarkably built a nest and fell asleep in it only when it contained bobcat urine. Because defensive burying and grooming responses to predator odor in *Dbh -/-* mice did not differ from those to plain water, it was not initially clear whether *Dbh -/-* mice could even detect bobcat urine, but the nestlet result refutes that possibility. Instead, our findings suggest that suppression of DBH produces aberrant responses of rodents to threats. Crucially, NE transmission is generally anxiogenic and mediates aversive responses to stress, whereas DA signaling is a key component of many appetitive/approach behaviors (Iordanova et al 2021). Thus, we speculate that the loss of NE suppresses adaptive fearful/defensive behaviors, while the increased DA reverses the valence of predator odor from aversive to appetitive. Further experiments combing genetic and pharmacological manipulations of catecholamine signaling and circuits with real-time and conditioned place preference/aversion approaches will help test this idea.

4.5 Neuroanatomical substrates of NE and DA action

Results from the c-fos immunostaining experiment provide clues about where NE and DA released from noradrenergic neurons may drive predator odor-induced behaviors (**Fig. 8**). *Dbh -/-* mice had fewer c-fos+ neurons in a field of NET+ fibers in the ACC, dBNST, PAG, and LS, suggesting that NE is required in one or more of these brain regions for predator odor-induced

defensive behaviors (i.e. burying, arousal). These regions were chosen for examination because they are activated by predator odor, are innervated by noradrenergic neurons, and/or are key nodes in defensive behavior circuits (Janitzky et al 2015, Jhang et al 2018, McGregor et al 2004). Because the contribution of these regions and circuits to predator odor responses is vast and has been reviewed elsewhere (Dielenberg & McGregor 2001, Rosen et al 2008, Takahashi 2014), we will only highlight a few relevant findings with particular relevance to our data here. For example, microinfusion of either βAR or α1AR antagonists into the LS reduced defensive burying in the shock probe test, while intra-BNST application of the α2AR agonist clonidine suppressed TMT-induced NE release and fear responses (Fendt et al 2005). Furthermore, BNST inactivation not only suppresses aversive responses to cat urine in rats but increases duration of contact with the odor source, suggesting enhanced affiliative behavior similar to those observed in *Dbh -/-* mice (Xu et al 2012). The PAG is generally implicated in defensive behaviors and fear responses and is of particular interest here because we previously identified a noradrenergic LC-PAG circuit regulating arousal that may be engaged to maintain vigilance under threatening conditions (Porter-Stransky et al 2019). The lack of NE in *Dbh -/-* mice impairs this circuit and leads to reduced arousal, which could explain why the knockouts fall asleep in the presence of bobcat urine. Interestingly, inactivation of the ACC increases predator odor-induced freezing, while activation has the opposite effect (Jhang et al 2018). It is possible that the increased activation of the ACC in our control mice was permissive for active coping (defensive burying) at the expense of passive coping (freezing). We did not observe a high degree of freezing in *Dbh -/-* mice despite the reduced c-fos response, suggesting that other brain regions responsible for grooming dominated in the knockouts.

Two regions, the MeA and LC, had an increased c-fos response in *Dbh -/-* mice. The MeA receives direct and indirect input from the olfactory system, as well as DA neurons in the VTA. Dopaminergic signaling within the MeA mediates social reward and has even been

implicated in human bonding (Atzil et al 2017, Hu et al 2021, Takahashi 2014). D1 receptor-expressing MeA neurons are activated by cat urine, and facilitating VTA dopaminergic transmission or activating D1 MeA GABAergic outputs to the BNST causes animals to display increased approach to a predator odor threat (McGregor et al 2004, Miller et al 2019, Vincenz et al 2017).

These data are consistent with the idea that bobcat urine elicits paradoxical appetitive rather than aversive responses in *Dbh -/-* mice because of ectopic DA release from LC afferents in the MeA. Because α2AR inhibitory autoreceptors limit LC activation, it is likely that the increased c-fos response in the LC of *Dbh -/-* mice is due to their lack of NE and α2AR engagement (Paladini et al 2007).

4.6 Implications for T. gondii infection and translational significance

As a parasite that reproduces in cats as part of its life cycle, *T. gondii* impairs the innate fear of cat odor in rodents, increasing the chance that the parasite reaches its host (Kannan et al 2010). Our results provide additional evidence that the *T. gondii*-induced reduction of DBH may lead to suppression of defensive behaviors that would increase a mouse's chance of escaping a predator. Indeed, the apparent affiliation of *Dbh -/-* mice to feline scents (i.e. sitting on domestic cat fur, nesting in bobcat urine-soaked cotton) may actually attract them to danger and would make them easy prey in the wild.

Consistent with our ~~intriguing~~ finding of increased c-fos in the MeA of *Dbh -/-* mice, reversal of *T. gondii*-induced epigenetic and gene expression signatures in this structure normalized defensive behavior and abolished the gain of attraction to cat odor (Hari Dass & Vyas 2014), further indicating a potential neuroanatomical substrate for the excessive DA in *Dbh -/-* mice. Humans can also be infected with *T. gondii*, and epidemiological studies have indicated

increased risk for schizophrenia, OCD, and ASD in people with latent *T. gondii* infection (Contopoulos-Ioannidis et al 2022, Nayeri et al 2020, Torrey & Yolken 2003, Virus et al 2021). Thus, further study of NE and DA circuitry underlying *T. gondii*-induced behavioral changes in rodents and of catecholaminergic drugs as therapies for disorders with stress-responsive repetitive behaviors are warranted.

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Figure legends

Fig. 1. Effects of DBH knockout on behavioral responses to predator odor. *Dbh* +/- (n=7) and *Dbh* -/- (n=7) mice were exposed to water or bobcat urine for 10 min, and the amount of time engaged in defensive burying (**A**) and grooming (**B**) were assessed. Shown are mean ± SEM. ***p< 0.001, ****p<0.0001.

Fig. 2. Effects of adrenergic drugs on behavioral responses to predator odor in control mice. *Dbh* +/- (n=8) mice were pretreated with saline, the α1AR antagonist prazosin (0.5 mg/kg), the α2AR antagonist atipamezole (1 mg/kg), or the βAR antagonist propranolol (5 mg/kg) and exposed to bobcat urine 30 min later for 10 min. Shown is the mean ± SEM time engaged in defensive burying (**A**-**C**) and grooming (**D**-**F**). *p<0.05.

Fig. 3. Effects of pharmacological DBH inhibition on behavioral responses to predator odor. *Dbh* +/- mice (n=10) were pretreated with saline or the DBH inhibitor nepicastat (100 mg/kg) and exposed to bobcat urine for 10 min, and the amount of time engaged in defensive burying (**A**) and grooming (**B**) were assessed. Shown are the mean ± SEM. *p< 0.05, **p<0.01.

Fig. 4. Effects of dopaminergic drugs on predator odor-induced grooming in *Dbh* -/- mice. *Dbh* -/- mice were pretreated with saline (n=7-13), the non-selective DA receptor antagonist flupenthixol (0.25 mg/kg; n=7), the D1 antagonist SCH-23390 (0.003 mg/kg; n=7), or the D2 antagonist L-741,626 (10 mg/kg; n=8) and exposed to bobcat urine 30 min later for 10 min. Shown is the mean ± SEM time engaged in grooming. **p<0.01.

Fig. 5. Effects of dopaminergic drugs on behavioral responses to predator odor in control mice. *Dbh* +/- mice (n=7) were pretreated with saline or the D1 antagonist SCH-23390

(0.003 mg/kg) and exposed to bobcat urine 30 min later for 10 min. Shown is the mean ± SEM time engaged in defensive burying (**A**) and grooming (**B**). *p<0.05, **p<0.01.

Fig. 6. Effects of DBH knockout on arousal and nestlet shredding during predator odor exposure. *Dbh* +/- and *Dbh* -/- mice were exposed to lavender oil (**A**-**B;** n=7,8) or bobcat urine (**C**-**D**; n=8,9) for 90 min, and the fraction of animals that shredded their cotton nesting material (**A**,**C**) and fell asleep (**B**,**D**) were assessed. *p< 0.05, **p<0.01.

Fig. 7. Regional c-fos responses to predator odor in *Dbh* +/- and *Dbh* -/- mice. *Dbh* +/- (n=8) and *Dbh* -/- (n=9) mice were exposed to bobcat urine for 90 min. Shown are (A) representative images and (B) the mean ± SEM number of c-fos+ (magenta) cells in the locus coeruleus (LC), anterior cingulate cortex (ACC), dorsal bed nucleus of the stria terminalis (dBNST), periaqueductal gray (PAG), paraventricular nucleus of the hypothalamus (PVN), lateral septum (LS), and medial amygdala (MeA). Images are counterstained with the nuclear marker DAPI (blue) and the noradrenergic terminal marker norepinephrine transporter (NET; green). *p<0.05, ***p<0.001, ****p<0.0001.

Fig. 8. Proposed noradrenergic circuit for defensive behavioral responses to predator odors. The PVN hypothalamus is activated by ancient odorant pathways, in turn exciting the LC by releasing CRH (black arrow). The LC projects to a constellation of brain regions implicated in defensive behavior, where it releases NE in DBH-sufficient animals (blue arrows). Loss of NE results in hypoactivity in PAG, ACC, LS, and dBNST (regions without circles). Interestingly, loss of NE did not affect neuronal activity in the PVN (green circle), suggesting this pathway remains intact. Further, NE deficiency resulted in hyperactivity in the LC and the MeA (magenta circles). Image created with Biorender.

Fig. 1

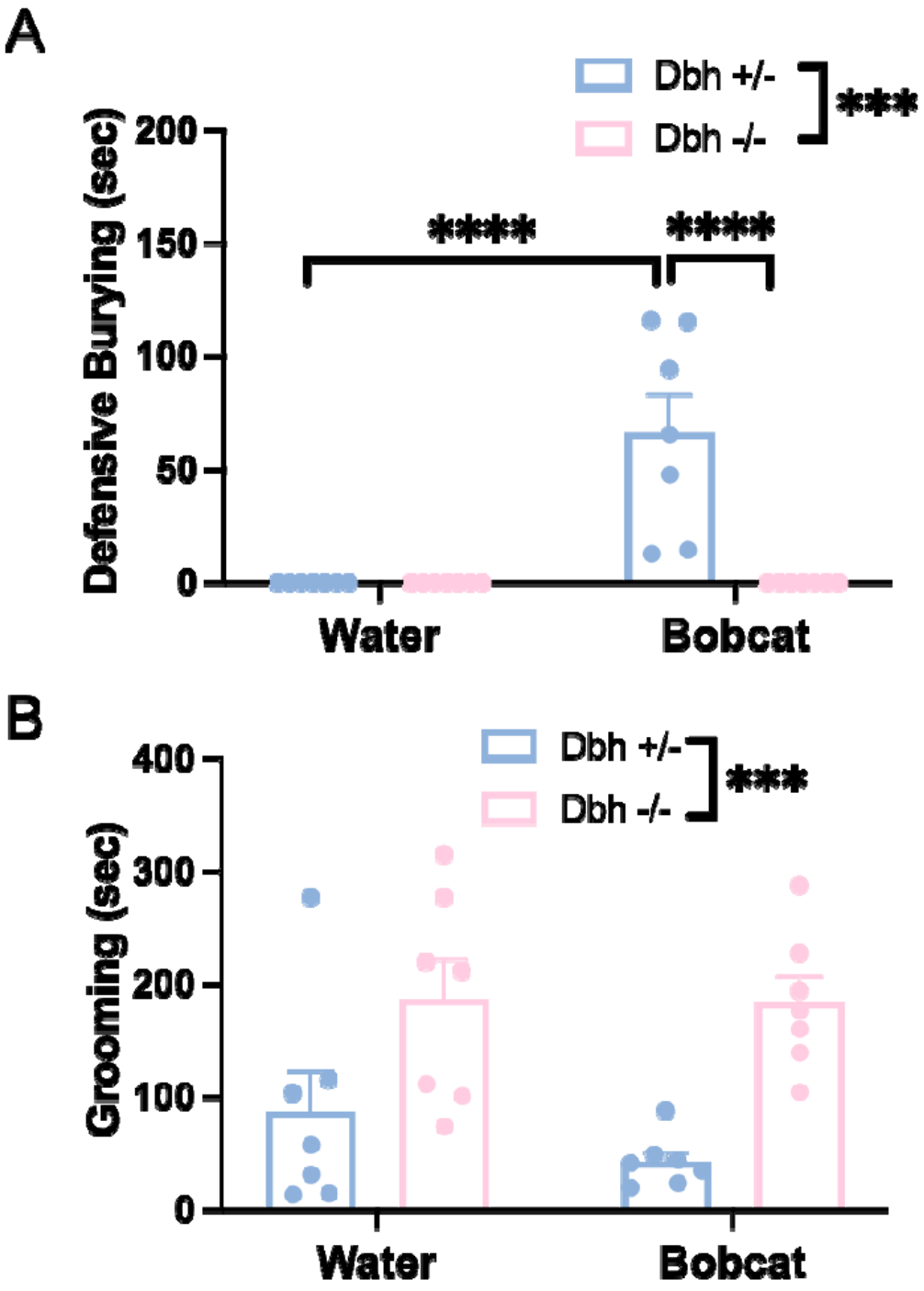

A
Dbh +/-
Dbh -/-
***
****
****
Defensive Burying (sec)
200
150
100
50
0
Water
Bobcat
B
Dbh +/-
Dbh -/-
***
Grooming (sec)
400
300
200
100
0
Water
Bobcat

Fig. 2

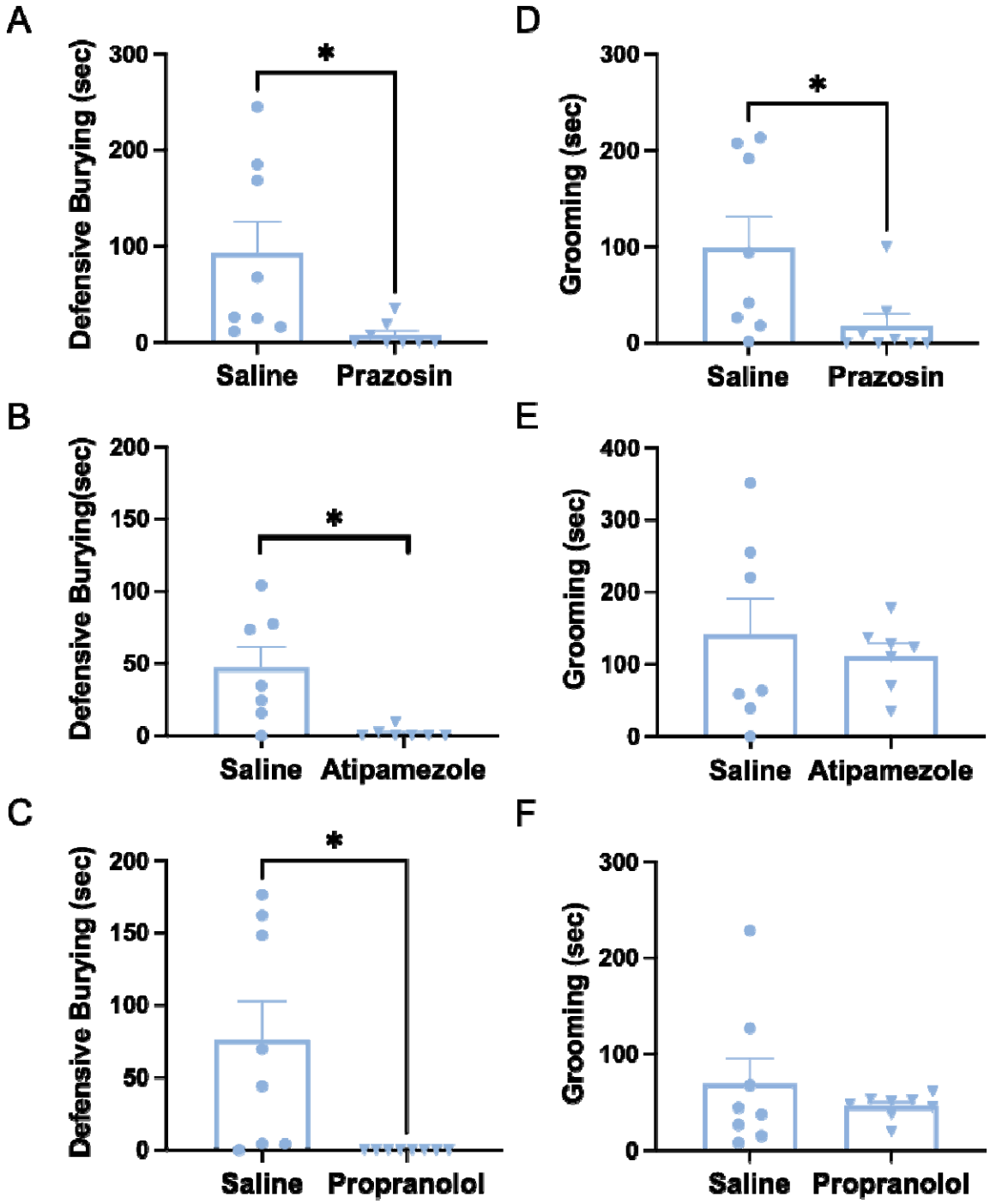

A
Defensive Burying (sec)
300
200
100
0
*
Saline
Prazosin
B
Defensive Burying(sec)
200
150
100
50
0
*
Saline
Atipamezole
C
Defensive Burying (sec)
200
150
100
50
0
*
Saline
Propranolol
D
Grooming (sec)
300
200
100
0
*
Saline
Prazosin
E
Grooming (sec)
400
300
200
100
0
Saline
Atipamezole
F
Grooming (sec)
300
200
100
0
Saline
Propranolol

Fig. 3

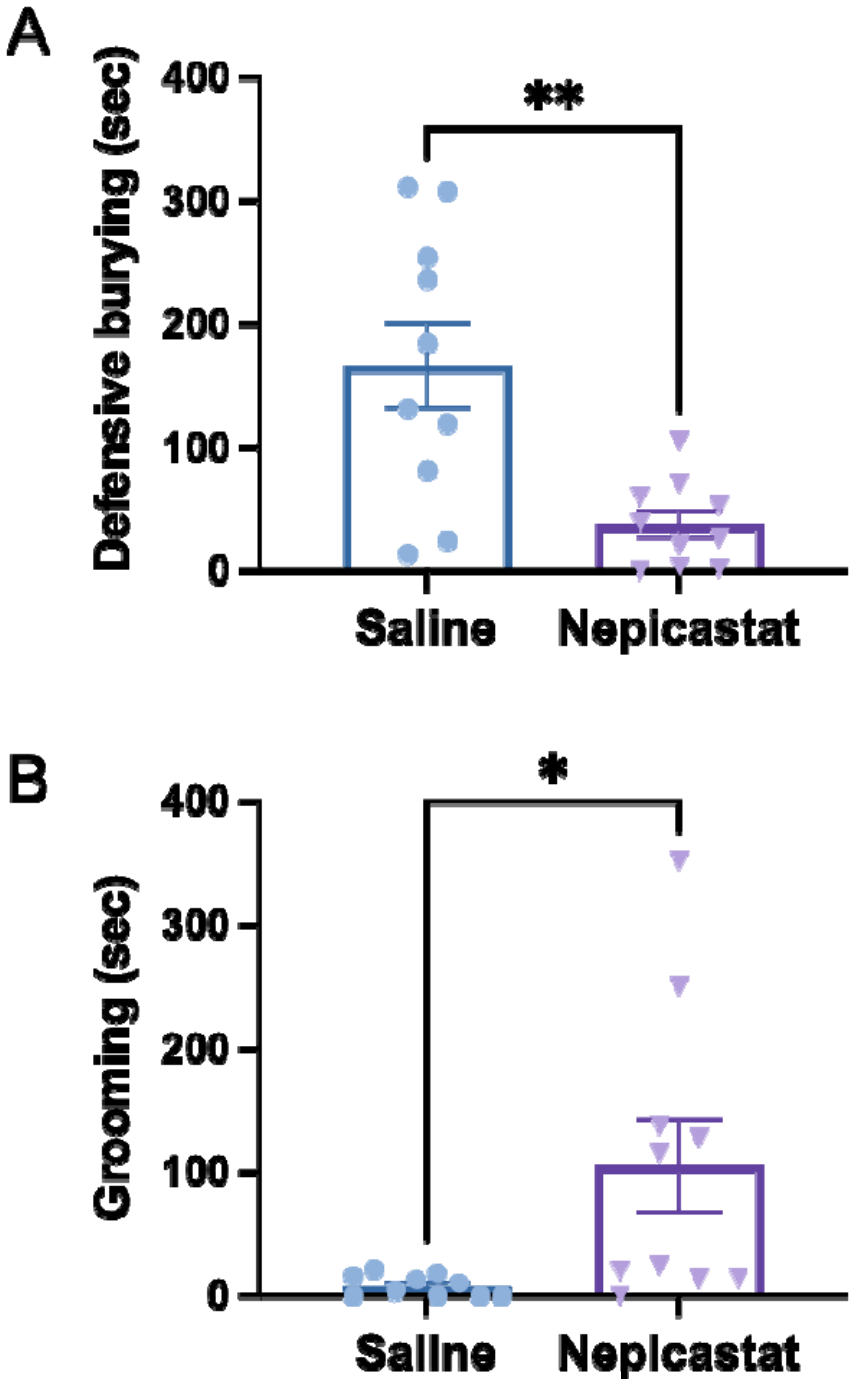

A
Defensive burying (sec)
400
300
200
100
0
**
Saline
Nepicastat
B
Grooming (sec)
400
300
200
100
0
*
Saline
Nepicastat

Fig. 4

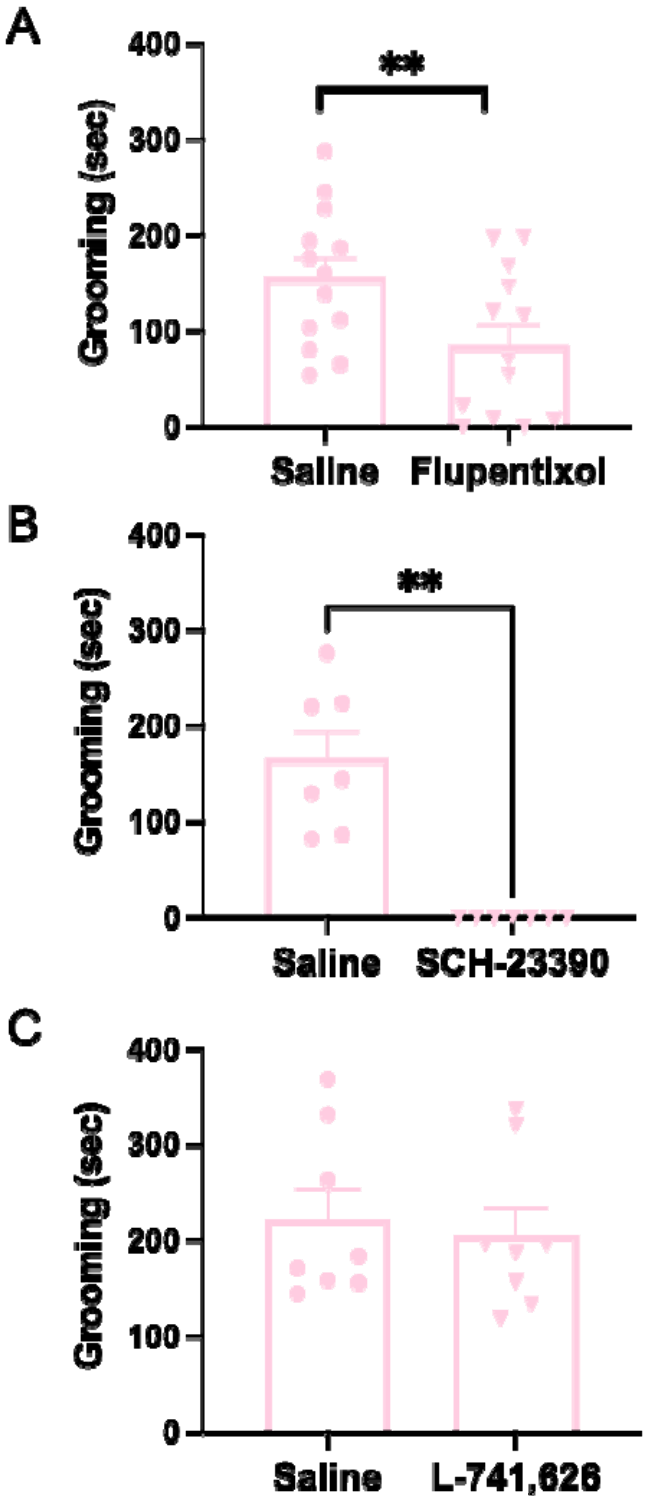

A
400
300
200
100
0
Grooming (sec)
**
Saline
Flupentixol
B
400
300
200
100
0
Grooming (sec)
**
Saline
SCH-23390
C
400
300
200
100
0
Grooming (sec)
Saline
L-741,626

Fig. 5

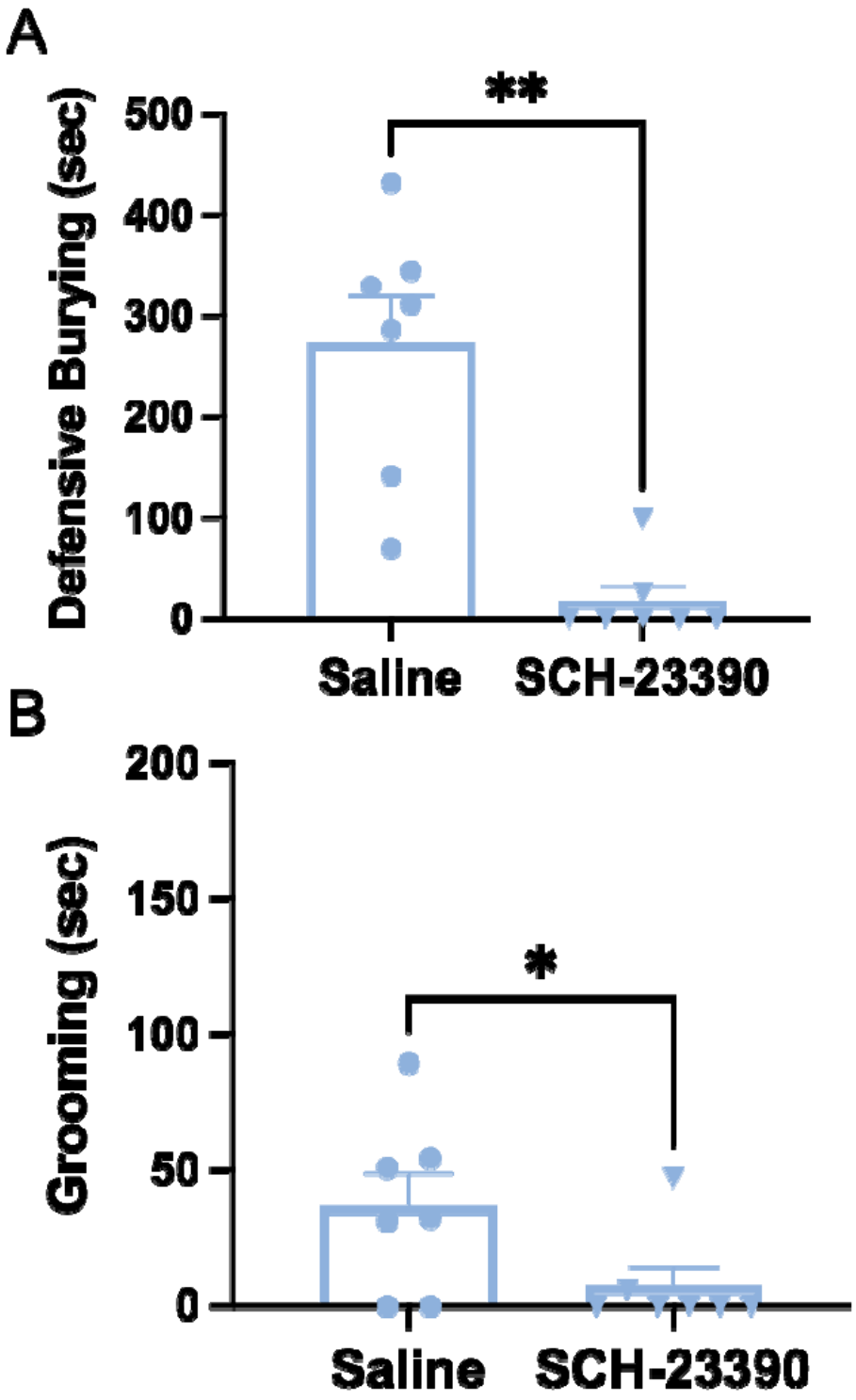

A
Defensive Burying (sec)
500
400
300
200
100
0
**
Saline
SCH-23390
B
Grooming (sec)
200
150
100
50
0
*
Saline
SCH-23390

Fig. 6

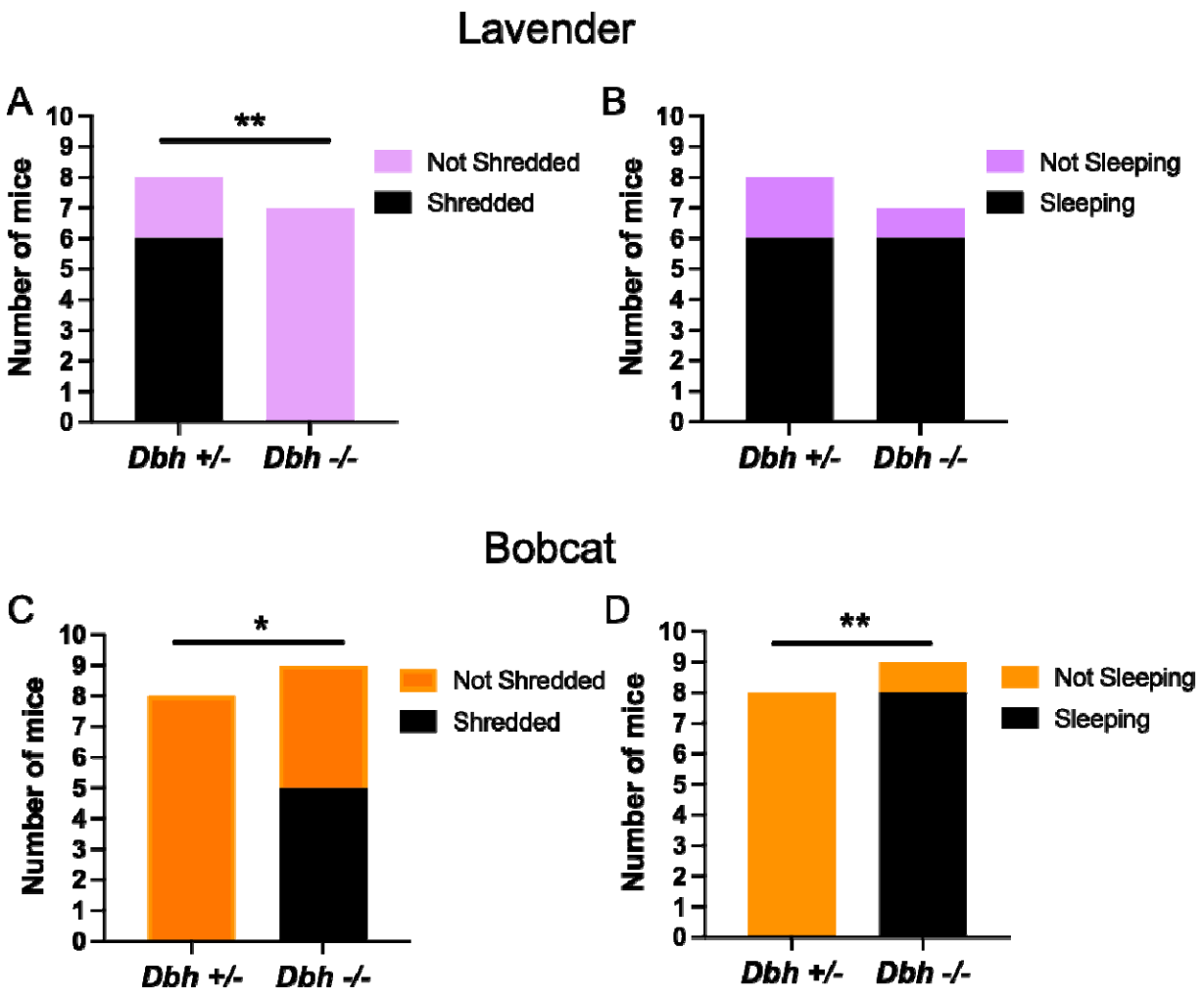
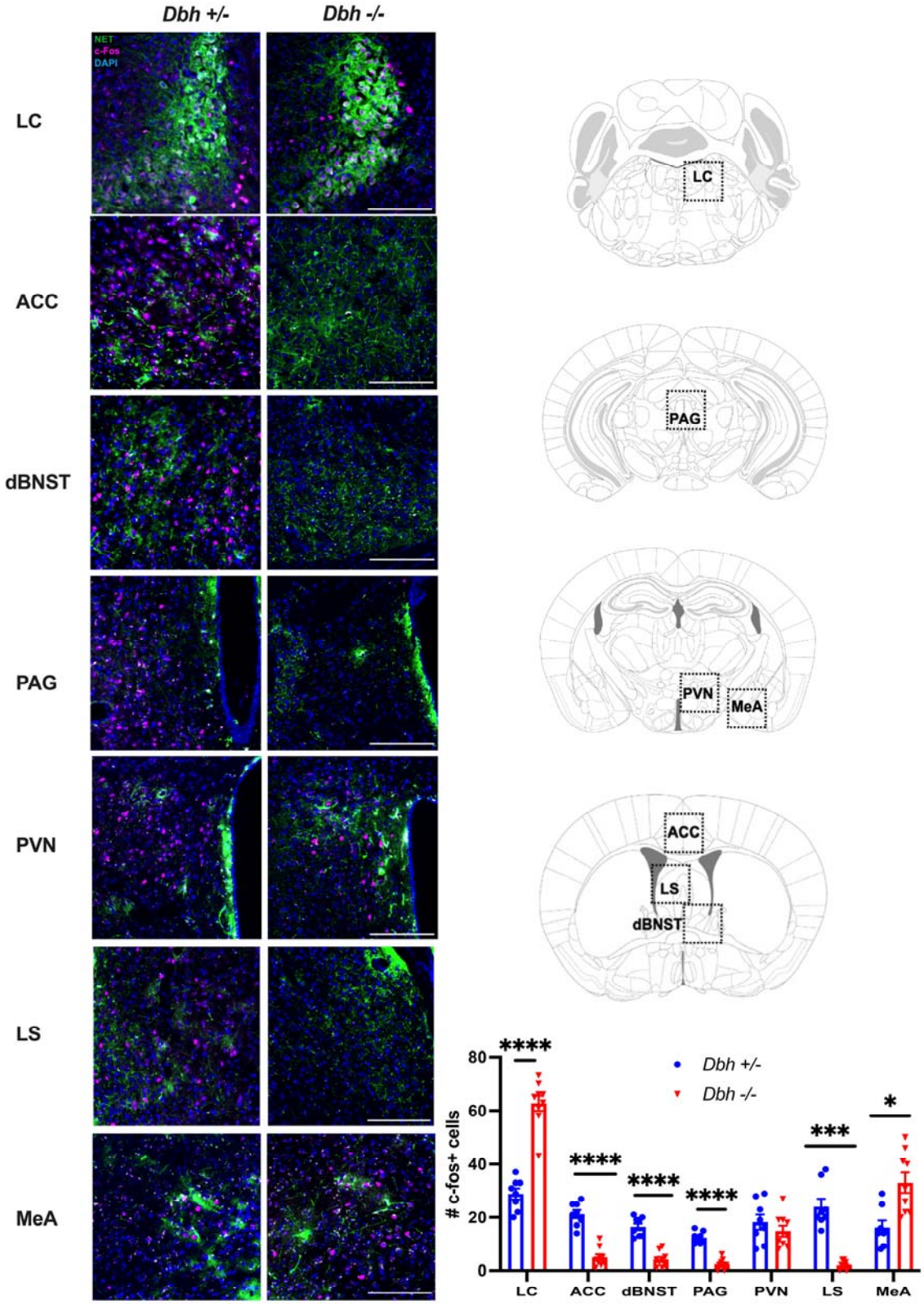

Lavender
A
B
Number of mice
**
Not Shredded
Shredded
Not Sleeping
Sleeping
Dbh +/-
Dbh -/-
Bobcat
C
D
*
**
Not Shredded
Shredded
Not Sleeping
Sleeping
Dbh +/-
Dbh -/-

Fig. 7


Dbh +/-
Dbh -/-
NET
c-Fos
DAPI
LC
ACC
dBNST
PAG
PVN
LS
MeA
LC
PAG
PVN
MeA
ACC
LS
dBNST
Dbh +/-
Dbh -/-
c-fos+ cells
80
60
40
20
0
****
****
****
****
***
*
LC
ACC
dBNST
PAG
PVN
LS
MeA

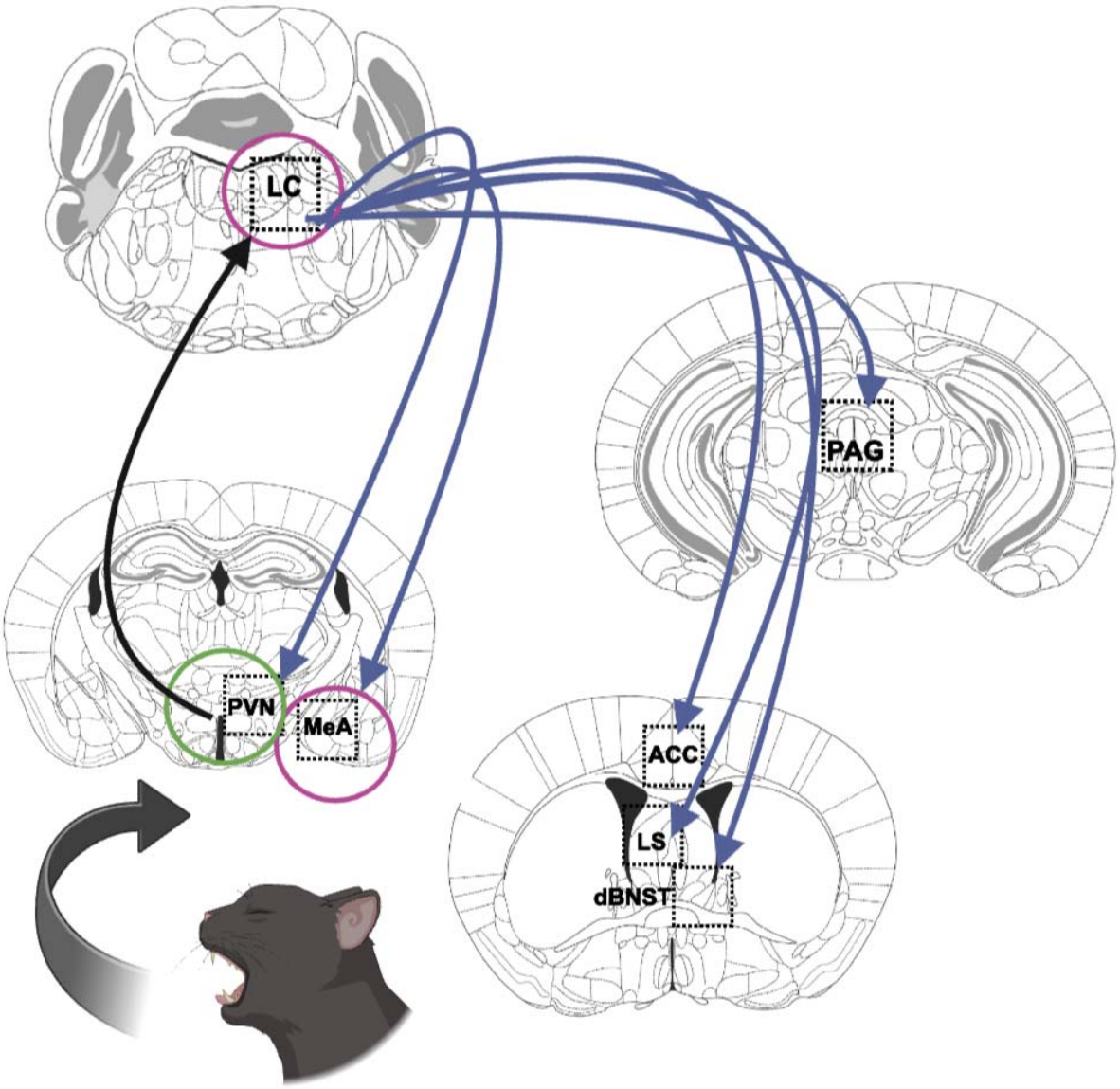



Fig. 8