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# Oxytocin Modulates Female Sociosexual Behavior through a Specific Class of Prefrontal Cortical Interneurons

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## SUMMARY

**Human imaging studies have revealed that intranasal administration of the "prosocial" hormone oxytocin (OT) activates the frontal cortex, and this action of OT correlates with enhanced brain function in autism. Here, we report the discovery of a population of somatostatin (Sst)-positive, regular spiking interneurons that express the oxytocin receptor (OxtrINs). Silencing of OxtrINs in the medial prefrontal cortex (mPFC) of female mice resulted in loss of social interest in male mice specifically during the sexually receptive phase of the estrous cycle. This sociosexual deficit was also present in mice in which the *Oxtr* gene was conditionally deleted from the mPFC and in control mice infused with an Oxtr antagonist. Our data demonstrate a gender-, cell type-, and state-specific role for OT/Oxtr signaling in the mPFC and identify a latent cortical circuit element that may modulate other complex social behaviors in response to OT.**

## INTRODUCTION

Complex behaviors in mammals are generated by the cerebral cortex in response to dynamic sensory cues and internal state. Although the neocortex can be subdivided into architectonic areas that conform generally to the sensory modality providing their input, and into associative areas that process information from multiple cortical and subcortical structures to generate appropriate behavioral outputs (Kandel, 2013), in each area the balance of excitation and inhibition governs its contributions to behavior and sensory perception (Haider et al., 2006; Isaacson and Scanziani, 2011). Disturbances in this balance are thought to play a role in the social impairments at the core of psychiatric disorders such as autism and schizophrenia (Kehrer et al., 2008; Markram and Markram, 2010; Rubenstein and Merzenich, 2003). Recent evidence has indicated altered inhibition may be especially important in the pathophysiology of these disorders (Chao et al., 2010; Oblak et al., 2011; Takahashi et al., 2013).

Inhibition in the cortex is generated by a large variety of cortical interneurons that release the neurotransmitter γ-aminobutyric acid (GABA). To gain insight into the circuit functions of inhibitory neurons in the cerebral cortex and to understand how their contributions to behavior can be modulated by external and internal cues, it is necessary to identify discrete cortical interneuron types and investigate their physiological and molecular properties (Fishell and Heintz, 2013; Kepecs and Fishell, 2014; Pfeffer et al., 2013). For example, detailed knowledge of neurotransmitter and neuropeptide profiles of defined neurons in the crab stomatogastric ganglion (Marder, 2012), and identification neuropeptide receptors expressed in *Caenorhabditis elegans* neurons (Flavell et al., 2013), have guided electrophysiological and interventional studies that revealed state-dependent contributions of these cells to behavior. Given these precedents, and the evolving concept of latent circuits that contribute to behavior in response to internal modulatory influences (Bargmann, 2012; Bargmann and Marder, 2013), it is important to identify cortical interneuron populations responding to specific neuromodulators and understand the contributions of these neurons to complex behaviors.

GABAergic cortical interneurons emerge from one of two embryonic subcortical progenitor zones, the medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE) (Fishell and Rudy, 2011), and diversify within the developing cerebral cortex to generate an as yet undetermined number of functionally distinct cell types (Ascoli et al., 2008; Fishell and Rudy, 2011; Nelson et al., 2006). While it is clear that they can be broadly categorized based on expression of calcium binding proteins and neuropeptides (Kubota et al., 1994; Markram et al., 2004), abundant evidence that these "cardinal" interneuron types can be further subdivided into functionally relevant subtypes has been obtained (Kvitsiani et al., 2013; Xu et al., 2013). However, molecular definition of these classes of cortical interneurons and determination of their contributions to cortical function and behavior has been difficult.

To identify and characterize an interneuron population that is involved in modulation of a complex behavior, we employed TRAP translational profiling for comparative analysis of the "cardinal" interneuron populations in the mouse cerebral cortex (Doyle et al., 2008; Heiman et al., 2008). This resulted in the discovery of a novel population of somatostatin expressing regular spiking interneurons that express the oxytocin receptor (Oxtr). To understand the role of these neurons in the complex social behaviors modulated by oxytocin, transgenic mice expressing Cre recombinase in Oxtr-expressing interneurons were generated (OxtrCre) and shown to target this small population of

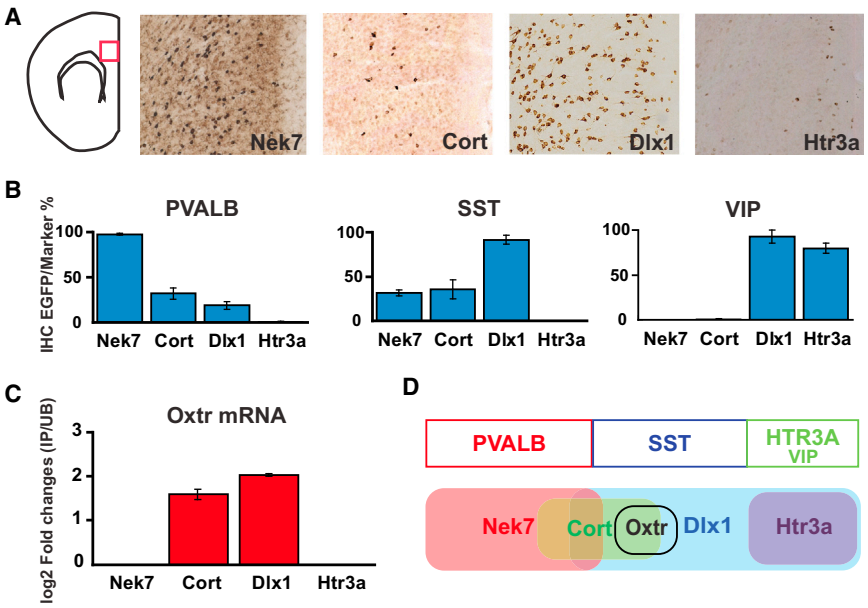


**Figure 1. Expression of the Endogenous *Oxtr* Gene in New bacTRAP Transgenic Lines Targeting Cortical Interneuron Populations**

(A) DAB immunohistochemistry (IHC) with anti-EGFP antibody on each mouse line in the medial prefrontal cortex (mPFC) reveals a unique and different pattern of expression for the EGFP-L10a transgene.

(B) Summary of immunofluorescence studies using anti-EGFP and anti-Pvalb, anti-Sst, or anti-Vip antibody for each line. The y axis is the percentage of specific neuronal markers (Pvalb, Sst, Vip) that is also positive for EGFP. Error bars represent SEM.

(C) Enrichment of *Oxtr* mRNA is observed in TRAP profiling data collected from Cort and Dlx1 interneurons, but not *Nek7* RNA or *Htr3a* RNA. Error bars represent SD.

(D) Diagram indicating that the *Oxtr* mRNA is present in a novel population of Sst-expressing cortical interneurons.

See also Figure S1.

oxytocin responsive cortical interneurons. Silencing of neurotransmission in OxtrCre interneurons in the medial prefrontal cortex (mPFC) resulted in a female-specific social deficit during estrus (but not during diestrus) that did not occur upon silencing of other interneuron subtypes or pyramidal neurons in the mPFC. The loss of normal sociosexual behavior in mice carrying OxtrCre neurons that have been silenced in the mPFC was also evident in wild-type (WT) female mice infused with an Oxtr antagonist and in female mice in which the oxytocin receptor gene (*Oxtr*) was conditionally deleted from the mPFC. Taken together, our data identify a novel subpopulation of Oxtr-expressing cortical interneurons that play a gender- and state-specific role in oxytocin signaling in the medial prefrontal cortex. They reveal a critical function for these neurons in a latent cortical circuit that governs female sociosexual behavior and suggest that this circuit may modulate other complex social behaviors.

## RESULTS

### Oxtr Is Expressed in a Novel Subpopulation of Sst-Positive Cortical Interneurons

To begin to probe further the diversity of interneurons in the cerebral cortex, we employed the translating ribosome affinity purification (TRAP) approach (Doyle et al., 2008; Heiman et al., 2008) in BAC transgenic mice expressing the EGFP/L10a ribosomal fusion protein (bacTRAP) targeting broad categories of interneurons. bacTRAP mice were generated using bacterial artificial chromosomes (BAC) containing the well-characterized interneuron markers distal-less homeobox 1 (Dlx1) (Cobos et al., 2005) and the serotonin receptor 3A (Htr3a) (Rudy et al., 2011) and the never in mitosis gene a (NIMA)-related expressed kinase 7 (Nek7) genes, as described previously (Doyle et al., 2008; Gong et al., 2003). The Nek7 gene was chosen as an alternative to parvalbumin (Pvalb) because its expression significantly overlaps in fast-spiking cortical interneurons and because this locus performs very well as a driver in transgenic mice.

It has been reported that three nonoverlapping marker populations parvalbumin (Pvalb), somatostatin (Sst), and serotonin receptor 3A (Htr3a) can account for nearly 100% of neocortical interneurons, and that all vasoactive intestinal peptide (Vip)-positive neurons are included within the Htr3a group (Rudy et al., 2011). Therefore, we examined the colocalization of Pvalb, Sst, and Vip proteins in these bacTRAP lines by immunofluorescence (Figure 1B). Consistent with previous studies (Cobos et al., 2005; Férézou et al., 2002), these bacTRAP mice targeted different, partially overlapping, GABAergic interneuron subtypes in the cortex. The Dlx1 GM520 line labeled nearly all Vip-positive (92.9%) or Sst-positive (91.6%) neurons in the cortex, but only 18.8% of the Pvalb-expressing cortical interneurons. The Nek7 MN733 bacTRAP line targeted nearly all Pvalb-positive (98.8%) neurons. The Nek7 line also labeled a subpopulation of Sst-expressing neurons (30%), although Nek7-expressing neurons did not express Vip. In contrast, the Htr3a GM433 line is expressed in the majority of caudal ganglionic eminence (CGE)-derived neurons (including Vip-positive neurons), but it does not label the medial ganglionic eminence (MGE)-derived Pvalb- or Sst-positive interneurons. Finally, to provide further discrimination in our comparative analysis, we generated a bacTRAP line for cortistatin (Cort GM130), which is expressed in subpopulations of both Pvalb- and Sst-positive neurons (de Lecea et al., 1997), but is not expressed in Vip-expressing cortical interneurons.

To identify more specific interneuron-expressed genes that might define a functionally coherent cortical interneuron cell type, TRAP translational profiles were collected from each of these lines and compared (Figure S1 available online). As expected, previously studied conventional interneuron markers were enriched in the TRAP expression data for each line, consistent with both our characterization of these lines (Figure 1) and studies of the complex patterns of expression of these genes in the cerebral cortex (Cobos et al., 2005; de Lecea et al., 1997; Férézou et al., 2002). Although many novel interneuron

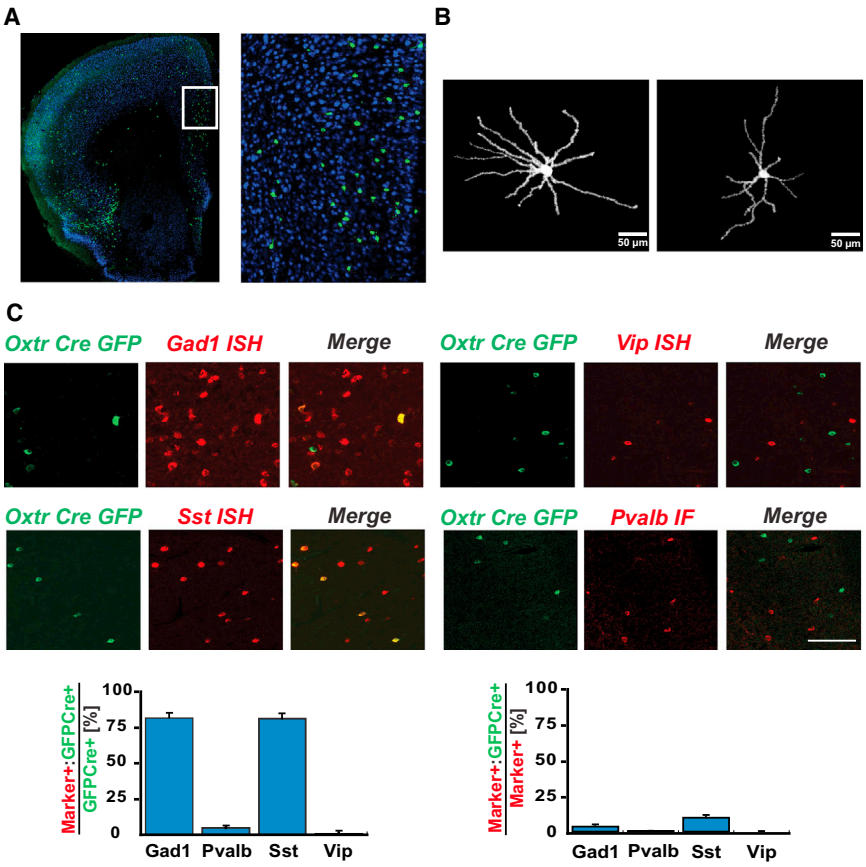


**Figure 2. Recombination in OxtrCre BAC Transgenic Mice Is Accurately Targeted to a Subpopulation of *Sst*-Expressing Cortical Interneurons**

(A) Immunofluorescence image showing EGFP-L10a expressing OxtrCre-positive neurons in the medial prefrontal cortex (mPFC). The right panel is a higher magnification image of the area indicated by the box in the left panel.

(B) Stellate morphology of ChR2-mCherry expressing OxtrCre neurons in mPFC.

(C) Immunofluorescence for EGFP (green) and in situ hybridization (ISH) or immunofluorescence images (red) for known interneuron markers (scale bar represents 100 μm). The EGFP signal overlapped with the *Gad1*/*Sst* ISH signal, but rarely with *Vip* ISH or Pvalb IF signal. Bar graphs showing quantitation of these results (n = 4): the majority of EGFP-positive cells (left panel) are positive for *Gad1* (81.8%) and *Sst* (81.3%), but do not express Pvalb (4.9%) or *Vip* (0.7%); EGFP-positive cells constitute only a small fraction of the total population of *Gad1*-positive interneurons (3.4%) or of the *Sst*-positive (9.5%) subpopulation of interneurons (right panel). Summary data are presented as mean ± SEM.

See also Figures S2, S3, and S4.

expressed genes are evident in these data, the biological role of oxytocin in mammalian social behaviors (Gimpl and Fahrenholz, 2001; Insel, 2010) and the unique expression pattern of the oxytocin receptor gene (*Oxtr*) in our data sets stimulated our interest in investigation of Oxtr-expressing cortical interneurons. Thus, the enriched expression of the *Oxtr* gene in the Dlx1 and Cort neurons but not the Nek7 or Htr3a TRAP profiles, and its absence in expression profiles collected from cortical pyramidal neurons (Schmidt et al., 2012), strongly suggested that Oxtr is expressed in a specific subpopulation of Sst-positive cortical interneurons (Figures 1C and 1D).

## An Oxtr Cre Driver Line Targeting a Specific Population of Cortical Interneurons

To investigate further the properties and function of Oxtr-expressing interneurons, a BAC transgenic Cre driver line was prepared as previously described (Figure 2) (Gerfen et al., 2013). The Oxtr Cre line was then crossed to an EGFP/L10a reporter line (Stanley et al., 2013) to characterize Cre-expressing cells in the cerebral cortex and determine their expression profile. OxtrCre recombination was evident in a scattered cell population in the mPFC (Figure 2A). There were no gender differences in the number of Oxtr Cre-positive neurons in the mPFC (Figure S2). Upon injection of a Cre-dependent AAV virus encoding ChR2-mCherry into the mPFC, OxtrCre neurons displayed a stellate morphology with multiple dendrites extending from the cell soma (Figure 2B).

Consistent with the comparative TRAP data used to select the *Oxtr* gene to target this class of neurons, EGFP/L10a recombination and expression in the Oxtr Cre line was evident in a subpopulation of *Gad1*-positive (81.8%) or *Sst*-positive (81.3%) interneurons (Figure 2C, left panels). These cells represented only a small minority of cortical interneurons, as we observed that only 3.4% of *Gad1*-expressing neurons and 9.5% of *Sst*-positive cortical neurons coexpressed EGFP/L10a (Figure 2C, right panels). Although expression of *Oxtr* was evident by in situ hybridization (ISH) in ~60% of the OxtrCre/EGFPL10a neurons (Figure S3), the very low signal-to-noise ratio in the *Oxtr* ISH studies prevented accurate quantitation of these results.

To provide further insight into the molecular nature of OxtrCre/EGFPL10a neuron population, TRAP expression profiles were collected from OxtrCre/EGFPL10a neurons (Figure S4A). These results support the anatomical studies: expression of *Gad1*, *Gad2*, *Oxtr*, and *Sst* are all enriched in the TRAP data from the OxtrCre neurons, whereas *Htr3a* and *Vip* are not enriched. Markers for cortical pyramidal cells (e.g., Emx1) are depleted in these data. Furthermore, comparative analysis of TRAP data collected from independently generated additional cortical interneuron and pyramidal cell bacTRAP lines (Schmidt et al., 2012), confirmed that expression of the endogenous *Oxtr* gene is evident in the OxtrCre/EGFPL10a, Cort, and Dlx1 TRAP data and not detected in the Nek7 and Htr3a interneuron or the S100a10 pyramidal cell data (Figure S4B). That the OxtrCre/EGFPL10a line reflected accurately the known markers for specific classes of cortical neurons is evident from the enrichment or depletion of known markers. Thus, *Pvalb* is expressed in Nek7,

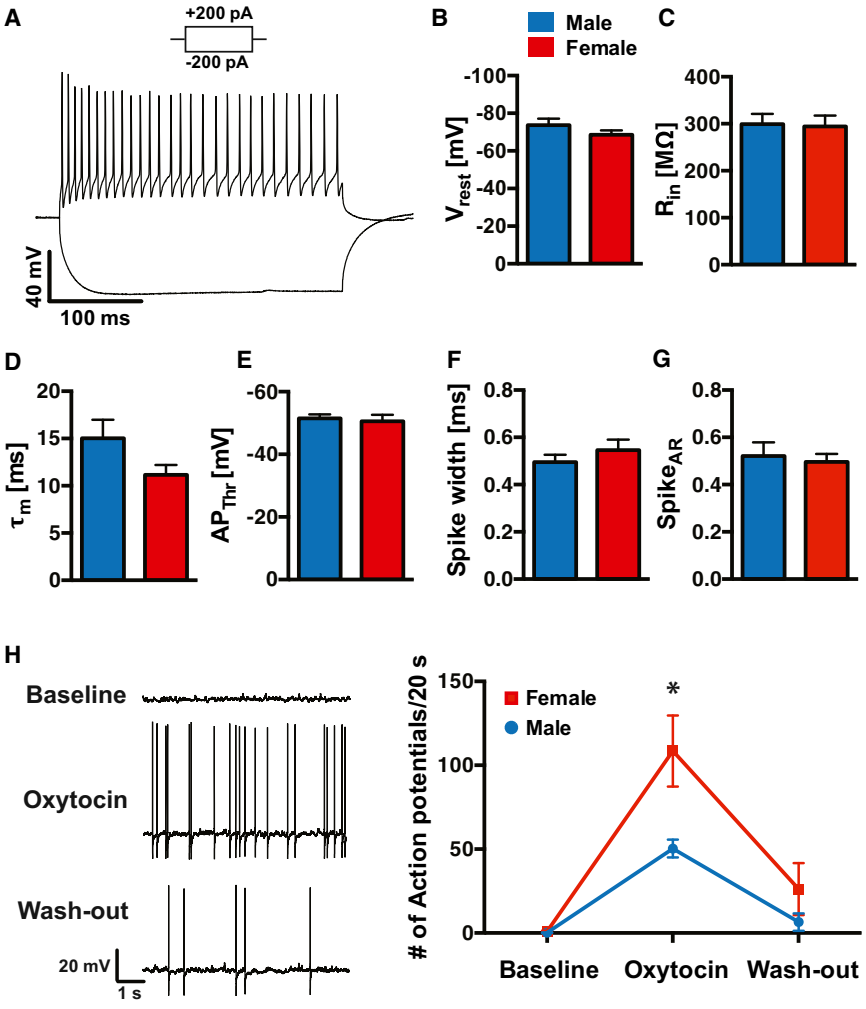


**Figure 3. OxtrCre Interneurons Initiate Action Potential Firing in Response to Oxytocin**

(A) A representative trace of OxtrCre interneuron activity upon a +200 pA and −200 pA current pulse at resting membrane potential.

(B–H) Plots showing the resting potential ($V_{rest}$) (B); the input resistance ($R_{in}$) (C); the membrane time constant ($\tau_m$) (D); the action potential threshold ($AP_{Thr}$) (E); the spike width (F); and the spike adaptation ratio ($Spike_{AR}$) (G) in OxtrINs from male (blue bars) and female (red bars) animals. Oxytocin receptor-positive neurons at $V_{rest}$ were not spontaneously active, but initiated action potential firing upon bath application of 100 nM oxytocin (H, left panel). This effect was more pronounced in females (108.5 ± 21.25 APs/20 s, n = 10), compared to males (50.3 ± 5.32 APs/20 s, n = 6; p = 0.024); wash-out of oxytocin partially reduced action potential firing to baseline level in both groups (H, right panel). Summary data are presented as mean ± SEM.

See also Table S1.

Cort, and Dlx1 interneurons and not detected or depleted in Oxtr and Htr3a TRAP data; *Sst* is enriched in Oxtr, Cort, and Dlx1 interneurons and depleted in Nek7, Htr3a, and S100a10 data; *Htr3a* is expressed in the Htr3a and Dlx1 TRAP data, but not the Oxtr, Nek7, Cort or S100a10 expression data; and *Emx1* is uniquely expressed in the TRAP data set collected from a pyramidal cell bacTRAP line (S100a10) (Figure S4C). Taken together, the immunofluorescence analysis of the Cre-expressing cells, the enrichment and depletion of conventional interneuron and pyramidal cell markers in the TRAP expression data prepared from the OxtrCre-targeted cell population, and the comparative studies of *Oxtr* expression in bacTRAP lines targeting known populations of interneurons and pyramidal cells identify the targeted neurons in this OxtrCre line as a novel subpopulation of Sst-positive cortical interneurons that express the endogenous *Oxtr* gene.

## OxtrINs Display a Distinct Physiological Profile and Spontaneously Fire upon Oxytocin Application

The novel molecular profile of OxtrINs suggests that their electrophysiological properties might also be distinctive. To determine whether this is the case, we performed whole-cell current-clamp recordings from OxtrINs from male and female mice to analyze their basic physiological properties, their action-potential (AP) firing pattern (Figure 3A) and potential gender differences in their physiology. In contrast to the previously studied Sst-positive Martinotti, X94, X98, or GIN interneuron types (Ma et al., 2006; Xu et al., 2013), no $I_h$-currents were observed in OxtrINs in either gender (Figure 3A). These currents are typically driven by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, which were not enriched in the OxtrIN TRAP data. Similar to Martinotti cells, OxtrINs displayed continuously adapting, regular AP-firing upon 200 pA current injections (Figures 3A and 3G; spike-adaptation rate [$Spike_{AR}$] = 0.52 ± 0.06 for male, 0.50 ± 0.03 for female), although their resting membrane potential was significantly more negative than Martinotti cells ($V_{rest}$ = −73.7 ± 3.39 mV for male, −68.6 ± 2.38 mV for female; Figure 3B) (Xu et al., 2013). Thus, although the general electrophysiological parameters of OxtrINs (input resistance [$R_{in}$]; membrane time constant [$\tau_m$]; AP-threshold [$AP_{Thr}$]; Spike width) (Figures 3C–3F; Table S1) are consistent with their identity as a subpopulation of Sst-positive cortical interneurons, their properties are not shared by previously characterized cortical interneuron types.

As predicted from their identification as interneurons that express the oxytocin receptor, OxtrINs respond robustly to OT. Thus, patch-clamped recordings of OxtrINs under basal conditions reveal that they are inactive. However, upon bath-application of oxytocin (100 nM) to the slice, the cells begin to fire APs (Figure 3H). Interestingly, although general physiological properties were not sexually dimorphic between males and females (Table S1), the oxytocin effect on AP-firing was more pronounced in OxtrINs from female, compared to male mice

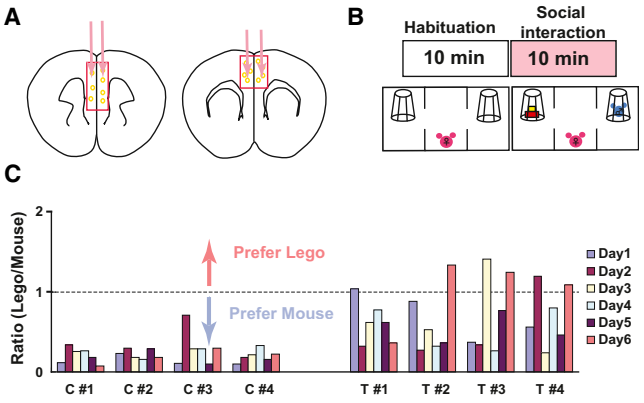


**Figure 4. Silencing of OxtrCre in the mPFC of Female Mice Results in Variations in Social Preference toward Male Mice over a 6-Day Period**

(A) Schematic diagram showing four injection sites of Cre-dependent AAV viruses encoding membrane-tethered toxins that block neurotransmission (APC/MPE) or control AAV viruses expressing EGFP.

(B) Diagram indicating the social interaction test. After 10 min habituation to the apparatus, a subject female mouse is exposed to a Lego (novel object, left panel) and a strange WT male mouse (stranger mouse, right panel) in wire cups for 10 min.

(C) Results of the social interaction at 6 consecutive days of testing for four control virus-injected OxtrCre female mice (C1–C4) and four t-toxin virus-injected OxtrCre female mice (T1–T4). The ratio of exploration of the novel object relative to the stranger male mouse indicated on the y axis (Lego cup/Mouse cup). A ratio of 1 indicates equal preference for the Lego and the stranger mouse, whereas values below 1 indicate a preference for the mouse versus the Lego. Control mice (C1–C4) prefer interaction with the male stranger mouse on all days of testing. OxtrCre interneuron silenced mice (T1–T4) lost their preference for social interaction with the male stranger mouse on certain test days. See also Figure S5.

(female: 108.5 ± 21.25 APs/20 s, n = 10; male: 50.3 ± 5.32 APs/20 s, n = 6; p = 0.024; Figure 3H). The resting membrane potential did not significantly change over the course of the experiment, and a 10 min wash-out of oxytocin partially restored initial baseline conditions in both genders (Figure 3H). Taken together, these data identify OxtrINs as a novel population of continuously adapting cortical interneurons, with a gender-specific response to oxytocin.

## OxtrCre Neurons in the Medial Prefrontal Cortex Are Required for Normal Female Sociosexual Behavior during Estrus

Oxytocin is a nonapeptide hormone that is known to play important roles in a variety of mammalian social behaviors, including maternal care, pair bonding, social recognition, stress, and anxiety (Gimpl and Fahrenholz, 2001; Insel, 2010). Given our demonstration that the action of oxytocin on OxtrCre neurons is to stimulate their activity, and recent studies showing that manipulation of the general balance of excitation and inhibition in the medial prefrontal cortex (mPFC) alters social behavior (Yizhar et al., 2011), we next tested directly the impact of OxtrCre neurons in the mPFC in mouse social interactions. To do so, we chose the viral-mediated tethered toxin (t-toxin) strategy for chronic inhibition of neurotransmission (Auer et al., 2010; Warner-Schmidt et al., 2012) in mPFC OxtrCre interneurons and the three chamber mouse social interaction test (Moy et al., 2004). Adult OxtrCre mice were injected stereotactically at four sites in the mPFC (Figure 4A) with AAV2/8 viruses that express tethered peptide toxins that inhibit the Cav2.1 and Cav2.2 calcium channels only after recombination in Cre-expressing neurons (thus blocking neurotransmission from mPFC OxtrCre neurons) or control viruses expressing EGFP alone. Virus infection was evident in cingulate, prelimbic, infralimbic, and medial orbital cortex (from bregma 2.5 to bregma −0.5 mm). Given the known roles of oxytocin in female sociosexual behavior (Ross et al., 2009) and the increased physiological response in female OxtrINs, we were particularly interested in female social interactions with male mice. Thus, following habituation to the three chamber social interaction box, virus-injected OxtrCre mice were given a choice to explore a chamber containing an unknown mouse or one containing a plastic Lego block (Figure 4B). Initial results from the tests were quite variable (Figure 4C). Although no effect of silencing was observed in the social interaction tests for any of the male mice tested (Figure S5), a strong but quite variable effect was observed in female mice injected with the silencing viruses (Figure 4C).

To ascertain whether this variability in female social approach behavior might result from interference with the actions of oxytocin in the mPFC during a specific time in their estrous cycle, new cohorts of OxtrCre females were injected with the t-toxin and control viruses in the mPFC, their social interactions measured, and vaginal smears collected and examined to determine whether the mice were in the estrus or diestrus phase of their cycle (McLean et al., 2012). As shown in Figure 5, silencing of OxtrCre neurons in the mPFC had a major effect on social interactions with male mice during the sexually responsive, estrus phase of their cycle. Thus, female mice in which these interneurons were silenced lost interest in male mice during estrus. However, these same animals retained their social interest in female mice during estrus and in male mice during diestrus (Figure 5). There was no impact of OxtrCre neuron silencing on the distance traveled (Figure S6) or in the novel object recognition test (Figure S7), demonstrating that the loss of social preference we observed is not due to hyperactivity. We conclude that Oxtr-expressing interneurons in the mPFC play an important role in the regulation of male-directed, female social behavior specifically during the sexually responsive phase of the estrous cycle.

To understand whether this phenotype occurred specifically as a result of altering the activity of this specific class of cortical interneurons, or whether it might result from an general imbalance in excitation/inhibition in the mPFC that causes a less specific alteration in social behavior, we measured the effects of silencing of several additional cell types on female-specific, estrus-specific social interest in males. Injections of the AAV t-toxin viruses into Htr3a (Chittajallu et al., 2013), Pvalb (Hippenmeyer et al., 2005), and Emx1 (Gorski et al., 2002) Cre driver lines had no impact on female-specific social behavior during estrus (Figure 6). Interestingly, Sst Cre-injected (Taniguchi et al., 2011) mice displayed a mild phenotype in the social interaction test. Although their social response to male mice during estrus was blunted, they retained interest in female mice as expected. This supports the finding that Sst-positive interneurons can be

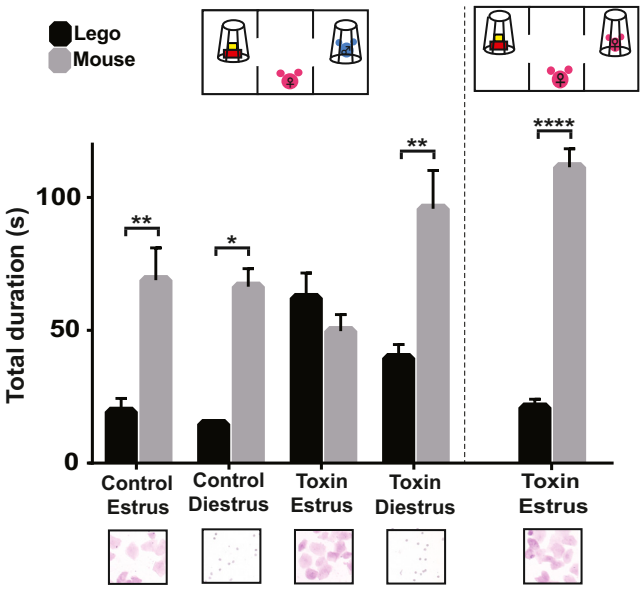


**Figure 5. Silencing of OxtrCre Interneurons in the mPFC of Female Mice Causes an Estrous Cycle-Dependent Loss of Social Interest in Male Mice**

Control virus injected in OxtrCre female mice (littermates of the experimental group) showed normal preference to male mice regardless of the stage of the estrous cycle (estrus, n = 11 **p ≤ 0.01; diestrus, n = 6; *p ≤ 0.05). T-toxin virus-injected OxtrCre female mice showed no preference to WT male mouse during estrus, the sexually responsive stage of the estrous cycle (n = 14). However, during diestrus (when they are not sexually receptive), these same animals display significantly stronger interest in male mice (n = 9, **p ≤ 0.01). T-toxin-injected mice display normal interest in female mice during estrus (right diagram and columns), indicating that general social recognition is not impaired during this phase of their cycle (n = 10, ****p ≤ 0.0001). Below each column is a photograph of cell shapes evident in vaginal smears taken from a representative female mouse in the test group. Summary data are presented as mean ± SEM. See Figures S6 and S7.

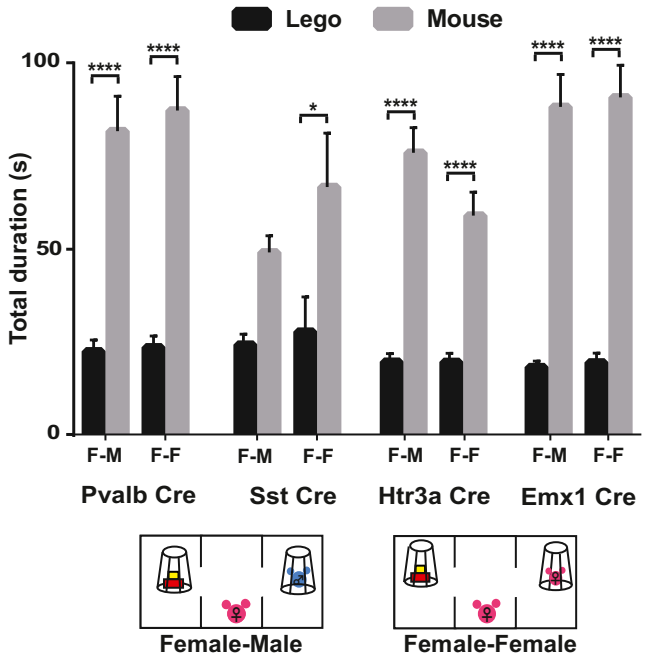


**Figure 6. The Sociosexual Behavioral Deficit Resulting from T-Toxin Silencing of OxtrCre Interneurons in the mPFC is Cell-Type-Specific**

Female-male (F-M) and female-female (F-F) social interactions (see diagrams below figures) during estrus in additional Cre driver lines injected with the T-toxin virus in the mPFC: Parv interneurons (Pvalb Cre), Sst interneurons (Sst Cre), Htr3a interneurons (Htr3a Cre), and pyramidal cells (Emx1 Cre). No statistically significant social deficit is observed in animals in which populations of cortical neurons that do not overlap with OxtrINs have been silenced: Pvalb Cre (n = 9, ****p ≤ 0.0001, n = 15, ****p ≤ 0.0001), Htr3a Cre (n = 9, ****p ≤ 0.0001, n = 8, ****p ≤ 0.0001), Emx1Cre (n = 11, ****p ≤ 0.0001, n = 12, ****p ≤ 0.0001). However, silencing of the parental interneuron population for OxtrINs in female mice (Sst Cre) resulted in a less robust social interaction with male mice during estrus (n = 9) while maintaining normal interactions with female mice (n = 11, *p ≤ 0.05). Summary data are presented as mean ± SEM. See also Figure S7.

subdivided into subtypes with distinct functions (Xu et al., 2013). These data suggest that OxtrCre neurons regulate specifically female sociosexual behavior, and demonstrate that a general imbalance in excitation/inhibition in the mPFC is not sufficient to alter this aspect of female sociality.

## Oxytocin Action in the mPFC Is Required to Regulate Female Sociosexual Behavior

The known roles of oxytocin in regulating female social behaviors (Campbell, 2008; McCall and Singer, 2012), and the present demonstration that OxtrCre neuron activity in mPFC is required for female social approach to male mice during estrus, strongly suggest that oxytocin acts within the mPFC to regulate female social behavior. To test directly this hypothesis, two approaches were employed. First, Cre recombinase expressing AAV viruses were injected into the mPFC of mice carrying a conditional allele of *Oxtr* gene (Lee et al., 2008) to delete *Oxtr* (and hence signaling by oxytocin) specifically in the mPFC. As shown in Figure 7A, genetic ablation of *Oxtr* in the mPFC of female mice again resulted in loss of social interest in male mice specifically during estrus, having no effect on social approach during diestrus (Figure 7A). To confirm this result, a second set of experiments were conducted using a specific antagonist of the Oxtr (Dölen et al., 2013). Thus, Oxtr-A (L-368,899 hydrochloride, 10 nM, and 0.5 μl) was infused bilaterally into the mPFC of female mice through a cannula to block pharmacologically the Oxtr during the social interaction test. Control mice infused with saline displayed normal sociality in these assays, whereas those females infused with Oxtr-A lost interest in male mice in the social approach test during estrus (Figure 7B). Taken together, the aberrant social approach behavior evident in the three chamber social interaction test as a result of either genetic or pharmacological disruption of oxytocin signaling in the mPFC proves that oxytocin action in the mPFC is required for normal female sociosexual behavior.

## DISCUSSION

A mechanistic understanding of mammalian brain function must include identification of cell types and circuits that control specific aspects of behavior and analysis of the internal and external cues that regulate their functions. In this study, we have

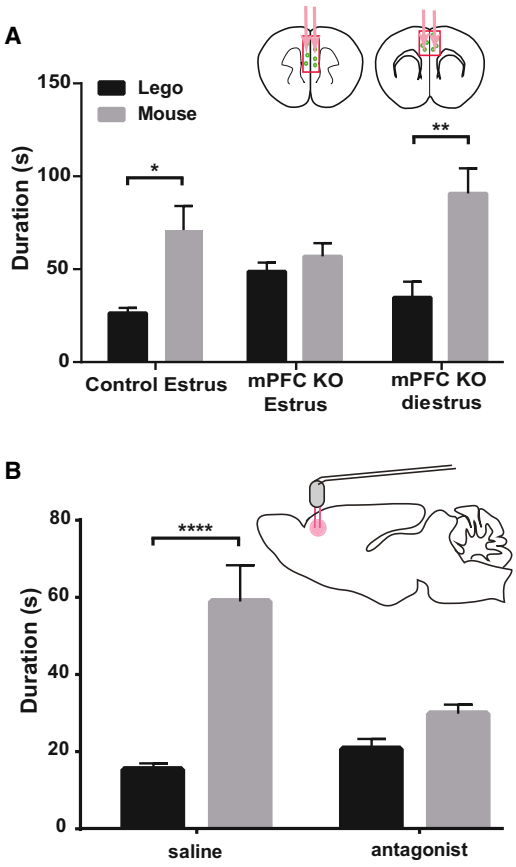


**Figure 7. Female Social Interactions with Male Mice during Estrus Are Oxytocin Dependent**

Two approaches were used to disrupt oxytocin signaling in the mPFC and measure its effects on female social behavior.

(A) Injection of an AAV Cre virus into the mPFC of female *Oxtr* conditional knockout (mPFC *Oxtr* KO) mice results in loss of social interest in male mice during estrus. Female mPFC *Oxtr* KO mice injected with the control virus (n = 7) displayed normal social behavior during estrus (*$p \leq 0.05$). mPFC *Oxtr* KO female mice at the estrus stage (n = 8) failed to show preference for male mice, however, they retained their social interest in male mice (n = 7) during diestrus (**$p \leq 0.01$).

(B) Wild-type female mice injected with the Oxtr antagonist into the mPFC (L-368,899 hydrochloride) (n = 11), also lost the social preference for male mice during estrus. Saline-injected control mPFC-injected female mice retained normal behavior during estrus (n = 9, ****$p \leq 0.0001$). Social interactions were tested using the three chamber social interaction test. Summary data are presented as mean ± SEM.

employed TRAP translational profiling (Doyle et al., 2008; Heiman et al., 2008) of cardinal interneuron populations in the cerebral cortex (Rudy et al., 2011) to identify a subpopulation of somatostatin-positive, oxytocin-responsive, regular spiking interneurons. Chronic silencing of this interneuron population (OxtrINs) in the mPFC revealed that it modulates female social interactions with male mice during estrus, the sexually receptive phase of the estrous cycle. Deletion of the *Oxtr* gene in the mPFC, or pharmacological inhibition of Oxtr activity at this site, demonstrated that modulation of this aspect of female social behavior requires oxytocin. Taken together, our data identify a novel interneuron population in the mouse prefrontal cortex that regulates an important aspect of female sociosexual behavior and suggest that OxtrINs in the mPFC may be important for the modulation of social interactions in response to the changing levels of oxytocin that occur in a variety of contexts in nonhuman primates (Moscovice and Ziegler, 2012) and humans (Heinrichs and Domes, 2008).

## Are OxtrINs a New Cell Type?

It is generally agreed that identification of new central nervous system (CNS) cell types must include data demonstrating their anatomical and molecular properties, their physiological characteristics, and their contributions to CNS function (Ascoli et al., 2008; Markram et al., 2004). In this study, we have determined that OxtrINs in the mPFC express a distinctive set of molecular markers, that they are continuously adapting, regular spiking GABAergic neurons, that they respond to oxytocin by increasing their basal firing rate, and that oxytocin action on these neurons modulates a very specific aspect of female social behavior. These properties, particularly the specific function of OxtrINs relative to other Sst-positive subpopulations and Htr3a- or Pvalb-expressing interneurons, allow us to conclude provisionally that OxtrINs are a novel, functionally coherent cell type. However, there are two issues that preclude a definitive conclusion regarding OxtrIN identity. First, we have not provided detailed anatomical data placing the OxtrINs within cortical circuitry, or examined possible variations in OxtrINs in other regions of the cerebral cortex. Second, while our comparative data demonstrate that OxtrINs share a gene expression signature in their ground state that is distinct from other known cortical interneuron populations (Figure S4), these analyses have been done using data from broad classes of interneurons that are not sufficiently well defined to allow us to assign a unique molecular identity to OxtrINs. Nevertheless, the data that we have collected thus far, and the fact that there is only one known receptor for oxytocin (Gimpl and Fahrenholz, 2001), suggest that further molecular and functional studies of OxtrINs may confirm our provisional definition of these cells as a coherent, modulatory cell type that participates in a latent cortical circuit controlling specific oxytocin-dependent behaviors (Bargmann, 2012).

## Oxytocin Function during the Estrous Cycle

A central finding of our study is that experimental disruptions of oxytocin responsive interneurons in the mPFC, or of oxytocin signaling to these neurons, results in loss of female social approach to male mice in sexually receptive estrus females but not in sexually unresponsive diestrus females. In a general sense, this finding fits in well with previous studies of prosocial behavioral effects of the hormone (Donaldson and Young, 2008; Insel and Young, 2001) and with the variations in peripheral oxytocin levels that are known to occur during estrus in a variety of species. Studies of rodents (Crowley et al., 1978; Van Tol et al., 1988), nonhuman primates (Perlow et al., 1982), and women (McCarthy and Altemus, 1997; Richard and Zingg, 1990) have provided evidence that there is an estrogen-dependent surge in oxytocin in the periovulatory period that facilitates female sexual receptivity. Recent evidence in freely cycling female baboons in the wild (Moscovice and Ziegler, 2012), or in human couples

(Scheele et al., 2012), have provided the additional insight that oxytocin acts primarily in these contexts to improve interactive reciprocity between consorting partners. However, the levels of OT binding varies between brain area and during estrus (Dumais et al., 2013), and Greer et al. (1986) have reported that OT concentrations in the paraventricular hypothalamus were significantly greater during diestrus. In spite of the apparent complexities in OT regulation during the estrous cycle, we believe the simplest interpretation of our results is that OxtrINs respond to the fluctuations in oxytocin levels that occur during the estrous cycle, and that they modulate latent cortical circuits that play a fundamental role in the female receptivity to males. Our observation that OxtrINs in the mPFC of female mice respond to OT more robustly than those in male mice (Figure 3) suggests that female mice may be more sensitive generally than male mice to changing levels of OT. Although OxtrINs have not been identified or studied in the cortex of other mammals, it seems probable that this new class of interneurons and their associated circuitry may modulate preferentially female social interactions.

### Oxytocin and the Cerebral Cortex

A large number of studies in experimental animals and humans have established that oxytocin regulates many aspects of mammalian social behavior, from the regulation of affiliate behaviors between individuals to social behaviors in groups (Anacker and Beery, 2013). *Oxtr* knockout mice display deficits in social memory (Lee et al., 2008) and social approach behavior (Nishimori et al., 2008). It is apparent that oxytocin production in the hypothalamus is regulated in response to social interactions in many species (Leng et al., 2008), that oxytocin-expressing neurons project widely (Knobloch et al., 2012; Knobloch and Grinevich, 2014), and Oxtr is expressed in many brain structures (Gimpl and Fahrenholz, 2001; Yoshida et al., 2009). The fact that OxtrINs are dispersed throughout the cerebral cortex, and that within the mPFC these cells are important for female interactions with male mice specifically during the peak of peripheral oxytocin that occurs around estrus, suggests that these neurons participate in a latent cortical circuit (Bargmann, 2012) that can modulate behavior in a state-dependent manner. It seems probable that this circuit will be activated in response to elevated oxytocin levels in a variety of contexts, and that it will mediate the contributions of the cerebral cortex to oxytocin-dependent behaviors. Recent studies of the effects of intranasal oxytocin administration in children and adults with autism spectrum (ASD) disorder are of particular interest. Although it remains unclear whether oxytocin therapy will be safe and effective for long term use in the treatment of social deficits associated with ASD (MacDonald et al., 2011), encouraging results in clinical trials have emerged recently (Gordon et al., 2013; Guastella and MacLeod, 2012; Watanabe et al., 2014). Our studies strongly implicate the mPFC and other cortical areas in the behavioral effects of oxytocin administration and treatment and suggest that further investigation of the contributions of OxtrINs to behavior in a variety of social contexts and definition of the exact mechanisms responsible for activation of this interesting circuit may provide insights into ASD and other social behavioral disorders.

### Activation of OxtrINs

Oxtr is expressed in many brain structures and in distinct cell types (Yoshida et al., 2009). The cortical OxtrINs identified here are nearly silent under basal conditions in acute mPFC brain slices, and they respond to oxytocin by initiating action potential firing in response to bath application of the hormone (Figure 3). In the nucleus accumbens, presynaptic Oxtr on serotonergic neurons from the dorsal raphe modulate long term depression, thus contributing to social reward behaviors (Dölen et al., 2013). In the median raphe nucleus, oxytocin acts through its receptors on serotonergic neurons to induce serotonin release and reduce anxiety-related behaviors (Yoshida et al., 2009). In the hippocampus, oxytocin increases the firing of fast-spiking interneurons and improves the fidelity of information transfer from these neurons to pyramidal cells (Owen et al., 2013). Although there is only one Oxtr, it can couple to different G proteins and, therefore, lead to different functional effects within the cell (Gimpl and Fahrenholz, 2001; Stoop, 2012). Given the variety of cell types expressing Oxtr, the different cellular mechanisms that are engaged by Oxtr depending on its G protein coupling, and the distinct physiological effects of oxytocin on receptor bearing neurons, two areas of investigation would significantly improve our understanding of the functions of OxtrINs. First, additional studies of Oxtr signal transduction in OxtrINs can provide additional insights into its mechanism of action and its possible interactions with other neuromodulators. Second, detailed neuroanatomical studies of the connectivity of OxtrINs in the cerebral cortex, and studies of the influence of these neurons on their targets, would shed important light on the circuit mechanisms that are engaged by oxytocin in the cerebral cortex to regulate behavior.

### Conclusions

In this study, we have characterized a novel interneuron population in the cerebral cortex (OxtrINs) that regulates a gender-specific, estrus-specific social behavior. The modulation of these neurons by oxytocin and their state-dependent contributions to behavior identify them as components of a circuit that is activated in response to an internal physiological cue to regulate an important aspect of female sociosexual behavior. Thus, our data extend the concept of latent circuits that has arisen from studies on neuromodulation in crustaceans, flies, worms, and mammalian sensory neurons (Bargmann, 2012) to the cerebral cortex and suggest that external social stimuli that stimulate oxytocin production will recruit this newly identified circuit to help coordinate the complex behavioral responses elicited by changing social situations in all mammals, including humans (Gimpl and Fahrenholz, 2001). Finally, we identify OxtrINs as an important focus for investigation of cellular and molecular mechanisms that can play a role in normalization of the aberrant cortical circuit activities evident in ASD and other human social disorders (Kehrer et al., 2008; Markram and Markram, 2010; Rubenstein and Merzenich, 2003).

## EXPERIMENTAL PROCEDURES

### Animals

All procedures involving animals were approved by the Rockefeller University Institutional Animal Care and Use Committee and were in accordance with the

NIH guidelines. The Dlx1 GM520, Nek7 MN733, Cort GM130, and Htr3a GM443 bacTRAP mice, and OxtrCre ON82 and Htr3aCre NO152 mice were generated and maintained at the Rockefeller University (Doyle et al., 2008; Schmidt et al., 2012). Conditional *Oxtr* KO mice, Pvalb-Cre, Sst Cre and Emx1 Cre mice were purchased from Jackson Laboratory (008471, 008069, 013044, 005628). EGFP/L10a reporter mice (Stanley et al., 2013) were a generous gift from the laboratory of J. Friedman.

### Immunofluorescence Staining and In Situ Hybridization

Immunofluorescence staining was carried out on 35 μm thick sections using chicken anti-GFP (1:1000, Abcam), rabbit anti-Pvalb (1:1000, Swant), rat anti-Sst (1:100, Chemicon), and rabbit anti-Vip (1:500, Immunostar) followed by Alexa-fluor conjugated secondary antibodies (Invitrogen). Fluorescence in situ hybridization (FISH) was performed as described previously (Ishii et al., 2004; Serizawa et al., 2003). Detailed protocols are in the Extended Experimental Procedures.

### Immunohistochemistry

For diaminobenzidine (DAB) staining, brains were processed identically with MultiBrain Technology (Neuro Science Associates) with a 1:75,000 dilution of goat anti-EGFP serum (Heiman et al., 2008) according to the Vectastain elite protocol (Vector Labs).

### TRAP Translational Profiling

TRAP was conducted as described previously (Doyle et al., 2008; Heiman et al., 2008). Whole cortices were dissected and homogenized rapidly. Collected polysomes were immunoprecipitated with a mix of two monoclonal antibodies (19C8, 19F7) against EGFP. For microarray analysis, 20 ng of total RNA was amplified and hybridized to GeneChip Mouse Genome 430 2.0 microarrays (Affymetrix). Detailed protocols are in the Extended Experimental Procedures. Raw data are available from Gene Expression Omnibus with accession number GSE56996.

### Slice Preparation and Electrophysiological Recordings

Patch pipettes had resistances of 4–8 MΩ when filled with a solution containing (in mM): 105 K-gluconate, 30 KCl, 10 HEPES, 10 phosphocreatine, 4 ATP-Mg2+, 0.3 GTP (pH adjusted to 7.2 with KOH). Electrophysiological responses were recorded at 33°C–36°C. Recordings were basically analyzed as described in Xu et al. (2013). Oxytocin was bath applied at 100 nM.

### Behavioral Testing

Virgin female mice were used for behavioral experiments. The three-chamber social interaction test was conducted as described previously (Moy et al., 2004). Results from each time investigating each cup and the distance they traveled was recorded manually and automatically using Ethovision v7.0 (Noldus). Statistical comparisons were made by ANOVA using Prism 5 software (GraphPad). Detailed descriptions of behavioral methods can be found in the Extended Experimental Procedures.

## ACCESSION NUMBERS

The Gene Expression Omnibus accession number for the TRAP data reported in this paper is GSE56996.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.09.020.

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