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Exploring Neuropeptides Implicated in Social Anxiety-related Behaviors

By

PEI LUO
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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in

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in the

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of the

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DAVIS

Approved:

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Brian Trainor, Chair

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Committee in Charge

2023

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Abstract

Social Anxiety Disorder (SAD) is one of the most prevalent and debilitating stress-related affective disorders across nations. Despite its high prevalence, current treatments, such as selective serotonin reuptake inhibitor and cognitive behavioral therapy, only work in about half of the patients. Thus, there is a strong need for novel therapeutics that address the underlying biological mechanisms of SAD. This dissertation explores the roles of neuropeptides oxytocin and hypocretin in social behaviors and stress responses, highlighting their potential as treatment targets for SAD.

Chapter 1 examines the anxiogenic effects of oxytocin receptor (OTR) signaling. We find that OTR-Gq signaling in the bed nucleus of the stria terminalis (BNST) promotes social avoidance and social vigilance behaviors in female and male California mice. Chapter 1 also provides analyses of published BNST and nucleus accumbens (NAc) single cell RNA-seq data and reveals a diverse expression pattern of OTRs across numerous cell types.

Chapter 2 broadens the scope to include the hypocretin (Hcrt) system, typically associated with arousal and wakefulness, but also implicated in stress responses. This chapter presents a comprehensive review of how Hcrt may contribute to individual differences in stress coping strategies, especially in response to social stress. We propose that Hcrt differentially facilitates active and passive coping behaviors in response to social stress by acting in different brain regions and cell types.

Chapter 3 focuses on understanding sex differences in SAD since women are twice as likely to be diagnosed with an anxiety disorder. Experimental findings from the study of California mice demonstrate that the effects of Hcrt on social behaviors are anatomically and sex

specific. In female but not male mice, the Hcrt receptor signaling regulates social approach and vigilance in the NAcshell but not anterodorsalBNST.

Taken together, these chapters provide a more comprehensive understanding of the biological mechanisms contributed to SAD and highlight the importance of considering individual differences in the development of treatment.

Chapter 1:

Oxytocin receptor behavioral effects and cell types in the bed nucleus of the stria terminalis

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Abstract

Oxytocin is a neuropeptide that can produce anxiolytic effects and promote social approach. However, emerging evidence shows that under some conditions, oxytocin can instead induce anxiety-related behaviors. These diverse effects of oxytocin appear to be mediated by circuit-specific actions. Recent data showed that inhibition of oxytocin receptors (OTRs) in the bed nucleus of the stria terminalis (BNST) was sufficient to increase social approach and decrease social vigilance in female California mice (*Peromyscus californicus*) exposed to social defeat stress. As a member of the G-protein coupled receptor family, OTRs can induce distinct downstream pathways by coupling to different G-protein isoforms. We show that infusion of carbetocin, a biased OTR-Gq agonist, in the BNST reduced social approach in both female and male California mice. In both females and males, carbetocin also increased social vigilance. To gain insight into cell types that could be mediating this effect, we analyzed previously published single-cell RNAseq data from the BNST and nucleus accumbens (NAc). In the NAc, we and others showed that OTR activation promotes social approach behaviors. In the BNST, *Oxtr* was expressed in over 40 cell types, that span both posterior and anterior subregions of the BNST. The majority of *Oxtr*-expressing neurons were GABAergic. In the anterior regions of BNST targeted in our carbetocin experiments, *Cyp26b1*-expressing neurons had high average *Oxtr* expression. In the NAc, most *Oxtr+* cells were D1 dopamine receptor-expressing neurons and interneurons. These differences in *Oxtr* cell type distribution may help explain how activation of OTR in BNST versus NAc can have different effects on social approach and social vigilance.

Introduction

Oxytocin is traditionally considered to promote affiliative behaviors and has been put forth as a potential treatment for social deficits, such as those associated with autism spectrum disorder (Ford and Young, 2021; MacDonald and MacDonald, 2010; Meyer-Lindenberg et al., 2011; Striepens, 2011). Oxytocin can facilitate pair bonding, parental care, and social play across a wide range of species (Bosch and Neumann, 2012; Bredewold et al., 2014, 2014; Hammock and Young, 2006; Keverne and Kendrick, 1992; Klatt and Goodson, 2013; Leng et al., 2008; Romero et al., 2015). It is also implicated in human research to increase trust, empathy, and in-group cooperation (De Dreu and Kret, 2016; Geng et al., 2018; Kosfeld et al., 2005; Van IJzendoorn and Bakermans-Kranenburg, 2012). Indeed, many studies show that oxytocin signaling can promote social approach behaviors (Dölen et al., 2013; Lukas et al., 2011). However, emerging studies have reported that administration of oxytocin can generate avoidance of social contexts (Beery, 2015). For example, intranasal oxytocin reduced social interaction in female California mice (Steinman et al., 2016) while intracerebroventricular infusion of oxytocin did not increase social approach in female rats exposed to social defeat (Lukas and Neumann, 2014). In humans, intranasal oxytocin increased self-reported perceived social stress among male participants (Eckstein et al., 2014). The mixed results suggest that oxytocin has a more complex role than promoting affiliative behaviors *per se*.

The social salience hypothesis proposes that oxytocin enhances the salience of both positive or negative social contexts (Shamay-Tsoory and Abu-Akel, 2016). It has been hypothesized that distinct neural circuits may mediate diverse behavioral effects of oxytocin (Steinman et al., 2019). Oxytocin acting in the nucleus accumbens (NAc) and ventral tegmental area (VTA) has been found to promote social reward and enhance social approach (Borland et

al., 2018; Dölen et al., 2013; Hung et al., 2017; Peris et al., 2017; Song et al., 2016; Yu et al., 2016). In contrast, oxytocin has been found to produce anxiogenic effects in the bed nucleus of the stria terminalis (BNST) (Duque-Wilckens et al., 2020; Janeček and Dabrowska, 2019). The BNST plays an important role in modulating fear and anxiety-related behaviors (Fox and Shackman, 2019; Walker et al., 2003) and expresses high levels of oxytocin receptors (OTR) (Tribollet et al., 1992). For instance, infusion of an OTR antagonist in the BNST impaired acquisition of cued fear in male rats (Moaddab and Dabrowska, 2017) and attenuated the effects of social defeat stress in female California mice (Duque-Wilckens et al., 2018). While there is a strong literature describing how oxytocin receptors modulate behaviors, less is known about the molecular pathways that mediate these effects.

Oxytocin receptors belong to the G-protein coupled receptor (GPCR) family, which is the target of over one-third of FDA-approved drugs (Rask-Andersen et al., 2011). An important property of these receptors is that they are capable of modulating diverse signaling pathways and pathological processes. Oxytocin receptors can induce distinct downstream pathways through coupling to either excitatory G_q or inhibitory $G_{i/o}$ subunits (Busnelli et al., 2012; Gimpl and Fahrenholz, 2001; Rosenbaum et al., 2009; Strader et al., 1994). Although the molecular pathways of oxytocin receptor signaling are well-studied *in vitro*, it is less clear how the differential G-protein signaling translates into behavioral phenotypes (Jurek and Neumann, 2018). A previous study from our group demonstrated that infusion of biased agonists for OTR-G_q but not OTR-G_i pathway in the NAc of stressed female California mice increased social approach and decreased social vigilance, a behavior in which an individual orients towards an unfamiliar conspecific while simultaneously avoiding it (Williams et al., 2020). Recent data showed that infusion of oxytocin into the anteromedial BNST (BNSTam) reduced social

approach and increased vigilance (Duque-Wilckens et al., 2020), so we decided to test whether infusion of G_q-biased OTR agonist in the BNST would facilitate behavioral responses related to social anxiety. We also explored whether the oxytocin receptor gene (*Oxtr*) is expressed in different cell types in the BNST compared to the adjacent NAc. Differences in the cell-type expression of *Oxtr* across brain regions could contribute to variability in circuit-specific actions of oxytocin.

To address these questions, we first microinjected the functionally selective OTR-G_q agonist carbetocin into the anterior BNST and assessed social interaction behaviors. We used California mice, a species that is unique in that both males and females are aggressive. This has allowed for the study of social defeat stress in both sexes (Kuske and Trainor, 2021). To determine *Oxtr* cell types we analyzed recently published single cell RNA sequencing datasets from the BNST (Welch et al., 2019) and NAc (Chen et al., 2021) in *Mus musculus*.

Methods

Animals

All experiments on California mice (*Peromyscus californicus*) were in accordance with and approval of the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis. Adult male (n=58) and female (n=26) California mice from our laboratory colony were co-housed in same-sex groups of 2. Mice were kept on a 16:8 Light:Dark cycle and fed *ad libitum* (2016 Teklad global 16% protein rodent diets). Sani-chip bedding, cotton nestlets, and enviro-dri (Newco Distributors) were provided in all cages. Drug infusion and behavioral

tests were performed during the dark cycle. Previous studies have demonstrated that estrus cycle does not affect behaviors during the social interaction test (Trainor et al., 2013, 2011).

Cannulation and Carbetocin Infusion

Males and females were implanted with 26-gauge bilateral cannula guides aimed at the anterior BNST (A-P: +0.45 mm; M-L: ±1.0 mm; D-V: +5.6 mm). The mice were single housed and given a 7-day recovery period after surgery. The animals received daily subcutaneous injection of carprofen as anti-inflammatory from day 1 to 3 and handled daily for 1 minute to get used to scruffing. On the testing day, female mice were randomly assigned to receive 200nL bilateral infusion of either artificial cerebrospinal fluid (aCSF) vehicle, 200ng carbetocin, or 1μg carbetocin. In bioluminescence resonance energy transfer (BRET) assays, carbetocin selectively induces OTR/Gq coupling (Passoni et al., 2016). Although this specificity has never been demonstrated directly *in vivo*, indirect evidence suggests that carbetocin acts via a similar mechanism *in vivo*: when microinected in the NAc, atosiban, which blocks OTR/Gq coupling while activating OTR/Gi coupling (Busnelli et al., 2012), reduced social approach in stress naïve mice whereas carbetocin increased social approach in stressed female California mice (Williams et al., 2020). Male mice were randomly assigned to receive 200nL bilateral infusion of either vehicle, 200 ng carbetocin, or 1μg carbetocin. Twenty minutes following the infusion, mice were tested for social interaction. After behavior testing, mice were perfused, and brains were collected for Nissl stain to confirm successful cannula placement.

Behavioral Test

The social interaction test consists of 3 phases, each lasting 3 minutes (Greenberg et al., 2014). Mice were introduced into an empty arena (89 x 63 x 60cm) and allowed to freely explore during the open field phase. During the acclimation phase, an empty wire cage was placed against one side of the arena for habituation. For the social interaction phase, a same-sex unfamiliar target mouse was placed into the wire cage. Distance traveled, time in the center zone (located 14cm from the sides), and time that the focal mouse spent within the interaction zone (within 8cm of the wire cage) were recorded and analyzed using AnyMaze. Time that the focal mouse spent outside of the interaction zone while its head oriented towards the target mouse was defined as social vigilance and scored manually.

Statistical analyses

Behavioral data analyses were performed in RStudio. The Shapiro-Wilk's test was used to test for data normality and the Fligner-Killeen test was used to assess homogeneity of variance. One-way ANOVA was used to detect group differences in female and male mice, respectively. Pairwise comparisons with Bonferroni correction were used for post-hoc analyses. For data that did not meet the assumptions of normal distribution or homogeneity of variance (i.e., vigilance), Kruskal-Wallis one-way analysis was used followed by Dunn's test with Bonferroni adjustment. Cohen's d was calculated to reflect the effect size of the significant results.

Single-cell RNA Sequencing Data Analyses

Single-cell sequencing data were analyzed in RStudio using Seurat v4.0.4 (Hao et al., 2021). For BNST analysis, we accessed previously published data from a *Mus musculus* BNST single nucleus RNA-seq data containing a total of 204,737 cells across 7 adult female and 8 adult male biological replicates (Welch et al., 2019) from GEO: GSE126836, and loaded these data into a Seurat object (Stuart et al., 2019). We used the Welch et al. 2019 cluster identity, replicate, and sex data as metadata features for each Seurat object. Cells with unique features under 200 and over 7500 were filtered out. To characterize *Oxtr* cells, cells with > 0 *Oxtr* counts were considered as *Oxtr+* and subsetted from the main Seurat object. The percent expression of the following gene markers: glutamate decarboxylase 1 (*Gad1*) and glutamate decarboxylase 2 (*Gad2*) for GABAergic neurons, vesicular glutamate transporter 2 (*Slc17a6*) and vesicular glutamate transporter 3 (*Slc17a8*) for glutamatergic neurons and glial fibrillary acidic protein (*Gfap*) for glial cells were calculated in *Oxtr+* cells. These categories made up over 96% of all cell types expressing *Oxtr*. We also calculated percent expression of dopamine receptor D1 (*Drd1*) in *Oxtr+* cells to make direct comparisons with the NAc data. To visualize *Oxtr* expressing neurons, gene counts were normalized and scaled from the main Seurat object. Linear dimensionality reduction was performed by principal component analysis (PCA). BNST *Oxtr-* expressing clusters were visualized with UMAP, using the same number of dimensions as PCA (runUMAP, dims = 10). The expression level of *Oxtr* across different neuron clusters was quantified and visualized using the DotPlot function. We also visualized the expression of other transcripts including thyrotropin-releasing hormone receptor (*Trhr)*, dopamine receptors (*Drd1, Drd2, Drd3),* serotonin receptors (*Htr1a, Htr1b*, *Htr2a*, *Htr2c)*, neuropeptide Y receptors (*Npy1r*, *Npy2r)*, opioid receptors (*Oprk1*, *Oprd1*, *Oprm1)*, and tachykinin precursor (*Tac1)* in a Dotplot to compare with *Oxtr*.

The same approach was used for the NAc analysis. We accessed published data (Chen et al., 2021) from 11 adult male *Mus musculus* NAc single cell RNA-seq data containing 47,576 total cells, organized into 21,842 neuronal and 25,734 non-neuronal cells from GEO: GSE118020 along with cluster identities. UMAP (runUMAP, dims = 1:10) was used to visualize NAc clustering. A total of 199 *Oxtr+* cells were subsetted and analyzed for cell type composition. In addition, the expression level of *Oxtr* across different NAc cell types and interneuron types (*Sst, Pvalb, Chat*) was visualized using the DotPlot function.

Results

Female California mice that received 1µg carbetocin spent less time interacting with a novel target mouse ($p<0.01$, $d = 2.39$, Fig.1A) and showed increased vigilance behavior ($p<0.05$, $d = 1.56$, Fig.1B) compared to controls. Females that received 200 ng carbetocin or infusion outside of the BNST did not show significant differences from the controls (all p's > 0.05, Fig.1A, B). Representative heat maps demonstrated the location of one mouse per treatment group during the interaction phase (Fig.1C). No differences were observed in the distance traveled ($F_{3,22}=0.658$, $p =0.587$, Fig.1D), time spent in center during the open field phase ($F_{3,22}=0.454$, $p =0.717$, Fig. 1E) or time spent in the interaction zone during the acclimation phase ($F_{3,22}=0.745$, $p =0.537$, Fig. 1F) across different treatment groups. Histology was used to confirm successful placement of cannula guides and location of the needle tracts (Fig. 1G)
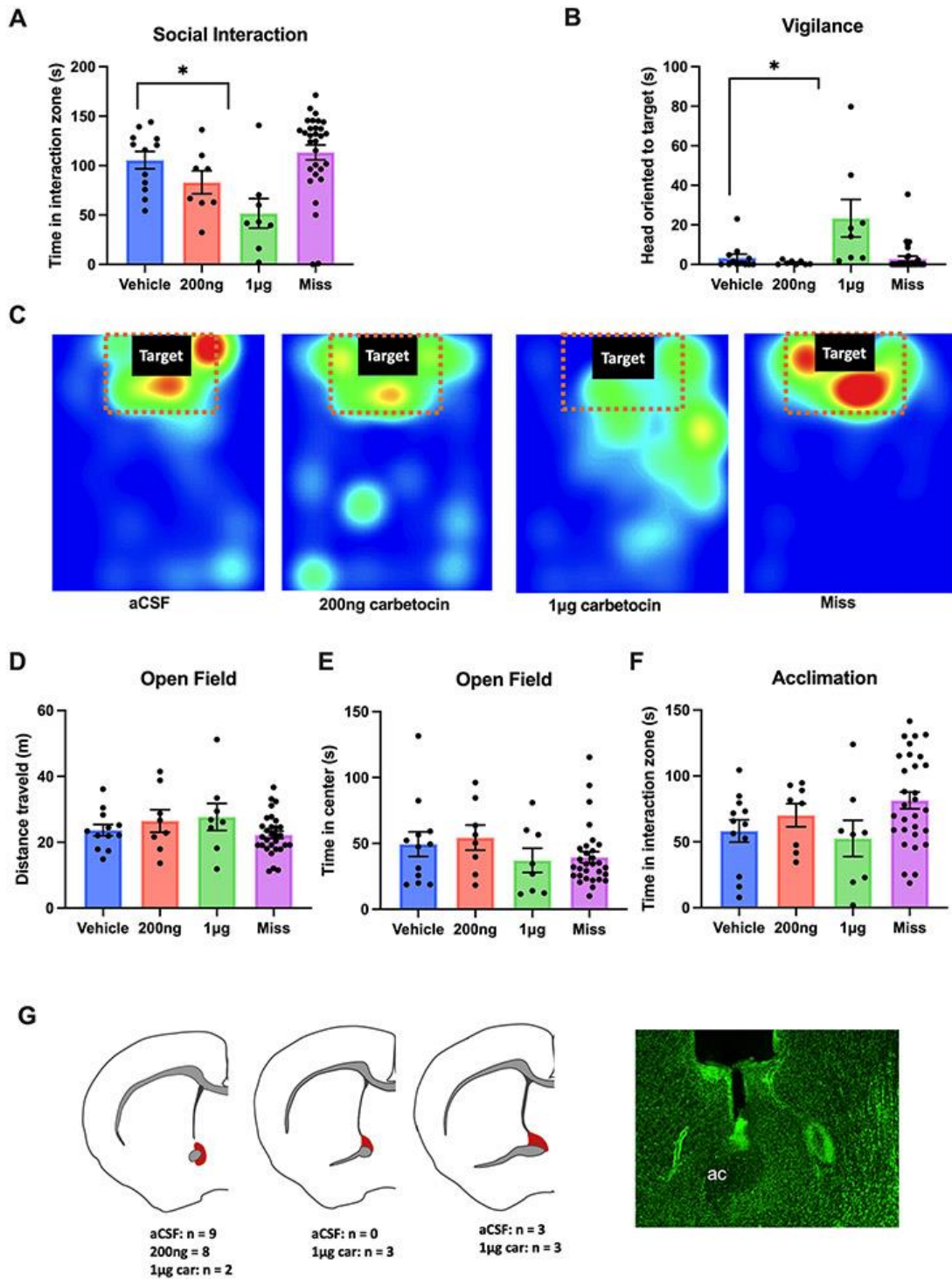
Figure 1. Intra-BNST carbetocin infusion reduced social approach and increased vigilance in female California mice. Infusion of 1µg carbetocin in the anterior BNST (n=8), but not 200ng cabetocin (n=7) or misplaced infusion ("Miss",n=4), decreased social approach to a novel

target mouse (a) and increased social vigilance (b) compared with controls (n=7). Representative heatmaps showed that during the interaction phase, a mouse that received the higher dose of carbetocin spent less time in the interaction zone compared with the other treatment groups (c). There were no significant differences across all treatment groups during the open field or the acclimation phases (d,e,f). Brain slices were Nissl stained to confirm successful injection sites (red shading).

Similar results were observed in males. Infusion of 1μg carbetocin decreased social interaction time (p<0.05, d = 1.47, Fig.2A, C) and increased vigilance behavior (p<0.05, d=1.03, Fig.2b). Males that received 200ng carbetocin or infusion outside of the BNST did not show significant differences from the controls (all p's> 0.05, Fig. 2A, B). Again, the mice did not show differences in the distance traveled ($F_{3,54}$=1.462, p =0.235, Fig. 2D), center time ($F_{3,54}$=1.064, p = 0.372, Fig. 2E) or time investigating the empty cage ($F_{3,54}$=2.406, p =0.0773, Fig. 2F) across different treatment groups**.**
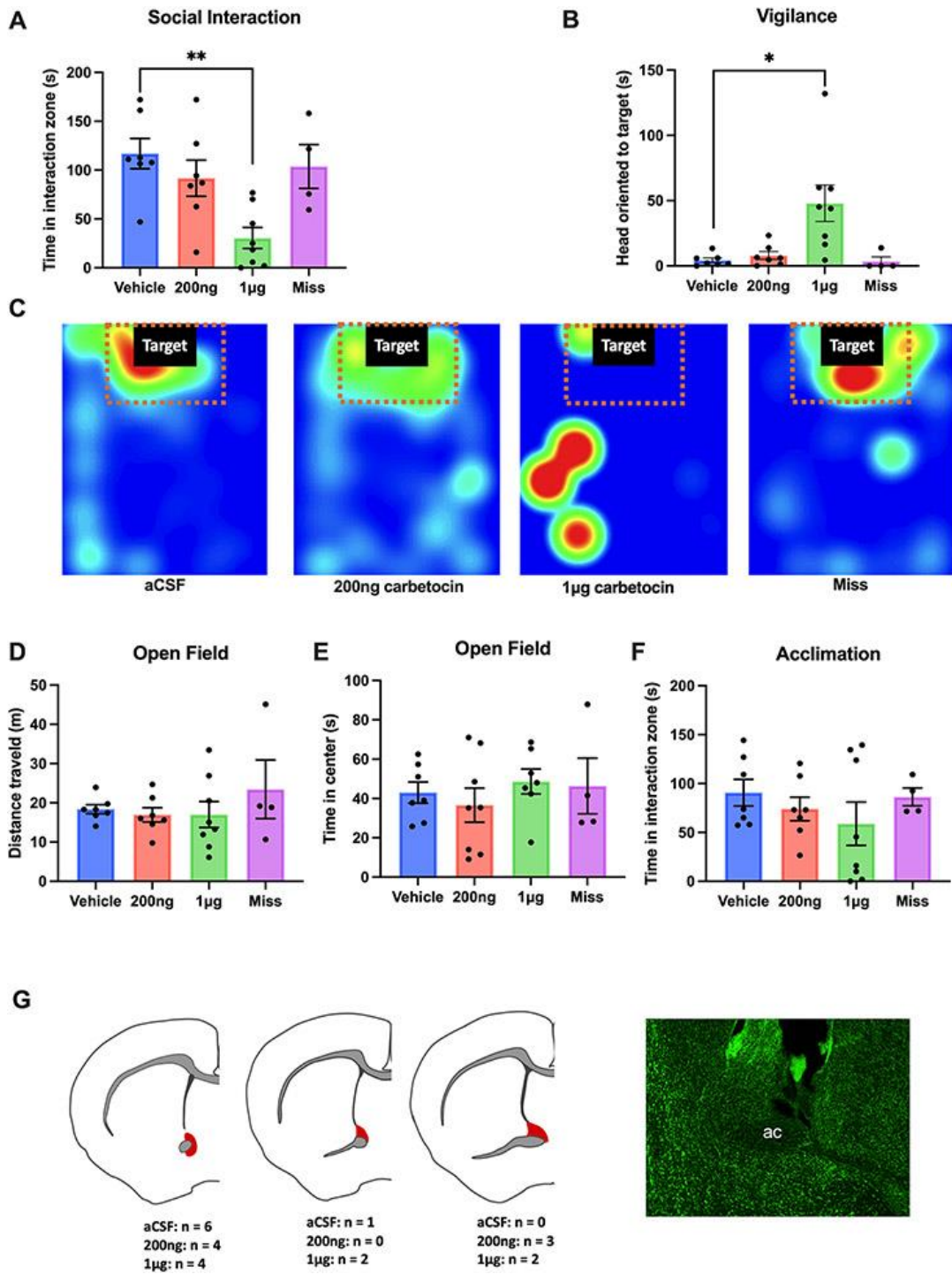
Figure 2. Intra-BNST carbetocin infusion reduced social approach and increased vigilance in male California mice. Infusion of 1µg carbetocin in the anterior BNST (n=8), but not misplaced infusions (n=30), decreased social interaction time with a novel target mouse (a) and

increased social vigilance (b) compared with controls (n=12). Representative heatmaps showed that during the interaction phase, a mouse that received the drug spent less time in the interaction zone compared with the other treatment groups(c). There were no significant differences across all treatment groups during the open field or the acclimation phase(d,e,f). Brain slices were Nissl stained to confirm successful injection sites (red shading).

In adult *Mus* BNST, more than 90% of *Oxtr+* cells were GABAergic neurons (Fig 3). In females, 5.02% of *Oxtr+* cells only expressed *Gad1*, 31.22% only expressed *Gad2* and 58.96% expressed both. Similarly, in males, 5.24% of *Oxtr+* cells only expressed *Gad1*, 31.44% only expressed *Gad2* and 56.77% expressed both. A small percentage of *Oxtr+* cells expressed the glutamatergic neuronal markers *Slc17a6* or *Slc17a8* (2.4% in females and 3.06% in males). An even smaller percentage of *Oxtr+* cells expressed glial cell maker (*Gfap*) or none of the gene markers. To visualize *Oxtr*-expressing neurons, UMAP was constructed using 41 original cluster IDs acquired from Welch et al.,2019 (Fig.4A). *Oxtr* was expressed across various neuron types and had similar expression patterns between males and females (Fig.4b). Most *Oxtr+* cells were found in the posterior BNST, especially in the *Ror*1 cluster. In general, *Oxtr* expression was less abundant in the BNST (1.18% of transcripts in females and 1.27% in males) compared to other transcripts, such as *Htr2c* and *Oprm1* (Supplementary Fig. 1). In both sexes, *Oxtr* had the highest average expression (2.5 standard deviation above mean expression) in the BNSTal_*Cyp26b1* (Cytochrome P450 Family 26 Subfamily B Member 1) cluster as well as the highest percent expression in the same cluster (6.94% of *Cyp261* cells for females and 6.78% for males) (Fig.4C). *In situ* hybridization data acquired from the Allen Brain Map showed that *Cyp26b1* expression in the anterior BNST overlapped with our microinjection sites (Fig. 4D) (Lein et al.,

2007). *Oxtr* was also highly expressed (over 1.5 SD above mean expression in either sex) in *Ror1* (receptor tyrosine kinase-like orphan receptor 1)*, Sst* (somatostatin) and *Ebf1* (early B-cell factor 1) clusters in the posterior BNST (Fig. 4C).
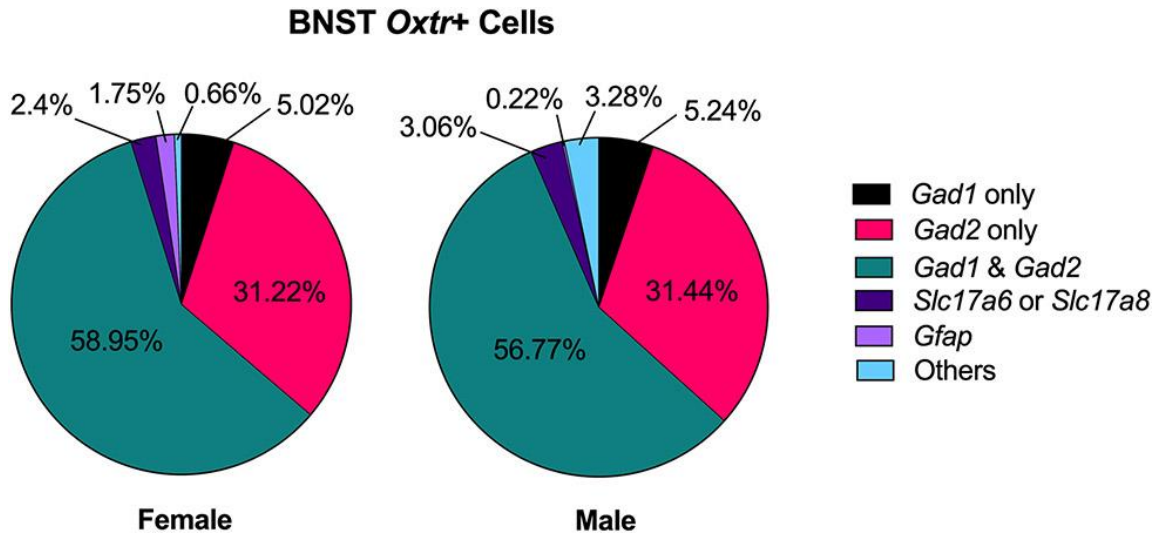

BNST *Oxtr*+ Cells

Female
Male

Gad1 only
Gad2 only
Gad1 & Gad2
Slc17a6 or Slc17a8
Gfap
Others

Figure 3. Characterization of *Oxtr*+ cells in adult *Mus musculus* BNST. Percentage expression of gene markers for inhibitory neurons (*Gad1* and *Gad2*), excitatory neurons (*Slac17a6* or *Slc17a8*) and glial cells (*Gfap*) in female (left) and male (right) *Oxtr*-expressing cells.
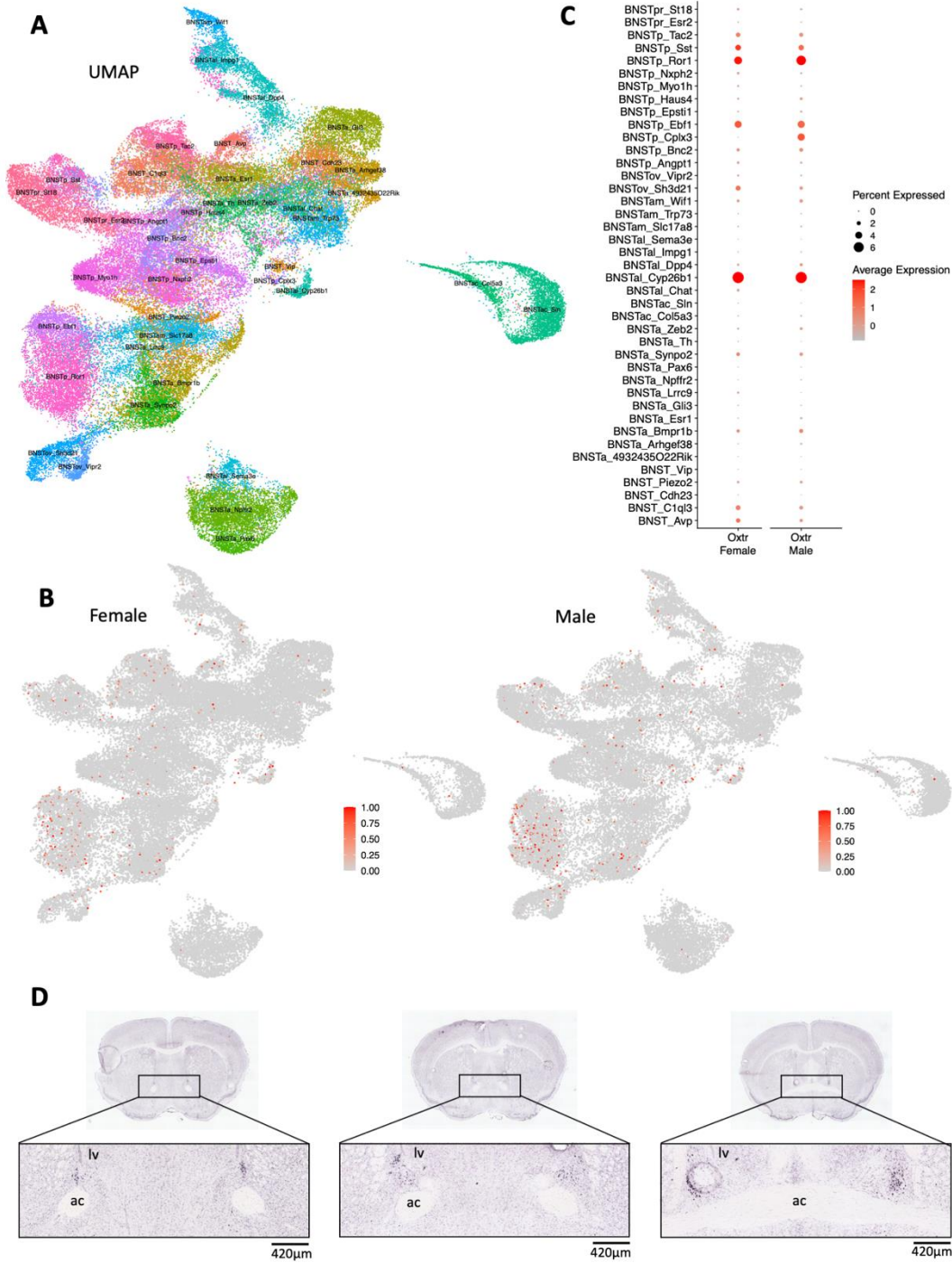
Figure 4. *Oxtr* and *Cyp26b1* expression in adult *Mus musculus* BNST. *Oxtr* expression across

different cell types and BNST subregions (BNSTa, BNSTac, BNSTal, BNSTam, BNSTov,

BNSTp, BNSTpr) were visualized with UMAP and Dotplot (a,b,c). Male and female animals

showed similar expression patterns of *Oxtr* (a, b). *Oxtr* had the highest percent and average

expression in the BNSTal *Cyp26b1* cluster of both sexes (c). *In situ* data also showed that *Cyp26b1* is expressed in both the oval and anterior BNST. The expression of *Cyp26b1* in the anterior region overlaps with our carbetocin microinjection sites (d). Image credit: Allen Institute. URL: https://mouse.brain-map.org/experiment/show/79568022.

Adult male *Mus* NAc cells were grouped into 9 different clusters: astrocytes, oligodendrocytes, endothelial cells, microglias, interneurons, dopamine receptor 1-expressing cells, dopamine receptor 2-expressing cells, oligodendrocyte progenitor cells, neuro stem cells and neuroblasts (Fig. 5A). The majority of the *Oxtr+* cells were either D1 medium spiny neurons (65.83%) or interneurons (24.62%) (Fig. 5A). These results contrast with cell types in the BNST, where only 15.28% of female and 14.85% of male *Oxtr+* cells co-express *Drd1*. The remaining NAc *Oxtr* cell types were expressed in D2 medium spiny neurons (5.53%), oligodendrocytes (2.01%), OPC (1.01%), astrocytes (0.5%) and endothelial cells (0.5%). Although most *Oxtr+* cells were D1 medium spiny neurons, *Oxtr* had the highest percent expression (3.68%) and average expression (2.5 standard deviation above mean expression) within the interneurons (Fig. 5B). We also visualized the expression of *Oxtr* across different interneuron subtypes (Fig. 5C) due to the heterogeneity of this cell type. *Oxtr* expression overlapped with somatostatin (*Sst*)-expressing interneurons and was generally absent from parvalbumin (*Pvalb*) and cholingeric (*Chat*) interneurons (Fig 5D). Calbindin1 (*Calb1*) and *Calb2* were expressed in all interneurons.
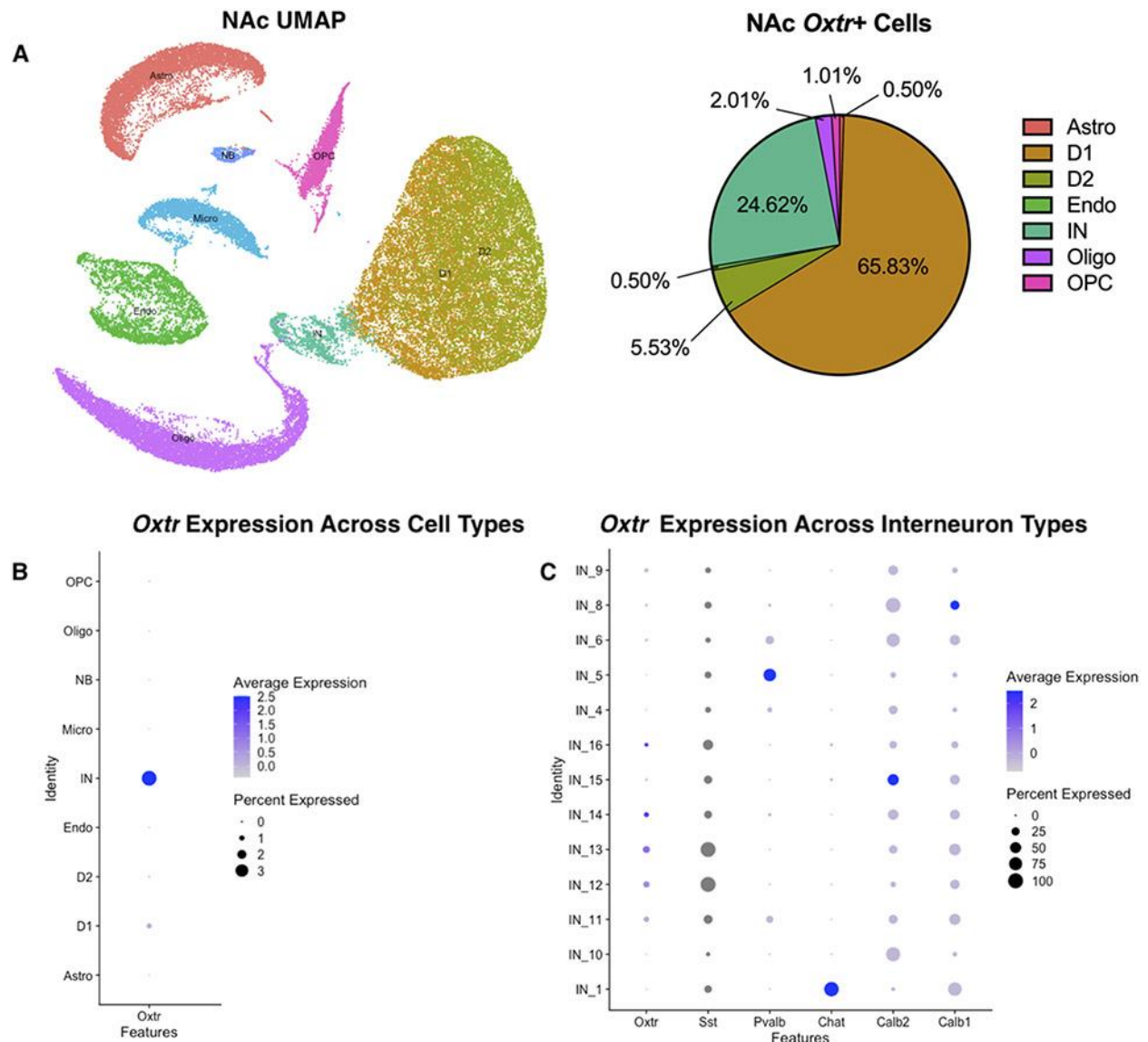
Figure 5. NAc *Oxtr*+ cell type characterization and *Oxtr* expression across clusters. The
UMAP shows 9 different cell types within the NAc: astrocytes (Astro), oligodendrocytes
(Oligo), endothelial cells (Endo), interneurons (IN), dopamine receptor 1-expressing cells (D1),
dopamine receptor 2-expressing cells (D2), and oligodendrocyte progenitor cells (OPC),
microglias (Micro), neuro stem cells and neuroblasts (NB) (A). *Oxtr* is expressed primarily in the
dopamine D1 receptor medium spiny neurons and interneurons (A). A Dotplot across all cell

types (B) shows *Oxtr* has the highest average and percent expression in the interneurons (IN). When examining interneuron subtypes (C), a Dotplot was shows that *Oxtr* expression occurs primarily in somatostatin (*Sst*) expressing interneurons. Comparatively few *Oxtr* transcripts were expressed in *Pvalb* or *Chat* neurons. Dots for *Sst* appear gray due to its high abundance relative to other transcripts.

Discussion

We demonstrated that activating OTR via carbetocin in the anterior BNST reduces social approach and increases social vigilance in stress naïve males and females. These behavioral responses mirror behavior patterns observed in mice exposed to social defeat (Duque-Wilckens et al., 2020), suggesting that these are social anxiety-related responses. Although the mechanism of action for carbetocin has never been demonstrated *in vivo*, prior work in the NAc, suggests that OTR-Gq signaling is most likely the mechanism of action. Our results from the BNST complement observations from the NAc, where social approach is reduced by OTR-G_q antagonists and promoted by carbetocin (Williams et al., 2020), and suggest that oxytocin is more likely to regulate social behaviors in a circuit-dependent manner than through different G-protein coupled signaling. Analyses of single cell RNAseq data from *Mus* show that in the BNST, *Oxtr* is expressed by many types of GABAergic neurons. Meanwhile in the NAc, *Oxtr* is mainly expressed in both D1 dopamine receptor neurons and interneurons. Anatomical variation in *Oxtr* cell-type expression may be an important factor contributing to circuit-specific effects of *Oxtr* on behavior.

Effects of Oxytocin Receptors in the BNST on Behavior

Microinjection of the highest dose of carbetocin (1 mg) induced social avoidance and social vigilance in females and males. *In vitro*, carbetocin can activate V1a receptors at high concentrations (Passoni et al., 2016). However, it is unlikely that decreases in social approach and increases in social vigilance were driven by V1a receptors. Infusion of a highly selective V1a receptor antagonist into the BNST reduced social approach in unstressed males and females (Duque-Wilckens et al., 2016). On the contrary, infusion of a selective oxytocin receptor antagonist into the BNST increased social approach and reduced social vigilance in stressed females (Duque-Wilckens et al., 2018). If carbetocin infusions activated V1a receptors, we would expect to see enhanced social approach, which is the opposite of what we observed. Together, these results suggest that the anxiogenic behavioral effects of carbetocin in the anterior BNST are mediated by oxytocin receptors. Similarly, in a non-social context, OTR neurotransmission in the dorsolateral BNST facilitated acquisition of cued fear response in male rats (Martinon et al., 2019; Moaddab and Dabrowska, 2017). We also observed that intra-BNST administration of carbetocin induced similar behavioral phenotypes in both sexes. Sex differences have been reported in neural and behavioral responses to intranasal oxytocin in human (Domes et al., 2010; Rilling et al., 2014) and animal research (Duque-Wilckens et al., 2018; Steinman et al., 2016). However, sex differences were not observed in OTR expression across a wide range of brain regions and oxytocin infusions into anterior BNST of California mice had similar behavioral effects in both sexes (Duque-Wilckens et al., 2020). These results suggest that sex differences in oxytocin release may be a key driver for sex differences in stress responses.

Previous studies have demonstrated that systemic and central administration of carbetocin could reverse stress-induced depression and anxiety-related behaviors (Chaviaras et al., 2010; Klenerova et al., 2010, 2009; Meng et al., 2016). In one study, two weeks of intraperitoneal (i.p.) injection of carbetocin blocked social withdrawal, sucrose anhedonia and learned helplessness in stressed tree shrews (Meng et al., 2016). In another study, either acute intravenous, intraperitoneal or intracerebroventricular injection of carbetocin reduced immobility during forced swim tests in male rats (Chaviaras et al., 2010). These studies indicated that both chronic and acute administration of carbetocin could induce anti-depressant or anxiolytic effects. Instead, our results showed that intra-BNST infusion of carbetocin decreased social approach and increased social vigilance. There is growing evidence that the behavioral effects of OTR are circuit-specific (Steinman et al., 2019). For example, microinjection of carbetocin in the NAc, which is adjacent to the anterior BNST, induced an opposite behavioral phenotype by increasing social approach and decreasing vigilance in stressed female California mice (Williams et al., 2020). These results were consistent with previous findings that oxytocin acting in the NAc could interact with serotonin and dopamine receptor signaling to enhance social approach responses (Dölen et al., 2013; Liu and Wang, 2003). Similarly, oxytocin infusion into the dorsal lateral septum blocked social fear responses after conditioning (Zoicas et al., 2014). Taken together, our results support the hypothesis that the behavioral effects of OTR signaling are brain region-specific. Although many mechanisms, likely contribute to circuit specific actions of OTR, one contributing factor could be *Oxtr* expression in different cell types across the brain. To examine *Oxtr* cell types in a more systematic way than has been performed previously, we analyzed single-cell RNAseq data from the BNST and NAc.

Oxtr cell types in BNST and NAc

In the Welch *et al.* 2019 BNST data, the overwhelming majority of *Oxtr+* cells were GABAergic neurons expressing either *Gad1, Gad2,* or both transcripts. In both the *Mus* RNAseq data and *in situ* hybridization analyses of *Oxtr* in BNSTam California mice (Duque-Wilckens et al., 2020), about 60% of *Oxtr+* neurons co-expressed *Gad1*. Interestingly, almost all *Gad1* expressing cells also expressed *Gad2*, while an additional 31% of *Oxtr+* neurons in the BNST only expressed *Gad2*. This suggests that *Gad2* may be a better marker for GABAergic neurons in the BNST. It is also important to consider the heterogeneity of neuronal subtypes in the BNST (Beyeler and Dabrowska, 2020). Over 40 cell types were identified in Welch et al. 2019 dataset, and many of these cell types expressed low or moderate levels of *Oxtr* mRNA. Several *Oxtr+* cell types are predominant in more anterior regions of BNST, which were targeted in our carbetocin experiment. One cell type that had the most abundant *Oxtr* expression was *Cyp26b1-*expressing neurons. *Cyp26b1* encodes a type of retinoic acid degradation enzyme (White et al., 2000). A recent sequencing study of BNST observed increased expression of *Cyp26b1* in male mice that exhibited increased social approach after chronic social defeat (resilient) compared to mice that exhibited reduced social approach (susceptible) (Gururajan et al., 2022). Although clinical and preclinical research suggests that retinoic acid signaling can modulate depression and anxiety-related behaviors (Bremner and McCaffery, 2008), no studies have manipulated *Cyp26b-1* function in the extended amygdala. This transcript could be an interesting target for functional studies, especially given that *Cyp26b1* is part of a group of transcripts that distinguish the anterior basolateral amygdala (BLA) from the posterior BLA (Hintiryan and Dong, 2022). Intriguingly, retrograde tracing studies show a strong connection between the caudal anterior BLA and the anteromedial BNST, the primary target of the carbetocin experiments.

Our analyses also showed that *Oxtr* is expressed in several cell types located in the posterior BNST. Posterior subregions of BNST are sexually dimorphic (Campi et al., 2013), and well known for modulating sexual behavior and aggression (Flanigan and Kash, 2020). The Welch et al. 2019 dataset is unusual in that cells from both males and females were included. *Oxtr* had similar expression patterns across different neuron clusters in males and females. While these observations are consistent with a lack of reported sex differences in OTR binding within the anterior BNST subnuclei (Duque-Wilckens et al., 2018), previous studies have reported sex differences in OTR binding in the posterior BNST (Dumais and Veenema, 2016; Smith et al., 2017). It is possible that RNA transcript expression level may not be linearly correlated with the protein expression level. This may help explain how *Oxtr* in the BNST is behaviorally active even though the relative abundance of *Oxtr* transcripts was relatively low compared with transcripts for TRH, dopamine receptors, NPY and opioid receptors in the *Mus* BNST.

In the NAc, *Oxtr* was primarily observed in D1 medium spiny neurons and interneurons. Conversely, few BNST *Oxtr+* neurons expressed D1 receptors. This result provides new insights into previous behavioral pharmacology experiments that identified coordination between oxytocin and dopamine signaling in the NAc. For example, in female prairie voles the formation of pair bonds requires activation of both OTR and D2 receptors (Liu and Wang, 2003). The fact that almost no D2 neurons express *Oxtr* suggests that pair bond formation in voles could be mediated by the coordinated action of D2 neurons and either D1 neurons or interneurons. Although there are many fewer interneurons than medium spiny neurons, interneurons have significant effects on behavior (Castro and Bruchas, 2019; Robison and Nestler, 2011). Increased activity of somatostatin interneurons enhances the rewarding effects of cocaine in a place preference assay (Ribeiro et al., 2018). Interestingly, optogenetic manipulations of somatostatin

interneurons in the absence of cocaine or another salient context had no effects on place preference or locomotor behavior. Behavioral effects of oxytocin are also generally stronger in social contexts (e.g., enhancing social salience), suggesting that somatostatin interneurons may enhance the salience of biologically important experiences. Also notable is that in male mice *Oxtr* positive somatostatin interneurons in the medial prefrontal cortex promote interest in females during estrus (Nakajima et al., 2014) . We also observed *Oxtr* positive somatostatin neurons in the BNST, which suggests that *Oxtr*/somatostatin cell types may be present more broadly across the brain.

Conclusions

Oxytocin can promote diverse behavioral effects by acting in different neural circuits. The availability of single cell RNA-seq methods allows for the ability to determine how *Oxtr* is expressed in different cell types in different brain regions. In the BNST, *Oxtr* was distributed across several types of GABAergic neurons while in the NAc, *Oxtr* was confined primarily to D1 medium spiny neurons and interneurons. While some BNST neurons have electrophysiological properties (inward rectification in response to current injection) that are similar to medium spiny neurons, they have different input resistances and resting membrane potentials (Egli and Winder, 2003). Taken together, *Oxtr* is expressed in substantially different cell types in the BNST versus NAc. Our analyses of *Oxtr* mRNA have important implications for interpreting mechanisms of oxytocin action. Previous work highlighted the role of pre-synaptic OTR on dorsal raphe terminals in the NAc in promoting salience in a social reward task (Dölen et al., 2013). That *Oxtr* mRNA was detected in single cell (NAc) and single nucleus (BNST) datasets implies the importance of post-synaptic OTR action may also be important. For example, in male rats,

oxytocin excites Type I interneurons within the dorsolateral BNST through a post-synaptic mechanism (Francesconi et al., 2021). Excitation of these interneurons suppresses the activity of adjacent Type II neurons that project to the central nucleus of the amygdala. This observation is especially interesting as optical excitation of different populations of BNST projection neurons can produce different behavioral effects (Kim et al., 2013). The complexities of how oxytocin modulates different brain circuits likely contribute to the mixed results observed in clinical studies using intranasal oxytocin as a therapeutic treatment for anxiety (Leppanen et al., 2018; MacDonald and MacDonald, 2010) or other social deficits (Ford and Young, 2021). Future studies should explore potential interaction between region-and-cell-type-specific effects of OTR signaling. Development of therapeutics that could target specific cell types or circuits could be more effective than existing systemic treatments.

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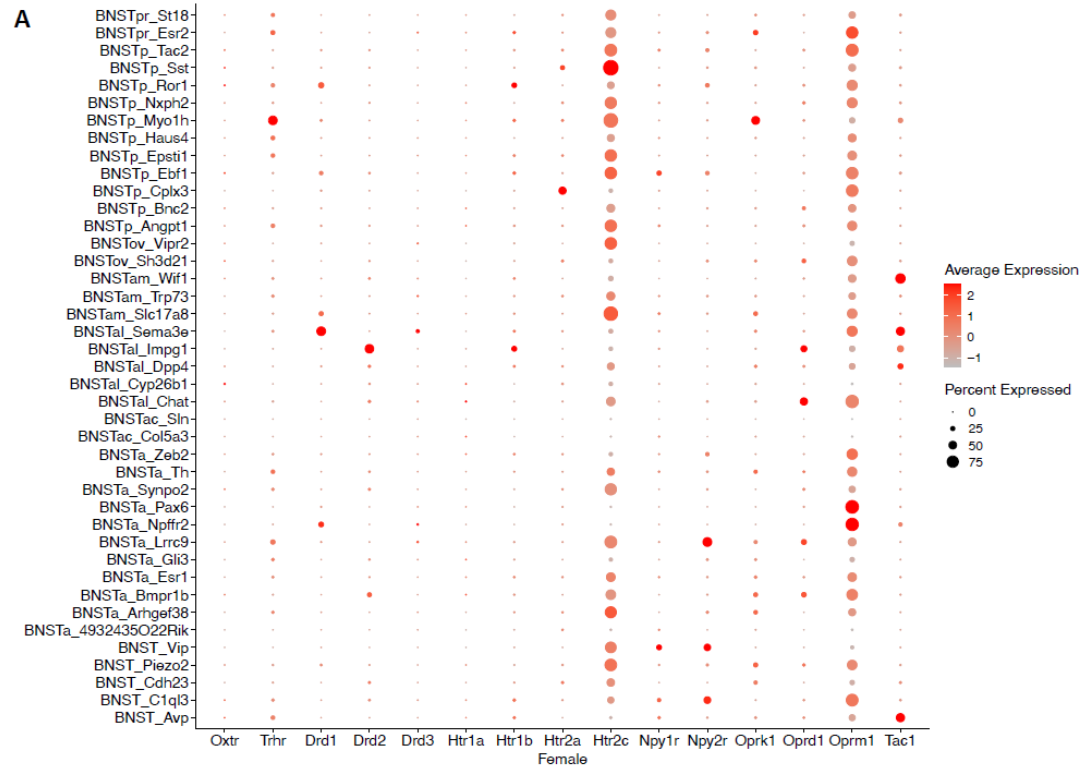
Supplementary Figure 1. Comparison of *Oxtr* expression with other abundant transcripts in the BNST. *Oxtr* expression appeared to be less abundant in the BNST compared to other

transcripts, such as serotonin receptor *Htr2c* and opioid receptor *Oprm1*. *Oxtr* is less abundant in

BNST neurons expressing dopamine receptor D1 (*Drd1)* compared with the nucleus accumbens.

Chapter 2:

The hypocretin system modulates coping strategies in response to social stress

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Abstract

Best known for promoting wakefulness and arousal, the neuropeptide hypocretin (Hcrt) also plays an important role in mediating stress response, including social stress. However, central and systemic manipulation of the Hcrt system has produced mixed behavioral outcomes in animal models. In this review, we will first examine inconsistent results in current literature where similar manipulation of the Hcrt system led to divergent coping behaviors and hypothesize that Hcrt contributes to individual differences in stress response. We propose that Hcrt differentially facilitates active and passive coping behaviors in response to social stress by acting in different brain regions and cell types. We will then focus on region and cell type-specific effects of Hcrt in the ventral pallidum, lateral habenula, nucleus accumbens, bed nucleus of the stria terminalis, and amygdala.

Introduction

The neuropeptide hypocretin (Hcrt), or orexin, is best known for its function of promoting wakefulness and arousal (Berridge and España, 2005; Carter et al., 2009). The Hcrt system plays an important role in modulating stress responses, and may serve as a potential treatment target for stress-related affective disorders, such as major depressive disorders and general anxiety disorders (Fagan et al., 2023; James et al., 2017; Summers et al., 2020). It has been suggested that Hcrt is essential for facilitating adaptive behaviors when an organism faces acute stress, whereas chronic stress may lead to Hcrt dysregulation and suboptimal behavioral responses (Giardino and de Lecea, 2014; Grafe and Bhatnagar, 2018; Sargin, 2019). In this review, we will consider the role of Hcrt in regulating stress coping behaviors, particularly in response to social stress.

The behavioral effects of Hcrt have been studied across a range of stress paradigms, including restraint and forced swim. Social stress paradigms, such as resident-intruder, are among the most naturalistic and ecologically valid methods to model stress in a laboratory setting and exerted strong effects on animals (Blanchard et al., 1990; Tamashiro et al., 2005). Individual variation in susceptibility and coping behaviors are also well reflected in social interaction tests (Krishnan et al., 2007). This review proposes that the Hcrt system plays a pivotal role in shaping these individual differences in stress coping strategies. First we examine the literature assessing the behavioral effects of systemic or central manipulation of the Hcrt system and address diversity in behavioral outcomes. The focus will then shift to the interplay between Hcrt and social stress response. Wet will delve into studies that used region or circuit-specific manipulations and hypothesize that Hcrt promotes both active and passive coping behaviors through its action in different brain regions and cell types.

Overview of the Hypocretin System

Hypocretin-1 (Hcrt1) and hypocretin-2 (Hcrt2), are cleaved from the same prepro-hypocretin (*prepro-hcrt*) gene and are highly conserved across mammalian species (Soya and Sakurai, 2020). Hcrt1 contains 33 amino acids and Hcrt2 contains 28 amino acids. The two peptides share similar C-terminal regions but their N-terminal halves are more divergent (Sakurai et al., 1998). There are also two hypocretin G-protein coupled receptors HcrtR1and HcrtR2 (de Lecea et al., 1998; Sakurai et al., 1998). HcrtR1 preferentially binds Hcrt1 over Hcrt2 while HcrtR2 binds both peptides with similar affinities (Gotter et al., 2012). At a molecular level, Hcrt receptors share high structural similarities and could both couple to G_q, G_s and $G_{i/o}$ proteins to activate diverse downstream pathways (Kukkonen and Leonard, 2014; Thompson et al., 2014). Hcrt producing neurons are restricted to a few subregions within the hypothalamus, including the lateral (LH), dorsomedial (DMH) and perifornical (PeFH) areas but project widely throughout the brain (Baldo et al., 2003; Peyron et al., 1998) and receive reciprocal inputs from most of the projected regions (Sakurai et al., 2005).

The Hcrt system was independently discovered by two groups in the 1990s. One group named the neuropeptides hypocretin for their hypothalamic origin and structural similarities to secretin (de Lecea et al., 1998). The other group adopted the name orexin, which means "appetite" in Greek, since initial findings showed strong effects on feeding behaviors (Sakurai et al., 1998). However, these initial studies were conducted in rodents during the light phase. When studies were repeated in the dark phase, effects of Hcrt on appetite were muted, suggesting that the results may have been confounded by effects on arousal (Berridge et al., 2010). Other work showed that *hcrtr2* mutated dogs (Lin et al., 1999) and *prepro-hcrt* knockout mice (Chemelli et al., n.d.) exhibited disrupted sleep cycles and episodes of cataplexy or behavioral arrests. Follow-

up clinical studies revealed that Hcrt deficiency is observed in human narcolepsy patients, suggesting the essential role of Hcrt system in regulating sleep-wake cycles (Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000). In addition to maintaining wakefulness and arousal, the Hcrt system is also implicated in various physiological and behavioral functions, including mediating stress response.

Hypocretin and Stress Response

Exposure to acute stress generally activates the Hcrt system. Preclinical rodent studies reported that a single episode of immobilization(Ida et al., 2000), cold stress (Ida et al., 2000) and foot shock (Chen et al., 2014) increased Hcrt mRNA expression in the hypothalamus. Short-term forced swim also increased Hcrt1 protein levels in the cerebrospinal fluid (Martins et al., 2004) and c-Fos expression in Hcrt neurons (Chang et al., 2007). Wakefulness, exploration and re-exposure to footshock box all increased c-Fos expression in Hcrt neurons compared with resting phase (Furlong et al., 2009). The short-term increase in Hcrt activity is often associated with elevated hypothalamic-pituitary-adrenal (HPA) axis (Winsky-Sommerer, 2004; Winsky-Sommerer et al., 2005) and sympathetic nervous system functions (Shirasaka et al., 1999; Smith et al., 2002). In a rat panic model, where animals were administered panic-inducing sodium lactate, Hcrt neurons showed increased c-Fos expression along with increased locomotion, heart rate and artery pressure (Johnson et al., 2010). The physiological and behavioral panic response were attenuated by knocking down *prepro-hcrt* gene (Johnson et al., 2012).

Acute activation of the Hcrt system is essential for an organism to shift from a basal to arousal state in order to mobilize energy and facilitate adaptive behaviors in face of immediate

challenges (Berridge and España, 2005; Mahler et al., 2014). At the same time, Hcrt system is also altered in patients diagnosed with stress-related affective disorders. For instance, clinical studies have found elevated Hcrt1 protein in the cerebrospinal fluid and blood samples of patients with anxiety symptoms (Akça et al., 2020; Johnson et al., 2012) as well as depressive patients (H. Li et al., 2021; Salomon et al., 2003; Wang et al., 2023) compared to healthy controls. Chronic use of non-selective Hcrt receptor antagonist Suvorexant has been shown to improve comorbid depressive symptoms in insomnia patients (Nakamura and Nagamine, 2017; Shigetsura et al., 2022), although a case of acute worsening of symptoms was also reported (Petrous and Furmaga, 2017).

In animal studies, systemic and central manipulation of the Hcrt system produced mixed results. For example, i.c.v. injection of Hcrt1 induced anxiogenic effects in stress naïve mice during light-dark box (LDB) and elevated plus maze (EPM) tests (Suzuki et al., 2005). In contrast, i.c.v injection of Hcrt1 in rats previously exposed to predator scent stress produced anxiolytic effects during EPM (Cohen et al., 2020). One study examined a wide range of anxiety and depression-related behaviors in male HcrtR1 null mice and discovered that knocking out HcrtR1 led to decreased immobility during forced swim but increased immobility during tail suspension (Abbas et al., 2015). Table 1 summarizes how central and systemic activation or inhibition of the Hcrt system produced mixed results in various behavioral paradigms. One possible explanation for diversity in Hcrt action is that activation of Hcrt receptors in different brain regions exert different effects on stress coping behaviors, as has been described for oxytocin receptors (Steinman et al., 2019). Since recent reviews have summarized how the Hcrt system integrates physiological and behavioral responses to stress stimuli (Grafe and Bhatnagar,

2018; Sargin, 2019), this current review will focus on the relationship between Hcrt and social

stress.

Table 1. Literature summary of central/systemic manipulation of the Hcrt system.

Activation/ Inhibition	Behavioral effects	Citations	Manipulation	Behavioral paradigm
Activation of the Hcrt system	↑ Stress Phenotype	Suzuki et al., 2005	i.c.v. Hcrt1 injection	LDB; EPM
		Chung et al., 2014	i.c.v.Hcrt1/2 injection	SI
		Flores et al., 2014	i.c.v. Hcrt1 injection	FC
		Heydendael et al., 2014	optogenetic stimulation of Hcrt neurons	SI
		Eacret et al., 2019	DREADDS stimulation of Hcrt neurons	SI
	↓ Stress Phenotype	Chung et al., 2014	i.c.v.Hcrt1/2 injection	SI
		Staton et al., 2018	i.c.v. Hcrt2 injection	SAM
		Cohen et al., 2020	i.c.v. Hcrt1 injection	PSS
		Han et al., 2021	i.c.v. Hcrt1 injection	SPS
		Kim et al., 2023	optogenetic stimulation of Hcrt neurons	LHT
Inhibition of the Hcrt system	↑ Stress Phenotype	Lutter et al., 2008	Hcrt knockout;	FST; SI
		Scott et al., 2011	HcrtR2 knockout	FST; TS
		Abbas et al., 2015	HcrtR1 knockout	TS; OFT; EPM
		Staton et al., 2018	i.c.v. HcrtR2 antagonist injection	SAM
		Eacret et al., 2019	oral HcrtR2 antaognist	SI
		Faesel et al., 2021	Hcrt knockout	SNT
		Dawson et al., 2023	optogenetic inhibition of Hcrt neurons	SI
	↓ Stress Phenotype	Johnson et al., 2010	i.p. HcrtR1 antagonist injection	SI
		Scott et al., 2011	HcrtR1 knockout; HcrtR1 antagonist i.p. injection	FST; TS
		Nollet et al., 2011	i.p. non-selective HcrtR antagonist injection	TS
		Sears et al., 2013	i.c.v. HcrtR1 antagonist injection	FC

		Flores et al., 2014	i.p. HcrtR1/R2 antagonist injection; HcrtR1 knockout	FC
		Abbas et al., 2015	HcrtR1 knockout	FST
		Grafe et al., 2017	DDREADS inhibition of Hcrt neurons	RRS
		Grafe et al., 2018	DDREADS inhibition of Hcrt neurons	SI, FST

(LDB: light-dark box; EPM: elevated plus maze; SI: social interaction; FC: fear conditioning; SAM: stress alternative model; PSS: predator scent stress; SPS: single prolonged stress; LHT: learned helpless test; FST: forced swim test; OFT: open field test; SNT: social novelty test; TS: tail suspension; RRS: repeated restraint stress)

Hypocretin mediates individual differences in response to social stress

Hypocretin is pivotal in modulating social behaviors. Fiber photometry recording from both male and female mice revealed that hypothalamic Hcrt neurons are more activated when investigating a social context compared with a non-social context. Optogenetic inhibition of Hcrt neurons also decreased social approach to novel conspecifics in male mice without affecting locomotion or behaviors during the EPM test (Dawson et al., 2023). Hcrt modulates social memory (Eacret et al., 2019; Yang et al., 2013), sexual behaviors(Di Sebastiano et al., 2010; Luan et al., 2023; Muschamp et al., 2007) and parental behaviors (D'Anna and Gammie, 2006; Rivas et al., 2016). While social interactions can be rewarding, they can sometimes be a source of stress and the Hcrt system has important behavioral effects during stressful social situations.

Individual variation in stress coping strategy has been well documented across both human and animal literature (Korte et al., 2005). Although not every coping behavior follows a

bimodal distribution (e.g. attack latency), most research classifies coping styles into either active/proactive or passive/reactive coping (Koolhaas et al., 2007). For instance, during resident-intruder test, the intruder animal with an active coping style may exhibit longer defeat latency and reciprocal aggression, whereas the intruder animal with a passive coping style may show shorter defeat latency, increased freezing and social vigilance behaviors (Franklin et al., 2012; Wood et al., 2010; Wright et al., 2020). In response to a territorial intruder, the resident animal with an active coping style may show high aggression and low HPA axis reactivity whereas the resident with a passive coping style may show immobility and high HPA reactivity (Koolhaas et al., 1999). Sources of variation in coping response are still under investigation. Pre-existing differences in dispositions (Calvo et al., 2011; Duclot et al., 2011) and environmental conditions (Lehmann and Herkenham, 2011; Ruis et al., 1999) could both contribute to divergent coping behaviors. From a neurobiological perspective, corticotropin-releasing hormone, dopamine, and neuropeptide Y are suggested to mediate individual differences in coping styles (Wood and Bhatnagar, 2015). In addition, the Hcrt system also plays an important role in modulating individual differences in response to social stress (Summers et al., 2020).

Animals with varying coping styles demonstrate differences in Hcrt expression. In one study, active coping rats displayed longer defeat latency and lower *prepro-hcrt* expression compared with passive coping rats (Grafe et al., 2018). A negative correlation emerged between defeat latency and hypothalamic *prepro-hcrt* mRNA level. DREADDs inhibition of Hcrt neurons prior to defeat sessions increased social approach in passive coping rats but had no effects in active coping or stress-naïve rats. In another study, mice were subjected to 10-day social defeat stress and categorized as resilient or susceptible based on the subsequent social interaction test (Chung et al., 2014). Similar to the rat study, resilient mice showed significantly decreased

prepro-hcrt mRNA expression in hypothalamus. If an overall lowered Hcrt expression predicts resilient phenotype, we would expect administration of Hcrt to decrease social approach. Instead, i.c.v. infusion of Hcrt1 increased social approach in susceptible mice and had no effects in resilient mice. Meanwhile, i.c.v. infusion of a mixture of Hcrt1 and Hcrt2 decreased social approach in resilient mice but had no effects in susceptible mice. Such findings suggest that overall expression of Hcrt and activation of Hcrt neurons may not be the best indicator for behavioral phenotypes, as Hcrt neurons project widely throughout the brain and could signal both stress and reward depending on the circuits (Giardino and de Lecea, 2014; Peleg-Raibstein and Burdakov, 2021).

Indeed, the Hcrt system could contribute to variation in coping strategy by differentially activating Hcrt receptors expressed in distinct brain regions and cell types. The relative activation level of the complimentary neural circuits leads to either active or passive coping phenotypes.

Region and cell type-specific effects of the hypocretin system

In Figure 1, we summarized a hypothetical model of how Hcrt mediates coping behaviors in a region and cell type dependent manner. we focus on a few candidate regions, including the ventral pallidum, lateral habenula, nucleus accumbens, bed nucleus of the stria terminalis and amygdala.

A few studies have proposed that HcrtR1 and HcrtR2 may produce opposite behavioral effects (Arendt et al., 2014; Scott et al., 2011; Staton et al., 2018; Summers et al., 2020; Yaeger et al., 2020). Again, HcrtR1 preferentially binds Hcrt1 over Hcrt2 while HcrtR2 binds both peptides with similar affinities. Both HcrtR1 and HcrtR2 express widely across the brain but

show noticeably different distribution patterns (Trivedi et al., 1998). In one study, chronic social

defeat increased HcrtR1 but decreased HcrtR2 mRNA expression in the basolateral amygdala in

susceptible male mice (Arendt et al., 2014). As will be discussed in the following sections,

HcrtR1 and HcrtR2 may promote divergent coping behaviors through differential expression in

competing neural circuits in some regions but not across the whole brain.
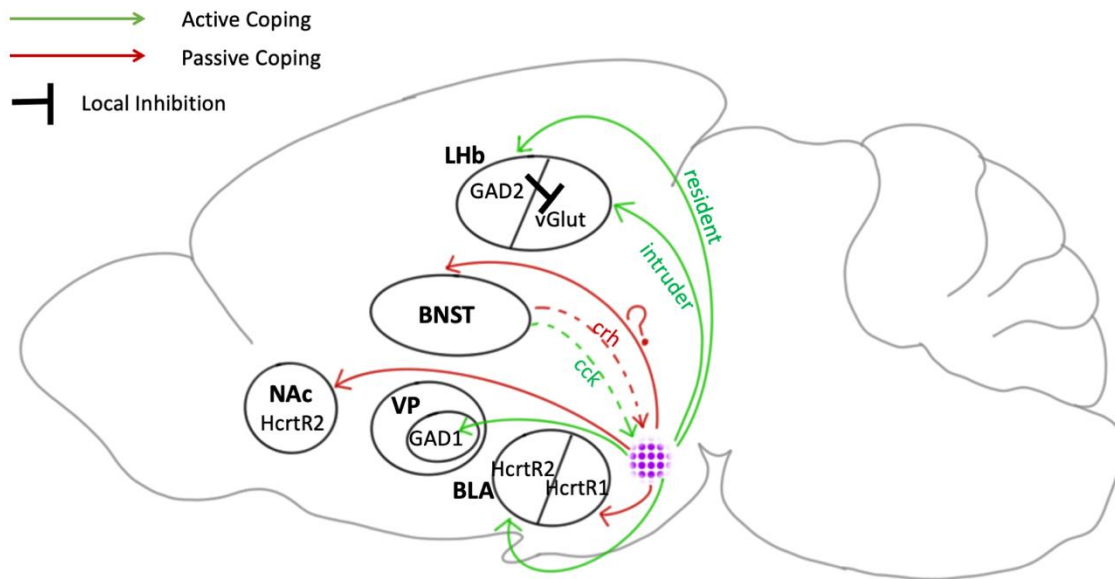
(**Figure 1.** A hypothetical model of how Hcrt differentially promotes active and passive coping

response. Hcrt neurons from the hypothalamus directly project to the VP, LHb, NAc, BNST and

Amygdala to promote either active or passive coping behaviors. The BNST also sends two

parallel projections back to the hypothalamic Hcrt neurons to facilitate either approach or

avoidance. The projections facilitating active versus passive coping styles are featured as parallel

circuits in this figure, but it is not clear if there is any overlapping or crosstalk. VP: ventral

pallidum; LHb: lateral habenula; NAcSh: Nucleus accumbens shell; BNST: bed nucleus of the

stria terminalis; BLA: basal lateral amygdala.)

Ventral Pallidum

The ventral pallidum (VP) receives dense projection from hypothalamic Hcrt neurons (Peyron et al., 1998) and is a possible site where Hcrt facilitates active coping behaviors. In a rat study, the dominance ranking between two males was first determined through the tube test (Ji et al., 2019). Knocking down HcrtR1 in the VP suppressed social approach of a subordinate male to a dominant male but not a dominant male to a subordinate during subsequent social interaction test. Avoidance of a dominant conspecific was not observed in rats without Hcrt knockdown, which suggests that endogenous HcrtR1 signaling in the VP is necessary for approach behaviors only when the animals are exposed to potential social stress. This observation is in line with other evidence suggesting that behavioral effects of Hcrt are often context-specific (Heydendael et al., 2014). For instance, DREADDS activation of Hcrt neurons decreased social interaction time only in defeated but not non-defeated male rats (Eacret et al., 2019).

Whole-cell patch clamp recordings in rat brain slices showed that Hcrt receptors in the VP directly excite local GABAergic neurons (Ji et al., 2019). In a different study, inhibition of VP GABAergic neurons caused mice to pursue a small but safe food reward over a large but dangerous one (Farrell et al., 2021). Perhaps Hcrt facilitates high-risk adaptive behaviors in a stressful context (e.g. approach a dominate conspecific) through activation of VP GABAergic neurons.

Lateral Habenula

The Lateral Habenula (LHb) receives direct projections from Hcrt neurons (Peyron et al., 1998). The region is crucial for facilitating adaptive behaviors when the current situation does not meet the expectation (Hu et al., 2020; Mizumori and Baker, 2017). Both rodent and primate

studies suggest that the LHb plays an important role in behavioral flexibility and strategy adjustment (Baker et al., 2015; Kawai et al., 2015). Existing evidence in rodent research shows that Hcrt receptor signaling in the LHb may promote active coping behaviors in a context dependent manner.

In male C57BL/6J mice exposed to chronic social defeat stress, microinjection of Hcrt1 in the LHb rescued social avoidance phenotype whereas blocking HcrtR2 exacerbated the avoidance of a unfamiliar male CD1 mice (Wang et al., 2021). Optogenetic activation of the hypocretinergic terminals in the LHb also alleviated social avoidance (Wang et al., 2021). In defeated animals, separate calcium imaging in Hcrt-cre mice and Vglut2-cre mice revealed a strong activation of LH Hcrt neurons and LHb glutamatergic but not GABAergic neurons when the animals were attacked. Interestingly, another study using resident-intruder paradigm showed that HcrtR2 signaling in LHb GAD2 neurons promoted aggression in resident mice (Flanigan et al., 2020). The same population of GAD2 neurons provides local inhibition of the whole LHb. Taken together, these results support the notion that behavioral effects of the Hcrt system could be context specific. On one hand, Hcrt neurons may innervate LHb glutamatergic neurons in intruder mice to facilitate active coping by alleviating social avoidance. On the other hand, Hcrt neurons may innervate GABAergic neurons in resident mice to facilitate active coping behaviors by promoting aggression.

Nucleus Accumbens

Nucleus accumbens (NAc), a core region within the ventral striatum, plays a pivotal role in mediating motivation and reward-related behaviors (Salgado and Kaplitt, 2015; Wise, 2004). At the same time, NAc is implicated in regulating both social approach and social avoidance (Kohls et al., 2013; Williams et al., 2020). Unpublished data from our lab showed that

microinjection of Hcrt1 in the NAc shell (NAcSh) reduced social interaction in stress-naïve female but not male California mice. At the same time, inhibition of HcrtR2 in the NAcSh of stressed female California mice attenuated defeat-induced social avoidance. These results are in line with Li et al.(2021), where antagonizing NAcSh HcrtR2 in male rats exposed to restraint stress rescued anxiety-like behaviors in EPM and LDB tests. It is likely that activation of HcrtR2 in the NAcSh produces anxiogenic effects in both social and non-social situations. However, the rat study also found that intra-NAcSh Hcrt1 infusion increased center time during OF test, which was not observed in California mice. It is worth investigating whether the discrepancy is resultant from species differences.

Hcrt potentially regulates stress coping behaviors through interaction with dopamine signaling in the NAc. Hcrt has been shown to modulate dopamine release and dopamine receptor expression in the NAc (Kawashima et al., 2022; Morales-Mulia et al., 2020). Analysis of NAc single cell RNA sequencing data (Chen et al., 2021) revealed that a large percentage of Hcrt receptors are expressed in median spiny neurons (unpublished data). Electrophysiology recording and calcium imaging studies suggested that increased activation of D1, but not D2, medium spiny neurons are associated with a resilient phenotype in male mice subjected chronic social defeat stress (Francis et al., 2015; Muir et al., 2018). On the other hand, activating D1 neurons in female but not male California mice within NAcSh induced social withdrawal(Campi et al., 2014), which is similar to when the animals received Hcrt1 infusion. In male mice, optogenetic stimulation of Hcrt neuron terminals in the NAcSh activates local D2 neurons and the excitatory Hcrt-NAcD2 circuit is necessary for innate predator odor avoidance (Blomeley et al., 2018). It is worth investigating wether Hcrt interacts with D1 and D2 medium spiny neurons within the NAc to differentially promote active versus passive coping behaviors.

Bed Nucleus of the Stria Terminalis

It is reasonable to suspect that Hcrt receptor signaling in the bed nucleus of the stria terminalis (BNST) might promote passive coping response considering that the region is implicated in modulating anxiety-related behaviors (Fox and Shackman, 2019; Walker et al., 2003). In one study, microinjection of Hcrt1 into the BNST reduced social interaction in male rats (Lungwitz et al., 2012). In a different study, blocking HcrtR1 in the BNST increased social interaction time in male rats administered panic-inducing sodium lactate (Johnson et al., 2010). Meanwhile, unpublished data from our group did not find significant effects of intra-BNST infusion of Hcrt1 (same dose as in the Lungwitz et al., 2012) on social interaction behaviors in either male or female California mice. HcrtR1 antagonist also did not rescue social avoidance in mice that were exposed to social defeat. In a non-social context, Hcrt1 infusion in the BNST produced anxiogenic effects in male rats during EPM (Lungwitz et al., 2012) but not in male mice during OF (Chung et al., 2014). The BNST has many subregions with different subtypes (Dong et al., 2001). Activation of different subregions within the BNST could produce opposite behavioral effects (Steinman et al., 2018). For instance, activation of the oval BNST is anxiogenic whereas activation of the anterodorsal BNST is anxiolytic (Kim et al., 2013). Future studies focusing on Hcrt action in the BNST should address the anatomical differences between the subregions.

The BNST not only receives dense inputs from the hypothalamic Hcrt neurons but also sends direct reciprocal outputs (Giardino et al., 2018). Giardino and colleagues revealed two distinct populations of GABAergic BNST neurons, labeled by corticotropin releasing hormone (*Crh*) versus cholecystokinin (*Cck*) that directly innervate hypothalamic Hcrt neurons. Optogenetic activation of the *Crh* neurons promote avoidance whereas activation of the *Cck*

neurons promoted approach in real-time place preference (Giardino et al., 2018). It would be interesting to investigate whether the parallel projections from BNST to LH Hcrt neurons promote distinct behaviors in aversive versus appetitive social contexts.

Amygdala

The amygdala expresses both Hcrt receptors (Marcus et al., 2001) and receives direct inputs from the hypothalamic Hcrt neurons (Peyron et al., 1998). A human microdialysis study showed that the Hcrt1 level in the amygdala was maximal when the subjects reported positive emotions, negative emotions as well as during social interaction (Blouin et al., 2013). Preclinical evidence also suggests that Hcrt acting in the amygdala could potentially promote either active or passive coping behaviors (Arendt et al., 2014; Kim et al., 2013).

In a recent study, Yaeger and collogues (2022) classified the mice into resilient versus susceptible phenotypes based on whether they chose to escape or stay, respectively, when introduced with a novel larger aggressor. During aggressive encounters, infusion of HcrtR1 antagonist in the basolateral amygdala (BLA) decreased freezing behaviors in susceptible mice whereas HcrtR2 antagonist increased freezing behaviors in resilient mice (Yaeger et al., 2022b). Similar findings have been reported by the same group where either inhibition of HcrtR1 (Yaeger et al., 2022a) or activation of HcrtR2 (Staton et al., 2018) in the BLA mitigated stress phenotypes in susceptible mice by decreasing freezing time and promoting attention towards escape route. Further in-situ hybridization analysis revealed that Hcrt receptor 1 and 2 expression had little overlap within the BLA(Yaeger et al., 2022b). This could be an example where Hcrt regulates distinct coping behaviors by activating different receptor types expressed in competing circuits.

Intra-CeA infusion of Hcrt1 increased time in the open arms during elevated plus maze and time in the center during open field(Pan et al., 2020). The anxiolytic effects are blocked by HcrtR1 but not HcrtR2 antagonist, suggesting that the anxiolytic effects were mediated by HcrtR1 signaling in the CeA. Whether such behavioral effects are translatable to a social context requires further investigation.

Limitations and Future Directions

Women are at significantly higher risk than men to suffer from stress-related affective disorders (Altemus et al., 2014; Gater et al., 1998) and the Hcrt system possibly plays a role in female vulnerability to stress(Grafe and Bhatnagar, 2020). Both human and rodent research has revealed sex differences in the Hcrt system. *Prepro-hcrt* mRNA expression is two times higher in female rats (Jöhren et al., 2002). Female depressive patients also exhibit higher Hcrt1 level in the hypothalamus than male patients (Lu et al., 2017). In addition, sexually dimorphic expression of the Hcrt receptors are found in some brain regions (Jöhren et al., 2002; Loewen et al., 2017). However, many Hcrt studies were done in only male subjects. Limited studies that examined both sexes suggest that higher Hcrt activity in females might contribute to susceptibility to repeated stress exposure (Grafe et al., 2017; Grafe and Bhatnagar, 2020; James et al., 2014). Yet, little is known about how the Hcrt system mediates sex differneces in stress response at a circuit-level. Future Hcrt research should include both female and male subjects.

Hcrt has far-reaching behavioral and physiological impacts beyond mediating stress responses per se. It is implicated in arousal, homeostatic regulation, cognitive function, and reward seeking, etc (Mahler et al., 2014). When interpreting the behavioral outcomes of Hcrt

manipulation, we should keep in mind that the effects could potentially result from disruption of other biological functions. Including multiple behavioral measurements may help us interpret ambiguous data. For example, is the increase in social avoidance due to anhedonia or heightened social vigilance? These two phenotypes represent distinct arousal states and can be differentially modulated (Williams et al., 2020). Future research examining chronic stress exposure might also consider including sleep analysis as Hcrt plays a major role in promoting and maintaining wakefulness.

Conclusion

In this review, we proposed that the Hcrt system could promote either active or passive coping strategies in response to social stress across different brain regions and cell types. Previous stress exposure (e.g. social defeat) modulates Hcrt and Hcrt receptor expression and re-exposure to stress-related context activates the Hcrt system to facilitate stress coping behaviors. We only focused on five candidate regions, but other regions, such as the locus coeruleus (LC), ventral tegmental area (VTA), paraventricular nucleus (PVN), and paraventricular nucleus of the thalamus (PVT), are also involved in Hcrt-mediated stress response (Giardino and de Lecea, 2014; Li and de Lecea, 2020).

It has been suggested that the Hcrt could promote opposing coping strategies by differentially activating HcrtR1 versus HcrtR2, (Giardino and de Lecea, 2014; Summers et al., 2020; Yaeger et al., 2020). This is supported by evidence in the BLA, where activation of HcrtR1 promotes passive coping whereas activation of HcrtR2 promotes active coping(Yaeger et al., 2022b). However, in the NAc, activation of HcrtR2 is anxiogenic whereas activation of

HcrtR1 did not affect anxiety-related behaviors (B. Li et al., 2021). In the CA1 region of hippocampus, administration of either HcrtR1 or HcrtR2 antagonists was able to attenuate forced swim and food deprivation induced morphine reinstatement (Edalat et al., 2018). Although HcrtR1 and HcrtR2 could potentially promote opposing coping styles by expressing in competing circuits, such organization is not generalized to the whole brain. It is the specific cell types and circuits that Hcrt receptors are expressed in that drive the divergent coping strategies. For instance, Hcrt neurons may innervate glutamatergic neurons in the LHb to promote social approach in intruder animals but innervate GABAergic neurons in the LHb to promote aggression in resident animals, and both effects were mediated by HcrtR2 action (Flanigan et al., 2020; Wang et al., 2021).

In addition, the behavioral effects of the Hcrt system is more far-reaching than regulating individual differences in response to social stress. Unlike restraint or predator scent exposure, which are innately stressful, social interactions could be either rewarding or stressful depending on the context. Although we only discussed stressful social contexts in this review, the Hcrt system could also modulate individual differences in rewarding social behaviors, such as social play (Reppucci et al., 2020).

With the availability of new technology, such as single cell RNA sequencing, Hcrt-Cre mice and genetically encoded Hcrt sensor (Duffet et al., 2022), circuit-specific manipulation will help fill in the gaps of how endogenous Hcrt orchestrates a wide range of brain regions and cell types to facilitate diverse coping strategies.

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Chapter 3:

Hypocretin regulates social approach and social vigilance in a region and sex specific manner

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Abstract

Social anxiety disorder (SAD) is more prevalent in women than men but the mechanisms underlying such sex differences are unknown. The hypocretin (Hcrt) system is proposed as a novel treatment target for stress-related affective disorder that may also contribute to female susceptibility to SAD. Here, we examined the effects of Hcrt on social behaviors acting in the nucleus accumbens shell (NAcSh) and anterodorsal bed nucleus of the stria terminalis (adBNST) in male and female California mice (*Peromysucs californicus*). In female but not male California mice, intra-NAcSh infusion of Hcrt1 decreased social approach and increased social vigilance, a behavior in which an individual orients towards an unfamiliar conspecific while simultaneously avoiding it. The selective Hcrt receptor 2 antagonist infused into the NAcSh attenuated social avoidance in females previously exposed to social defeat stress but had no effects on social vigilance. In contrast, infusion of Hcrt1 in the adBNST had no behavioral effects in either male or female California mice. While Hcrt receptor 1 antagonist infused into the adBNST of stressed females also had no effects. Analyses of published single cell RNAseq datasets in *Mus muculus* detected distinct expression patterns of Hcrt receptor 1 versus Hcrt receptor 2 in the NAc and BNST. Our findings demonstrate that activation of Hcrt receptors in the NAc are sufficient to drive social anxiety-related behaviors and that Hcrt receptor 2 in the NAc are necessary for stress-induced social avoidance but not vigilance.

Introduction

Social anxiety disorder (SAD) is one of the most prevalent mental disorders across

nations(Stein et al., 2017). However, existing treatments, such as cognitive behavioral therapy and

selective serotonin reuptake inhibitors, are only effective in about half of the patients (Stein and

Stein, 2008). SAD is also more common in women than men, but the mechanisms contributing to

sex differences are still under investigation (Asher et al., 2017; Williams and Trainor, 2018). The

hypocretin (Hcrt), or orexin, system has been proposed as a novel treatment target for stress-

related mental disorders, including SAD (Summers et al., 2020), that may also underlie female

vulnerability to stress (Grafe and Bhatnagar, 2020).

One of the key symptoms of SAD is avoidance of social contexts (Stein et al., 2004).

Although the Hcrt system is best known for its effects on wakefulness and arousal (Berridge and

España, 2005; Carter et al., 2009), preclinical evidence suggests that it also regulates social

approach and avoidance behaviors. For instance, chemogenetic inhibition of Hcrt neurons

increased social approach in male rats that were previously exposed to social defeat stress and

showed short defeat latency (Grafe et al., 2018). However, optogenetic inhibition of Hcrt

neurons decreased social approach to novel conspecifics in male mice (Dawson et al., 2023).

Central manipulation using pharmacological approaches also showed that Hcrt could generate

either prosocial or antisocial effects (Chung et al., 2014; Eacret et al., 2019; Johnson et al., 2010;

Staton et al., 2018).

One explanation for the diverse behavioral outcomes is that the effects of Hcrt are brain

region-specific. The neuropeptide Hcrt is present in two isoforms, hypocretin-1 (Hcrt1) and

hypocretin-2 (Hcrt2), both cleaved from the same prepro-hypocretin (*prepro-hcrt*) gene (Soya

and Sakurai, 2020).There are also two Hcrt G-protein coupled receptors, hypocretin receptor 1

(HcrtR1) and hypocretin receptor 2 (HcrtR2) (de Lecea et al., 1998; Sakurai et al., 1998). HcrtR1 preferentially binds Hcrt1 over Hcrt2 while HcrtR2 binds both peptides with similar affinities (Gotter et al., 2012). Hcrt neurons are restricted within the lateral, dorsomedial and perifornical hypothalamus but project widely throughout the brain (Baldo et al., 2003; Peyron et al., 1998). The nucleus accumbens (NAc) and bed nucleus of the stria terminalis (BNST) are two candidate regions where Hcrt might promote social avoidance. Both regions receive direct input from hypothalamic Hcrt neurons (Peyron et al., 1998) and are implicated in regulating anxiety-related behaviors. For instance, microinjection of Hcrt1 in the nucleus accumbens shell (NAcSh) of male rats promoted anxiety-like behaviors during open field, light dark box and elevated plus maze tests (Li et al., 2021). In another study, intra-BNST infusion of Hcrt1in male rats reduced social interaction and increased time in the closed arms during elevated plus maze (Lungwitz et al., 2012).

However, existing research that investigates the behavioral effects of Hcrt acting in the NAc and BNST was only conducted in male animals (Johnson et al., 2010; Kim et al., 2023; Li et al., 2021; Lungwitz et al., 2012). Both human and rodent research has revealed sex differences in Hcrt peptide and receptor expression (Jöhren et al., 2002; Loewen et al., 2017; Lu et al., 2017), and suggests that higher Hcrt activity in females might contribute to the susceptibility to stress exposure (Grafe et al., 2017; Grafe and Bhatnagar, 2020). In this current study, we investigated both region- and sex-specific effects of Hcrt on social anxiety-like behaviors in California mice (*Peromyscus californicus*). The California mouse is a unique species in that both male and females are aggressive, and females show more prominent social avoidance after exposure to social defeat stress (Trainor et al., 2011). First, we respectively infused Hcrt1 in the NacSh and anterodorsal BNST (BNSTad) in both sexes and assessed subsequent social interaction

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behaviors. Next, we infused selective HcrtR2 antagonist in the NAcSh or selective HcrtR1

antagonist in the BNST of females previously exposed to social defeat stress to investigate

effects of endogenous Hcrt. The selective Hcrt receptor antagonists were used based on previous

in situ hybridization study, which suggests that NAc largely expresses HcrtR2 while BNST

largely expresses HcrtR1 (Marcus et al., 2001). We also analyzed published NAc and BNST

single cell RNA sequencing (scRNA-seq) data of *Mus musculus* to examine Hcrt receptor

expression in both regions.

Methods

Animal

All experiments on California mice were approved by the Institutional Animal Care and

Use Committee (IACUC) at the University of California, Davis. Adult male and female

California mice from our laboratory colony were co-housed in same-sex groups of 4. Mice were

kept on a 16:8 Light: Dark cycle and fed *ad libitum* (2016 Teklad global 16% protein rodent

diets). Sani-chip bedding, cotton nestlets, and Enviro-dri (Newco Distributors) were provided in

all cages. Drug infusion and behavioral tests were performed during the dark cycle.

Cannulation and Site-specific Injections

Males and females were implanted with 26-gauge bilateral cannula guides aimed at either

the NAcSh (A-P: +0.85 mm; M-L: ±1.1 mm; D-V: +5.85mm) or the adBNST (A-P: +0.0 mm; M-

L: ±1.0 mm; D-V: +5.1 mm) and given a 7-day recovery period. During recovery, the animals

received subcutaneous injections of carprofen as anti-inflammatory from day 1 to 3 and handled daily for 1 minute to get habituated to scruffing. During microinjection, 33-gauge internals that projected 1mm past the guides were used. All animals received 300nL bilateral infusions lasting 2 minutes and the internals were left in for an additional 30 seconds. Stress-naïve mice were randomly assigned to receive either saline (vehicle) or Hcrt1 (300ng or 30ng in the NacSh; 300ng in the adBNST; OrexinA, Tocris). The dosage selection was based on previous rodent studies (Li et al., 2021; Lungwitz et al., 2012).

A separate cohort of female mice were exposed to social defeat stress one week prior to surgery. Mice with guides aimed at the NAcSh received either saline (vehicle) or $13\mu g$ HcrtR2 antagonist (TCS OX2 29, Tocris). Mice with guides aimed at the adBNST received either 20% DMSO (vehicle) or or $0.3\mu g$ HcrtR1 antagonist (SB 334867, Tocris). The dosage selection was based on previous rodent studies (Heydendael et al., 2014; Li et al., 2021; Pan et al., 2020).

Social interaction test was performed 20 minutes following the infusion. Brains were collected for histology to confirm successful cannula placement and vaginal lavage was performed on all female mice to determine estrous stages and after behavioral testing.

Social Interaction Test

The social interaction test consists of 3 phases, each lasting 3 minutes (Greenberg et al., 2014). Mice were introduced into an empty arena (89 x 63 x 60cm) and allowed to freely explore during the open field phase. During the acclimation phase, an empty wire cage was placed against one side of the arena for habituation. For the social interaction phase, a same-sex unfamiliar target mouse was placed into the wire cage. Distance traveled, time in the center zone

(located 14cm from the sides), and time that the focal mouse spent within the interaction zone (within 8cm of the wire cage) were recorded and analyzed using AnyMaze. Time that the focal mouse spent outside of the interaction zone while its head oriented towards either an empty cage or target mouse was defined as vigilance and scored manually.

Social Defeat Stress

Mice assigned to social defeat were placed in the homecage of an aggressive same-sex mouse. Each defeat episode lasted 7 min or until the resident attacked the focal mouse 7 times, whichever occurred first. The intruder mice were immediately returned to their homecage following the defeat.

Statistical analyses

Behavioral data analyses were performed in Rstudio. The Shapiro-Wilk's test was used to test for data normality and the Fligner-Killeen test was used to assess homogeneity of variance. For experiment 1, two-way ANOVA (sex*treatment) followed by pairwise comparisons were used to analyze normally distributed behavioral data. For non-normal vigilance data, Kruskal-Wallis test was used followed by Dunn's test. For experiment 2 & 3, Welch t test was used for normal data and Mann-Whitney U test was used for non-normal data. Cohen's d was calculated to reflect effect size of the significant results. Animals with misplaced cannula guides were included in the analysis as anatomical controls when the sample size is larger than 6. No main effects or interaction effects (estrous*treatment) of estrous stage were detected (Supplementary table 1).

Single-cell sequencing data were analyzed in Rstudio using Seurat v4.0.4 (Hao et al., 2021). We accessed published data (Chen et al., 2021) from 11 adult male *Mus musculus* NAc single cell RNA-seq data containing 47,576 total cells, organized into 21,842 neuronal and 25,734 non-neuronal cells from GEO: GSE118020 along with cluster identities. A total of 57 *Hcrtr1+* and 464 *Hcrtr2+* cells were subsetted, analyzed for cell type composition, and visualized using the DotPlot function. The same approach was applied to the *Mus musculus* BNST snRNA-seq data containing 76,693 neurons across 7 adult female and 8 adult male biological replicates (Welch et al., 2019) from GEO:GSE126836).

Results

Experiment 1: Hcrt1 infusion in the NAcSh drives avoidance of a novel cage and social target.

There was a main effect of treatment in stress naïve animals that received intra-NAcSh Hcrt1 injection ($F_{3,52}$ = 8.775, p<0.001). In female California mice, microinjection of 30ng and 300ng Hcrt1 in the NAcSh of decreased social approach of a novel same-sex conspecific (p< 0.001, d= 2.18 for 30ng; p< 0.001, d= 1.54 for 300ng) (Fig. 1B). The effects on social approach behaviors were sex specific ($F_{3,52}$ = 8.775, p<0.001) and not observed in males. No interaction effect between treatment and sex was found ($F_{3,45}$= 2.035, p= 0.120). Females treated with 30ng, but not 300ng, Hcrt1 also exhibited increased social vigilance behaviors (p< 0.05, d= 1.38) (Fig. 1C). Such effects were not found in males (all p's> 0.05).

Infusion of Hcrt1 also induced avoidance of a novel empty cage during the acclimation phase in both males (p< 0.05, d= 0.90 for 30ng; p< 0.05, d= 1.43 for 300ng) and females (p< 0.01, d= 1.15 for 30ng; p< 0.01, d= 1.36 for 300ng) (Fig. 1D). A main effect of sex was observed

for time spent in the interaction zone during both acclimation phase ($F_{1,52}= 5.53$, $p < 0.05$) No differences were observed in the vigilance behavior during the acclimation phase ($H(3)= 3.02$, $p= 0.39$ for males; $H(3)= 1.33$, $p= 0.72$ for females), distance traveled during the open field phase ($F_{3,52}= 0.936$, $p= 0.43$), or time spent in the center of the open arena ($F_{3,52}= 0.201$, $p = 0.90$) (Fig. 1E-G) across treatment groups. And social interaction phase ($F_{1,52}= 4.65$, $p < 0.05$). No interaction effects (treatment*sex) were found (all p's > 0.05) for acclimation or open field phase. Animals with misplaced cannula guides did not show significant differences from controls (All p's > 0.05).
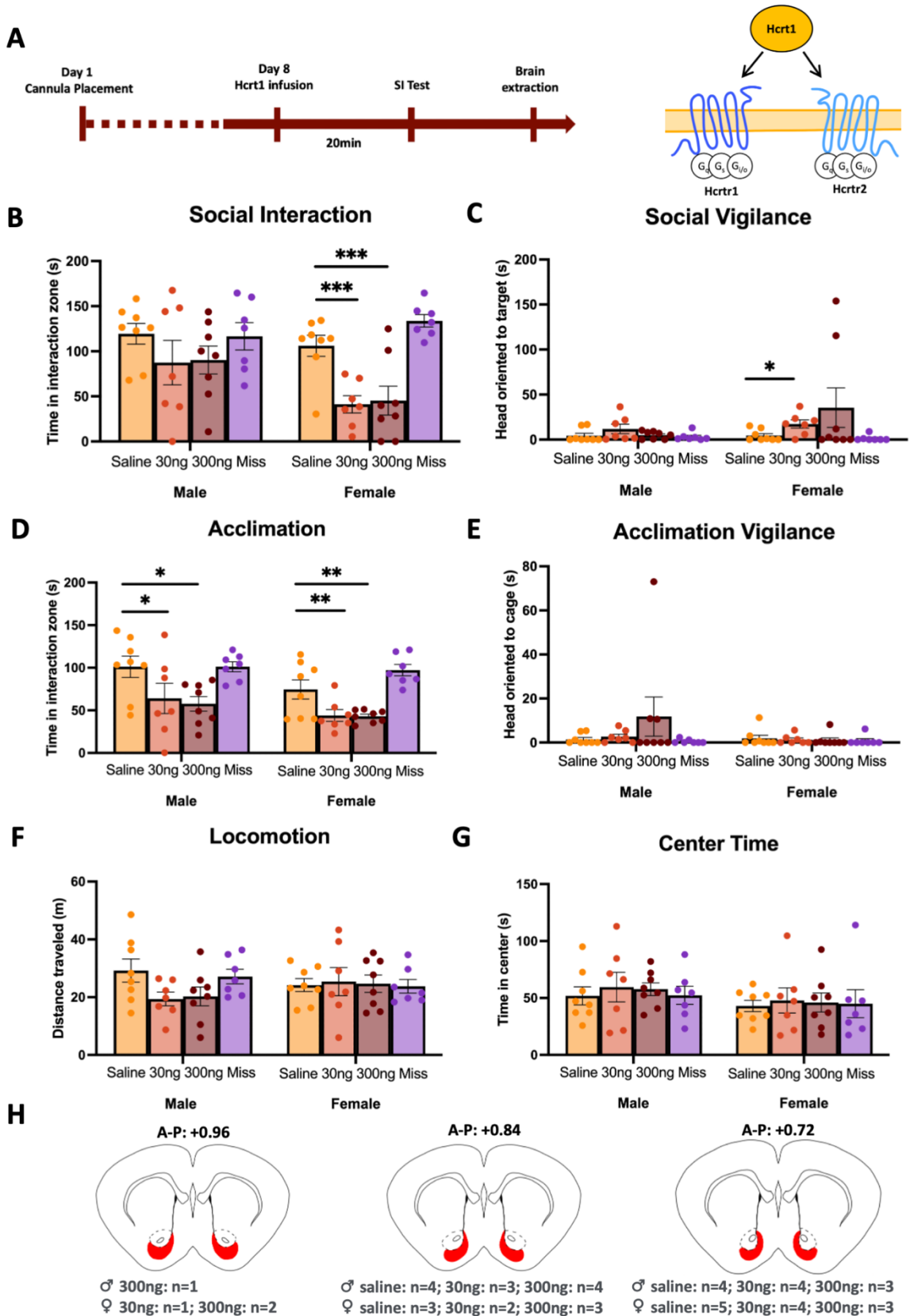
A Day 1 Cannula Placement — Day 8 Hcrt1 infusion — SI Test — Brain extraction (20min)

Hcrt1 → Hcrtr1 / Hcrtr2 (Gq, Gs, Gi/o)

B Social Interaction

C Social Vigilance

D Acclimation

E Acclimation Vigilance

F Locomotion

G Center Time

H

A-P: +0.96
♂ 300ng: n=1
♀ 30ng: n=1; 300ng: n=2

A-P: +0.84
♂ saline: n=4; 30ng: n=3; 300ng: n=4
♀ saline: n=3; 30ng: n=2; 300ng: n=3

A-P: +0.72
♂ saline: n=4; 30ng: n=4; 300ng: n=3
♀ saline: n=5; 30ng: n=4; 300ng: n=3

Figure 1. Hcrt1 infusion in the NAcSh promoted social avoidance and vigilance in stress-naïve female California. Timeline of experiment and schematic of mechanism of action for Hcrt1 (a). Infusion of both 30ng and 300ng Hcrt1 in the NAcSh reduced social approach in female, but not male, California mice (b). Infusion of 30ng but not 300ng Hcrt1 also increased social vigilance in females and had no effects in males (c). Intra-NAcSh infusion of 30ng and 300ng Hcrt1 decreased approach of a novel empty cage during the acclimation phase in both sexes (d) but had no effects on vigilance behavior towards the cage. Infusion of Hcrt1 in the NAcSh had no effect on distance traveled (f) or time spent in the center (g) during the open field phase. Schematic representing injection sites (red shading) of successful cannula placement in the NAcSh (h).

Experiment 2: Blocking HcrtR2 in the NAcSh alleviated social avoidance in stressed females.

Adult male *Mus musculus* NAc cells were grouped into 9 different clusters astrocytes, oligodendrocytes, endothelial cells, microglias, interneurons, dopamine receptor 1-expressing cells, dopamine receptor 2-expressing cells, oligodendrocyte progenitor cells, neuro stem cells and neuroblasts. Out of 47,576 NAc cells, 57 expressed *Hcrtr1* and 464 expressed *Hcrtr2*. The majority of *Hcrtr1+* neurons were D1 medium spiny neurons (40.35%) and interneurons (26.32%), while the majority of *Hcrtr2+* neurons were D1 and D2 medium spiny neurons (Fig. 3A). *Hcrtr1+* and *Hcrtr2+* also have distinct expression patterns across cell types. *Hcrtr1* had the highest percent expression (3.68%) and average expression (2.12 standard deviation above mean expression) within the interneurons, whereas *Hcrtr2* had the highest percent (1.13%) and average expression (1.96 standard deviation above mean expression) within D1 medium spiny neurons (Fig. 2B).

Stressed female California mice received selective HcrtR2 antagonist infusion in the NAcSh showed increased social interaction compared with stressed females received vehicle (t (15.96) = 2.49, p<0.05, d= 1.18) (Fig 2D). However, there were differences between groups in social vigilance behaviors (W=21.5, p=0.10) or during the open field and acclimation phases (all p's >0.05) (Fig 2 E-I).
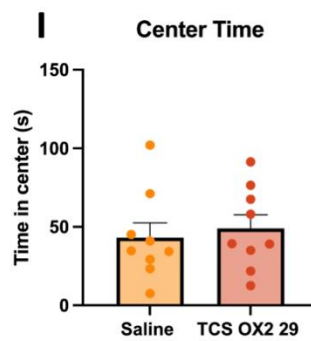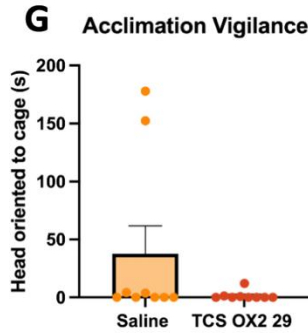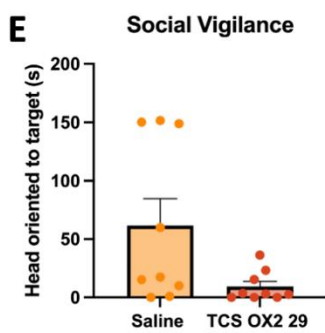
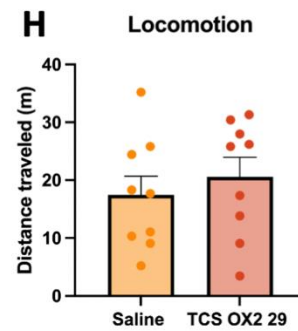**A** NAc HcrtR1+ Cells

- D1 — 40.35%
- D2 — 14.04%
- Endo — 1.75%
- IN — 26.32%
- Oligo — 17.54%
- Astro

NAc HcrtR2+ Cells

- 3.45%
- 0.22%
- 1.51%
- 0.43%
- D2 — 28.66%
- 65.73%

B

Percent Expressed
- 0.0
- 0.5
- 1.0
- 1.5
- 2.0

Average Expression
- 2
- 1
- 0

OPC, Oligo, NB, Micro, IN, Endo, D2, D1, Astro — Hcrtr1, Hcrtr2

C

Day 1-3 Social Defeat | Day 11 Cannula Placement | Day 18 HcrtR2 Antagonist Infusion | SI Test | Brain extraction

20min

TSC OX2 29

Hcrtr1 — G_q G_s $G_{i/o}$
Hcrtr2 — G_q G_s $G_{i/o}$

D Social Interaction

Time in interaction zone (s)

Saline — TCS OX2 29

*

F Acclimation

Time in interaction zone (s)

Saline — TCS OX2 29

H Locomotion

Distance traveled (m)

Saline — TCS OX2 29

E Social Vigilance

Head oriented to target (s)

Saline — TCS OX2 29

G Acclimation Vigilance

Head oriented to cage (s)

Saline — TCS OX2 29
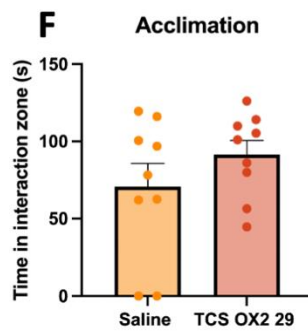
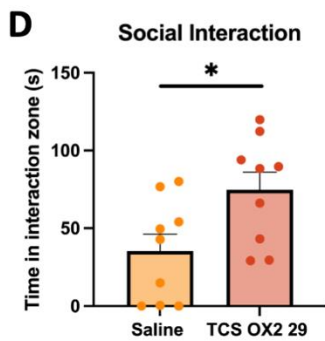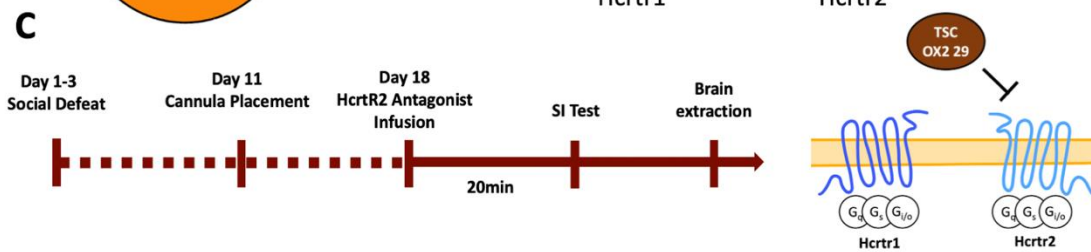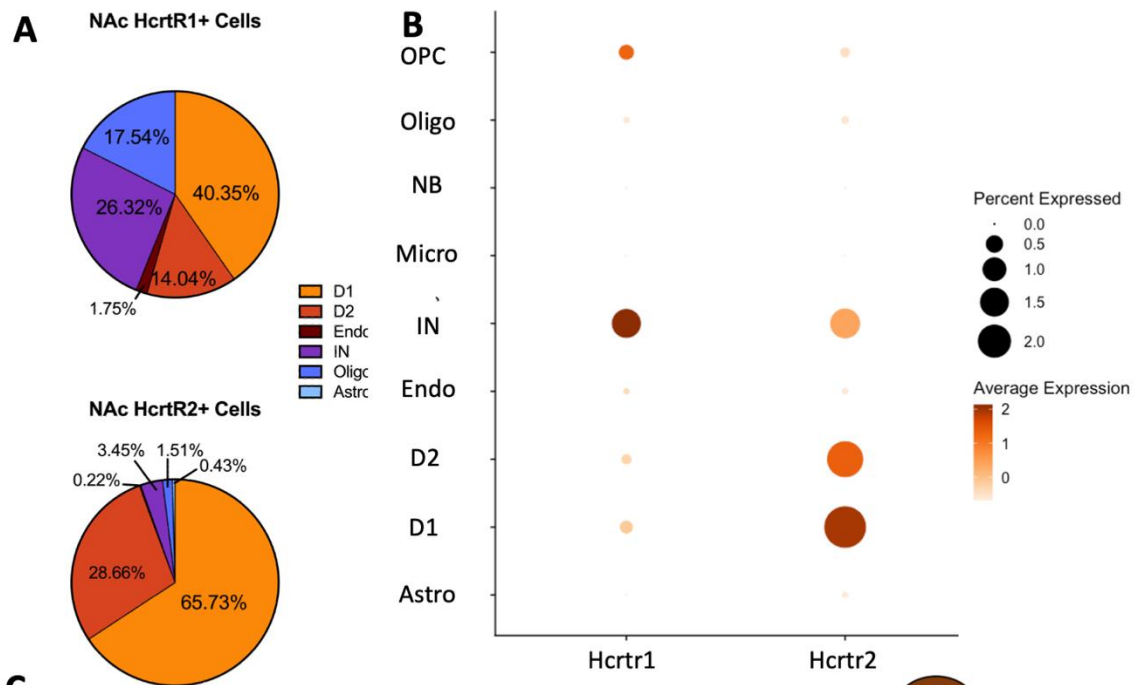**I** Center Time

Time in center (s)

Saline — TCS OX2 29

75

Figure 2. Selective HcrtR2 antagonist infusion in the NAcSh increased social approach but did not affect social vigilance behaviors in stressed female California mice. In male *Mus*, NAc cells were organized into 9 cells types: astrocytes (Astro), oligodendrocytes (Oligo), endothelial cells (Endo), interneurons (IN), dopamine receptor 1-expressing cells (D1), dopamine receptor 2-expressing cells (D2), and oligodendrocyte progenitor cells (OPC), microglias, neuro stem cells and neuroblasts (not shown in the figure). *Hcrtr1* is primarily expressed in the dopamine D1 receptor medium spiny neurons and interneurons, whereas *Hcrtr2* is primarily expressed in the D1 and D2 medium spiny neurons (a). A Dotplot shows that *Hcrtr1* and *Hcrtr2* have distinct expression patterns across all cell types in male *Mus*. *Hcrtr1* has the highest average and percent expression in the interneurons, whereas *Hcrtr2* has the highest average and percent expression in D1 neurons (b). Timeline of experiment in stressed female California mice and schematic of mechanism of action for selective HcrtR2 antagonist TSC OX2 29 (c). Infusion of TSC OX2 29 in the NAcSh of females California mice previously exposed to social defeat stress increased social approach (d) but had no effects on social vigilance behavior, behaviors during the acclimation phase (f, g), locomotion (h) or center time during the open field phase (i).

Experiment 3: Hcrt1 or HcrtR2 antagonist infusion in the adBNST had no effects on approach or vigilance behaviors.

Intra-BNST infusion of 300ng Hcrt1 did not affect behaviors during social interaction, acclimation, or open field phase in male or female California mice (Fig 3. B-G). Infusion of selective HcrtR2 antagonist also had no effects in stressed females during the 3-phase social interaction test (Supplementary Fig1. D-I).

In adult *Mus* BNST, *Hcrtr2* was more abundant than *Hcrtr1* (Supplementary Fig. 1A). In male *Mus*, 14.09% BNST cells express *Hcrtr2*, 2.94% express *Hcrtr1* and only 0.49% express both receptors. In female *Mus*, 13.35% BNST cells express *Hcrtr2*, 1.49% express *Hcrtr2* and only 0.28% express both receptors. Adult *Mus* neurons were grouped into 41 clusters based on Welch et al., 2019. Both receptor types were expressed across multiple neuron types and had similar expression patterns between males and females (Supplementary Fig. 1B). In both males and females, *Hcrtr2* had the highest average expression (2.5 standard deviation above mean expression) and the highest percent expression (87.68% of *Cplx3* cells for males and 87.32% for females) in the *Cplx3* (complexin 3) cluster found in the posterior BNST. In contrast, *Hcrtr1* had the highest average expression (2.5 standard deviation above mean expression) the highest percent expression (16.73% of *Esr2* cells for males and 7.91% for females) in the *Esr2* cluster in the principal subdivision of BNST.
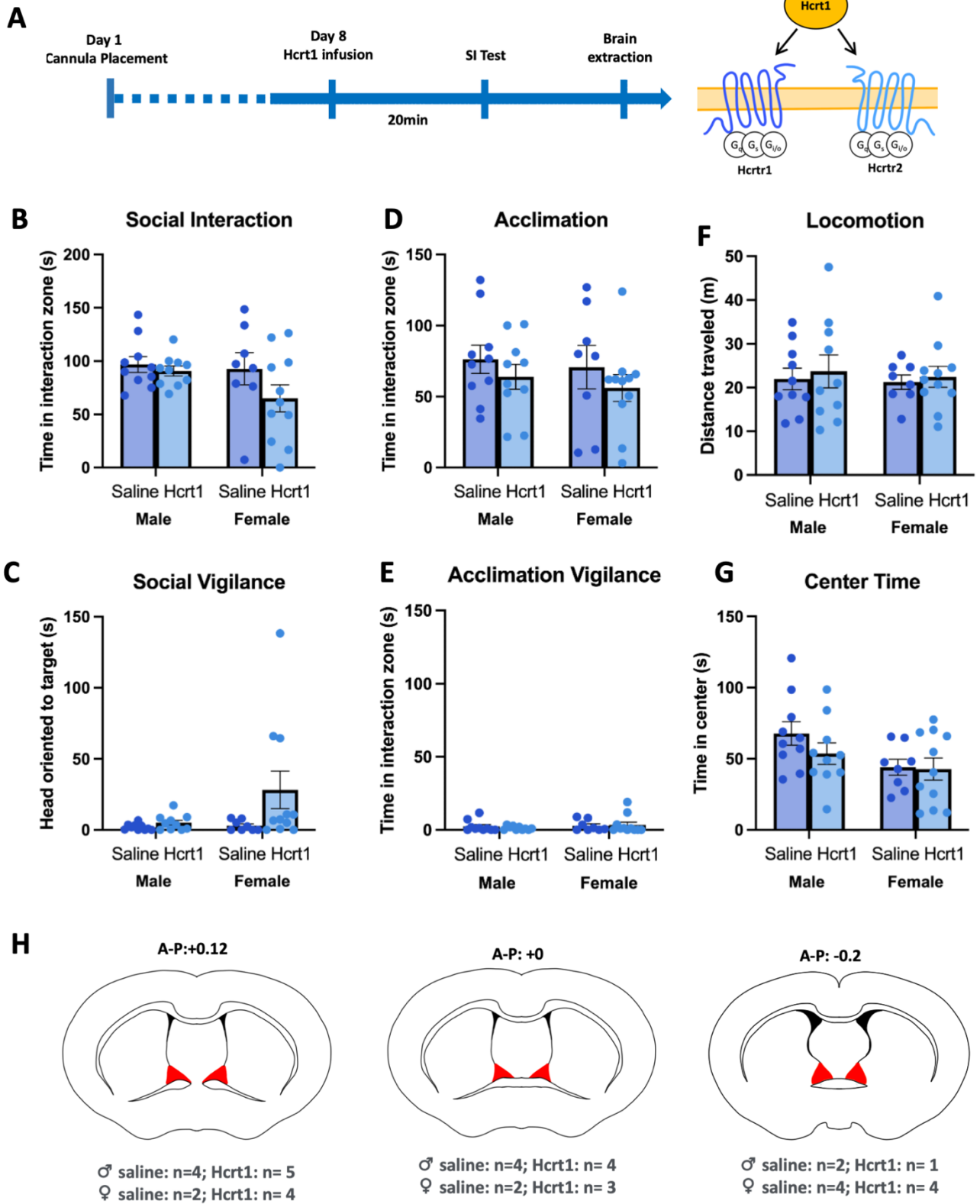
A Day 1 Cannula Placement — Day 8 Hcrt1 infusion — SI Test — Brain extraction (20min)

Hcrt1 → Hcrtr1 (Gs, Gs, Gi/o), Hcrtr2 (Gs, Gs, Gi/o)

B Social Interaction
Time in interaction zone (s)
Saline Hcrt1 — Male; Saline Hcrt1 — Female

D Acclimation
Time in interaction zone (s)
Saline Hcrt1 — Male; Saline Hcrt1 — Female

F Locomotion
Distance traveled (m)
Saline Hcrt1 — Male; Saline Hcrt1 — Female

C Social Vigilance
Head oriented to target (s)
Saline Hcrt1 — Male; Saline Hcrt1 — Female

E Acclimation Vigilance
Time in interaction zone (s)
Saline Hcrt1 — Male; Saline Hcrt1 — Female

G Center Time
Time in center (s)
Saline Hcrt1 — Male; Saline Hcrt1 — Female

H
A-P:+0.12
♂ saline: n=4; Hcrt1: n= 5
♀ saline: n=2; Hcrt1: n= 4

A-P: +0
♂ saline: n=4; Hcrt1: n= 4
♀ saline: n=2; Hcrt1: n= 3

A-P: -0.2
♂ saline: n=2; Hcrt1: n= 1
♀ saline: n=4; Hcrt1: n= 4

Figure 3. Hcrt1 infusion in the adBNST had no behavioral effects during the social interaction test in male or female California mice. Timeline of experiment and schematic of mechanism of action for Hcrt1 (a). Infusion of 300ng Hcrt1 in the adBNST in male and female California mice had no effects on social approach (b), social vigilance (c), approach or vigilance towards a novel empty cage (d, e), locomotion (f) or center time during the open field (g). Schematic representing injection sites (red shading) of successful cannula placement in the NAcSh (h).

Discussion

Using site-specific injection of Hcrt1 and Hcrt receptor antagonists, our study demonstrates that the effects of Hcrt on social approach and avoidance behaviors are anatomically and sex specific. Infusion of Hcrt1 into the NAcSh promotes social avoidance and social vigilance behaviors in female but not male California mice. Inhibition of HcrtR2 in stressed females increased social approach but had no effects on social vigilance. Intriguingly, intra-NAcSh infusion of Hcrt also induced avoidance of a novel empty cage in both sexes, while pharmacological manipulations of Hcrt receptors in adBNST had no effects on the behavior. Analyses of scRNA-seq data in NAc and BNST showed that HcrtR1 and HcrtR2 are expressed in different cell types, which likely contributes to anatomically specific effects of hypocretins on behavior.

Sex differences in the Hcrt system have been reported in both human and rodent literature. In women but not men diagnosed with depression, more Hcrt1 immunoreactive neurons were observed in the hypothalamus compared to healthy controls (Lu et al., 2017). In rats exposed to 5-day repeated restraint stress, female rats exhibited increased hypothalamic Hcrt

expression and increased Hcrt/c-fos colocalizations compared to males (Grafe et al., 2017). Sex differences in Hcrt receptor mRNA expression were also found in the brain. HcrtR1 expression in the hypothalamus (Jöhren et al., 2001) and HcrtR2 expression in the paraventricular nucleus (Loewen et al., 2017) were higher in female rats than males. Thus, it has been hypothesized that increased Hcrt receptor activation might contribute to increased female susceptibility to stress (Grafe and Bhatnagar, 2020). However, both optogenetic inhibition of Hcrt neurons and systemic administration of HcrtR1 antagonist decreased social interaction time with a novel conspecific in male but not female C57/BI6 mice (Dawson et al., 2023). Taken together, the source of sex specific effects of Hcrt system might be more complex than differences in overall Hcrt expression and activation *per se*. Brain region specific manipulation of receptor signaling will aid in understanding the mechanism of sex differences in Hcrt function. In the current study, intra-NAcsh infusion of Hcrt1 induced social avoidance and vigilance in female but not male California mice, which demonstrated that Hcrt receptor signaling in the NAcSh is a possible pathway where Hcrt regulates social anxiety-related behaviors in a sex dependent way.

Microinjection of HcrtR2 antagonist in the NAcSh of socially defeated female mice attenuated stress-induced social avoidance behaviors. The anxiogenic effects of NAc HcrtR2 signaling has been observed in non-social contexts. Intra-NAcSh infusion of selective HcrtR2 antagonist (but not HcrtR1 antagonist) increased exploratory behaviors during open field, elevated plus maze and light dark box tests in male rats exposed to acute restraint stress (Li et al., 2021). The anxiogenic effects of HcrtR2 signaling could be dependent on dopamine release. Electrophysiology recording in male rat brain slices showed that simultaneous application of Hcrt2 and dopamine to NAcSh slices increased the firing rate of a subpopulation of NAcSh neurons more than application of Hcrt2 alone (Mori et al., 2011). In male rats, infusion of Hcrt2

in the NAcSh potentiated D1 and D2 agonist-induced turning behaviors, while Hcrt2 alone did

not elicit such turning behaviors (Kotani et al., 2008). These findings are line with our single cell

RNA-seq analysis of male *Mus* that HcrtR2 is largely co-expressed with D1 and D2 receptors.

Interestingly, infusion of D1 agonist in the NAcSh reduced social interaction in unstressed

female but not male California mice, while infusion of D1 antagonist increased social approach

in stressed females (Campi et al., 2014). The sex-specific behavioral effects of D1 agonist and

antagonist mirror those of Hcrt1 and HcrtR2 administration, suggesting a possible interaction

between Hcrt2 receptors and D1 medium spiny neurons. However, it should be noted that the

single cell RNA sequencing analysis was done in male *Mus*, so future sequencing data are

needed from both sexes and in different species.

Interestingly, although Hcrt1 infusion in the NAcSh induced both social avoidance and

vigilance behaviors, HcrtR2 antagonist only significantly affected social approach in stressed

female California mice but had no significant effect on social vigilance. Previous findings from

our group showed that social avoidance and vigilance can be differentially regulated in the NAc

(Williams et al., 2020). Oxytocin receptor antagonist infusion into NAc core decreased social

approach without affecting vigilance behaviors in male and female California mice, whereas

activation of oxytocin receptor G_q signaling increased social approach and reduced social

vigilance in stressed females. In our current study, it is possible that endogenous Hcrt release in

the NAcSh was not involved in modulating social vigilance in stressed females. It is also

possible that social vigilance behavior is regulated by HcrtR1 instead of HcrtR2 signaling, since

previous studies in mice suggested that HcrR1 and HcrtR2 signaling could have differential

behavioral effects (Arendt et al., 2014; Summers et al., 2020; Yaeger et al., 2020). Future
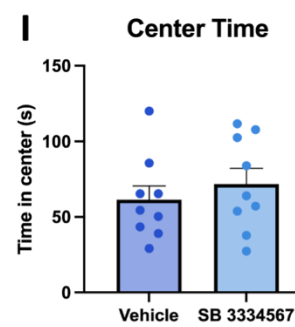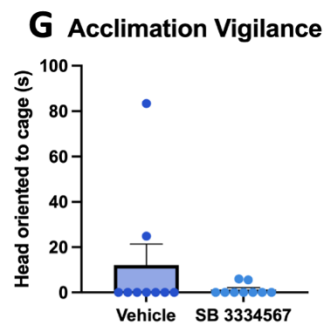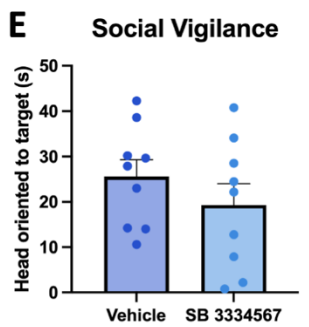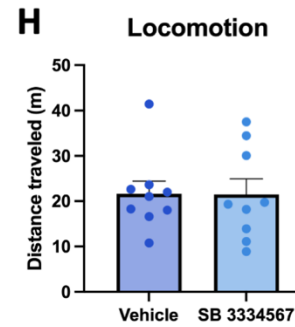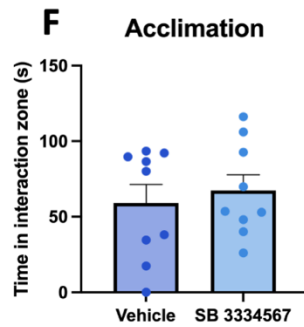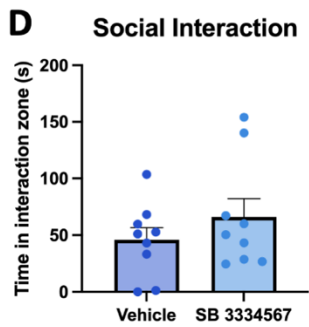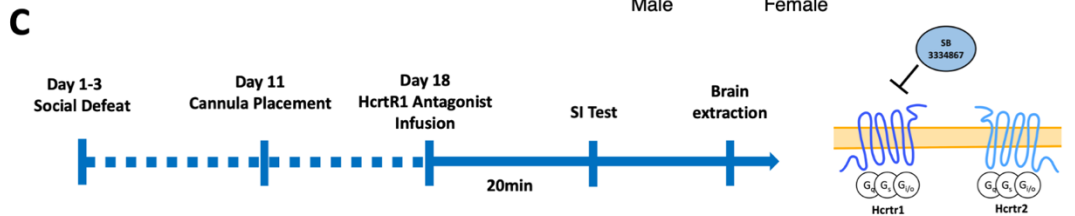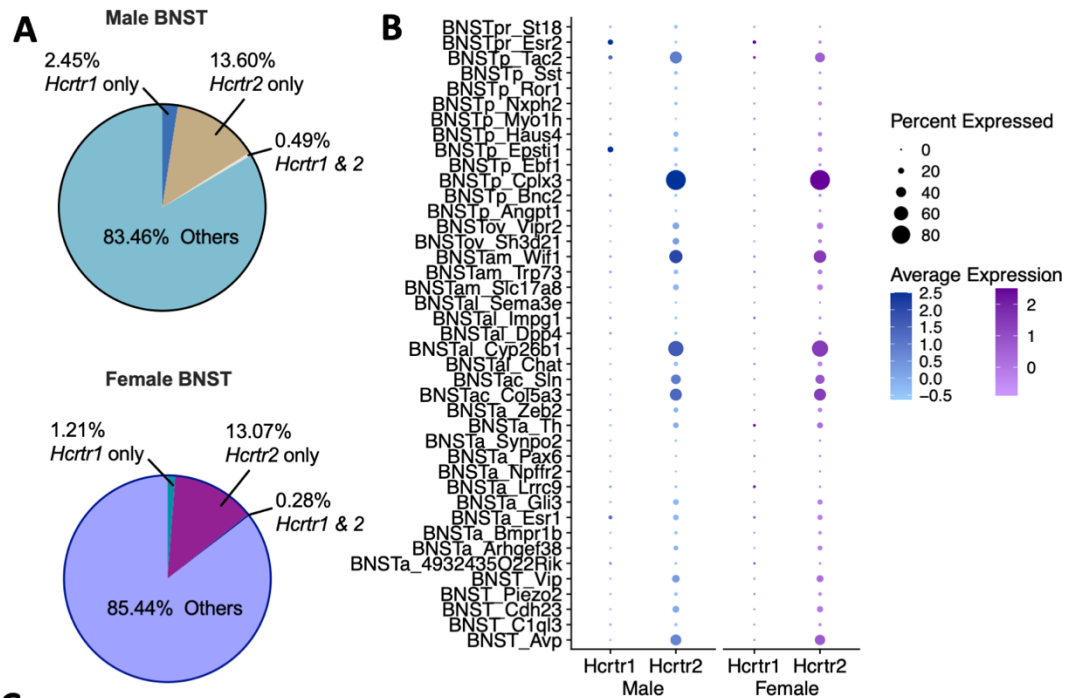
research should investigate how HcrtR1 signaling in the NAcSh may affect social anxiety-related behaviors.

Previous research shows that Hcrt signaling in the NAc is also involved in regulating general anxiety-related behaviors. Male rats received intra-NAcSh infusion of Hcrt spent less time in the open arms during elevated plus maze, light box during light-dark box, and center area during open fireld tests (Li et al., 2021). Although infusion of Hcrt1 in the NAcSh did not affect time in the center of an open field in California mice, both males and females showed decreased approach of a novel empty cage during the acclimation phase. Application of Hcrt1 or Hcrt2 to rat brain slices reduced NMDA receptor mediated currents in the NAc (Martin et al., 2002). In male rats, infusion of NMDA receptor antagonists in the NAc decreased the approach of novel objects, e.g. pens, cups and cans, (Maldonado-Irizarry and Kelley, 1994; Mogenson and Nielsen, 1984). Perhaps Hcrt1 infusion in the NAc decreased approach of the novel empty cage in California mice by inhibiting NMDA currents. It would be interesting to examine how administration of NMDA receptor antagonist alone and co-administration of Hcrt and NMDA receptor agonist in the NAc will affect exploratory behaviors of a novel object. Although intra-NAcSh infusion of Hcrt1 reduced approach of both a novel cage and a novel conspecific, in stressed females, HcrtR2 antagonist only increased interaction time with a novel mouse but not an empty cage. Other evidence suggests that the behavioral effects of Hcrt are often context-specific (Heydendael et al., 2014; Ji et al., 2019). For instance, chemogenetic activation of Hcrt neurons decreased social interaction time in defeated but not stress-naïve male rats (Eacret et al., 2019). Endogenous Hcrt might only be released in the NAcSh in a stressful context (i.e. presence of a social target) but not during a non-stressful context (i.e. presence of a novel object) to drive

avoidance behaviors, since social defeat only elicits avoidance of a social target in female California mice (Trainor et al., 2011).

Unlike NAcSh, microinjection of Hcrt1in the anterodorsal BNST did not affect social approach or social vigilance behaviors in either male or female California mice. These data contrast with previous studies which observed anxiogenic effects of BNST Hcrt receptors(Johnson et al., 2010; Lungwitz et al., 2012). The variability between our findings and previous work could be driven by Hcrt receptors having different behavioral effects in different subregions of the BNST. The BNST is an anatomically complex and functionally diverse region consisting of several subregions (Dong et al., 2001). Optogenetic studies in male mice showed that oval BNST activity promotes anxiety-like behaviors whereas anterodorsal BNST activity attenuates anxiety-like behaviors (Kim et al., 2013). In one study, Hcrt1 infused into both anterodorsal and anteroventral BNST of male rats decreased social interaction time in stress-naïve males (Lungwitz et al., 2012). In a different study, unilateral infusion of selective HcrtR1 antagonist in the principal nucleus of the posterior BNST increased social interaction time in male rats treated with panic-inducing sodium lactate (Johnson et al., 2010) Our sequencing analyses suggest that the principal subregion of posterior BNST has the highest average and percent expression of *Hcrtr1*. It is possible that Hcrt produces anxiogenic effects in some BNST subregions, such as anteroventral and the principal subregion, but not others, such as adBNST. Another interesting finding from the single-cell RNAseq data is that HcrtR2 is more abundantly expressed than HcrtR1 in adult *Mus musculus* of both sexes, which is the opposite from previous in-situ hybridization findings (Marcus et al., 2001). Future studies are needed to assess the behavioral effects of HcrtR2 signaling in the BNST.

In conclusion, Hcrt acting in the NAcSh but not adBNST induces social avoidance and social vigilance behaviors in stress-naïve female but not male California mice. HcrtR2 signaling in the NAcSh is necessary for expression of social avoidance but not social vigilance in stressed females. Taken together, our results suggest that Hcrt regulates social approach and vigilance behaviors in a brain region and sex dependent manner. Future studies should distinguish the behavioral effects of HcrtR1 and HcrtR2 signaling considering that the two receptor types have distinct expression patterns in the NAc and BNST. It will also be interesting to explore crosstalk between the Hcrt and other neurotransmitter systems, such as dopamine and NMDA, and how the interplay affects social anxiety-related behaviors. The current study suggests that the Hcrt system likely contributes to sex differences in SAD and may serve as a potential treatment target.

A

Male BNST

2.45%
Hcrtr1 only

13.60%
Hcrtr2 only

0.49%
Hcrtr1 & 2

83.46% Others

Female BNST

1.21%
Hcrtr1 only

13.07%
Hcrtr2 only

0.28%
Hcrtr1 & 2

85.44% Others

B

Percent Expressed
0
20
40
60
80

Average Expression

C

Day 1-3
Social Defeat

Day 11
Cannula Placement

Day 18
HcrtR1 Antagonist
Infusion

SI Test

Brain
extraction

20min

SB
3334867

Hcrtr1   Hcrtr2

D Social Interaction

F Acclimation

H Locomotion

E Social Vigilance

G Acclimation Vigilance

I Center Time

Supplementary Figure 1. Selective HcrtR1 antagonist infusion in the adBNST did not affect social interaction behaviors in stress female California mice. In both male and female *Mus musculus* BNST, *Hcrtr2* is more abundant than *Hcrtr1* and only a small percentage of the cells express both receptor types (a). Both receptor types were expressed across multiple neuron types and had similar expression patterns between males and females (b). Timeline of experiment in stressed female California mice and schematic of mechanism of action for selective HcrtR1 antagonist SB 334867 (c). Infusion of SB 334867 in the adBNST of females California mice previously exposed to social defeat stress had no effects on behaviors during the 3-phase social interaction test (d-i).

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