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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; Jane ek 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/G_q 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 microinjected in the NAc, atosiban, which blocks OTR/G_q coupling while activating OTR/G_i 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)

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.

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 *Ror1* cluster (Supplementary Data 1). 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, Supplementary Data 2). *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) (Supplementary Data 2).

Adult male *Mus* NAc cells were grouped into 8 different clusters: astrocytes, oligodendrocytes, endothelial cells, interneurons, dopamine receptor 1-expressing cells, dopamine receptor 2-expressing cells, and oligodendrocyte progenitor cells (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, Supplementary Data 3). We also visualized the expression of *Oxtr* across different interneuron subtypes (Fig. 5C, Supplementary Data 4) due to the heterogeneity of this cell type. *Oxtr* expression overlapped with somatostatin (*Sst*)-expressing interneurons and was generally absent from parvalbumin (*Pvalb*) and cholinergic (*Chat*) interneurons (Fig 5D). Calbindin1 (*Calb1*) and *Calb2* were expressed in all interneurons.

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-G_q 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 µg) 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 *Cyp26b1* 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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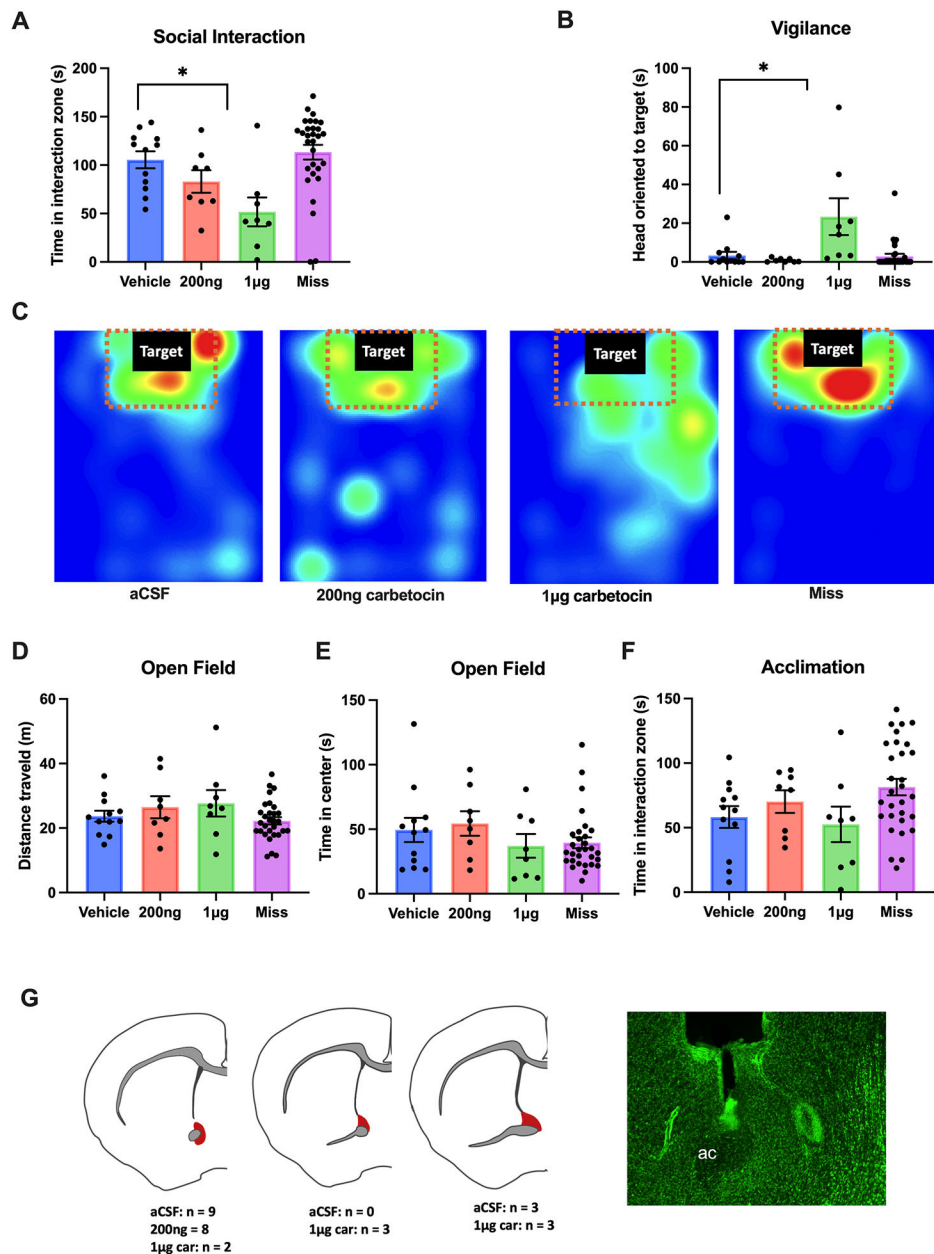


Figure 1. Intra-BNST carbetocin infusion reduced social approach and increased vigilance in female California mice.

Infusion of 1µg carbetocin in the anterior BNST (n=8), but not 200ng carbetocin (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).

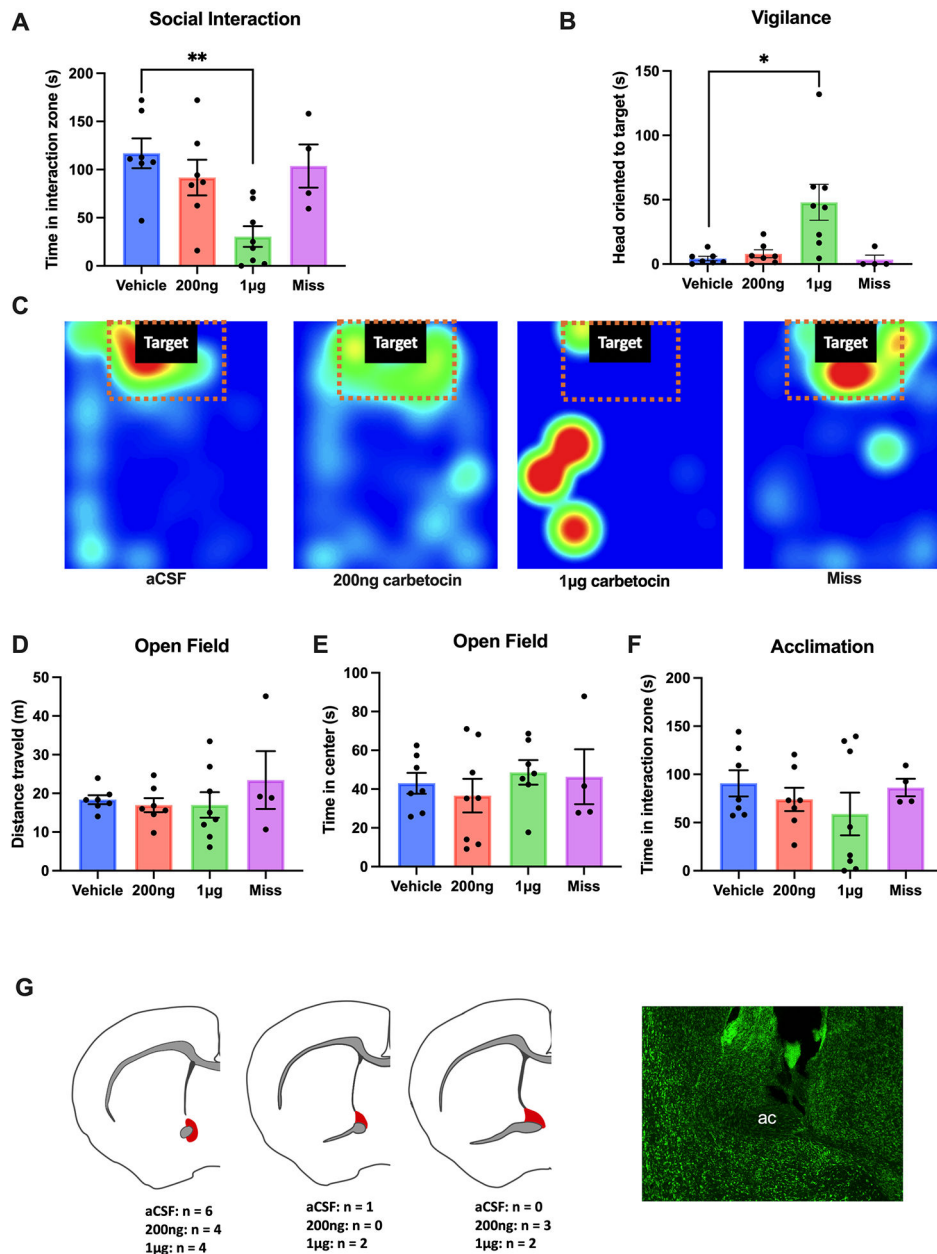


Figure 2. Intra-BNST carbetocin infusion reduced social approach and increased vigilance in male California mice.

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).

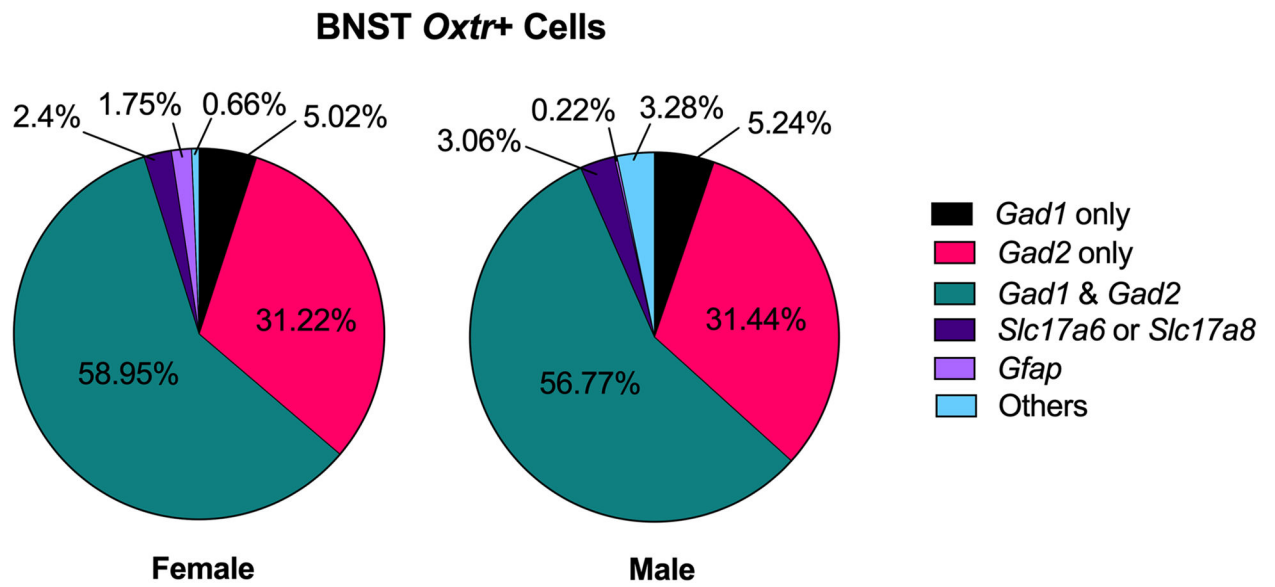


Figure 3. Characterization of *Oxtr*+ cells in adult *Mus musculus* BNST. Percentage expression of gene markers for inhibitory neurons (*Gad1* and *Gad2*), excitatory neurons (*Slc17a6* or *Slc17a8*) and glial cells (*Gfap*) in female (left) and male (right) *Oxtr*-expressing cells.

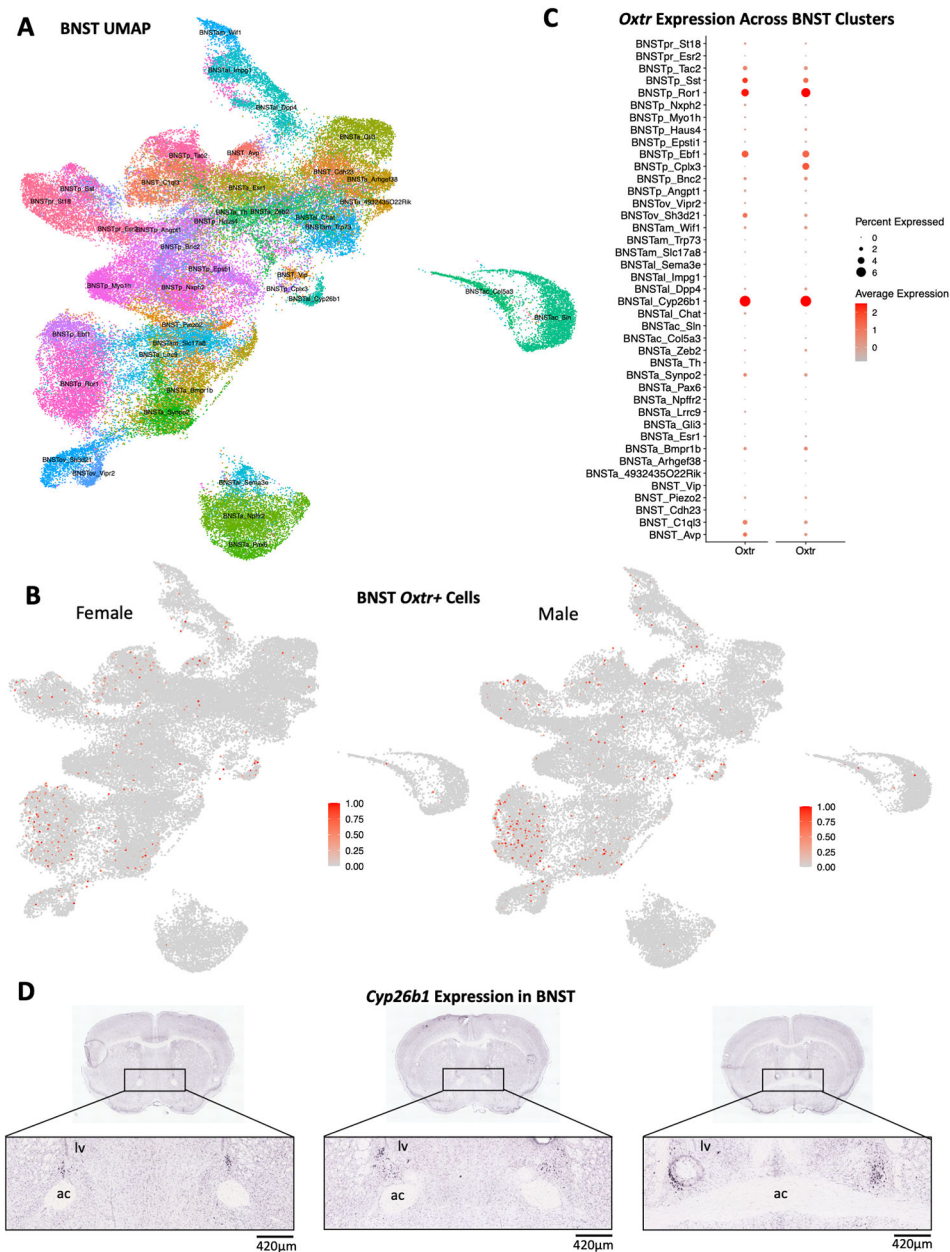


Figure 4. *Oxt* and *Cyp26b1* expression in adult *Mus musculus* BNST.

Oxt 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 *Oxt* (a, b). *Oxt* 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>.

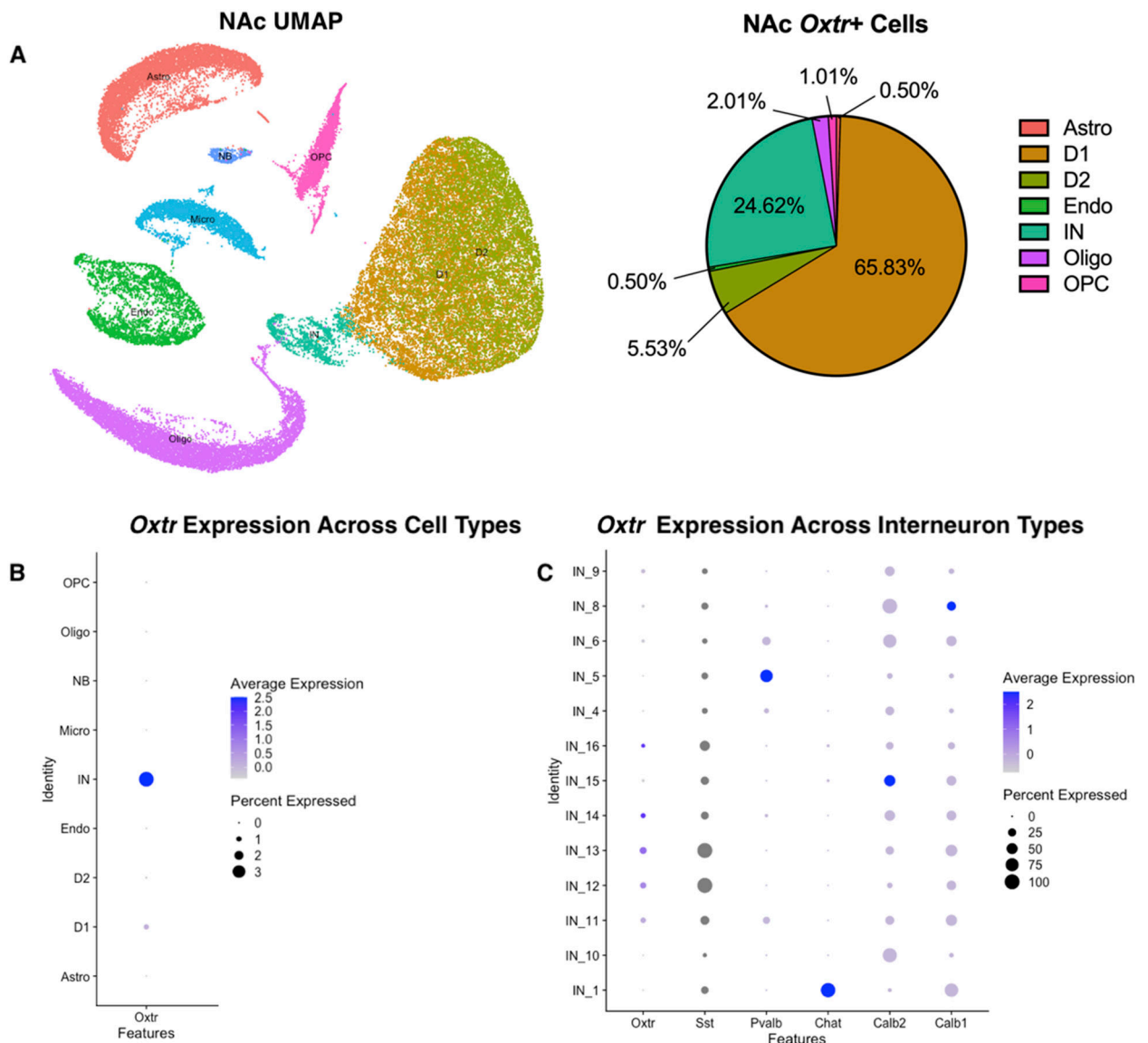


Figure 5. NAc *Oxtr*+ cell type characterization and *Oxtr* expression across clusters.

The UMAP shows 8 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) (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 shown 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.