CRISPR-mediated knockdown of oxytocin receptor in extended amygdala reduces stress-induced social avoidance and vigilance

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ABSTRACT

Oxytocin receptors (OTR) within the extended amygdala and nucleus accumbens have been implicated in modulating social behaviors, particularly following stress. The effects of OTR could be mediated by modulating the activity of pre-synaptic axon terminals or via post-synaptic neurons or glia. Using a viral-mediated CRISPR/Cas9 gene editing system in California mice (*Peromyscus californicus*), we selectively knocked down OTR in the anteromedial bed nucleus of the stria terminalis (BNST) or the nucleus accumbens (NAc) to examine their roles modulating social approach and vigilance behaviors. Knockdown of OTR in the BNST attenuated stress-induced decreases of social approach and increases of social vigilance behaviors in adult female California mice, similar to prior pharmacological studies. These effects were more prominent in the large arena social interaction where mice could control proximity to a social target with a barrier (wire cage). In a small arena interaction test where focal mice freely interacted with target mice, effects of BNST OTR knockdown were muted. This suggests that within the BNST OTR are more important for modulating behavioral responses to more distal stimuli versus more proximal social contexts. In mice with OTR knockdown in the NAc, few behavioral changes were observed which is consistent with previous findings of the importance of presynaptic OTR, which were unaffected by our gene editing strategy, driving social approach behaviors in the NAc. Interestingly, BNST OTR knockdown increased exploratory behavior toward a non-social stimulus after stress, pointing to a potentially broader role for BNST OTR function. Our findings highlight the region- and context-specific functions of OTR in social behavior and the advantages of using a selective gene-editing tool to dissect the neural circuits that influence social and stress-related responses.

INTRODUCTION

Individuals affected by anxiety disorders experience significant distress in social situations that can lead to self-isolation and avoidance behaviors (Abramowitz & Deacon, 2010). Women are twice as likely to develop anxiety disorders compared to men (Kessler et al., 2012). While there are existing pharmaceutical and behavioral therapeutic interventions but, ~40% do not respond to these treatments (Williams & Trainor, 2018). Oxytocin is a neuropeptide that modulates social and emotional behaviors (Gimpl & Fahrenholz, 2001) and has been considered as a potential alternative therapeutic to existing antidepressants (MacDonald & Feifel, 2013 and 2014; Modi and Young., 2012; Gutkowska & Jankowski, 2012; Giovanna et al., 2020; van Zuiden et al., 2017; Schultebraucks et al., 2022; Rashidi et al., 2025). However, the field has struggled to explain findings where intranasal oxytocin sometimes decreases anxiety symptoms but in other cases can increase anxiety and other psychiatric symptoms (Tabak et al., 2022; Schultebraucks et al., 2022). A potential explanation for these results is the social salience hypothesis of oxytocin (Bartz et al., 2011; Shamay-Tsoory & Abu-Akel, 2016). This proposes that oxytocin can modulate behaviors by enhancing attention to both positive and negative social contexts. Several lines of evidence indicate that oxytocin promotes salience by acting in discrete neural circuits to drive these dynamic responses (Steinman et al., 2019).

In appetitive social contexts, activation of oxytocin receptors (OTR) in the mesolimbic dopamine system plays a key role in promoting social approach behaviors. The nucleus accumbens (NAc) modulates motivational and learning processes (Salgado & Kaplitt, 2015), and activation of OTR within the NAc facilitates social approach (Williams et al., 2020), social learning (Nardou et al., 2019), pair bonding (Liu & Wang, 2003; Rigney et al., 2025) and social novelty seeking (Smith et al., 2017). Activation of OTR in the ventral tegmental area (VTA) has also been found to modulate social motivation (Borland et al., 2017) and social learning (Hung et al., 2017). It was hypothesized that oxytocin acting in the mesolimbic dopamine system facilitates social salience in aversive social contexts (Shamay-Tsoory & Abu-Akel, 2016). However, most evidence suggests that OTR in the extended amygdala may play a more important role during stressful social contexts. Social defeat stress activates a population of oxytocin neurons in the medioventral bed nucleus of the stria terminalis (BNST) in *Peromyscus californicus* (California mice) (Steinman et al., 2016) and C57Bl/6J mice (Nasanbuyan et al., 2018). The BNST modulates anxiety-related and social behaviors (Lebow & Chen, 2016) and several subregions have strong molecular (Gegenhuber et al., 2022) and anatomical (Campi et al., 2013; Allen & Gorski, 1990) sex differences. Antisense knockdown of oxytocin in the BNST prevented defeat stress-induced social avoidance and social vigilance in female California mice (Duque-Wilckens et al., 2020). These BNST oxytocin neurons project to the anteromedial BNST (BNSTam) where activation of OTR promotes avoidance and vigilance (Duque-Wilckens et al., 2018; Luo et al., 2022). Oxytocin can also promote aversive responses in non-social contexts, as oxytocin in the dorsolateral BNST (BNSTdl) enhanced fear-potentiated startle (FPS) response in rats (Moaddab & Dabrowska, 2017). Together these data suggest that distinct neural circuits mediate the effects of oxytocin in appetitive and aversive social contexts (Steinman et al., 2019).

All of the studies reviewed above relied on pharmacological manipulations to target OTR function. Pharmacological approaches target both locally expressed post-synaptic receptors and pre-synaptic receptors.

Importantly, at least some behavioral effects of OTR can be mediated by pre-synaptic receptors. Dolen and colleagues showed that the behavioral effects of OTR antagonists infused into the NAc were mediated by OTR expressed on presynaptic terminals originating from the dorsal raphe nucleus (Dölen et al., 2013; Nardou et al., 2023). These studies were performed in C57Bl/6J mice and utilized transgenic mouse lines to specifically target different populations of OTR. The CRISPR/Cas9 gene-editing systems provides an exciting opportunity to perform similar manipulations in species for which transgenic lines are not available. A viral-mediated CRISPR/Cas9-based tool was developed to target OTR expression across a variety of rodent species used as models to study social behavior (Boender et al., 2023). When a guide RNA targeting the *Oxtr* gene was expressed with Cas9, OTR binding was reduced across six different rodent species, including California mice. We used this gene editing system to determine whether locally expressed OTR in the BNST or NAc modulate social approach and social vigilance in female California mice. This viral construct was recently used in a male spiny mouse model in the NAc, which reduced huddling behaviors with strangers as well as some consumption behaviors identifying a role for OTRs in non-reproductive contexts (Fricker et al., 2025).

California mice are monogamous rodents in which paired males and females defend joint territories. The high levels of aggression in female California mice make this species ideal for studying the effects of social defeat stress (SDS) in both males and females without the use of CD1 aggressive male mice generally used in C57Bl/6J models (Steinman & Trainor, 2017; Lake & Trainor, 2024). In this study we assessed the impact of *Oxtr* knockdown on behavior before and after stress using two behavioral assays. Focal mice were tested in a large arena with an unfamiliar female stress-naïve target mouse confined to a small wire cage, which allows for precise measurements of social approach and social vigilance. Social vigilance is a risk assessment behavior in which an individual avoids but attends to a social context (Walker et al., 2023; Wright et al., 2020). Focal mice were also tested in a smaller arena with freely moving stress-naïve and aggressive SDS target mice. This test is ideal for the observation of affiliative and defensive behaviors in different contexts. The primary goal for this study was to determine whether the behavioral effects of OTR are mediated by local *Oxtr* expression within the BNST or NAc. Previous studies showed that social approach and social vigilance are modulated by OTR acting within complementary networks that include the BNST and NAc. Here we use CRISPR-mediated OTR knockdown to determine the effects of locally expressed OTR on social behavior before and after social defeat stress. We used two behavioral assays to distinguish specific roles of OTR in proximal versus distal social interactions, further shedding light on the dynamics of how oxytocin modulates behavior.

MATERIALS AND METHODS

Animals. All studies were conducted using California mice at the University of California, Davis (UCD). Protocols were approved by the UCD Institutional Animal Care and Use Committee (IACUC). Adult (PND 90) female California mice from our laboratory colony were utilized. Mice were housed with same-sex cagemates after weaning in groups of 2 to 4 within clear polypropylene cages. Cages were set up with Sani-Chip bedding (Harlan Laboratories, Indianapolis, IN, USA) and 2 nestlets per cage (Ancare, Bellmore, NY, USA). Mice were

kept on a 16L:8D light cycle and had *ad libitum* access to food and water. Viral infusion/surgery were conducted in light cycle, while behavioral testing and stress exposure were performed during the dark cycle.

Intracranial surgeries. Anesthesia was induced via exposure to 5% isoflurane mixture with oxygen and then maintained at 2-3.5%. With a stereotaxic apparatus, mice were bilaterally injected 300nL with either the knockdown *Oxtr* virus or a scramble sequence virus. Mice were randomly assigned to treatment group (CTRL/KD) and region group (NAc/BNST). Coordinates were calculated using the online California mouse brain atlas at brainmaps.org. For NAc we used, AP: +0.51, ML: ±1.5, DV: -6.0. For BNST we used, AP: + 0.29, ML: ± 1.1, DV: -5.85. Mice assigned to *Oxtr* knockdown received viral cocktail consisting of an AAV9 vector expressing Streptococcus pyogenes Cas9 under the RSV promoter (AAV9-RSV-spCas9), and a second AAV9 vector encoding a guide RNA targeting Oxtr (AAV9-U6-gRNA(ΔOXTR.1)-CMV-eGFP, 5′-GGTGCTTCATGAAAAAGAAG-3′), as described in Boender et al., 2023.This guide RNA targets a sequence of the *Oxtr* gene that is evolutionarily conserved across several rodent species. Control mice received a cocktail consisting of the Cas9 vector and a scrambled gRNA sequence. All mice are given a carprofen injection at the beginning of surgery (1.45 mg/kg dosage) and then 3 daily doses during recovery. Skin staples were used to close the skin, and were removed 7 days post-surgery.

Behavioral timeline: Three weeks after surgery, all mice were tested in a Large Arena Social Interaction test. One day later mice were tested in a small arena social interaction test. In this test target mice are not caged, allowing direct contact with focal mice. Three days after these behavioral tests all focal mice underwent three days of social defeat on consecutive days. One week later both the large and small arena social interaction tests were repeated as described above. Mice were never tested with the same Target or aggressive Target mouse (except for post-stress interaction with aggressive Target) in the large or small arena tests.

Large arena test. This test consisted of 3 phases: Open Field, Acclimation, and Interaction. Focal mice were placed in the large rectangular arena, made opaque white acrylic (89 × 63 × 60 cm) Each phase lasted 3 min and was recorded via AnyMaze software. In the Open Field phase, mice were allowed to explore the empty arena. Time spent in the center of the arena and total distance traveled was recorded. This was followed by the Acclimation phase where an empty wire cage was placed on one end of the arena. Finally, in the last phase a “Target” unfamiliar stress-naïve female mouse was placed inside the wire cage. This test was repeated 1 week after Social Defeat Stress.

Small Arena test. The small arena test was conducted in an acrylic chamber (51 × 25.4 × 76 cm) connected to a small chamber with a sliding door for introducing target mice. In the Open Field phase, focal mice entered through the attached chamber (13 × 10 × 18 cm) and explored the empty arena for 3 minutes. We then assessed interactions with target mice with (aggressive) or without (naïve) prior experience winning aggressive encounters (Wright et al., 2023; Luo et al., 2024). First a female target mouse was introduced to the arena and interactions with the focal mouse were recorded for 3 minutes. The target mouse was removed and then an

aggressive female target mouse was introduced to the arena for final 3 minutes. All videos were scored using BORIS by a single trained observer without knowledge of the treatment group status of the focal mouse. Here affiliative (nose-to-nose sniffing, anogenital sniffing, and bodily sniffing), coping (auto-grooming and flipping), defensive (lunging), and behaviors were recorded. Flipping behavior is a unique behavior in the California mouse species that we identify as a coping behavior.

Social defeat stress. All mice were assigned to receive social defeat stress and controls (with no viral manipulation) were not utilized due to pre-stress and post-stress behavioral testing. Mice undergo 3 consecutive days of social defeat stress as previously described (Greenberg et al., 2014). Throughout the 3 days, mice are placed into the home cage of an aggressive resident same-sex mouse. These mice are our Vasectomy Paired (VP) mice. Due to the similar level of aggression both male and female California mice, same-sex defeat can be easily conducted. Defense of territory is higher in mated pairs, however, to avoid unexpected litters males are vasectomized. This allows for VP mice to be purely dedicated to social defeat testing. Each episode of this defeat lasts 7 minutes or until the intruder/experimental mouse is attacked/defeated a total of 7 times, whichever comes first. After testing, experimental mice are immediately placed back in their homecage. Post-stress testing will not be conducted until a week after the last session. This 3-day protocol ensures our mice have reduced risk to physical injury and its substantial to induce stress-induced behaviors for up to 10 weeks after social defeat (Steinman et al., 2016).

Autoradiography. After post-stress behavior testing mice were anesthetized with isoflurane, decapitated and brains were removed to be flash frozen on dry ice. Brains were then cut with a cryostat at 20µm and mounted directly on frost plus slides. Slides were stored at -80°C until ready for autoradiography processing to determine *Oxtr* binding using an established protocol for rodent brain tissue (as in Hartman et al., 2018). Briefly, slides were removed from -80℃ storage, thawed at room temperature for 1 h, fixed in a light 0.1% paraformaldehyde (pH 7.4) solution, and washed twice in Tris Buffer, before being incubated in the tracer buffer at room temperature for 60 min. Tracer buffer consisted of 50 mM Tris-HCl buffer (7.4 pH) with 10 mM $MgCl_2$, 0.1% bovine serum albumin, and 50 pM of radiotracer. For OTR binding, [125I]-ornithine vasotocin analog [^{125}I-OVTA] [vasotocin, d(CH_2)5[Tyr(Me)2, Thr4, Orn8, (125I)Tyr9-NH2]; 2200 Ci/mmol] was used (Revvity, Waltham, MA, USA). Following the incubation period, unbound radioligand was removed by 4 washes in 50 mM Tris buffer and 2% $MgCl_2$, pH 7.4, and then dipped in dH_20 and air dried.

The slides were dried overnight and exposed to Cytiva Amersha, Hyperfilm (Cytiva Life Sciences, Washington, DC, USA) for 4 days with a set of ^{125}I standard microscales (American Radiolabeled Chemicals, St. Louis, MO, USA). We then used MCID Core Digital Densitometry system (Cambridge, UK) to quantify *Oxtr* binding in each target brain. For each specimen, optical binding density (OBD) values were calculated for each region of interest (ROI), as well as one background area where no binding was detected. Three separate measurements for each ROI were taken per specimen and averaged. For each specimen, the average background binding value was subtracted from each average ROI measurement to yield normalized OBDs

across specimens. There were 2 samples that were damaged in the slide processing and treatment designation for knockdown was determined with GFP photomicrographs of coronal sections of NAc and BNST.

Statistical analyses: Autoradiography and behavioral data were analyzed using one-way ANOVA with planned comparisons to compare treatment groups with controls. Social vigilance data were square root transformed to correct for heterogeneous variation between groups.

RESULTS

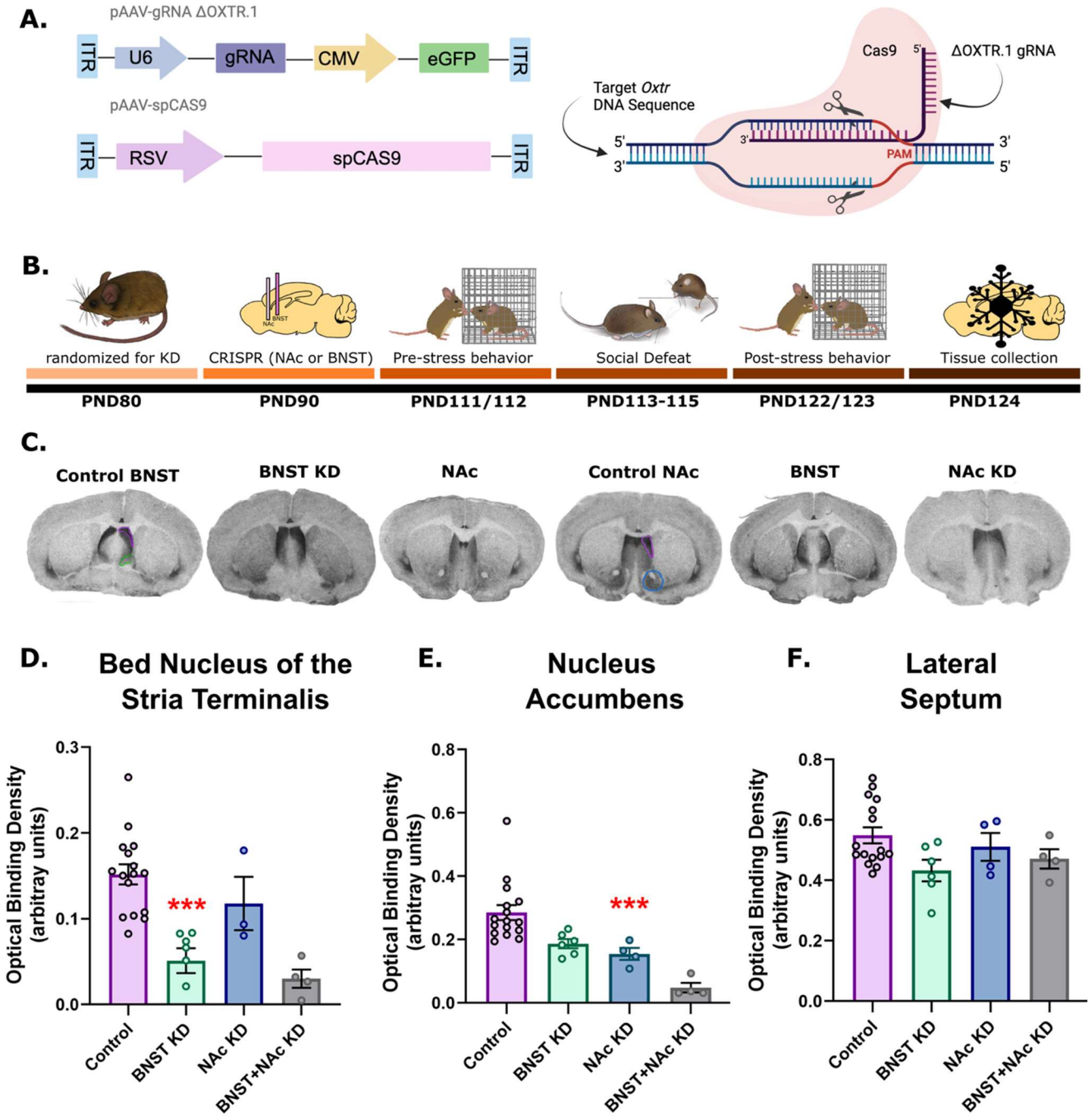

Figure 1: The CRISPR/Cas9 genome-editing system can selectively inhibit *Oxtr* expression in female *Peromyscus californicus* in the extended amygdala and nucleus accumbens. (A) Schematic of AAV plasmids and CRISPR mechanism of action. (B) Experimental timeline (C) Representative images of *Oxtr* manipulation in the BNST and NAc with OTR autoradiography. Control panels show regions quantified for NAc (blue), BNST (green) and LS (purple). (D) Control female mice compared to CRISPR-based knockdown of BNST and other treatment groups. Controls are significantly higher than the BNST KD group (P= <0.001). (E) Control female mice compared to CRISPR-based knockdown of NAc and other treatment groups. Controls are significantly higher from the NAc KD group (P<0.001). (G) Controls compared to all groups for density comparison in "control" region, the lateral septum. *** P < 0.0001 control vs. knockdown.

OTR Knockdown in the BNSTam and NAc in Female California Mice. To determine how locally expressed OTR modulates behavior, we performed CRISPR based knockdown in either the BNSTam or NAc using the CRISPR/Ca9 gene-editing system to specifically target OTR expression (Fig 1A). After behavior testing (Fig 1B) we then used autoradiography to determine the extent of OTR knockdown (Fig. 1C). Autoradiography analysis showed that the *Oxtr* CRISPR construct was effective in knocking down OTR binding in the BNSTam ($F_{3,26}$ = 13.69, p<0.001) and NAc ($F_{3,26}$= 12.18, p<0.001). Mice assigned to BNST knockdown had reduced OTR binding in BNST (Fig. 1D, planned comparison, P<0.001, Cohen's d =2.36) but not NAc (Fig. 1E). In contrast mice assigned to NAc knockdown had reduced binding in NAc (Fig. 1E, planned comparison p<0.001, d=0.48) but not BNST (Fig 1D). We noted that in some animals binding was reduced in both the BNST and NAc, so these animals were included as a separate group. There were no differences in OTR binding in the LS (Fig. 1F), indicating the manipulation was site-specific.

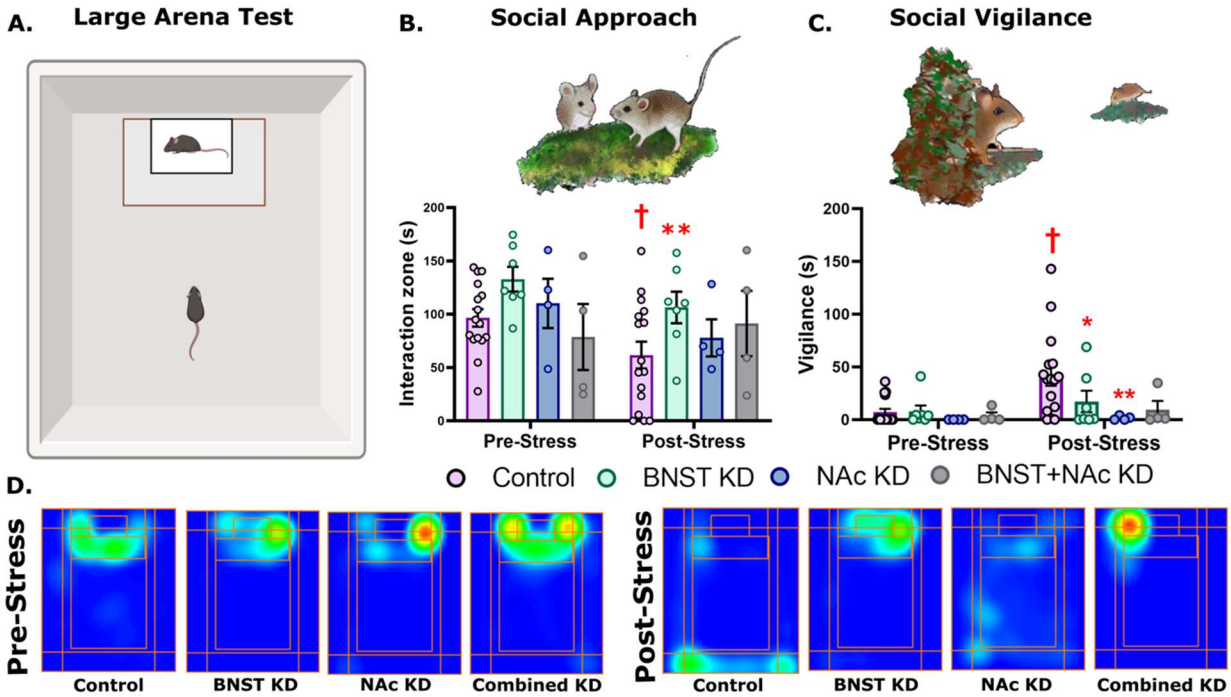

Figure 2: Oxytocin receptor knockdown increases social approach and decreases vigilance after stress in the large arena test. (A) Schematic illustration of the Large Arena Social Interaction test. (B) Effects of OTR knockdown on social approach before and after social defeat stress (BNST KD n= 7, NAc KD n= 4, BNST + NAc KD, n= 4, Control n= 16). (C) Effects of OTR knockdown on social vigilance before and after social defeat stress (E) Representative heatmaps for the interaction phase showing reduced social approach determined by time spent in the interaction zone in controls but not other treatment groups. * P < 0.05, ** P < 0.01, † paired significant comparison. Drawings by Dr. Natalia Duque-Wilckens.

OTR Knockdown within the BNSTam Prevents Stress-Induced Changes in Social Approach and Vigilance in the Large Arena. Three weeks after bilateral CRISPR injections, adult female California mice were tested in the large arena test to measure baseline behaviors before and after social defeat stress (Fig. 2A). In pre-stress observations there were no differences in social approach (Fig. 2B) or vigilance (Fig. 2C). After stress, there was evidence that OTR knockdown affected social approach ($F_{3,27}$ = 2.96, p = 0.05) and vigilance ($F_{3,27}$ = 3.50, p = 0.03). Compared to controls, BNST knockdown mice had significantly higher levels of social approach (Fig. 2B, p <0.01, d= 0.37) and lower levels of vigilance (Fig. 2C, p<0.05, d= 0.73 after stress). Mice with NAc knockdown did not differ from controls in social approach but did show lower vigilance (p=0.01, d=1.44). Mice with knockdown in both NAc and BNST did not differ from controls.

During the acclimation phase when the target mouse was absent, there were no difference in approach to the empty cage before stress (Fig. 3D) but there were effects of knockdown after stress ($F_{3,27}$=4.3, p=0.01). Mice with BNST KD had higher levels of approach to the empty cage compared to control (p<0.01, d=1.31) whereas NAc KD and BNST+NAc KD mice did not differ from controls. There were no differences in time spent in the center of the arena or total distance in the open field phase (Fig 3B and C).

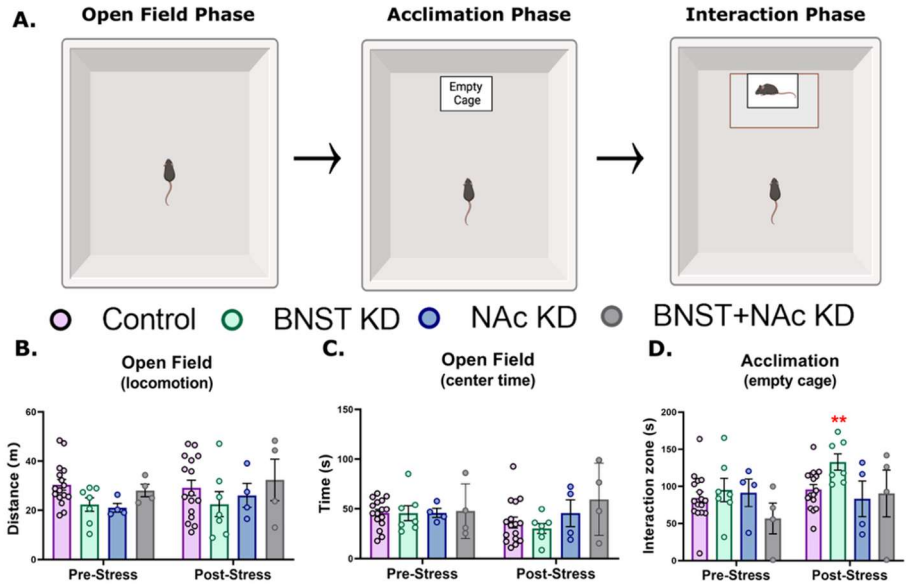

Figure 3: Large arena test open field and acclimation phase data. (A) Schematic illustration of the large arena test and the 3 phases: Open Field, Acclimation, and Interaction. (B) Open field locomotion activity for all treatment groups before and after stress (C) Open field center time plotted for all treatment groups before and

after stress (D) Acclimation interaction time with an empty cage for all treatment groups before and after stress.** P < 0.01

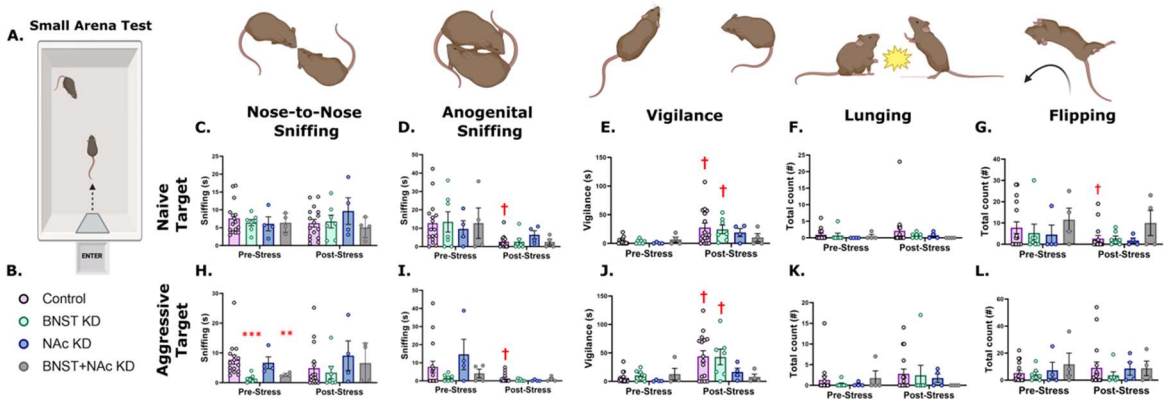

Figure 4: Small arena test social behaviors before and after stress (A) Schematic illustration for the small arena test. Legend for treatment groups. Nose-to-nose sniffing (C), anogenital sniffing (D), social vigilance (E), lunging (F), and flipping (G) were scored with non-aggressive naïve target mice and then aggressive target mice. Tests were conducted once before social defeat and once after social defeat.

OTR Knockdown Does Not Blunt Effects of Stress in the Small Arena. There were no differences in more affiliative nose-to-nose sniffing with naïve target mice (Fig. 4C) but differences were observed pre-stress with aggressive target mice (Fig. 4H, $F_{3,27}$ =17.84, p=5e-04). Lower nose-to-nose sniffing with aggressive target mice was observed in BNST (p<0.001, d=1.48) and BNST+NAc KD (p<0.01, d=1.21). There were no effects of OTR knockdown on more assertive anogenital sniffing data before stress although anogenital sniffing was significantly reduced post-stress in controls with the naïve target mice (Fig. 4D, paired-t_{15}=3.42, p<0.05, d=1.14). A similar effect of stress on anogenital sniffing was observed post-stress with aggressive target mice (Fig 4I, t_{15}=2.16, p<0.05, d=0.70). There were also no treatment effects on vigilance before or after stress (Fig. 4E and 4J). After stress, vigilance directed towards naïve target mice was increased in control (t_{15}=3.13, p<0.05, d=1.07) and BNST KD (t_6=3.05, p<0.05, d=1.51) mice compared to pre-stress observations. Similar results were observed with vigilance directed towards aggressive target mice, as stress increased vigilance in was increased in control (t_{15}=4.09, p<0.05, d=1.33) and BNST KD (t_6=2.37, p<0.05, d=1.43) mice. No differences were observed in lunging behaviors (Fig. 4F, 4K). There were no effects of OTR knockdown on flipping behavior (Fig. 4G, 4L) although reduced flipping was observed post-stress in control animals interacting with naïve target mice (t_{15}=2.26, p<0.05, d=0.59).

DISCUSSION

Using a CRISPR/Cas9 approach to manipulate OTR expression in California mice, we show that receptors expressed locally within the BNST are necessary for the expression of stress-induced decreases in social approach and increases in social vigilance in a large arena testing. These results mirrored prior effects of OTR antagonists in the BNST that increased social approach and decreased vigilance in stressed females (Duque-Wilckens et al., 2018). Similarly, OTR agonists infused into BNST decrease social approach and increase vigilance in stress naïve male and female California (Duque-Wilckens et al. 2020; Luo et al., 2022). Pharmacological manipulations can be mediated by pre- or post-synaptic receptors, but our viral manipulations targeted *Oxtr* expressed locally within the BNST or NAc. Thus, the behavioral effects we observed in large arena tests cannot be mediated by OTR derived from axon terminals originating outside of the BNST. Intriguingly OTR knockdown in the NAc had no effects on social approach in the large arena, even though previous results showed that OTR antagonists infused into NAc reduced social approach in males and females (Williams et al., 2020). While there is some evidence that post-synaptic OTR in the NAc can modulate social behaviors (Borie et al. 2022), our current results align more closely with prior work highlighting the importance of pre-synaptic OTR within the NAc in promoting social learning and approach (Dolen et al., 2013). When focal mice were in close proximity to target mice in the small arena, effects of *Oxtr* knockdown were more limited. This suggests that the behavioral effects of OTR in the BNST are modulated by proximity to a social stimulus.

Behavioral effects of locally expressed OTR in BNST

Sequencing analyses in C57Bl6/J mice showed that the BNST contains about 40 different cell types that express *Oxtr,* the majority of which are GABAergic (Luo et al., 2022). In the BNSTam, pharmacological activation of OTR drives social avoidance and vigilance behaviors in both male and female California mice (Duque- Wilckens et al., 2018 and 2020; Luo et al., 2022). Curiously, the effects of OTR knockdown in BNSTam were more limited when focal mice could freely interact with target mice in the small arena social interaction test. Knockdown of OTR in the BNST reduced nose-to-nose sniffing of aggressive target mice before stress, but did not affect any of the other behaviors we observed. Lesion studies suggested that the BNST responded preferentially to more diffuse or remote threats (Davis et al., 2010). However, more recent work indicates that the BNST can respond to more immediate threats (Shackman & Fox, 2016). For example, a calcium imaging study in California mice showed that social threats increased neural activity within medioventral BNST (BNSTmv, Wright et al., 2023). Although the BNSTmv is distinct from the BNSTam, it contains stress-sensitive oxytocin neurons (Steinman et al., 2016) that project to the BNSTam (Duque-Wilckens et al., 2020). Together these results suggest that while the BNST can respond to proximal social threats, the behavioral responses appear to be less dependent on OTR. Stress can also induce long-term changes in BNST function. Social defeat increases ΔFosB (da Costa et al., 2023) and brain derived neurotrophic factor (Greenberg et al., 2014) expression in male Swiss-Webster mice and female California mice respectively. These molecules play an important role in modulating long-term plasticity in neural signaling. To further elucidate the functional specificity of OTR within BNST subregions, findings from non-

social contexts can provide important insights into how OTR signaling influences distinct behavioral responses in rodent models.

In non-social contexts, activation of OTR in the dorsolateral BNST (BNSTdl) has context dependent effects on behavior. Oxytocin receptors in BNSTdl enhances the acquisition of cued fear responses (Martinon et al., 2019) but not non-cued fear potentiated startle fear-potentiated startle responses in rats (Moaddab & Dabrowska., 2017). These behavioral effects of OTR in BNST may be due to post-synaptic OTR; patch-clamp recordings from BNST slices of male rats showed that OTR agonists increased the intrinsic excitability of Type I interneurons, suggesting a post-synaptic mechanism of action (Franesconi et al., 2021). These interneurons were found to inhibit Type II BNST GABAergic neurons that project to the central amygdala. While these data point towards the importance of post-synaptic OTR within the BNST, our observed behavioral effects of OTR knockdown could be mediated by OTR on neurons or glial cells. In our study the *Cas9* expressing virus used a non-specific promoter, meaning that any cell type expressing *Oxtr* in the BNST was targeted. Thus, while we can conclude that locally expressed OTR in BNST drive stress-induced changes in social approach and vigilance, future studies are needed to determine whether these effects are driven by neurons or other cell types such as glia. Behavioral effects of OTR in glial cells have been observed. In the lateral central nucleus of the amygdala (CeL), of which 70% of *Oxtr* expressing cells are neurons while 18% are astrocytes (Wahis et al., 2021). Using a neuropathic pain model, OTR agonists reduced anxiety-like behavior in the elevated plus maze and promoted conditioned place preference. However, when *Oxtr* was ablated in astrocytes, behavioral effects of oxytocin in the CeL were lost. This is a critical finding that highlights how a relatively small number of cells could exert significant behavioral effects.

Curiously, OTR knockdown in the BNST also increased approach to an empty wire cage after stress exposure. In California mice, social defeat stress generally does not affect approach to the empty wire cage. However, when unstressed male or female California mice were treated with an infusion of 1 ng of oxytocin into the anteromedial BNST approach to an empty cage was reduced (Duque-Wilckens & Trainor unpublished data). A similar but non-statistically significant effect was observed following an infusion of OTR agonist carbetocin in the anteromedial BNST (Luo et al., 2022). This suggests that OTR activation can affect behavior outside of social contexts. Oxytocin receptor could also interact with other neuropeptide signaling systems in the BNST. For example, pituitary adenylate cyclase-activating polypeptide (PACAP) activates PAC1 receptors in the BNST to modulate stress-induced behaviors (Boucher et al., 2021). Adult male rats exposed to chronic variable stress had showed higher levels of approach to a novel object if treated with a PAC1 antagonist in the dorsolateral BNST (Roman et al., 2014). In cultured hypothalamic cells, PACAP can facilitate oxytocin release (Lutz-Bucher & Koch, 1996). Together, these findings suggest that oxytocin could interact with PACAP signaling in the BNST to modulate behavior in non-social contexts.

Behavioral effects of locally expressed OTR in NAc

Previous studies in California mice suggested that locally expressed OTR in the NAc might modulate social approach. Social defeat stress decreases OTR binding (Duque-Wilckens et al., 2018) and *Oxtr* mRNA

(Williams et al., 2020) in the NAc of male and female California mice while OTR antagonists infused into the NAc reduced social approach (Williams et al., 2020). However, we observed no effects of OTR knockdown in the NAc on social approach either before or after stress. This suggests that the effects of NAc OTR on social approach may be mediated by pre-synaptic OTR, as has been reported in other species. In juvenile male C57BL/6, presynaptic OTR in the NAc facilitate the formation of social place preferences (Nardou et al., 2019). Similarly, in prairie voles, CRISPR based OTR knockdown in the NAc did not significantly affect social approach during a social interaction test with female prairie voles (Nerio-Morales et al., 2024). However, social investigation was reduced when OTR knockdown was performed in the anterior cingulate cortex or basolateral amygdala. In contrast, OTR knockdown did reduce huddling with unfamiliar conspecifics in adult male spiny mice (Fricker et al., 2025). Spiny mice differ from house mice, California mice, and prairie voles in that this species lives in large groups and expresses low levels of aggression. These results suggest that there may be important species differences in the mechanisms through which OTRs modulate social behavior. It would be interesting to compare *Oxtr* expression in different cell types within the NAc across species. For example in C57Bl6/J, *Oxtr* is expressed in D1 but not D2 medium spiny neurons (Luo et al., 2022) while in prairie voles Oxtr is expressed in both D1 and D2 spiny neurons (Inoue et al., 2022). Overall, these studies suggest that combining pharmacological and genetic manipulations of OTR function can provide complimentary insights into mechanisms of social approach.

CONCLUSIONS

These results demonstrate that locally expressed OTR in the extended amygdala are necessary for stress-induced decreases in social approach and increases in social vigilance in situations where the focal mouse can control its proximity to an unfamiliar target mouse. In contrast, OTR knockdown in the NAc did not have expected effects on social approach and avoidance behaviors. Although the NAc sample size was smaller, these results are consistent with other studies demonstrating the importance of pre-synaptic OTR function in the NAc. Notably, BNST OTR knockdown increased exploratory behavior toward an empty cage after stress, a finding that aligns with work showing that OTR within the BNST can modulate behavioral outputs in non-social contexts. This suggests that neuropeptide signaling within the BNST, including oxytocin and PACAP, may constrain exploratory behaviors after stress and disrupting this signaling can restore approach behaviors even in non-social contexts. This study highlights the utility of CRISPR-based gene editing for dissecting the region-specific roles of OTR and raises questions of both pre- and post-synaptic mechanisms that shape stress-induced behaviors. Future studies could further investigate how distinct cell population within the BNST influence behavioral changes after stress.

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