Sexually Dimorphic Oxytocin Receptor-Expressing Neurons In The Anteroventral Periventricular Nucleus Regulates Maternal Behavior

Kaustubh Sharma

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SEXUALLY DIMORPHIC OXYTOCIN RECEPTOR-EXPRESSING NEURONS IN THE ANTEROVENTRAL PERIVENTRICULAR NUCLEUS REGULATES MATERNAL BEHAVIOR

A Dissertation
Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in
The Department of Biological Sciences

by
Kaustubh Sharma
B.S. Louisiana State University, 2011
December 2020
To my parents, Dr. Sharad and Shobha Sharma.

For making me believe in myself and that anything was possible.
ACKNOWLEDGEMENTS

To my advisor, Dr. Ryoichi Teruyama, thank you for your encouragement, support, and unwavering belief in me all through this challenging endeavor. You have challenged me to learn concepts and techniques that I never thought I would be capable of. I have watched myself develop as a student, as a scientist, and as a person under your guidance, and I am extremely indebted for your patience and willingness to help every step of the way. In you, I have found not just a brilliant mentor but also a friend and I will forever be grateful.

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<th>Definition</th>
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<tbody>
<tr>
<td>3V</td>
<td>third ventricle</td>
</tr>
<tr>
<td>AC</td>
<td>anterior commisure</td>
</tr>
<tr>
<td>Arc</td>
<td>arcuate Nucleus</td>
</tr>
<tr>
<td>AVPV</td>
<td>anteroventral Periventricular Nucleus</td>
</tr>
<tr>
<td>BNST</td>
<td>bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNO</td>
<td>clozapine-N-oxide</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DREADD</td>
<td>designer-receptors exclusively activated-by-designer drugs</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ER&lt;sub&gt;α&lt;/sub&gt;</td>
<td>estrogen receptor- alpha</td>
</tr>
<tr>
<td>ER&lt;sub&gt;β&lt;/sub&gt;</td>
<td>estrogen receptor – beta</td>
</tr>
<tr>
<td>ICV</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>MPOA</td>
<td>medial preoptic area</td>
</tr>
<tr>
<td>OT</td>
<td>oxytocin</td>
</tr>
<tr>
<td>OXTR</td>
<td>oxytocin receptor</td>
</tr>
<tr>
<td>OVLT</td>
<td>the vascular organ of lamina terminalis</td>
</tr>
<tr>
<td>OVX</td>
<td>ovariectomized</td>
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</tbody>
</table>
PPD  postpartum depression
PPD1 postpartum Day 1
POA  preoptic area
PR  progesterone receptor
PVN  paraventricular nucleus
SON  supraoptic nucleus
SCN  suprachiasmatic nucleus
TH  tyrosine hydroxylase
TH+ cells immunoreactive to TH
VP  vasopressin
VTA  ventral tegmental area
WT  wild type
ABSTRACT

The neurohypophysial hormone oxytocin is involved in the regulation of social behaviors, including social recognition, pair bonding, and sex-specific parental behaviors in a variety of species. Oxytocin triggers these social behaviors by binding to oxytocin receptors (OXTR) in various parts of the brain. Oxytocin-induced sex-typical behavior, therefore, suggests a sexual dimorphic distribution of OXTR in the brain. In recent years, the oxytocin system in the brain received tremendous attention as a potential pharmacological target for treatment of many psychiatric disorders, such as anxiety, autism spectrum disorders, and even sex-specific psychiatric disorder like postpartum depression (PPD). An important problem and a critical barrier to progress in the field is that despite the importance, the cellular characterization and distribution of the OXTR expressing neurons in the brain are still largely unknown. The overall long-term objective of this project is to elucidate the physiological and behavioral significance of the estrogen-dependent, sexually dimorphic OXTR in the AVPV. This study was conducted to map OXTR expressing neurons in the hypothalamic medial preoptic area (MPOA), a region known as sexually dimorphic and hormone-sensitive area. We revealed using OXTR-reporter mice in which a part of the OXTR gene was replaced with fluorescent protein, Venus, that OXTR neurons are predominantly present in the anteroventral periventricular nucleus (AVPV) in the MPOA of females, but not of males. Moreover, the expression of OXTR in neurons of the AVPV in females depends upon estrogen. Ovariectomy (OVX) resulted in total loss of OXTR-Venus neurons in the AVPV. Because the onset of proper maternal behavior at parturition requires activation of OXTR in the MPOA, the female specific expression of OXTR in neurons of the AVPV implies that these neurons are involved in the induction of maternal behavior. The dopaminergic neurons in the AVPV are known to play a role in the regulation of maternal
behavior. Our study showed that a fraction of the OXTR neurons are immunoreactive to tyrosine hydroxylase, an enzyme essential for dopamine synthesis, implying that some of the OXTR neurons are dopaminergic as well. Using immunocytochemistry and fluorescence microscopy, the exact distribution of OXTR-expressing dopaminergic neurons was shown and the changes in their expression during pregnancy and postpartum states were quantified. Using a chemogenetic approach to specifically inactivate OXTR neurons, this study shows that OXTR neurons in the AVPV are critical to regulate maternal behavior. Understanding sex differences in the oxytocin system in the brain could ultimately lead to sex-specific pharmacological interventions that could possibly treat sex-typical psychiatric disorders like PPD.
INTRODUCTION TO ANIMAL MODEL

The concept of unity in biology, “Anything found to be true of *E. coli* must also be true of elephants,” is the basis for the use of model organisms in biological research (Monod, J. & Jacob, F., 1961). However, identifying the properties of the organism that are similar to organisms of other species is essential while choosing a model organism.

*Mus musculus*, house mice, are commonly used as models for understanding human biology and diseases because mice share similar phylogenetic, cellular, anatomical, physiological, and social behavior to humans. Remarkable genetic homologies between mice and humans are highlighted through genomic studies (Cheng, Y. et al., 2014; Lin, S. et al., 2014; Mouse Genome Sequencing, C. et al., 2002). Genomic studies, along with the progress for creating transgenic, knockin, and knockout mice have provided an additional incentive for mouse research. Female wild mice reach reproductive maturity in 6-8 weeks, have a short gestation length of 20-21 days, produce a litter of 5-8 pups, and produce multiple litters in a year. In addition, the dramatic surge in the use of mice as model organism is reinforced by the ease of maintenance, breeding in captivity, and the accessibility to many inbred strains (Perlman, R.L., 2016). Studies on mice have thus contributed immensely since the 17th century when William Harvey used mice to study reproduction and blood circulation.

For research purposes, inbred mice have been essential as they improve the reproducibility and reliability of the experimental results. The C57BL/6, also known as B6, C57, or black 6, is the most commonly used inbred strain of laboratory mice, with approximately 23,000 articles detailing its use on Pubmed. The B6 strain has been a popular choice as it is physically active, capable of learning, and breeds frequently in captivity. However, this popularity has led to the establishment of many colonies and many substrains around the world.
As part of the nomenclature, Laboratory or vendor codes are added at the end to distinguish different substrains. The two most commonly used are the C57BL/6J, in which “J” is the code for The Jackson Laboratory, and C57BL/6N, where “N”, represents the colony established at the National Institute of Health (NIH). Significant behavioral, genotypic, and phenotypic differences between the two substrains have been found (Bryant, C.D. et al., 2008; Keane, T.M. et al., 2011; Matsuo, N. et al., 2010). The Jackson Laboratory, however, uses the genetic stability program and the genetic quality control program to limit genetic drift. Thus, for our mouse colony and this study we chose the C57BL/6J as a wild-type mouse. Any genetically engineered mouse that has been brought to our colony will therefore undergo backcrossing with the C57BL/6J mice for at least 10 generations. This allows the same genetic background between the control and the mutant group.

The OXTR-Venus and OXTR-T2A-Cre are the two mutant models used for this study. The generation of these mice will be discussed in Chapters 2 and 3 respectively. All measures to replace, refine, and reduce the number of animals used has been taken into consideration.
CHAPTER 1
REVIEW OF LITERATURE

1.1 Introduction

Sexually dimorphic circuitry of the brain has profound effects on the sex-typical physiological and behavioral manifestations. A body of evidence suggests gonadal hormones are key regulators for such sexual dimorphism. Oxytocin, a neurohypophysial hormone, has been well studied in both male and female. Oxytocin has been well-known for its role in the contraction of the uterus during labor and mammary glands during lactation. However, decades of research studies have shown that it is also associated in the regulation of social behavior, including social recognition, pair bonding, and sex-specific parental behavior, in a variety of species. Oxytocin induced sex-specific physiological and behavioral response therefore suggests a sexually dimorphic oxytocin system in the brain. As with any ligand, a specific receptor molecule is required to exert its effects, and oxytocin almost exclusively binds to oxytocin receptors.

This study identifies a sexually dimorphic population of estrogen dependent oxytocin receptor-expressing neurons in the anteroventral periventricular nucleus, a preoptic region of the hypothalamus. The implications of which will be discussed in chapters 2 and 3. This chapter will review the important findings of the oxytocin system through a century of research. All aspects from gene regulation of oxytocin and its receptor to physiological and behavioral response of such interaction will be explored.
1.2 The Oxytocin System

Evolution of oxytocin and related peptides

The story of neurohypophysial hormones began in 1895 when Oliver and Schäfer first discovered that the extracts from the pituitary gland raised blood pressure when injected into mammals (Oliver, G. & Schafer, E.A., 1895). This action was later found to be contributed by the posterior lobe of the pituitary or the neurohypophysis (Howell, W.H., 1898). Following this finding, biological activities from the extracts of posterior pituitary were eminent, particularly the oxytocic or the uterine-contracting effect during parturition elucidated by Dale (Dale, H.H., 1906). Dale thus coined the name oxytocin derived from the Greek words meaning “quick birth.” More discoveries were later made such as the milk-ejecting effect by Ott and Scott (Ott, J. & Scott, J.C., 1910) and the antidiuretic effect in man by Von den Velden (von den Velden, R., 1913). The two neurohypophysial hormones, Oxytocin (OT) and Vasopressin (VP), were separately purified into pitocin and pitressin, respectively and made available for research (Kamm, O. et al., 1928).

As many researches followed, it became evident that all neurohypophysial hormones make up a family of peptides with nine amino acids that are structurally and functionally alike. These nonapeptides are connected by a disulfide bridge between Cys residues 1 and 6, and terminated by a carboxyl group that is converted to an amide (Table 1) (du Vigneaud, V. et al., 1953; Pierce, J.G. & du, V.V., 1950; Turner, R.A. et al., 1951). The two neurohypophysial hormone, OT and VP, only differ from each other based on the amino acids at position 3 (Isoleucine for OT and Phenylalanine for VP) and 8 (neutral amino acid, Leucine, for OT and Arginine for VP).Shortly after the discovery of its structure, OT was the first peptide hormone
to be chemically synthesized in a biologically active form (du Vigneaud, V. et al., 1954). Vincent du Vigneaud was awarded the Nobel prize in chemistry for his work in 1955.

Table 1. Oxytocin and similar peptides across various species.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>Origin</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Oxytocin</td>
<td>Cys</td>
<td>Tyr</td>
<td>Ile</td>
<td>Gln</td>
<td>Asn</td>
<td>Cys</td>
<td>Pro</td>
<td>Leu</td>
<td>Gly (NH₂)</td>
<td>Placentals</td>
<td></td>
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<tr>
<td>Mesotocin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Ile</td>
<td>-</td>
<td>Marsupials</td>
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</tr>
<tr>
<td>Isotocin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Ser</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Ile</td>
<td>-</td>
<td>Amphibians, Reptiles, Bird Marsupials</td>
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<tr>
<td>Glumitocin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Ser</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Gln</td>
<td>-</td>
<td>Lungfishes</td>
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<tr>
<td>Valitocin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Val</td>
<td>-</td>
<td>Spiny dogfish/Sharks</td>
<td></td>
</tr>
<tr>
<td>Aspargtocin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Asn</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Spiny dogfish/Sharks</td>
<td>8</td>
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<tr>
<td>Asvatocin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Asn</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Val</td>
<td>-</td>
<td>Spotted dogfish/Sharks</td>
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</tr>
<tr>
<td>Phasvatocin</td>
<td>-</td>
<td>-</td>
<td>Phe</td>
<td>Asn</td>
<td>-</td>
<td>-</td>
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<td>Val</td>
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<td>Arg</td>
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<tr>
<td>Vasopressin</td>
<td>-</td>
<td>-</td>
<td>Phe</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Arg</td>
<td>-</td>
<td>Mammals</td>
<td>11, 12</td>
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<tr>
<td>Vasotocin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Arg</td>
<td>-</td>
<td>Non mammalian vertebrate/Cyclostomes</td>
<td>13</td>
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<tr>
<td>Lysipressin</td>
<td>-</td>
<td>-</td>
<td>Phe</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Lys</td>
<td>-</td>
<td>Pig</td>
<td>12</td>
</tr>
<tr>
<td>Phenypressin</td>
<td>-</td>
<td>Phe</td>
<td>Phe</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Arg</td>
<td>-</td>
<td>Marsupials</td>
<td>14</td>
</tr>
</tbody>
</table>

The neuropeptides, OT and VP, are well conserved across phyla and nearly all vertebrate species possess an OT-like and VP-like peptide. Based on the presence of isotocin and vasotocin in bony fishes (Osteichthyes), predecessors of land vertebrates (Gimpl, G. & Fahrenholz, F., 2001), two evolutionary lineage of these molecules are proposed: isotocin-OT related to reproduction and vasotocin-vasopressin related to water homeostasis. Homologs of OT and VP have been identified in diverse organisms such as hydra, worms, insects, and vertebrates. These are believed to have existed at least 700 million years ago and are thought to have arisen from a gene-duplication event before the divergence of vertebrates. Within all the lineages, OT and VP genes are found close to one another separated by an intergenic region and in opposite transcriptional direction of the same chromosome which regulates the synthesis of peptides (Archer, R. & Chauvet, M.T., 1995; Caldwell, H.K. & YoungIII, W.S., 2006). Some identified OT-like and VP-like peptides are listed in Table 1.

**Gene structure and regulation**

The regulation of neurohypophysial hormones is controlled by a highly conserved DNA element where the genes are located on the same chromosomal locus separated by an intergenic region but transcribed in opposite direction (Fig. 1-1). These intergenic regions ranges from 3 to 12kb in length in mouse (Hara, Y. et al., 1990), rat (Mohr, E. et al., 1988), and

![Figure 1.1. Schematic of OT (shaded gray) and VP (shaded black) gene structure including cell-specific enhancers. Rectangular boxes represent exons 1, 2, and 3.](image-url)
human (Sausville, E. et al., 1985). As described earlier, this type of gene arrangement resulted from a common ancestral gene duplication that could result in inversion of one of the genes. The OT-neurophysin (carrier protein produced along with OT) I gene that encodes OT prepropeptide consists of three exons. The translocator signal, the hormone itself, the tripeptide processing center (gly-lys-arg, GKR), and the first nine residues of the neurophysin is encoded by the first exon. The second exon encodes residues 10-76 of neurophysin while the third exon encodes the COOH-terminal region (residues 77-93/95) of neurophysin (Ivell, R. & Richter, D., 1984b).

There are several lines of evidence that show an increase in OT mRNA in response to the activation of hypothalamo-neurohypophysial system during pregnancy, lactation, and dehydration (Carter, D.A. & Murphy, D., 1991; Zingg, H.H. & Lefebvre, D.L., 1989). Additionally, several members of the nuclear receptor family could interact and regulate the expression of OT gene (Parker, M.G. et al., 1993). Previous works have shown the stimulation of OT promoter through estrogen receptors α and β (Richard, S. & Zingg, H.H., 1990), thyroid hormone receptor α (Adan, R.A. et al., 1993), and the retinoic acid receptors α and β (Richard, S. & Zingg, H.H., 1991). The highly conserved hormone response element (Fig. 1) is located ~160 nucleotides upstream of the transcription site. Deletion of this region has resulted in loss of responsiveness to thyroid hormone, estrogen, and retinoic acid (Adan, R.A. et al., 1992). Before the onset of labor, there is a strong upregulation in OT mRNA, that ultimately increases uterine OT binding sites in the rat endometrial epithelium (Lefebvre, D.L. et al., 1994). This may be caused by the high levels of estrogen inducing the increase in OT mRNA facilitated through the estrogen response element.

In addition, cortiosterone also regulate the expression of OT. Detection of glucocorticoid and mineralocorticoid receptors in the OT neurons of the SON and PVN
(Di, S. et al., 2005; Haque, M. et al., 2015) supports a regulatory role of these steroids in the OT system.

Other genes such as paternally expressed gene 3 (Peg3) (Li, L. et al., 1999; Swaney, W.T. et al., 2007), are known to be expressed in the mouse hypothalamus. Their specific roles in OT-neurons are unknown, however, the number and/or function of OT-neurons are altered in Peg3 mutant mice (Li, L. et al., 1999).

**Neurobiology of Oxytocin**

Across species, OT is synthesized and expressed by magnocellular neurosecretory neurons primarily located in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN, Fig. 1-2) of the hypothalamus (Kelly, J. & Swanson, L.W., 1980; Rosen, G.J. et al., 2008). Previous studies also shows OT-synthesizing cells in the accessory nuclei (AN) of the hypothalamus in rats and humans (Meynen, G. et al., 2007; Wierda, M. et al., 1991) and the parvocellular neurons of the PVN (Buijs, R.M. & Swaab, D.F., 1979). The OT is also synthesized in the bed nucleus of the stria terminalis (BNST), medial preoptic area (MPOA), lateral (LH), and anterior (AH) hypothalamus (Brownstein, M.J. et al., 1980; Rhodes, C.H. et al., 1981; Wang, Y. et al., 2013; Xu, L. et al., 2010; Young, W.S., 3rd & Gainer, H., 2003).

Oxytocin is synthesized as a preprohormone and assembled on the ribosome along with its carrier protein, neurophysin, inside the cytoplasm. It is subsequently packaged into Golgi vesicles and post-translationally modified in their transport vesicles yielding three products: OT, neurophysin I, and a signal protein (SP) (Caldwell, H.K. & YoungIII, W.S., 2006; Gimpl, G. & Fahrenholz, F., 2001). Neurophysin, a 93-95 residue protein functions to properly target, package, and store OT within the large dense core vesicles (LDCV) before being released into
Figure 1.2. Fluorescent photomicrograph of coronal section from a female mouse brain. Immunocytochemistry was performed to label OT-synthesizing cells in the SON and PVN using anti-OT neurophysin antibody. 3V: third ventricle. Scale bar = 0.5mm

the bloodstream (Gimpl, G. & Fahrenholz, F., 2001; Johnson, Z.V. & Young, L.J., 2017). The OT prepropeptide matures and undergoes cleavage and other modifications as it is transported via large neurosecretory axons through the hypothalamo-hypophyseal tract into the posterior pituitary (Brownstein, M.J. et al., 1980). At the posterior pituitary, OT is stored within the Herring bodies (terminal ends of hypothalamic axons) until osmotic, reproductive, and/or social stimuli elicits its release (Lee, H.J. et al., 2009).

The magnocellular neurons release OT from LDCVs in three ways: endocrine, paracrine, or synaptic. Endocrine refers to the release of OT directly into the bloodstream, from axon terminals in the posterior pituitary, which contains fenestrated capillary bed, and circulated throughout the body to elicit peripheral effects. Paracrine release refers to the somato-dendritic release directly into the cerebrospinal fluid (CSF) that bathes the brain (Castel, M. et al., 1996) via diffusion between cell spaces. Synaptic release of OT occurs where axon terminals make synaptic connection to local neurons. OT released in such fashion are neurotransmitters, released more rapidly, and in small quantities (van den Pol, A.N., 2012). Thus, OT can also act as a
potent neuromodulator, rather than a peripheral hormone, targeting oxytocin receptors (OXTRs) located in various parts of the brain.

The release of OT, central and peripheral, can be either independent or coordinated (Neumann, I.D. & Landgraf, R., 2012). Several stimuli such as sexual activity, birth, suckling, and various stressors trigger both central and peripheral release, while social defeat solely triggers central release without affecting peripheral levels of OT (Landgraf, R. & Neumann, I.D., 2004). This implies the presence of a fine-tuned regulatory mechanism by which a hormone is compartmentally released from a single neuron. The somato-dendritic, but not axonal release of OT, is triggered by the depolymerization of filamentous (F)-actin to monomer G-actin. This change from F-actin to G-actin is caused by depolarization-induced release of calcium (Ca^{2+}) from intracellular stores. F-actin acts a barrier for LDCV and prevents exocytosis, whereas G-actin facilitates exocytosis of OT by allowing fusion of LDCV with the membrane (Tobin, V.A. & Ludwig, M., 2007). The magnocellular neurons display unique electrophysiological pattern that are associated with release pattern for the hormone. OT neurons showed increase in firing activity in response to hyperosmolarity, whereas the same neurons displayed synchronized bursts that are accompanied by pulsatile release that contract mammary smooth muscle for milk let-down (Renaud, L.P. & Bourque, C.W., 1991). The well-known peripheral effects of OT via action potential-driven axonal release from hypothalamic neurons into blood stream of posterior pituitary will be discussed later.

Central effects of oxytocin

As discussed earlier, OT gene is primarily expressed in the magnocellular neurons of the SON and the PVN in the hypothalamus. While all the neurons in the SON and the majority of magnocellular neurons from the PVN project to the posterior pituitary, collateral branches from a
single axon terminal project within the central nervous system (CNS). OT fibers and terminals were observed in various brain areas such as the dorsal and ventral hippocampus, dorsomedial hypothalamic nucleus, several thalamic regions, medial and lateral septal nuclei, amygdala, MPOA, BNST, and LH preoptic periventricular nucleus (Bales, K.L., Plotsky, P.M., et al., 2007; Dumais, K.M. et al., 2013; Wang, Z. et al., 1996). OT released in the brain modulates a wide variety of social behaviors (Bales, K.L. et al., 2013; Bales, K.L., van Westerhuyzen, J.A., et al., 2007; Ross, H.E. & Young, L.J., 2009) by binding to OXTRs widely distributed throughout the brain (Insel, T.R. & Shapiro, L.E., 1992a, 1992b).

Besides the pro-social effects, centrally released OT mediates a variety of autonomic and somatic effects. OT axons project to several brain stem nuclei that are involved in cardiovascular control. OT injected into the dorsal motor nucleus of the vagus reduced heart rate, while this effect was eliminated by the injection of OT antagonist (Rogers, R.C. & Hermann, G.E., 1985). Previous works showed that central OT also induces analgesic effects and modifies behavior associated with motor activity. Injection of OT into the lateral ventricles in rats caused analgesia (Kordower, J.H. & Bodnar, R.J., 1984). Another study showed that low doses of OT decreased peripheral locomotion, whereas high doses triggered analgesic effects (Uvnas-Moberg, K. et al., 1994). OT is also thought to play a role in thermoregulation (Lipton, J.M. & Glyn, J.R., 1980; Mason, G.A. et al., 1986), gastric motility (Flanagan, L.M. et al., 1992; Verbalis, J.G. et al., 1995), and osmoregulation (Blackburn, R.E. et al., 1995).

Peripheral effects of oxytocin

The OT gene is expressed in several peripheral organs including the uterus, ovary, mammary gland, placenta, testis, kidney, heart, and the vascular endothelium. The female reproductive system, especially the uterus, is one of the prime targets of OT. As OT is a potent
agent that triggers the contraction of the uterus, it is clinically used to induce labor. Accordingly, OT antagonist are equally important therapeutically to prevent preterm labor (Williams, P.D. et al., 1998). In rats, estrogen-induced increase in OT mRNA levels was found in the uterine epithelium (Lefebvre, D.L. et al., 1994). Although significant increases in OT levels before the onset of labor was not detected, a study showed that maternal OT concentration correlates with uterotonic activity. This correlation of OT concentration and uterotonic activity increases progressively during late pregnancy and delivery (Hirst, J.J. et al., 1993). The ovary is another site that is influenced by OT and possibly a site for local OT production (Ivell, R. & Richter, D., 1984a). Cumulus oophorous cells surrounding the oocyte express both OT and OXTR genes in humans (Furuya, K. et al., 1995) suggesting a role for OT in early embryonic development as well as fertilization.

A well-known role of OT is to stimulate milk letdown from the mammary glands. Suckling by infants stimulates the tactile receptors and the impulses are transmitted to the OT neurons in the hypothalamus (McNeilly, A.S. et al., 1983). OT released into the bloodstream is carried to the lactating breasts where it causes contraction of the myoepithelial cells lining the walls of the lactiferous ducts and the alveoli. This process of milk letdown works within a minute after a baby starts suckling. The essential role of OT in milk letdown has been confirmed in OT knock-out mice where a major shortfall of the females was failure to lactate (Nishimori, K. et al., 1996).

Oxytocin also influences some aspects of the male reproductive tract. In many species, a pulse of systemic OT release is linked with ejaculation, presumably by contracting the smooth muscles that line the reproductive tract. OT and OT-like peptide has been shown to exist in the testis of many mammalian species, birds (Pickering, B.T. et al., 1989), and marsupials (Bathgate,
R.A. et al., 1993). A body of evidence shows that OT is locally made, especially in humans, within the testis, epididymis, and prostate (Frayne, J. & Nicholson, H.D., 1998; Ivell, R. et al., 1997). Oxytocin peptide was also found in the interstitial cells of Leydig within rat testes (Nicholson, H.D. & Hardy, M.P., 1992). Interstitial cells are known to produce the main male hormone, testosterone, under the influence of luteinizing hormone (LH). The LH also increases the OT production; however, this effect was only seen during active spermatogenesis (Nicholson, H.D. & Jenkin, L., 1995). Thus, OT most probably plays a role in contraction of seminiferous tubules as well as modulation of steroidogenesis. OT present in concentrations higher than plasma was found in the prostate of rat, dog, guinea pig, and human (Bodanszky, M. et al., 1992; Frayne, J. & Nicholson, H.D., 1998). It is involved in contraction of the prostate expelling prostatic secretions during ejaculation.

Besides the male and female reproductive system, OT and OXTR are found in variety of other organs such as: the kidney (Arpin-Bott, M.P. et al., 1997; Schmidt, A. et al., 1990), heart (Gutkowska, J. et al., 1997; Jankowski, M. et al., 1998), thymus (Elands, J. et al., 1990; Geenen, V. et al., 1986), pancreas (Amico, J.A. et al., 1988), and adrenal gland (Ang, V.T. & Jenkins, J.S., 1984; Hawthorn, J. et al., 1987). OT influences activity in all the above listed peripheral organs. All things considered OT exerts a variety of effects on animal behavior and physiology that are in direct response of it binding to the OXTRs, which will be discussed below.

1.3. Oxytocin Receptor

Gene structure and regulation

The oxytocin receptor gene is differentially expressed in various tissues across various species. This expression is regulated at the transcriptional level (DNA methylation of a specific CpG site in the promoter (Mamrut, S. et al., 2013)) under different physiologic conditions (virgin
The gene encoding OXTR was first isolated in 1992 (Kimura, T. et al., 1992) and was mapped to the gene locus 3p25-3p26.2 in humans (Inoue, T. et al., 1994; Simmons, C.F., Jr. et al., 1995). The sequence encoding OXTR has been identified in mouse (Kubota, Y. et al., 1996), rat (Rozen, F. et al., 1995), pig (Gorbulev, V. et al., 1993), sheep (Riley, P.R. et al., 1995), cow (Bathgate, R. et al., 1995), and rhesus monkey (Salvatore, C.A. et al., 1998). The human OXTR gene promoter comprises of a variety of species-specific transcription factor binding sites and contains 4 exons and 3 introns (Fig. 1-3). Exon 1 and 2 form the 5′-untranslated region while exons 3 and 4 encodes the 389 amino acid long protein. Part of Exon 4 is also the 3′-untranslated region (Inoue, T. et al., 1994). Thus, the translated receptor is 389 amino acids long with 7 transmembrane domains that shares high levels of sequence identity among species, and belong to the class 1 G protein coupled receptor (GPCR; reviewed in (Gimpl, G. & Fahrenholz, F., 2001)).

In the human OXTR gene the transcription starts ~600 bp upstream of the START codon where a TATA-like motif and a transcription factor specificity protein 1 (SP-1) binding site are located. The 5′-flanking region contains GATA-1 motif, a c-Myb binding site, an AP-2 binding site, two AP-1 binding sites, but no complete estrogen response element (ERE). Instead, half palindromic sequences of ERE exists (Inoue, T. et al., 1994). As shown in the ovalbumin gene, the lack of classical EREs does not, however, affect the role of estrogen on gene expression, and the existing half palindromic sequence can mediate estrogen activation (Kato, S. et al., 1992).
The promoter region of the mouse *OXTR* gene lacks the TATA sequence and contains several half palindromic motifs, multiple interleukin-response elements, and a classical ERE (Kubota, Y. et al., 1996).

*In vivo* studies have shown an important influence of gonadal steroids on the accumulation of uterine OXTR mRNA. The OXTR expression correlates with the levels of sex steroids, particularly estradiol, in the uterus or hypothalamus. Ovariectomized rats that were administered estrogen showed markedly increased OXTR binding sites and mRNA accumulation in the uterus (Soloff, M.S. et al., 1983). Even though it is not clear if OXTR gene transcription is mainly regulated by estrogen, a previous study performed in knock-out mice showed that estrogen receptor- alpha (ERα) is essential for OXTR binding in the brain by estrogen (Young, L.J. et al., 1998). Based on our findings, the continuous presence of receptors in most of the brain areas even after ovariectomy suggests that there could be alternative machinery for OXTR expression and regulation.

Uterine sensitivity to OT increases significantly at the onset of labor. This is due to an upregulation of OXTR mRNA levels and a markedly increased density of myometrial OXTRs (Kimura, T. et al., 1992). This significant rise in the OXTR levels during labor to ~200 times that of non-pregnant state was found in rats, cows, and humans (Fuchs, A.R. et al., 1995; Fuchs, A.R. et al., 1984; Ivell, R. et al., 1995) and allows OT to stimulate uterine contractions that would have been futile in non-pregnant states. The uterine OXTR mRNA levels decrease rapidly, more than sevenfold within 24 hours after parturition (Zingg, H.H. et al., 1995). This downregulation is important to avoid unwanted contractions of the uterus during lactation. All this OXTR mRNA upregulation is likely a feature of the OXTR promoter as suggested by nuclear protein binding and transfection experiments (Ivell, R. et al., 1998). Transcriptional gene suppression is linked to
a genomic element within the third intron in human OXTR gene (Mizumoto, Y. et al., 1997).

This intronic region is hypomethylated in the uterus at term, when the gene is upregulated, but hypermethylated in non-expressing tissues. Altogether, sex steroids indirectly affect OXTR gene expression by modulating several transcription factors or cofactors that are involved in expression of OXTR (Ivell, R. & Walther, N., 1999; Robinson, I.C. & Jones, P.M., 1982).

Besides the gonadal steroids, corticosteroids also influence the OXTR system. Administration of OT decreased glucocorticoid receptor expression in the hippocampus (Petersson, M. & Uvnas-Moberg, K., 2003) and blood cortisol levels in female rats (Petersson, M. et al., 2005). In contrast, administration of dexamethasone, caused an increased OT binding in various regions of the brain such as BNST, lateral septum, and amygdala (Patchev, V.K. et al., 1993). Recent adrenalectomy experiment however, showed an increased stress induced peripheral OT secretion even in the absence of glucocorticoids (Torner, L. et al., 2017). Taken together, there seems to be a bi-directional link between the glucocorticoids and the OT system and whether one system excites or inhibits the other is area specific.

Additionally, several other genes possibly regulate the OXTR gene. For example, paternally expressed gene 3 (Peg3), an imprinting gene, acts as a repressor in the expression of the OXTR gene (Frey, W.D. et al., 2018). OXTR expression was found to markedly increase in mammary epithelia and the hypothalamus of Peg3 KO mice (Frey, W.D. et al., 2018).

**Oxytocin receptor-coupled signaling**

As stated previously, the neurohypophysial hormones, OT and VP share similar structure. OT and VP are both nonapeptide molecule with a tripeptide amidated C-terminal, and a cyclic part that is connected via a disulfide bridge. OT and VP receptors also share similar structure and belong to the rhodopsin-type class 1 GPCR family. Thus, OT can bind not only to OXTRs but
also to VP receptors (V1a, V1b, and V2). Conversely, VP can also bind to OXTR and initiate a cascade of events. The sequence identity between the vasopressin receptor 1 (V1) and mammalian OXTR is nearly 50%. Whereas, it is 40% identical between OXTR and V2R (Gimpl, G. & Fahrenholz, F., 2001). The extracellular loops and the transmembrane helices show highest levels of sequence identity between VP and OT receptor, whereas the N- and C- terminus have the least similarities. This is also the case for the intracellular loops, which allows OXTR to have weak ligand selectivity. Cyclic parts of different OT and VP-like neuropeptides bind with varying affinity to the OXTR (Gimpl, G. & Fahrenholz, F., 2001; Postina, R. et al., 1996). The cyclic part of OT is more important for binding selectivity to the second extracellular loop of OXTR than is the tripeptidic tail of OT in interacting with the N- terminal domain and first extracellular loop of the OXTR (Postina, R. et al., 1996). Specifically, the isoleucine in position 3 of OT can stimulate OXTRs more than the Phenlyalanine in position 3 of VP (Gimpl, G. & Fahrenholz, F., 2001).

Although there are several subtypes of VP receptors, only one OXTR is known to exist, and can be in high or low affinity state. The OXTR has an extremely high affinity for OT in the nanomolar range (Akerlund, M. et al., 1999), about tenfold higher than its affinity for VP (Kimura, T. et al., 1994; Postina, R. et al., 1996). VP only acts as a partial agonist of OXTR (Chini, B. et al., 1996; Gimpl, G. & Fahrenholz, F., 2001), and about a 100-fold higher concentration of VP would be needed to elicit the same response caused by OT activating OXTR (Chini, B. et al., 1996; Kimura, T. et al., 1994). Divalent cations, such as Mg$^{2+}$ or Mn$^{2+}$, and cholesterol are the two essential components for high-affinity binding of OT (Singewald, N. et al., 2015). Cholesterol stabilizes the OXTR in a high affinity state for agonists to bind (Gimpl, G. & Fahrenholz, F., 2001).
OXTRs are attached mostly to Gq/11α class of GTP binding proteins. Upon activation these proteins stimulate the action of phospholipase C (PLC) with beta-gamma dimer of G-protein (Gβγ). This leads to the breakdown of phosphatidylinositol biphosphate in the cell membrane to produce inositol-3-phosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to the IP₃-gated Ca²⁺-channels in the endoplasmic reticulum and triggers Ca²⁺-release sequestered within intracellular stores. DAG activates protein kinase C, which ultimately phosphorylates downstream targets. The increase in Ca²⁺ stimulates the Ca²⁺/calmodulin system that activate the myosin light chain kinase. In case of myometrial cells, activation of myosin light chain kinase, leads to contraction of smooth muscles during labor. However, OXTR may also bind to other G-proteins as well. For example, activation of OXTR elicited Ca²⁺ response via the activation of Gi/o protein (Phaneuf, S. et al., 1993). Since Gi-proteins inhibit adenylyl-cyclase, which leads to a reduction in cAMP, activation of OXTRs can result in differential downstream signaling. This dual regulation of G-protein pathway has been previously shown to exist even in the same brain regions (Gravati, M. et al., 2010).

The activation of OXTR and subsequent signaling predominantly involves Gaq, but OXTRs can regulate signaling through Gβγ subunits as well. A previous study revealed that activation of the extracellular signal regulated kinase 1/2 (ERK1/2) involves a PLC independent pathway mediated through the action of Gβγ subunits (Zhong, M. et al., 2003). This pathway is dependent upon epidermal growth factor receptor tyrosine kinase activation and Ca²⁺.

In addition, activation of OXTR in PVN neurons lead to the integration of transient receptor potential vanilloid type 2 (TRPV2) channels into the cell membrane that resulted in influx of Ca²⁺ from the extracellular space (van den Burg, E.H. et al., 2015). This influx increased cellular excitability via several Ca²⁺-dependent signaling cascades. Thus TRPV2
channels in the PVN are mediators of OT-induced anxiolytic effect and pharmacological blocking of these channels attenuated the anxiolytic effect (van den Burg, E.H. et al., 2015).

The expression of OXTRs and binding affinity for OT are both regulated. Like most GPCRs, OXTRs can be desensitized and subsequently internalized upon constant agonist stimulation (Evans, J.J. et al., 1997). Desensitizing of GPCRs includes uncoupling of the GPCR from its G-proteins and endocytosis of the receptor by clathrin-dependent pathway (Gimpl, G. & Fahrenholz, F., 2001). Previous study that worked on immortalized human myometrial (ULTR) cell line showed that the OXTR is desensitized already within a minute in the presence of OT (Willets, J.M. et al., 2009). However, exposure of OT to the OXTRs for 20 hours in human myometrial cells lead only to about 10-fold reduction in OT binding, while no changes in the OXTR protein level we observed (Phaneuf, S. et al., 1997).

Expression in the brain

OXTRs have been identified in various tissues in the body some of which have been mentioned above. This thesis work identified a group of OXTR expressing cells in the hypothalamus that was previously unknown. Thus, this portion of the review will focus on previously identified OXTRs in the brain.

The information about the distribution of OXTRs in the brain has been brought into light through receptor autoradiography studies that used very specific radioiodinated ligands for VP and OT (Johnson, A.E. et al., 1993; Tribollet, E., 1992; Tribollet, E., Barberis, C., et al., 1992). Commercially available antibodies against mouse OXTR was also used in a study; but, they lack the specificity to target endogenous OXTR (Torner, L. et al., 1999). Therefore, recent studies have used OXTR reporter mice (Hidema, S. et al., 2016; Li, K. et al., 2016) or quantified local OXTR mRNA (Dabrowska, J. et al., 2011; Yoshimura, R. et al., 1993) to detect OXTR.
expression in adult rodent brain. As a result, brain regions such as the olfactory bulb, nucleus accumbens, lateral septum, BNST, MPOA, PVN, amygdala, ventromedial nucleus of hypothalamus, hippocampus, striatum, periaqueductal gray, and ventral tegmental area have been known to express OXTRs (Grinevich, V. et al., 2014).

The PVN of mice and humans have relatively low expression of OXTRs (Jurek, B. et al., 2015; Loup, F. et al., 1991) and can be detected through single cell RT-PCR (Dabrowska, J. et al., 2011), or by upregulating OXTR expression using antagonists (Freund-Mercier, M.J. et al., 1987). OXTR expression is similar between males and virgin females (Mitre, M. et al., 2016) and remains stable throughout the estrous cycle and through mid-pregnancy (Young, L.J. et al., 1997). However, a study showed that during mid and late gestation (day 15-20), the OXTR mRNA in the PVN increases (Bealer, S.L. et al., 2006). The PVN is populated by oxytocinergic and vasopressinergic magnocellular neurons, and the majority of magnocellular cells do not express OXTRs. However, magnocellular cells that do express OXTRs are either corticotropin releasing factor (CRF)-positive or CRF-negative (Dabrowska, J. et al., 2011). Furthermore, a study showed that OXTR-expressing neurons in the PVN are exclusively glutamatergic (Dabrowska, J. et al., 2013).

The expression of OXTRs in the brain is also species and sex dependent. In contrast to the PVN, OXTR expression was higher in ventromedial hypothalamic nuclei of male than in female rats (Bale, T.L. & Dorsa, D.M., 1995). However, a study did not find any sex difference in OXTR expression in the ventromedial hypothalamic nuclei of golden hamsters (Dubois-Dauphin, M. et al., 1992) suggesting species-dependent difference. In the prefrontal cortex of female mice, OXTR expression was found to be higher in the left auditory cortex than in the right (Marlin, B.J. et al., 2015). Since pup-retrieval behavior requires the left but not the right
auditory cortex (Marlin, B.J. et al., 2015), this lateralization suggests a role in maternal behavior. In contrast, OXTR-expressing cortical neurons in the auditory cortex are involved in anxiety-like behavior in male counterparts. Similar to the mice studies, OXTRs have been detected in the prefrontal cortex of male and female rats (Sabihi, S., Durosko, N.E., et al., 2014).

Pharmacological studies have indicated involvement of these receptors to play a role in maternal behavior and anxiety-like behavior as well (Sabihi, S., Durosko, N.E., et al., 2014). For instance, blocking OXTR in the medial prefrontal cortex using OXTR antagonist caused impaired maternal care and increased maternal aggression (Sabihi, S., Dong, S.M., et al., 2014). Similarly, the medial prefrontal cortex of monogamous prairie voles has higher OXTR expression than in promiscuous montane voles (Smeltzer, M.D. et al., 2006) suggesting a role in modulating social interaction of the monogamous prairie voles.

OXTR expression was detected in all regions of the hippocampus (Dubois-Dauphin, M. et al., 1992; Yoshida, M. et al., 2009), the central, and medial amygdala of rats and mice (Gould, B.R. & Zingg, H.H., 2003; Terenzi, M.G. & Ingram, C.D., 2005; Young, L.J. et al., 1997). In the rat hippocampus, OXTR expression was the highest in CA1 pyramidal cells and played a major role in formation of spatial memory during lactation (Tomizawa, K. et al., 2003). The presence of OXTRs in the amygdala suggests a role in regulation of social behavior and fear expression. Indeed, studies showed that OXTR expression in the central amygdala is negatively correlated with social interest in female rats (Dumais, K.M. et al., 2013). Similarly, OXTR induced calcineurin signaling enhanced the anxiolytic effects of OT which negatively regulated the expression of fear (Huber, D. et al., 2005; Viviani, D. et al., 2011).

The OXTR expression and OT binding sites in the brain undergoes major changes in
development dependent pattern. Tribollet et al. (Tribollet, E., Dubois-Dauphin, M., et al., 1992) showed that only a fraction of OXTRs are constantly expressed throughout the development while some are transiently expressed in neurons during infancy or maturation. The two most critical periods during the development of male and female rats are the third postnatal week (before weaning) and during puberty (after postnatal day 35). Areas such as the cingulate or retrosplenial cortex showed high OT binding sites in infant brain, whereas undetectable OT binding were observed in the same brain regions in the adult (Tribollet, E., Dubois-Dauphin, M., et al., 1992). Similarly, OXTR binding sites in the lateral septum of pre-pubescent rats was higher than those in adult brains (Lukas, M. et al., 2010). Conversely, in areas such as the islets of Calleja, ventral pallidum, and ventromedial nuclei of the hypothalamus, OXTRs were undetectable before puberty, abundant in adult brain, but low again in aged rats (Arsenijevic, Y. et al., 1995; Tribollet, E., Dubois-Dauphin, M., et al., 1992). OXTR binding can be detected in the BNST and central amygdala of infants and this binding density further increased in adults (Tribollet, E., Dubois-Dauphin, M., et al., 1992). A variety of factors plays a role in such development-dependent pattern of OXTR expression and binding. One study showed a role of gonadal steroids in the expression of OXTRs in the olfactory tubercle and ventromedial nuclei of the hypothalamus, and testosterone treatment restored normal levels of OXTR in aging rats (Arsenijevic, Y. et al., 1995). In contrast, other studies suggest that these developmental changes in OXTR expression are due to age-dependent response to social demands (Bredewold, R. et al., 2014; Smith, C.J. et al., 2017).

**Effect of steroids on the oxytocin system**

Steroids play a major role in OT secretion, OXTR binding, and expression. All steroid hormones are derivatives of the 27-carbon atom, cholesterol. Cholesterol itself is required along
with Mg\textsuperscript{2+} or Mn\textsuperscript{2+} for high affinity OT binding to OXTRs (Singewald, N. et al., 2015). In a study that expressed OXTRs in insect cells, which naturally has low cholesterol content in the plasma membrane, very low affinity OT binding was seen. Addition of cholesterol converted some receptor from low to high affinity state increasing OT binding (Gimpl, G. et al., 1995). Furthermore, cholesterol was found to protect OXTRs from thermal degradation (Gimpl, G. et al., 2000). Cells that exhibit low and high affinity OXTRs could thus reflect the uneven amount of cholesterol distributed within its plasma membrane (Crankshaw, D. et al., 1990; Gimpl, G. et al., 1997). Therefore, cholesterol along with divalent cations Mg\textsuperscript{2+} or Mn\textsuperscript{2+} creates a beneficial interaction by stabilizing OXTRs in a high affinity state for agonists and antagonists to bind.

Estrogen, an 18-carbon cholesterol derivative, is a key regulator of OXTR expression. In fact, the two subtypes of estrogen receptor (ER), ER\textalpha and ER\textbeta, differentially regulate OT and OXTR expression. Studies showed that ER\textalpha is essential for OXTR expression, whereas activation of ER\textbeta induces transcription of OT in the mouse brain (Choleris, E. et al., 2003; Sharma, D. et al., 2012). Estrogen, binding to one of the two ERs, forms homo- or hetero-dimers that interact with the ERE in the promoter of OXTR to induce transcription. As mentioned earlier, mice and rats, but not humans have a complete ERE in OXTR promoter region. However, ER\textalpha and ER\textbeta can trigger G-protein signaling cascades that can activate transcription factors in OXTR promoter independent of ERE activation (Levin, E.R., 2015). Therefore, the lack of ERE in human OXTR promoter region does not hinder the effect of estrogen in OXTR expression. One factor that impedes the action of estrogen in OXTR expression is site/tissue-specific methylation of OXTR promoter. Indeed, a study performed in female rats showed that increase in OXTR mRNA through estrogen only occurs in areas of the brain sensitive to estrogen such as the hypothalamus (Breton, C. & Zingg, H.H., 1997).
Progesterone, a 21-carbon cholesterol derivative, is mainly synthesized by the corpus luteum. However, some regions of the brain, adrenal gland, and the placenta (during pregnancy) are also known to synthesize progesterone. The production of progesterone in high quantity is vital to maintain uterine quiescence thus preventing preterm birth. Burger et al. showed that high concentrations of progesterone blocked signaling in OXTRs and several other GPCRs (Burger, K. et al., 1999). This action of progesterone is possible due to its ability to interfere with the transport of cholesterol in the plasma membrane (Liscum, L. & Munn, N.J., 1999). As mentioned above, cholesterol is essential for OXTRs to be stable in high affinity state. The lack of cholesterol due to high concentrations of progesterone suggests low affinity of OT-OXTR binding causing inhibition of OXTR signaling. A study showed that progesterone withdrawal caused an increase in cholesterol levels, which converted the low affinity into high affinity state of OXTRs (Klein, U. et al., 1995). Progesterone also plays a role in inducing behavioral effects. Administration of progesterone caused an increase in OXTR expression in the posterior ventromedial hypothalamus, and an increase in female mating behavior (Tansey, K.E. et al., 2010).

1.4. Social Saliencies of Oxytocin

Maternal Behavior

Maternal behavior is a trait exhibited by all mammals. This behavior is elicited by a strong attraction or bond between the mother and infant that allows them to care and protect for their young. The OT-system plays an important role in exhibiting such behavior. Towards the end of pregnancy and during lactation, elevation in the hypothalamic OT synthesis and secretion allows to meet the physiological demands required during birth and lactation (Zingg, H.H. & Lefebvre, D.L., 1988). In addition, higher OXTR expression and density in several brain regions
such as the BNST, MPOA, PVN, hippocampus, and the olfactory bulb contributes to the high OT-OXTR interaction (Bosch, O.J. et al., 2010; Meddle, S.L. et al., 2007).

The increased availability of both OT and OXTR contributes to the rapid changes in behavior peri and post-partum (Insel, T.R. & Shapiro, L.E., 1992b; Kendrick, K.M. et al., 1997; Pedersen, C.A. & Prange, A.J., Jr., 1979). The OT signaling is necessary to establish maternal behaviors such as pup retrieval and nest building. Early studies used ovariectomized rats to test the effect of OT on maternal behavior. However, natural priming by steroid hormones are not possible in ovariectomized animals. Serum progesterone concentrations peak around 18 days after gestation, but declines drastically on the day before or the day of parturition. The estrogen concentrations however, drastically increase on the day of parturition. To mimic the changes of steroid hormones, ovariectomized animals are supplemented/“primed” with progesterone followed by estrogen. Intracerebroventricular (ICV) injections of OT in ovariectomized rats induced maternal behavior (Pedersen, C.A. & Prange, A.J., Jr., 1979). Studies performed in steroid primed sheep also showed that infusions of OT into the MPOA and BNST promoted maternal behavior (Kendrick, K.M. et al., 1992; Levy, F. et al., 1992). Conversely, ICV injections or infusions into the MPOA of OXTR antagonist delayed pup retrieval and nursing behavior in estrogen primed rats (Pedersen, C.A. et al., 1994). Furthermore, a body of evidence suggests the inhibitory role of nitric oxide (NO) by blocking the release of OT within the brain. Injections of the NO donor sodium nitroprusside also delayed maternal behavior in rats (Okere, C.O. et al., 1996; Srisawat, R. et al., 2000). Conclusive studies on OT induced maternal behaviors in humans have not been done so far. Nevertheless, breast-feeding immediately after birth, when circulating OT in mothers are high, was linked to mother-infant bonding (Kennell, J.H. et al., 1974). However, OT alone may not be sufficient for inducing and maintaining
maternal behavior. A study showed that female transgenic mice genetically deficient in OT displayed normal maternal behavior (Nishimori, K. et al., 1996). This could be due to OT-like ligands binding to OXTRs. A study performed in OXTR-KO mice showed that impairments in the OXTRs lead to disruption in the maternal behavior (Rich, M.E. et al., 2014). All together, these studies provide evidence that OXTR mediated signaling is vital to the onset of maternal behavior.

**Pair Bonding**

The selective preference towards a particular mate only exists in a few mammalian species. The North American prairie vole has been an important animal model to better understand the role of OT in pair bonding behavior (Insel, T.R. & Shapiro, L.E., 1992a; Young, L.J. & Wang, Z., 2004). These monogamous prairie voles exhibit pair bonding characteristics that include partner preference and aggression towards other conspecifics after mating.

The region-specific distribution of OXTR in the brain is predicted to be the basis of this monogamous pair bonding. Higher OXTR density was found in the nucleus accumbens and caudate putamen of prairie voles compared to that of the non-monogamous montane voles (Insel, T.R. & Shapiro, L.E., 1992a; Ophir, A.G. et al., 2012). A recent study generated a knock-in prairie vole that expresses Cre recombinase with OXTR using CRISPR mediated gene editing (Horie, K. et al., 2020). Using Cre dependent retrograde tracing, a species-specific circuitry of OXTR neurons was identified that was previously unknown from mice studies (Horie, K. et al., 2020). A previous study also showed OT-synthesizing neurons in the SON and PVN send long axonal projections to the nucleus accumbens, implying that OT is released in that region during mating (Ross, H.E. et al., 2009). Centrally released OT is suggested to act on the nucleus accumbens with high OXTR density. This OT-OXTR interaction activates the dopaminergic

Studies on OTs influence on pair bonding in humans have been limited. Neuroimaging studies showed that viewing the face of a romantic partner activated the nucleus accumbens and the ventral tegmental area (Bartels, A. & Zeki, S., 2004), regions associated with rewarding sensation. Intranasal application of OT in men was shown to increase neural responses in these regions only when viewing images of the female partner but not of a stranger (Scheele, D. et al., 2013). Collectively, these data suggest that OT-OXTR interaction stimulates a sense of reward in brain specific regions that contribute to pair bonding.

**Sexual behavior**

As mentioned earlier in the chapter, the main peripheral targets of OT are the uterus and mammary glands; both required for reproduction. However, OT plays a role in inducing sexual behavior in both genders. Females display sexual behavior by either soliciting (proceptivity) or being receptive (lordosis). Proceptive behaviors include vocalizations, investigation of male genitals, exposing body parts to the partner, and bouts of physical contact. The receptive dorsal-flexed position displayed by estrus female is also important behavior for male intromission (Veening, J.G. et al., 2015). Both, proceptivity and lordosis, are highly regulated by gonadal steroids but also, in part, by OT (Borrow, A.P. & Cameron, N.M., 2012).

Magnocellular OT neurons of the PVN are activated by sexual activity in females (Cameron, N. & Erskine, M.S., 2003; Flanagan, L.M. et al., 1993). Studies performed on sheep
(Kendrick, K.M. et al., 1988; Kendrick, K.M. et al., 1991), rats (Nyuyki, K.D. et al., 2011), and human females (Jong, T.R. et al., 2015) showed increase in plasma OT levels as well as OT release within the olfactory bulb, medial preoptic area, and the BNST. Thus, OT is an important factor in the expression of sexual behavior. Infusion of OT antagonist into the MPOA inhibited receptivity and increased partner rejection in female rats (Caldwell, J.D. et al., 1994).

Furthermore, ICV injections of OT into the MPOA or the ventromedial hypothalamus increased lordosis, whereas OT antagonist inhibited the behavior (Benelli, A. et al., 1994; Pedersen, C.A. & Boccia, M.L., 2006). These data, thereby, suggest that OT exerts its effects on sexual behavior by stimulating the MPOA and the ventromedial hypothalamus.

Evidences for the involvement of OT in the regulation of male sexual behavior was also identified. The male displays sexual behaviors such as chasing, sniffing, mounting, and intromission towards a receptive female (Veening, J.G. & Coolen, L.M., 2014; Veening, J.G. et al., 2014). These expressions of sexual behavior are also linked to the strong activation of the OT neurons in the SON and PVN of male rats (Nishitani, S. et al., 2004). Central infusion studies showed that OT induced penile erection and male sexual behavior, whereas OT antagonist inhibited these behaviors in male rats (Argiolas, A. et al., 1988; Argiolas, A. et al., 1985).

Altogether, OT signaling is vital for the expression of sexual behavior in both males and females.

**Oxytocin-mediated other social behaviors**

The OT system promotes a variety of other social behavior other than those discussed earlier. Decades of researches showed the influence of OT on aggression, feeding, grooming, memory, and learning.

In many species, aggressive behaviors are often displayed to not only defend their offspring, but also for territorial rights, food, and to attract mating partners. Resident-intruder
tests performed in rats, mice, and hamsters are an important method to quantify aggressive behavior towards unknown conspecifics (de Jong, T.R. et al., 2014; Koolhaas, J.M. et al., 2013). Female rats that displayed aggressive behavior towards the intruder had significantly lower levels of activity in the OT neurons in the PVN compared to the ones that displayed no aggression (de Jong, T.R. et al., 2014). Similarly, lower OT synthesis in the PVN neurons, but high OXTR binding in the central amygdala and BNST, was found in male rats that displayed high inter-male aggression (Calcagnoli, F. et al., 2014; Calcagnoli, F. et al., 2015). Studies performed in lactating hamsters also showed that a repeated local injection of OT in the amygdala enhanced maternal aggression towards male intruders (Ferris, C.F. et al., 1992). Thus, studies so far identified the OT-signaling in the central amygdala to play a prominent role in aggressive behavior. However, a study in female Syrian hamster also identified MPOA and the anterior hypothalamus as an important region for the regulation of aggression (Harmon, A.C. et al., 2002). With limited studies to identify the brain regions responsible, more research into gender and species dependent OT-mediated aggression is needed.

OT also acts as an important satiety hormone (reviewed in (Sabatier, N. et al., 2013). OT neurons in the SON and PVN have known to be activated soon after food intake (Johnstone, L.E. et al., 2006) and after injections of satiety peptide cholecystokinin (CCK)-8 (Caquineau, C. et al., 2010). In contrast, OT expression in the PVN decreased after a period of fasting (Kublaoui, B.M. et al., 2008). ICV or intraperitonelly injected OT reduced food intake and time spent eating (Arletti, R. et al., 1989), while pre-treatment with OT antagonist prevented these effects. These data suggests the inhibitory role of OT on feeding behavior.

Grooming, in rats and mice, is a behavior response to stress. Central administration of OT in mice has shown to elicit prominent self-grooming (Drago, F. et al., 1999). This study also
suggested that OT-mediated activation of dopamine D1-receptors in the mesolimbic pathway to promote grooming behaviors (Drago, F. et al., 1999).

Learning and memory involves acquisition and storage of new environmental and social stimuli based on the plasticity of certain brain circuitries. Some aspects that include mother-infant bonding and pair bonding are already discussed above. However, the role of OT in non-social and social memory is not discussed so far. As mentioned above, a study showed that OT acts on OXTRs in the dorsal hippocampus to improve spatial memory during lactation (Tomizawa, K. et al., 2003). This spatial memory was shown to involve OT-mediated facilitation and long-term potentiation of synapses from collateral fibers to the CA1 pyramidal cells in the hippocampus (Tomizawa, K. et al., 2003). However, the study also showed that ICV injections of OT improved spatial memory in virgin mice (Tomizawa, K. et al., 2003) suggesting that the effects of OT in spatial memory is not limited to postpartum period. The conclusion of this study was also challenged as OT-KO mice did not show any impaired spatial memory in a Y-maze test (Ferguson, J.N. et al., 2000). Furthermore, ICV injections of OXTR antagonist did not affect object recognition in the object discrimination test in male mice (Lukas, M. et al., 2013). Together, these data suggest that OT plays a negligible role (if any) in learning and processing of non-social information.

Social recognition and social discrimination tests provided great evidence to support the role of OT on the formation of social memory. In social recognition test, an animal was repeatedly exposed to a conspecific, which should result in a declined interest to investigate it. Similarly, in social discrimination test, an animal was simultaneously exposed to a familiar and a novel conspecific resulting in an increased investigation towards the novel one. ICV infusion of OT into the lateral septum and MPOA (Popik, P. et al., 1992) of male rats enhanced social
memory and juvenile recognition. In contrast, ICV infusions of OXTR antagonist immediately after being exposed to a conspecific impaired the maintenance of social memory (Lukas, M. et al., 2013). Furthermore, ICV injections of OXTR antagonist also affected the formation of olfactory memory in female mice (Engelmann, M. et al., 1998). A study conducted with OT-KO mice also supports the importance of OT system for social memory. These mice failed to recognize female mouse used as a social stimulus. Direct injection of OT into the medial amygdala rescued social memory in these OT-KO mice (Ferguson, J.N. et al., 2000), suggesting that OT and OT-OXTR interaction are necessary and sufficient for social recognition in mice. Therefore, these results showed that OT is an essential component to the formation of social memory in both sexes.

1.5. The Anteroventral Periventricular Nucleus

Organization of the AVPV

The anteroventral periventricular nucleus (AVPV) is located within the preoptic part of the periventricular zone, a region in the hypothalamus known to contain neurons that control the release of pituitary hormones. The AVPV occupies the ventral portion of the periventricular zone, along the wall of the third ventricle (Fig. 1-4), immediately caudal to the vascular organ of the lamina terminalis (OVLT). It is separated from the optic chiasm ventrally by a small group of fusiform cells in the suprachiasmatic nucleus (SCN). The morphology of the AVPV and SCN were first described in 1979 (Bleier, R. et al., 1979), and was referred to as a subdivision of the MPOA (Bleier, R. & Byne, W., 1985). However, several studies suggest that AVPV, and even the MPOA, are part of the periventricular zone of the hypothalamus on neurochemical basis (Simerly, R.B., 1989; Simerly, R.B. & Swanson, L.W., 1987; Simerly, R.B. et al., 1985; Simerly,
R.B. et al., 1989), connectivity (Gu, G.B. & Simerly, R.B., 1997), and functionality (Le, W.W. et al., 1999; Wiegand, S.J. & Terasawa, E., 1982).

Figure 1.4. Fluorescent photomicrograph showing OXTRs in the AVPV from a female OXTR reporter mouse. AC: Anterior commissure. Scale bar = 0.5mm.

Early retrograde (Simerly, R.B., 1998) and anterograde (Gu, G.B. & Simerly, R.B., 1997) tracing revealed some information on the afferents of the neurons in the AVPV, but detailed map is still not available. The AVPV receives strong inputs from the medial amygdala and BNST, which is suggested to play a role in conveying olfactory information (Simerly, R.B. et al., 1989). It is also heavily innervated by projections from the MPOA and the dorsomedial hypothalamus (Simerly, R.B. & Swanson, L.W., 1987). Afferents from several regions of the brainstem nuclei such as the nucleus of the solitary tract, ventral tegmental, and periaqueductal grey are also found in the AVPV (Simerly, R.B. & Swanson, L.W., 1986). It also provides direct inputs into regions of the OVLT and dopaminergic neurons found in the arcuate nucleus.
supporting its proposed role in the release of gonadotroopin releasing hormone (Gu, G.B. & Simerly, R.B., 1997; Simerly, R.B. & Swanson, L.W., 1986; Wiegand, S.J. & Terasawa, E., 1982). Furthermore, AVPV also receives strong inputs from the lateral septal region, which relays information from the hippocampus to the periventricular zone (Risold, P.Y. & Swanson, L.W., 1997a, 1997b). Most importantly, AVPV was shown to have bi-directional connection with parts of the PVN (Gu, G.B. & Simerly, R.B., 1997; Levin, M.C. et al., 1987), which suggest a role of AVPV neurons on the magnocellular neurons and vice-versa.

**Cell types in the AVPV and their function**

The MPOA and AVPV are known to be a sexually dimorphic area that contains a cluster of densely packed neurons. This thesis study also identified a novel group of sexually dimorphic OXTR-expressing neurons in the AVPV, which will be presented in Chapters 2 and 3. Thus this portion of the review will explore other known cell types identified in the AVPV so far.

Gonadal hormones exert profound effects on the development of the brain. In particular, regulation of the sexually dimorphic nuclei and organization of the neural circuitry (Matsumoto, A., 1991; Simerly, R.B., 1990). In addition, sex steroids regulate the development of sexually dimorphic connectivity in the forebrain regions (Matsumoto, A., 1991; Raisman, G. & Field, P.M., 1971). Steroids exert actions by binding to its nuclear receptors. Sexual dimorphism in the distribution of gonadal steroid receptors have been identified. In the AVPV and BNST, androgen receptors are generally higher in males and estrogen receptors are higher in females (Simerly, R.B., 1990). Estrogen binds to its two known receptor subtypes, ERα and ERβ. However, the effect of estrogen binding to one of the receptor subtypes has differential implication on neurophysiological functions. ERα is mainly associated with neuroendocrine and sexual behavior (Lindzey, J. et al., 1998; Rissman, E.F. et al., 1997), whereas ERβ is linked to learning and
memory (Jacome, L.F. et al., 2010; Rissman, E.F. et al., 2002), anxiety related behavior (Lund, T.D. et al., 2005), and stress response (Weiser, M.J. & Handa, R.J., 2009). Collective information from rodent studies implies that cells expressing ERα within the AVPV are critical in transmitting estrogen signaling to the GnRH neurons (Mayer, C. et al., 2010; Simonian, S.X. et al., 1999; Wintermantel, T.M. et al., 2006). Activation of GnRH neurons by estrogen stimulates luteinizing hormone (LH) surge that triggers ovulation. Early studies showed that electrostimulation of the preoptic area induced ovulation in rodents (Everett, J.W. & Radford, H.M., 1961). Similarly, direct implantation of estrogen into the preoptic area stimulated LH surge from the pituitary in ovariectomized rats (Goodman, R.L., 1978). Conversely, implantation of antiestrogens blocked estrogen induced LH surge (Petersen, S.L. & Barraclough, C.A., 1989). A study conducted with KO mice suggests LH surge is induced by estrogen binding to ERα. ERβ-KO mice exhibited normal preovulatory LH surge, while LH surge was absent in ERα-KO mice (Wintermantel, T.M. et al., 2006). In contrast, another study showed that expression of ERβ in the AVPV is sexually dimorphic. Significantly more ERβ expression was found in females, than in males, which plays an important role in LH surge by estrogen (Orikasa, C. et al., 2002). Since 83% of ERβ mRNA-positive cells were also immunoreactive to ERα (Orikasa, C. et al., 2002), it is possible that these receptors work in cohort or repress transcriptional activity of one another to exert alternative effects.

The surge of LH in mice is also progesterone dependent and progesterone receptors (PRs) have been found in the AVPV. Importantly, the expression of PRs in the AVPV depends on estrogen. Studies showed that estrogen increased the expression of PRs in the AVPV (Simerly, R.B. et al., 1996) whereas antiestrogenic compounds had the opposite effect (Shughrue, P.J. et al., 1997). Moreover, inhibition of PR in the AVPV with PR antagonist completely blocked both
GnRH and LH surges suggesting that estrogen and progesterone signaling in the AVPV is necessary for LH surge, and thus ovulation.

The presence of sexually dimorphic population of neurons in the AVPV maybe attributed to the influence of gonadal steroids on the AVPV. Two distinct, kisspeptin1 and dopaminergic, sexually dimorphic neuronal types were identified so far. The sexually dimorphic population of kisspeptin1 neurons in the AVPV is the key regulator for GnRH secretion and sexual reproduction (Semaan, S.J. et al., 2012). These kisspeptin1 express both ERα and ERβ (Kanaya, M. et al., 2019) implying that they may contribute to positive feedback input to GnRH neurons (Smith, J.T. et al., 2005). Kisspeptin signaling has been extensively studied over the past few years, especially for its role in ovulation (Kauffman, A.S. & Smith, C.J., 2013). Recently, a group of sexually dimorphic dopaminergic neurons were also discovered in the AVPV. These neurons innervate OT neurons in the PVN and regulate maternal behavior (Scott, N. et al., 2015). Dopamine and OT signaling plays a role in maternal behaviors and social behaviors. The presence of dopaminergic neurons and OXTR-expressing neurons in the AVPV suggests the possibility of such interactions to induce maternal behavior and will be discussed in Chapter 2 and 3.
CHAPTER 2
SEXUALLY DIMORPHIC OXYTOCIN RECEPTOR-EXPRESSING
NEURONS IN THE PREOPTIC AREA OF THE MOUSE BRAIN*

2.1. Introduction

The neurohypophysial hormone, oxytocin, is synthesized by magnocellular cells located
primarily in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. The
magnocellular cells send long axonal projections into the neurohypophysis where oxytocin is
released into the general circulation in response to physiological demands, such as milk let down
and parturition (Kelly, J. & Swanson, L.W., 1980; Rosen, G.J. et al., 2008). The release of
oxytocin also occurs within the brain and modulates many aspects of behaviors including but not
limited to maternal care (Bosch, O.J. & Neumann, I.D., 2008; Da Costa, A.P. et al., 1996; Insel,
Pedersen, C.A. & Prange, A.J., Jr., 1979; van Leengoed, E. et al., 1987), female sexual behavior
(Borrow, A.P. & Cameron, N.M., 2012; McCarthy, M.M. et al., 1994; Pedersen, C.A. & Boccia,
M.L., 2006), male sexual behavior (Argiolas, A. & Melis, M.R., 2013; Borrow, A.P. & Cameron,
N.M., 2012; Veening, J.G. et al., 2014), pair/social bonding (Carter, C.S. et al., 1992) (Insel,
T.R., 1992; Young, L.J. & Wang, Z., 2004), aggression (Arakawa, H. et al., 2015; Calcagnoli, F.
et al., 2013; de Jong, T.R. et al., 2014), anxiety (Menon, R. et al., 2018), and fear (Huber, D. et
al., 2005; Toth, I. et al., 2012).

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Oxytocin influences behaviors by binding to oxytocin receptors (OXTRs) that are widely distributed in various parts of the brain (Gimpl, G. & Fahrenholz, F., 2001; Jard, S. et al., 1987).


The present study was conducted to assess the sex differences in the distribution of OXTR neurons in the preoptic area (POA) using OXTR-Venus (an enhanced variant yellow fluorescent protein) mice. In contrast to transgenic reporter models which use random integration of a reporter gene that could end up anywhere in the host genome, this OXTR-Venus mouse line is an OXTR knock-in model in which Venus is inserted into the locus exactly where OXTR is normally located (Yoshida, M. et al., 2009). Therefore, Venus likely achieves natural expression patterns and levels, while ectopic expression less likely occurs. Unlike previously published reports on the localization of OXTRs in the brain that were conducted by either autoradiography

2.2. Materials and Methods

Animals

OXTR-Venus mice in which a part of the OXTR gene was replaced with Venus (a variant of the yellow fluorescent protein) cDNA (Yoshida, M. et al., 2009) were originally provided by Dr. Nishimori of the Tohoku University in Japan. A colony was established in facility at Louisiana State University, and OXTR-Venus mice were backcrossed with C57BL6J mice for at least 10 generations. Four breeder pairs of OXTR-Venus heterozygous (+/-) male and female gave 22 litters of pups that were used for this study. Only virgin female and male mice (6-10 weeks old) were used. The males and females were housed in separate cages (maximum 4 mice/cage) in the same room on a 12:12 h light/dark cycle with access to food and water available ad libitum. For genotyping, genomic DNA was isolated from tail snips by incubating the tissues with REDExtract-N-Amp™ Tissue PCR Kit (Sigma, St. Louis, MO). The isolated DNA was subsequently genotyped using the following two sets of primers: F (5’-GTTGGGAACAGCGGTGATTA-3’) and R (5’-GGCTCAGGCTTTCTCTACTT-3’). All protocols and animal experiments were approved by the Institutional Animal Care and Use Committee at Louisiana State University.

Immunocytochemistry

Mice (6-8 weeks old) were deeply anesthetized with Ketamine-Xylazine (9:1; 100 mg/kg; i.p.) and transcardially perfused with 0.01M sodium phosphate buffered saline (PBS; pH 7.2),
followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.2). The brains were extracted and postfixed in the same fixative for overnight. After infiltrated with 20% sucrose in 0.1 M PB for cryoprotection for 12 hr., coronal sections were transected at 40 µm by a sliding microtome (Leica SM2010R; Mannheim, Germany).

To enhance the signal of Venus, immunocytochemical localization of Venus with anti-green fluorescent protein (GFP) antibody (ab290; abcam, Cambridge, UK) was conducted in the brain sections obtained from adult virgin male and virgin female OXTR-Venus mice (8-10 week-old). The free-floating brain slices were incubated with the primary antibodies against GFP at dilutions of 1:10,000 in PBS containing 0.5% Triton X-100 (PBST) for overnight with continuous gentle agitation at 4°C. The brain sections were subsequently incubated with a secondary antibody (goat anti-rabbit) conjugated with DyLight 594 (Jackson ImmunoResearch, West Grove, PA) for 4 hr at room temperature. The sections were mounted in polyvinyl alcohol (PVA) with anti-fading agent 1,4-Diazabicyclo[2.2.2]octane (DABCO) that consists of 4.8 g PVA, 12 g glycerol, 12 ml dH2O, 24 ml 0.2 M Tris-HCl and 1.25 g DABCO. The specificity of the anti-GFP antibody was tested on brain sections from wild type mice that do not express Venus. Immunoreactivity to Venus was not observed in the brain sections from wild type mice. Thus, the anti-GFP antibody specifically recognizes Venus, which is a variant of GFP.

Fluorescence microscopic images (1280 x 1024) were acquired digitally (Eclipse 80i equipped with DS-QiMe, Nikon, Tokyo, Japan).

For double-fluorescence imaging of Venus and immunocytochemistry of estrogen receptor α (ERα), the brain sections were processed with polyclonal anti-ERα antibody (Cat#06-935; EMD Millipore, Billerica, MA) at dilutions of 1:8,000 in PBST for overnight with continuous gentle agitation at 4°C. The sections were subsequently incubated with secondary
antibody conjugated with fluorescence marker (AffiniPure Goat Anti-Rabbit IgG Alexa Fluor647; Jackson ImmunoResearch, West Grove, PA) at 1:400 dilution for 2-4 hr at room temperature. The sections were mounted in polyvinyl alcohol (PVA) with DABCO. Confocal fluorescence microscopic images (1,024 x 1,024; 1 µm optical section thickness) were acquired with a confocal microscope (Leica TCS SP2 spectral confocal microscope, Mannheim, Germany). Digital images were minimally altered in ImageJ software (Bethesda, NIH) with changes in dynamic range.

**Electrophysiology**

**Slice preparation**

Female mice were deeply anaesthetized using Ketamine-Xylazine (9:1, 100 mg/kg i.p.) and perfused through the heart with cold artificial cerebral spinal fluid (ACSF) in which NaCl was replaced by equiosmolar sucrose. The brains were then removed and coronal slices (250 µm) containing the hypothalamic periventricular nucleus were collected using a vibrating microtome (Leica VT1200; Leica, Mannheim, Germany). The brain slices were kept in ACSF (in mM: 125 NaCl, 2.5 KCl, 1 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, 20 D-glucose, 2 CaCl₂, 0.4 Ascorbic acid, pH of 7.3-7.4, with an osmolality of 290-300 mOsm/kg H₂O).

**Whole cell patch clamp recording**

OXTR-Venus neurons in the AVPV were identified with an epifluorescence microscope (Olympus BX50WI, Tokyo) equipped with a 40x water immersion lens (0.8 n.a.) and a CCD camera (ORCA-R², Hamamatsu Photonics, Hamamatsu). Whole-cell membrane potentials recordings were obtained with an Axopatch 700B amplifier (Molecular Devices, Foster City, CA). Traces were acquired digitally at 20 kHz and filtered at 5 kHz with a Digidata 1440A and in conjunction with PClamp 10 software (Molecular Devices, Foster City, CA). Patch electrodes
were drawn with a Flaming/Brown micropipette puller (Model P-1000, Sutter Instrument Novato, CA) from borosilicate capillary glass tubing (1.1 mm ID, 1.5 mm OD, Sutter Instruments, Novato, CA) to have resistance of 4-8 MΩ when filled with the pipette solution that contained (in mM): 140 K-Gluconate, 1 MgCl₂, 10 HEPES, 10 CaCl₂, 2 ATP(Mg⁺⁺), and 0.4 GTP (Na⁺) and 11 EGTA. ACSF was saturated with 95% O₂/5% CO₂ and was warmed to 33-34°C during the recordings. Picrotoxin (100 µM) and 6,7-dinitro-quinoxaline-2,3(1H,4H)-dione (DNQX; 10 µM) were also added to ACSF to block the synaptic activity. The estimated liquid junction potential was +9.2 mV; however, the data presented are not corrected for liquid junction potential.

**Data Analysis**

To observe the effect of oxytocin on OXTR-Venus cells, membrane potentials were recorded in the presence or absence of oxytocin (100 nM) while no current was injected. The mean membrane potentials were obtained by averaging the membrane potential in 30 sec time frames. These time frames were captured 30 sec before oxytocin was applied, 2 min after the application of oxytocin, and 3 min after washing. These sampling duration and point were selected because the peak response and plateauing of the membrane potential occurred at these time points in OXTR-Venus cells from heterozygous mice. Same parameters were used to measure the membrane potential of OXTR-Venus cells from homozygous mice that did not show apparent changes to the bath application of oxytocin.

Repeated measures ANOVA was used to assess the difference in basal membrane potential in the presence or absence of oxytocin for OXTR-Venus cells in heterozygous and homozygous mice. The changes in membrane potential caused by application of oxytocin between heterozygous and homozygous mice were analyzed with the Student's t test. Differences
were considered to be statistically significant at \( p < 0.05 \). Box and whisker plots were used to represent numerical data: mean and median are represented by a filled circle and a line, respectively; the box extends to the quartiles of the data points; the whiskers extend to the farthest data points.

**Single-Cell RT-PCR**

**Single cell harvest for single-cell RT-PCR**

The brain slices were prepared as described above in Slice preparation / Electrophysiology. Tissues containing the AVPV were carefully dissected out using a razor blade and were incubated in oxygenated ACSF (35°C) containing Protease Type XIV (1.2 mg/ml: Sigma Chemicals, St Louis, MO, USA) for 20-30 min, and washed in a sodium isethionate solution consisting of (in mM): 140 sodium isethionate, 2 KCl, 4 MgCl\(_2\), 23 glucose, 15 HEPES, pH 7.3 (adjusted with 1M NaOH). The tissue was then triturated in sodium isethionate solution using three successively smaller fire-polished pasteur pipettes to dissociate OXTR-Venus cells. The supernatant containing dissociated neurons was transferred to a plastic Petri dish (Nunc, Rochester, NY, USA) and allowed to settle for approximately 5 min. Borosilicated glass capillary tubes (inner diameter 1.1 mm, outer diameter 1.5 mm Sutter Instruments, Novato, CA) were pulled on a Flaming/Brown micropipette puller (Model P-1000, Sutter Instrument Novato, CA) to make micropipettes (tip inner diameter of ~5 \( \mu \)m) for cell harvest. Dissociated OXTR-Venus cells were identified by an inverted microscope equipped with epifluorescence (Olympus IX5, Tokyo). Each identified OXTR-Venus neuron was individually harvested by a micropipette filled with RNase free water on a micromanipulator (MP225, Sutter Instrument, Novato, CA). After aspiration, the contents of the micropipette were ejected into an ice-cold 0.5 ml PCR tube and stored at -80°C.
**RT-PCR**

Harvested cells were subjected to one step RT-PCR using iTaq Universal SYBR green One-step Kit (Bio-Rad, Hercules, CA) according to manufacturer’s protocol with ABI ViiA-7 sequence detection system (ABI Applied Biosystem, Grand Island, NY). The sequences of primer sets used to detect OXTR was Forward 5'-GTGCAGATGTGGAGCGTCT-3' & Reverse 5'-AGAGATGGCCCGTGAAGAG-3' and ERα (ESR1) was Forward 5'-CTGCCAAGGAGACTCGCTAC-3' & Reverse 5'-GCAACTCTTCTCCGGTTCT-3'. Identification of each cDNA of interest was based on the predicted size of each PCR product; 130 bp for OXTR and 179 bp for ERα.

**Ovariectomy and Estrogen replacement therapy**

Siblings of the female mice (6-8 weeks old, weighing 20-23g) were randomly assigned to intact female, OVX, and OVX+E groups. These female mice were anesthetized with 1.5-2% isoflurane (4% for induction) in oxygen at a rate of 2 L/m, the ovaries were removed bilaterally (OVX), and received an osmotic mini-pump (ALZET model 1004, DURECT, Cupertino, CA) under the back skin two weeks after ovariectomy. Each mouse received a subcutaneous injection of buprenorphine hydrochloride (Buprenex, 0.1 mg/kg) after surgery to control pain. Each osmotic mini-pump contained either 13.9 mM 17β-estradiol (E2, E8875, Sigma-Aldrich, St. Louis, MO) dissolved in a solvent containing dimethyl sulfoxide (DMSO) and 70% Ethanol (1:4, respectively) or vehicle only. The concentration of E2 was estimated to release 10 µg of E2 per day at 0.11 µl/hr. After 2 weeks, animals were deeply anaesthetized using Ketamine-Xylazine (9:1, 100 mg/kg i.p.) and their brains were extracted and sectioned as described above in Immunocytochemistry. The groups of mice were euthanized on the same day to avoid the age difference becoming a variable.
**Cell count and Statistics**

The group of OXTR-Venus neurons in the AVPV started appearing in the area surrounding the organum vasculosum laminae terminalis (OVLT) at the ventral end of the third ventricle (Bregma 0.5 mm in the mouse brain atlas) (Franklin, K.B.J. & Paxinos, G., 2008) and was continuously found in the next 22-25 consecutive 40 μm-thick coronal sections. The number of OXTR-Venus neurons in the AVPV was counted from the level of the OVLT (section -5) to the level of the suprachiasmatic nucleus (SCN, section 25) where most of OXTR-Venus neurons were found. Averaged cell number per section is presented as the means ± SE. The Student's *t* test was used for comparison of total number of OXTR-Venus neurons in the AVPV between sexes. The effects of OVX and E2 replacement therapy on number of OXTR-Venus neurons were also analyzed by the Student's *t* test. Differences were considered to be statistically significant at *p* < 0.05. Box and whisker plots were used to represent numerical data: mean and median are represented by a filled circle and a line, respectively; the box extends to the quartiles of the data points; the whiskers extend to the farthest data points.

**2.3. Results**

**The validation of the anti-GFP antibody**

To enhance and prolong the fluorescent signal of Venus (a variant of green fluorescent protein (GFP), immunocytochemistry was conducted using an antibody against GFP. Intense immunoreactive-cells were located in various part of the brain in sections from OXTR-Venus mice (Fig. 2.1A); however, no immunoreactive-cells were observed in brain sections from wild type mice (Fig. 2.1B). This finding suggests the antibody specifically recognized Venus in the brain sections.
Fig 2.1. Fluorescent photomicrographs of coronal sections from a female OXTR-Venus mouse and a female wild type mouse. Immunocytochemistry using an anti-GFP antibody was conducted to enhance and preserve the fluorescent signal from Venus. A. Numerous immunoreactive OXTR-Venus cells were observed in various regions in the section from an OXTR-Venus mouse. B. No immunoreactive-cells were observed in the brain section from a wild type mouse. Scale bar = 1 mm.

The overall distribution of OXTR-Venus cells in both sexes

Brains from young adult (6-8 weeks) virgin heterozygous (+/-) males (n=6) and females (n=8) were sectioned coronally from the beginning of the preoptic area to the end of the hypothalamus, and the distribution of OXTR-Venus cells was compared between the sexes. The overall distribution of OXTR-Venus cells was similar between sexes in all areas except within an area immediately along the third ventricle (3V) in the AVPV where OXTR-Venus cells were observed only in females, but not in males.

In coronal sections at the level of the organum vasculosum of the lamina terminalis (OVLT; Fig. 2.2A), OXTR-Venus cells were sparsely distributed in the dorsal-lateral part of the cortex including the cingulate (Cg1 & 2), motor (M1 & M2), and somatosensory (S1 & S2) cortex. In contrast to the dorsal-lateral regions of the cortex, clusters of OXTR-Venus cells were found in several areas in the piriform (Pir) and insular cortex, namely the dorsal endopiriform claustrum (DEn), the intermediate endopiriform claustrum (IEn), the dorsal and ventral parts of
the agranular insular cortex (AID & AIV). In the septum, lateral (LS) and medial (MS) regions along with the bed nucleus of the stria terminalis (BNST) medial division anterior part (STMA) had dense population of OXTR-Venus cells. A moderate population of OXTR-Venus cells were found in the MPOA surrounding the OVLT.

At the level of the anterior commissure (AC; Fig. 2.2B), clusters of OXTR-Venus cells were found in the lateral septum (both intermediate (LSI) and ventral (LSV) parts) and the BNST (medial division anterior part (STMA), the posterior-lateral (STLP), and ventral-lateral (STLV) divisions). In the preoptic areas, magnocellular preoptic nucleus (MCPO) and Septohypothalamic nucleus (Shy) had a cluster of OXTR-Venus cells, whereas a sparse population of OXTR-Venus cells was observed in the MPOA. A sexually dimorphic distribution of OXTR-Venus cells occurred in the AVPV where only females had considerable number of OXTR-Venus cells.

At the level of the SON (Fig. 2.2C), intense OXTR-Venus immunoreactivity was found in the amygdala along with areas in the insular and piriform cortex already described above. Within the amygdala, prominent OXTR-Venus cells were found in the anterior cortical amygdaloid nucleus (ACo), cortex amygdala transition (CxA), basomedial amygdala nucleus anterior (BMA), and central amygdala (capsular region (CeC), lateral division (CeL), and medial division (CeM)), and medial amygdala n. anterior dorsal (MeAD). In hypothalamic regions, OXTR-Venus cells were sparsely distributed in the anterior hypothalamus (AH). In addition, a moderate population of OXTR-Venus cells was present in the suprachiasmatic nucleus (SCN). OXTR-Venus cells were not observed in the SON. In the PVN, OXTR-Venus cells were located in the dorsal cap region. In the thalamus, a group of OXTR-Venus cells was present in the paraventricular thalamic nucleus (PVA).
Fig 2.2. Fluorescent photomicrographs showing OXTR-Venus immunoreactivity in coronal sections from a female (left panels) and a male (right panels) OXTR-Venus mouse. A. At the level of OVLT, OXTR-Venus cells were found in in the layers of the cortex (Cg1, Cg2, M1, M2, S1, and S2). Especially dense populations of OXTR-Venus cells were observed in the piriform cortex (Pir, DEn and IEn), insular cortex (AIP), lateral and medial septum (LS and MS), and anterior part of medial division of the bed nucleus of the stria terminalis (STMA). A moderate population of OXTR-Venus cells was found in the medial preoptic area (MPOA) surrounding the OVLT. B. At the level of the anterior commissure (AC), a dense population of OXTR-Venus cells was additionally found in the intermediate and ventral parts of the LS (LSI and LSV), and divisions of the BNST (STMA, STLP, and STLV). In the preoptic areas, magnocellular preoptic nucleus (MCPO) and Septohypothalamic nucleus (Shy) had a cluster of OXTR-Venus cells.

(fig. cont’d)
whereas not so dense a population of OXTR-Venus cells was observed in the medial preoptic area (MPOA). A sexually dimorphic distribution of OXTR-Venus cells occurred in the AVPV where it appeared only in the female; however, the difference is not very clear due to low magnification of images. C. At the level of the suprachiasmatic nucleus (SON), a prominent cluster of OXTR-Venus cells was additionally found in several areas in the amygdala (ACo, CxA, BMA, CeC, CeL, CeM, MeAD) and hypothalamus (AH, SCN, PVN). A sparse population of OXTR-Venus cells were also found in the thalamus (PVA). D. At the level of the posterior hypothalamus, a dense population of OXTR-Venus cells was observed in the VMH, while a sparse population was found in the CA1, CA2, and CA3 layers of the hippocampus. Overall, there is no obvious sex difference in the distribution of OXTR-Venus between sexes.
At the level of the posterior hypothalamus (Fig. 2.2D), a large cluster of OXTR-Venus cells was found in the ventro-lateral division of the ventromedial hypothalamic nucleus (VMH-VL) and in the arcuate nucleus (Arc). A sparse population of OXTR-Venus cells were found in the dorsal medial hypothalamic nucleus (DM). In the hippocampus, OXTR-Venus cells were located in the CA3 field. The overall distribution of OXTR-Venus cells was largely comparable to that of OXTR detected in the C57BL/6J mouse brain by autoradiography of OXTR bindings (Hammock, E.A. & Levitt, P., 2013b; Olazabal, D.E. & Alsina-Llanes, M., 2016) indicating that the expression of Venus occurred in native cell types that express OXTR.

**The sexually dimorphic distribution of OXTR-Venus cells in the AVPV**

The group of OXTR-Venus cells in the AVPV of females started to appear anteriorly at the level of the OVLT; however, the population became more prominent from the level of the anterior commissure (AC; Fig. 2.3 Aa) and was observed in the next 15 consecutive 40 µm sections posteriorly. The number of OXTR-Venus cells decreased considerably after 12-14 sections posteriorly from the AC.

To compare the distribution and number of OXTR-Venus cells between sexes, the number of OXTR-Venus cells in the AVPV in each brain section from 8 females and 6 males was counted and plotted (Fig. 2.3B i-a). The AVPV was defined as approximately the ventral two-third of an area within 130 µm from the edge of the 3V indicated by the dashed line in Fig. 2.3A. The anterior-posterior coordinate of each brain section was adjusted by the presence of the AC; the first section containing the "connected" AC in the midline (Fig. 2.3Aa) was designated as section #0 (Fig. 2.3B ii-a), whereas the OVLT and the SCN were observed on #5 and #25 sections, respectively. The mean number of OXTR-Venus cells in the AVPV on the anterior-posterior coordinated sections were plotted to better profile the population of the OXTR-Venus.
A. Female.

B. Male.

### ii-b

| Total # of OXTR-Venus cells in MPOA including AVPV |
|-----------------|-----------------|
| n=6             | n=8             |
| Male            | Female          |
| p<.0001         |                 |

### iii-b

| Total # of OXTR-Venus cells in MPOA excluding AVPV |
|-----------------|-----------------|
| n=6             | n=8             |
| Male            | Female          |
| p=.8234         |                 |
Fig 2.3. The sexually dimorphic distribution of OXTR-Venus cells in the AVPV. A. Fluorescent photomicrographs showing OXTR-Venus immunoreactivity in (a) the preoptic-hypothalamic regions at the level of the AC, (b) 5 sections (200 µm) posterior to the AC, and (c) 15 sections (600 µm) posterior to the AC from a female (top panels) and a male (bottom panels) OXTR-Venus mouse. B. The mean number of OXTR-Venus neurons in the AVPV (i-a), in the medial preoptic area (MPOA) including the AVPV (ii-a), and the MPOA without the AVPV (iii-a) on each brain section from 8 female and 6 male OXTR-Venus mice are plotted from the 10th section anterior from the AC to the 25th section posterior from the AC. The mean total number of OXTR-Venus cells in females was greater than in males in the AVPV (i-b) and in the MPOA including the AVPV (ii-b). There were no significant differences between sexes in the number of OXTR-Venus cells in the MPOA excluding the AVPV (iii-b). All numerical data are expressed as the mean ± SEM. AC: anterior commissure; POA: preoptic area. Scale bars = 200 µm.

cells in females and males (Fig. 2.3B i). Significantly more OXTR-Venus cells in the AVPV were observed on sections #3 to #25 of females than that of males (Fig. 2.3 i-a). Of six male mice examined, three mice had no detectable OXTR-Venus cell in their AVPV and the other three had 1 or 2 cells /section. The total number of OXTR-Venus cells in the AVPV was significantly greater in females (615 ± 43 cells) than in males (14 ± 2 cells) (Fig. 2.3B i-b; \(t_{12}=11.76, p<0.0001\)).

To assess whether the sexually dimorphic distribution of OXTR-Venus cells extends into the area surrounding the AVPV, the number of OXTR-Venus cells in the MPOA was also counted. The mean number of OXTR-Venus cells / section was significantly higher in females than in males on sections #3 to #10 (Fig. 2.3 ii-a). The total number of OXTR-Venus cells in the MPOA including the AVPV was significantly greater in females than in males (\(t_{12}=6.16, p<0.0001\)); however, the number of OXTR-Venus cells was significantly higher in sections #0-10 where clusters of OXTR-Venus cells were observed in the AVPV (Fig. 2.3B ii). The number of OXTR-Venus cells in the MPOA excluding the AVPV (Fig. 2.3 iii-a) was subsequently obtained by mathematical subtractions of Fig. 2.3B i-a from 2.3B ii-a. The number of OXTR-Venus cells was not significantly different on any sections between the sexes. Therefore, there
was no sex difference in the number of OXTR-Venus cells in the MPOA excluding the AVPV (Fig. 2.3B iii-b; t_{(12)}=0.23, p=0.823).

**The assessment of functional OXTR in OXTR-Venus cells in the AVPV**

Whenever a transgenic approach is used, it is always a concern that the expression of a transgene may interfere with the expression of a native gene or function of the native protein. In the OXTR-Venus mouse, a part of the OXTR was replaced by Venus. Therefore, the heterozygous (+/-) has a mono-allelic expression of both OXTR and Venus, while the homozygous (+/+) has bi-allelic expression of Venus without OXTR. Single-cell RT-PCR was performed to assess the expression of OXTR in OXTR-Venus neurons individually harvested from the dissected AVPV of heterozygous and homozygous OXTR-Venus female mice (Fig. 2.4 A). OXTR mRNA was detected from most of the heterozygous OXTR-Venus cells (38/48 cells from 8 virgin females), while OXTR mRNA was not detected from any homozygous OXTR-Venus cells (10 cells from 2 virgin females). In addition, to validate whether OXTR-Venus cells from heterozygous produce functional OXTRs, we performed patch-clamp electrophysiology. A bath application of oxytocin caused significant depolarization in all 10 OXTR-Venus cells examined in the AVPV from 5 female heterozygous OXTR-Venus mice (Fig. 2.4 Ba and Ca; F_{(2,8)}= 6.76; p=0.0003). The mean membrane potentials at the rest before application of oxytocin in heterozygous OXTR-Venus cells, during the application, and washing were -60.36 ± 5.97 mV, -52.76 ± 3.47 mV, and -60 ± 3.74 mV, respectively. In some instances, the application of oxytocin induced depolarization that caused repetitive firing of action potentials (Fig. 2.4 Bb). A bath application of oxytocin did not, however, cause any measurable change in the membrane potential of all 6 cells examined from 5 female homozygous OXTR-Venus mice, which do not express OXTR (Fig. 2.4 Bc and Ca; F_{(2,4)}=1.32; p=0.19). The mean membrane potentials at the
Fig 2.4. OXTR-Venus cells in the AVPV express functional OXTR. A. Single-cell RT-PCR analysis of OXTR expression in OXTR-Venus cells. Expression of OXTR mRNA was detected in 8 of 10 Venus cells harvested from OXTR-Venus heterozygous (+/-) females, but not detected from any of 4 OXTR-Venus cells obtained from homozygous (+/+) females. B. Examples of the effect of oxytocin on membrane potential and the firing pattern of OXTR-Venus (+/+) cells. a. Bath applications of oxytocin (100 nM) repeatedly caused membrane depolarization. b. The
oxytocin-mediated depolarization caused repetitive firing. c. Application of oxytocin had no effect on OXTR-Venus (+/+) cells. C. Analysis of oxytocin induced changes in membrane potential from electrophysiological recording. a. The mean basal membrane potential recorded with or without bath application of oxytocin. Oxytocin significantly depolarized OXTR-Venus (+/−) cells but had no effect on OXTR-Venus (+/+). b. The mean oxytocin effective membrane potential was significantly higher in OXTR-Venus (+/−) cells compared to OXTR_Venus (+/+). cells.

rest before application of oxytocin in homozygous OXTR-Venus cells, during the application, and washing were -67.57 ± 2.39 mV, -68.73 ± 2.35 mV, and -68.02 ± 2.24 mV, respectively. The oxytocin-induced changes in membrane potential was significantly greater (t(14)= -5.35; p<0.0001) in heterozygous OXTR-Venus cells (7.6 ± 1.004 mV) than in homozygous OXTR-Venus cells (-1.17 ± 1.3 mV). Since all electrophysiological recordings were performed under the presence of GABA and Glutamate receptor blockers, Picrotoxin and DNQX, the depolarization caused by application of oxytocin is most likely a result of oxytocin binding to OXTRs. These findings suggest that OXTR-Venus cells in the AVPV represent native OXTR cells.

The assessment of estrogen dependency of OXTR expression in the AVPV

Because the expression of OXTR-Venus cells in the AVPV occurred primarily in females, we hypothesized that expression of OXTR in the AVPV is supported by the female gonadal steroid, estrogen. To test this hypothesis, we conducted immunocytochemistry to examine whether the estrogen receptor α (ERα) is present in OXTR-Venus cells. ERα immunoreactive cells were found in the AVPV and its surrounding area of the MPOA in females and males (Fig. 2.5A). Double fluorescence confocal microscopy revealed that all OXTR-Venus neurons were immunoreactive with ERα in females (Fig. 2.5 A). The presence of ERα in OXTR-Venus cells was also confirmed by single-cell RT-PCR (Fig. 2.5 B). Of 58 OXTR-Venus cells (48 cells from heterozygous and 10 cells from homozygous females) that were individually
Fig 2.5. OXTR-Venus cells in the AVPV express ERα. A. Double confocal fluorescence photomicrographs of ERα immunoreactivity and OXTR-Venus from a female and a male OXTR-Venus (+/−) mouse. Many OXTR-Venus cells were seen in the AVPV from the female, whereas virtually no detectable OXTR-Venus cell was found in the AVPV from the male. The merged image shows that all OXTR-Venus cells were immunoreactive to ERα. B. Single-cell RT-PCR analysis of ERα expression in OXTR-Venus cells. The same set of cDNA derived from individually harvested OXTR-Venus cells used in Fig. 2.2A was used. ERα mRNA was detected from all cells except cell #4.

harvested from the AVPV, mRNA for ERα was detected in 57 cells. Moreover, ERα mRNA was detected in all cells in which OXTR mRNA was detected. Thus, these results indicate that OXTR-expressing neurons in the AVPV also express ERα.
To test whether the expression of OXTR in the neurons of the AVPV depends on estrogen, we first ovariectomized (OVX) virgin female mice to remove the source of estrogen and examined whether the expression of OXTR-Venus was affected. The number of OXTR-Venus cells in the AVPV was significantly fewer in brain slices collected two weeks after ovariectomy (n=6, 7.3 ± 2.25 cells) compared to that of intact virgin female siblings (n=8, 614.9 ± 43.2 cells, Fig. 2.6 A & B; t(12)=-12.04, p<0.0001). Next, we tested whether estradiol (E2) replacement therapy would restore the population of OXTR-Venus cells. Significantly more OXTR-Venus cells were observed in the AVPV of OVX mice that received two weeks of E2 therapy (OVX+E2; 100ng/day; n=6, 147.6 ± 14.9 cells) compared to OVX mice that received vehicle only (OVX+ Vehicle; n=6, 5.92 ± 1.24 cells; Fig. 2.6 C & D; t(10)=8.25, p<0.0001).

These findings suggest that expression of OXTR in the cells of the AVPV is supported by estrogen.

2.4. Discussion

Sexually dimorphic distribution of OXTR in the AVPV

Most of the previously published reports of the localization of OXTRs in rodent brains were conducted by in vitro receptor autoradiography with a selective radiolabeled OXTR ligand (Bales, K.L., Plotsky, P.M., et al., 2007; Beery, A.K. et al., 2008; Campbell, P. et al., 2009; Cao, Y. et al., 2014; Dubois-Dauphin, M. et al., 1992; Dumais, K.M. et al., 2013; Elands, J. et al., 1988; Hammock, E.A. & Levitt, P., 2013b; Insel, T.R. et al., 1991; Insel, T.R. et al., 1993; Olazabal, D.E. & Alsina-Llanes, M., 2016; Smeltzer, M.D. et al., 2006; Tribollet, E. et al., 2002; Uhl-Bronner, S. et al., 2005) or autoradiography of in situ hybridization of OXTR mRNA (Vaccari, C. et al., 1998; Yoshimura, R. et al., 1993). However, none of these studies reported the
Fig 2.6. Expression of OXTR in the sexually dimorphic OXTR-neurons is supported by estrogen. A. Plot of the average number of OXTR-Venus neurons in the AVPV/section in the serial sections from the OVLT to SCN in intact, OVX, OVX+E2, and OVX+Vehicle female mice. B. The mean total number of OXTR-Venus cells in the AVPV from intact female and OVX. The mean total number of OXTR-Venus cells in OVX was significantly lower than that of females. C. Fluorescence microscopic images of OXTR-Venus cells in the AVPV from an OVX+E2 and an OVX+Vehicle. The group of OXTR-Venus cells observed in the AVPV in the intact females was not observed in OVX+Vehicle; however, the group of OXTR-Venus cells was observed in the OVX+ E2 mouse. D. The mean total number of OXTR-Venus cells in the AVPV of OVX+E2 was significantly higher than that of OVX+Vehicle. All numerical data are expressed as the mean ± SEM.
presence of OXTR binding in the AVPV. Autoradiography lacks the ability to identify the precise distribution of OXTRs at the cellular level. Thus, the lack of detection of OXTR binding in the AVPV may be due to the marginal size of the AVPV, which may be difficult to be identified autoradiographically.

OXTR binding studies demonstrated that the distribution of OXTR binding is both brain region-specific and species-specific (reviewed in (Dumais, K.M. & Veenema, A.H., 2016). Whereas the species difference in the distribution of OXTR binding suggests the species-specific regulation of behavior by oxytocin, the sex differences in OXTR binding were also found in region- and species-specific manner. For example, sex-differences in the distribution of OXTR binding were found in various forebrain regions and within the hypothalamic VMH in rats (Dumais, K.M. et al., 2013; Uhl-Bronner, S. et al., 2005); however, such sex-differences were not observed in C57BL/6J mice (Olazabal, D.E. & Alsina-Llanes, M., 2016). The present study did not quantitatively analyze the density of OXTR-Venus cells in each brain area except the AVPV and MPOA, because such experiments were not within the scope of the study. Therefore, the sex-difference in the density of OXTR-Venus cells in other brain regions are yet to be determined. However, in stark contrast to all other brain areas where OXTR-Venus cells were found in both sexes, the presence of OXTR-Venus cells in the AVPV occurs nearly exclusively in females. Sex-differences in the distribution of OXTR cells in the AVPV are unique and strongly indicate the involvement of OXTR cells in the AVPV in the regulation of oxytocin induced sex-specific behaviors and/or physiology.

**Estrogen-dependent expression of OXTR in the AVPV**

Estrogen increases OXTR gene transcription (Mamrut, S. et al., 2013) in the uterus (Franczak, A. et al., 2002) and brain (Quinones-Jenab, V. et al., 1997). A study also showed that
the treatment with estrogen resulted in significant increase in OXTR binding in the MPOA and lateral septum of virgin female rats (Champagne, F. et al., 2001). The estrogen-induced expression of OXTR in the brain is, at least partly, mediated by the ERα (Ivell, R. & Walther, N., 1999). Estrogen-ERα regulates transcription of the OXTR gene through CG-rich SP1 transcription factor binding sites (Fleming, J.G. et al., 2006). These findings imply that DNA methylation affects the interaction between SP1, ERα and their binding sites in the OXTR promoter (Harony-Nicolas, H. et al., 2014). The present studies found all OXTR-Venus cells in the AVPV were ERα-immunoreactive. Moreover, the expression of OXTR in the AVPV is clearly estrogen dependent, because OXTR-Venus was not observed in the AVPV of ovariectomized females, whereas OXTR-Venus expression was restored in OVX that received estrogen therapy. Interestingly, OXTR expression in numerous other locations were not affected by OVX (data not shown) indicating not all OXTR cells in the brain are estrogen dependent.

Possible role of OXTR cells in the AVPV

The AVPV is a small cell group located immediately surrounding the area of the ventral half of the third ventricle at the anterior extreme. The AVPV is surrounded by the MPOA. Both the AVPV and MPOA are known to be sexually dimorphic areas (Cao, J. & Patisaul, H.B., 2011; Simerly, R.B., 1998; Yang, C.F. et al., 2013; Zuloaga, D.G. et al., 2014). The onset of maternal behavior at parturition in rats requires activation of OXTR in the MPOA (Insel, T.R. & Harbaugh, C.R., 1989; Pedersen, C.A. et al., 1994) by central oxytocin release that is activated by vaginocervical and suckling stimulations (Numan, M., 2006, 2007; Sheehan, T. et al., 2001). The MPOA neural activity is also necessary for maintenance of maternal behavior (Numan, M. & Stolzenberg, D.S., 2009). Moreover, it is speculated that one of the functions of estrogen is to stimulate the expression of OXTRs so that the critical neurons become responsive to oxytocin
thus allowing oxytocin to activate MPOA that regulates maternal behavior (Numan, M. & Stolzenberg, D.S., 2009). While these studies demonstrated the importance of OXTR in the general area of the MPOA for the regulation of maternal behavior, these previous investigations did not provide a detailed neural structure that express OXTR. Therefore, the presence of estrogen-dependent and sexually dimorphic distribution of OXTR neurons in the AVPV implies that OXTR-neurons in the AVPV are involved in the induction of maternal behavior; however, behavioral studies using specific knock-out of OXTR in the AVPV is required to confirm such a hypothesis.

Several female-biased sexually dimorphic characteristics in the AVPV were previously documented. A markedly larger number of tyrosine hydroxylase-immunoreactive (TH⁺: putative dopaminergic) neurons were found in the AVPV of females than in males (Simerly, R.B. et al., 1997; Yang, C.F. et al., 2013). More recently, the ablation of TH⁺ neurons in the AVPV was found to impair maternal behavior whereas optogenetic stimulation or increased tyrosine hydroxylase expression in these cells was found to enhance maternal care (Scott, N. et al., 2015). Moreover, TH⁺ neurons in the AVPV relay, a monosynaptic input to oxytocin neurons in the PVN (Scott, N. et al., 2015), suggesting that stimulation of oxytocin neurons by TH⁺ neurons from the AVPV is involved in induction of maternal behavior in females. Thus, it is possible that OXTR neurons interact with TH⁺ neurons within the AVPV to generate a positive feedback loop between TH⁺ neurons and oxytocin neurons to drive maternal behavior during pregnancy and lactation when the circulating level of estrogen is naturally high (Bridges, R.S., 1984). Interestingly, the same treatments, optogenetic stimulation or over expression of tyrosine hydroxylase in TH⁺ neurons, had no effect on parental care in males (Scott, N. et al., 2015). Thus, the lack of parental behavior in male mice to the extent of females (Brooks, R.J. &
may be due to the absence of OXTR neurons in the AVPV.
3.1. Introduction

The neurohypophysial hormone, oxytocin (OT), is known for its critical role in female reproductive physiology, specifically uterine contraction during labor and milk ejection while nursing. Oxytocin is synthesized by magnocellular neurons located primarily in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus (Kelly, J. & Swanson, L.W., 1980; Rosen, G.J. et al., 2008). The magnocellular neurons send long axonal projections into the neurohypophysis where OT is released into the general circulation in response to physiological demands. The centrally released OT influences several aspects of social behaviors, such as social recognition, maternal behavior, pair bonding, and grooming (Bales, K.L. et al., 2013; Bales, K.L., van Westerhuyzen, J.A., et al., 2007; Burkett, J.P. et al., 2016; Insel, T.R. & Shapiro, L.E., 1992b; Ross, H.E. & Young, L.J., 2009). These social behaviors are influenced by OT binding to the oxytocin receptor (OXTR) located in various parts of the brain (Gimpl, G. & Fahrenholz, F., 2001; Jard, S. et al., 1987). Furthermore, OT mediated behavioral effects is often sex-specific. For example, intracerebroventricular administration of oxytocin promotes pair bonding in female, but not in male prairie voles (Insel, T.R. & Hulihan, T.J., 1995). This suggests the presence of a sexually dimorphic circuitry that coordinates sexually dimorphic reproductive, physiological, and behavioral responses. However, the cellular characterization and distribution of OXTR-expressing neurons in the brain is still largely unknown.

Sexually dimorphic circuitry of the brain has profound effects on the sex-typical physiological and behavioral manifestations. The hypothalamus comprises of several sexually
dimorphic nuclei. In particular, the anteroventral periventricular nucleus (AVPV) possesses several female-biased sexually dimorphic characteristics (Scott, N. et al., 2015; Yang, C.F. et al., 2013). Studies demonstrated sex differences in the OXTR distribution in the brain (Dumais, K.M. et al., 2013; Hammock, E.A. & Levitt, P., 2013a); however, the presence of all-or-none sexually dimorphic distribution of OXTR was unknown. Using OXTR-Venus (a variant of yellow fluorescent protein) mice, our previous study identified a group of estrogen-dependent OXTR neurons in the AVPV of female but not male mice (Sharma, K. et al., 2019). The presence of all-or-none sexually dimorphic distribution of OXTR neurons within the preoptic area was found only in the AVPV and did not extend to the surrounding medial preoptic area (MPOA). Other studies also identified sexually dimorphic population of neurons in the AVPV. Dopaminergic neurons located within the AVPV plays a critical role in maternal care (Scott, N. et al., 2015). Ablating the dopaminergic neurons in the AVPV impaired maternal behavior, whereas optogenetic stimulation increased tyrosine hydroxylase (TH) expression in these cells and enhanced maternal behavior. In addition, estrogen receptor alpha (ERα)-expressing cells in the MPOA are key mediators for driving maternal behavior, especially pup approach and retrieval (Fang, Y.Y. et al., 2018). Chemogenetic inactivation of ERα-expressing cells in the MPOA impaired maternal behavior, whereas optogenetic activation induced immediate pup retrieval. Because the AVPV neural activity is necessary for the maintenance of maternal behavior (Numan, M. & Stolzenberg, D.S., 2009) and all the OXTR-expressing cells in the AVPV express ERα, we hypothesized that OT influences maternal behavior via OXTR neurons in the AVPV. To test this, the present study was conducted to investigate the contribution of OXTR cells in the AVPV to regulate maternal behaviors. To address this, we employed a
chemogenetic approach to specifically manipulate activity of OXTR neurons in the AVPV in vivo and ex vivo.

3.2. Materials and Methods

Animals

OXTR-Venus mice in which a part of the OXTR gene was replaced with Venus (a variant of the yellow fluorescent protein) cDNA (Yoshida, M. et al., 2009) were originally provided by Dr. Nishimori of the Tohoku University in Japan. A colony was established in facility at Louisiana State University by backcrossing OXTR-Venus mice with C57BL/6J (#000664, Jackson Lab, Bell Harbor, ME) mice for more than 10 generations. OXTR\textsuperscript{tm1.1(cre)Hze} mice, also known as OXTR-T2A-Cre-D, in C57BL/6J background were purchased from the Jackson Laboratory (#031303, Bell Harbor, ME). OXTR-T2A-Cre-D mice has a T2A sequence and a Cre recombinase gene inserted immediately at the OXTR translational stop codon so that the endogenous promoter drives Cre expression in OXTR-expressing cells (Daigle, T.L. et al., 2018). The OXTR-T2A-Cre-D mice can therefore be used for Cre dependent viral expression of DREADDs. OXTR-T2A-Cre-D mice were crossed with a floxed tdTomato reporter strain (Ai14, #007914, Jackson Lab, Bell Harbor, ME) for a set of experiment. For genotyping, genomic DNA was isolated from tail snips by incubating the tissues with REDExtract-N-Amp\textsuperscript{TM} Tissue PCR Kit (Sigma, St. Louis, MO). The isolated DNA was subsequently genotyped using the following sets of primers: F (5'-GTTGGGAACAGCGGTGATTA-3') and R (5'-GGCTCAGGCTTTCTCTACTT-3') for OXTR-Venus mice; F (5'-CATGGATCTACATGCTCTTCA-3'), R (5'-ACGTCTCCGCATGTCAGAAGACT-3'), and R (5'-GACCCTGTCATCTCCCATGGA-3') for OXTR-T2A-Cre-D mice. Only heterozygous mice were used for the experiments. The males and females were housed in separate cages (maximum
4 mice/cage) in the same room on a 12:12 h light/dark cycle (lights off at 15:00) with access to food and water available *ad libitum*. All protocols and animal experiments were approved by the Institutional Animal Care and Use Committee at Louisiana State University.

**Immunocytochemistry**

Mice (8-10 weeks old) were deeply anesthetized with Ketamine-Xylazine (9:1; 100 mg/kg; i.p.) and transcardially perfused with 0.01 M sodium phosphate buffered saline (PBS; pH 7.2), followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.2). The brains were extracted and postfixed in the same fixative for overnight. After infiltrated with 20% sucrose in 0.1 M PB for cryoprotection for 12 hr., coronal sections were transected at 40 µm by a sliding microtome (Leica SM2010R; Mannheim, Germany).

To enhance the signal of Venus, immunocytochemical localization of Venus with anti-green fluorescent protein (GFP) antibody (ab13970; abcam, Cambridge, UK) was conducted in the brain sections obtained from adult virgin male, virgin female, and lactating female OXTR-Venus age-matched siblings (9-11 week-old). The free-floating brain slices were incubated with the primary antibodies against GFP at dilutions of 1:10,000 in PBS containing 0.5% Triton X-100 (PBST) for overnight with continuous gentle agitation at 4°C. The brain sections were subsequently incubated with a secondary antibody (goat anti-chicken) conjugated with Alexa Fluor488 (103-545-155, Jackson ImmunoResearch, West Grove, PA) for 4 hr at room temperature. The anti-GFP antibody showed no specific binding in the brain sections from wild-type animals in our previous study (Sharma, K. et al., 2019).

For double-fluorescence imaging of Venus and immunocytochemistry of tyrosine hydroxylase (TH), the brain sections were processed with polyclonal anti-TH antibody (Cat#AB152; EMD Millipore, Billerica, MA) at dilutions of 1:5,000 in PBST for overnight with
continuous gentle agitation at 4°C. The sections were subsequently incubated with secondary antibody conjugated with fluorescence marker (AffiniPure Goat Anti-Rabbit IgG DyLight594; Jackson ImmunoResearch, West Grove, PA) at 1:250 dilution for 2-4 hr at room temperature. The sections were mounted in polyvinyl alcohol (PVA) with anti-fading agent 1,4-Diazabicyclo[2.2.2]octane (DABCO) that consists of 4.8 g PVA, 12 g glycerol, 12 ml dH2O, 24 ml 0.2 M Tris-HCl and 1.25 g DABCO. Confocal fluorescence microscopic images (1,024 x 1,024; 1 µm optical section thickness) were acquired with a confocal microscope (Leica TCS SP8 spectral confocal microscope, Mannheim, Germany).

To visualize the cellular distribution of Cre expressing cells, male and female brains from OXTR-T2A-Cre-D mice crossed with Ai14 (OXTR cre<sup>+</sup>tdTomato) were sectioned and mounted as described above. For a subset of experiments, the sections were also processed for the anti-TH antibody (Cat#AB152; EMD Millipore, Billerica, MA) at dilutions of 1:5,000 in PBST for overnight with continuous gentle agitation at 4°C. The sections were subsequently incubated with secondary antibody conjugated with fluorescence marker (AffiniPure Goat Anti-Rabbit IgG Alexa Fluor488; Jackson ImmunoResearch, West Grove, PA) at 1:250 dilution for 4 hr at room temperature. Fluorescence microscope images (1280 x 1024) were acquired digitally (Nikon Eclipse 80i equipped with DS-QiMc digital camera, Nikon, Tokyo, Japan).

**Cell Count and colocalization analysis**

OXTR-Venus and TH<sup>+</sup> neurons in the AVPV started appearing in the area surrounding the organum vasculosum laminae terminalis (OVLT) at the ventral end of the third ventricle, bregma 0.5 mm in the mouse brain atlas (Franklin, K.B.J. & Paxinos, G., 2013) and was continuously found in the next 20-25 consecutive 40 µm-thick coronal sections. The number of OXTR-Venus neurons in the AVPV was counted from the level of the OVLT (section -5) to the
level of the suprachiasmatic nucleus (SCN, section 20) where most of OXTR-Venus/ TH⁺
neurons were found. Detection of colocalization was performed using the ImageJ Plugin
application, JACoP (Bethesda, NIH). A Li’s IC value between 0.3 - 0.5 was considered as
colocalization.

The total number of OXTR-Venus and TH⁺ neurons in the AVPV from virgin female,
virgin male, and postpartum female mice were analyzed with ANOVA and differences between
each group were assessed by the Student's t test. Averaged cell number per section is presented
as the means ± SE.

**Viral Vectors**

Designer-receptors exclusively activated-by-designer drugs (DREADD) technology was
used for neuron-specific silencing of OXTR-cre neurons. pAAV-hSyn-DIO-mCherry (control,
2.1x 10¹³ vg/ml) and pAAV-hSyn-DIO-hM4D(Gi)-mCherry (DREADD-Gi, 2.3x 10¹³ vg/ml,
(Krashes, M.J. et al., 2011)) were gifts from Bryan Roth (Addgene viral prep #50459-AAV9 and
#44362-AAV9 respectively). AAV-FLEX-rev-ChR2-tomato (2.1x 10¹³ vg/ml, (Atasoy, D. et
al., 2008)) was a gift from Scott Sternson (Addgene viral prep #1817-AAV9). All viruses were
stored in aliquots at -80°C until use.

**Stereotaxic Injection**

Female mice (8 week-old) were deeply anesthetized using 1.5-2% isoflurane (4% for
induction) in oxygen at a rate of 2L/min and their heads immobilized within the frame of a
stereotaxic instrument (#940, David Kopf Instruments, Tujunga, CA). Prior to operation, mice
received Carprofen (5 mg/kg) and lactated ringers for hydration subcutaneously. Following
sterilization, an incision was made to expose the skull. Access holes were drilled and a guide
cannula (#C315G/SPC, Plastics One, Roanoke, VA) was inserted into the AVPV
(anteroposterior, 0.55 mm; mediolateral, ±0.15 mm; dorsoventral, -5.4 mm) according to the mouse brain atlas (Franklin, K.B.J. & Paxinos, G., 2013). An injector filled with virus and attached to a microliter syringe (#7000.5, Hamilton Company, Reno, NV) was threaded into the guide cannula and a volume of 120 nl/site was slowly infused into the target area at a rate of 20 nl/30s. The guide cannula and injector remained in place for 5 min before removal to prevent backflow. Skull access was sealed with bone wax (#DYNJBW25, Medline Industries, Northfield, IL) and incision was sutured. Buprenorphine (0.1 mg/kg) was injected subcutaneously every 12 hr as needed for pain management. After the surgery mice were single housed for two weeks to allow for recovery and viral expression before pairing them with males for breeding or for histological analysis. Cannula placement and viral spread were verified at the end of all experiments.

To validate the site of injection, brains from lactating mothers were harvested and sectioned immediately after behavior tests. The free-floating sections were mounted as described above and fluorescent microscopic images (1280 x 1024) were acquired digitally (Eclipse 80i equipped with DS-QiMc, Nikon, Tokyo, Japan). Digital images were minimally altered in dynamic range with ImageJ software (Bethesda, NIH).

For neuronal tracing experiment, AAV-FLEX-rev-ChR2-tdtomato (ChR2-tdtomato) was unilaterally injected into the AVPV of OXTR-Cre female mouse following stereotaxic injection procedure above. After the surgery mice were single housed for two weeks to allow for recovery, and co-housed with another female for an additional week to allow viral expression. Brains were collected and imaged as described above.
Behavior Analysis

Behavior annotations

Behavior tests were performed on postpartum day 1 (PPD1: a day after the birth). Pup approach was defined as the first step toward the pup. Pup contact was defined as close contact to any part of the pup by the nose or mouth of the female. Pup grooming was defined as close female and pup interaction that is accompanied by rhythmic up and down head movement of the female. Pup retrieval was defined as the moment the female opened her jaw or made clear contact with the pup to the moment when the pup was dropped inside the nest. Crouching over was defined as female situated herself quietly on top of the pups with an arched back and no obvious movement of any body parts. Nest building was defined as orally collecting the bedding and surrounding the nest. Self-grooming was defined as females licking or scratching parts of their own body. Feeding and drinking was defined as females picking or chewing on chow and licking the spout respectively. Two individuals blind to the study performed manual behavioral annotation on a frame-by-frame basis. The data collected was compared to the annotations from the experimenter for consistency.

Drugs

Activation of inhibitory DREADD was achieved by i.p. administration of Clozapine-N-Oxide (CNO, Cat. #4936, Tocris, Minneapolis, MN) dissolved in 0.9% saline at a dose of 3 mg/kg, 30 minutes before behavior testing. For control experiments, same volume of saline was injected.

Behavior recording

Animal behaviors were video recorded from the top for pup retrieval test and from the side of the cage for home cage test using CCD monochrome camera (Stingray F-145B, Allied
Vision, Exton, PA) and a commercial video acquisition software (StreamPix 8, Norpix, Quebec, Canada) in a semi-dark room with infrared illumination (< 5 lux) at a frame rate of 16 frames/s. All tests were performed within the first 4 hrs of dark cycle.

After becoming visibly pregnant, the female mice were individually housed (cage length 32 cm, width 17.5 cm, and height 14 cm), and monitored for pup birth. The pup retrieval assay was performed in the female's home cage containing bedding and a plastic dome. On the test day, animals were moved into the testing room and left untouched for an hour followed by an i.p. injection of saline or CNO. After 30 min post-injection, the mother and pups were briefly removed from their home cage, and the nest was disturbed with nesting material scattered throughout the cage. A new dome was placed in one corner and three pups were scattered on the opposite side. The mother was then reintroduced into the cage and her behavior was recorded for 10 min. Failure to retrieve all three pups within 10 min resulted in termination of the retrieval test and reported as no pups retrieved. All the pups from the litter were returned at the end of the retrieval test. Latency to retrieve the 1st, 2nd, and 3rd pup were quantified during a 10-min period.

Following pup retrieval test, the animals were left to acclimate for 5 min. Home cage observations were captured by the camera mounted to record the view from the side of the cage. Without disturbing the home cage, the mother’s behavior was recorded over a 1 hr period. Time spent inside the nest, pup directed licking, crouching over the pup (lactating position), self-grooming, eating, and drinking behaviors were quantified.

Behavioral assay to compare different strains of mice were analyzed using ANOVA and differences between each group were assessed by the Student's t test. Behavioral assays of AAV injected mice were analyzed first using the one way ANOVA with repeated-measures. Two way
ANOVA was used to compare multiple groups under multiple testing conditions. Post-hoc Student’s t test was used to compare differences between two groups. Differences were considered to be statistically significant at p < 0.05. Box and whisker plots were used to represent numerical data: mean and median are represented by a filled circle and a line, respectively; the box extends to the quartiles of the data points; the whiskers extend to the farthest data points.

**Ex vivo Electrophysiology**

**Slice preparation**

Following behavior recordings, lactating mice were deeply anaesthetized using Ketamine-Xylazine (9:1, 100 mg/kg i.p.) and perfused through the heart with cold artificial cerebral spinal fluid (ACSF) in which NaCl was replaced by equiosmolar sucrose. The brains were then removed and coronal slices (200 µm) containing the hypothalamic periventricular nucleus were collected using a vibrating microtome (Leica VT1200; Leica, Mannheim, Germany). The brain slices were kept in ACSF (in mM: 125 NaCl, 2.5 KCl, 1 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, 20 D-glucose, 2 CaCl₂, 0.4 Ascorbic acid, pH of 7.3-7.4, with an osmolality of 290-300 mOsm/kg H₂O).

**Whole cell patch clamp recording**

Patch clamp electrophysiology recordings were performed on virus injected slices containing the AVPV region. Labeled neurons in the AVPV were identified with an epifluorescence microscope (Olympus BX51WI, Tokyo) equipped with a 40x water immersion lens (0.8 n.a.) and a CMOS sensor camera (ORCA-Flash4.0LT, Hamamatsu Photonics, Hamamatsu). Whole-cell recordings were obtained with an Axopatch 700B amplifier (Molecular Devices, Foster City, CA). Traces were acquired digitally at 20 kHz and filtered at 5 kHz with a Digidata 1440A and in conjunction with PClamp 10 software (Molecular Devices, Foster City,
CA). Patch electrodes were drawn with a Flaming/Brown micropipette puller (Model P-1000, Sutter Instrument Novato, CA) from borosilicate capillary glass tubing (1.1 mm ID, 1.5 mm OD, Sutter Instruments, Novato, CA) to have resistance of 4-8MΩ when filled with the pipette solution that contained (in mM): 140 K-Gluconate, 1 MgCl₂, 10 HEPES, 10 CaCl₂, 2 ATP(Mg²⁺), and 0.4 GTP (Na⁺) and 11 EGTA. ACSF was saturated with 95% O₂/5% CO₂ and was warmed to 33-34°C during the recordings. Picrotoxin (100 µM) and 6,7-dinitro-quinoxaline-2,3(1H,4H)-dione (DNQX; 10 µM) were also added to ACSF to block the synaptic activity. Activation of DREADD was obtained using 5 µm CNO applied in the bath. The data presented are not corrected for liquid junction potential estimated to be +9.2mV.

**Data Analysis**

To observe the effect of oxytocin and CNO on OXTR-Cre cells, membrane potentials were recorded in the presence or absence of oxytocin (100nM) or CNO (500nM). Depending on the cell, a depolarizing or hyperpolarizing current was injected to hold the membrane potential slightly below the threshold. The mean membrane potentials were obtained by averaging the membrane potential in 30 sec time frame. These time frames were captured at 30 sec before the application of OT, 2 min after the OT application, and 4 min after the bath application of CNO. These sampling duration and points were selected because the peak response or plateauing phase of the membrane potential occurred at these time points. An ANOVA with repeated measures was used to assess the changes in membrane potential in response to oxytocin or CNO. Post-hoc student’s t test was used to analyze differences between each response. Differences were considered to be statistically significant at p < 0.05. Box and whisker plots were used to represent numerical data: mean and median are represented by a filled circle and a line, respectively; the box extends to the quartiles of the data points; the whiskers extend to the farthest data points.
3.3. Results

**Distribution of OXTR-Venus and TH immunoreactive (TH⁺) cells in the AVPV of virgin males, virgin females, and postpartum females.**

Brains from heterozygous (+/-) OXTR-Venus virgin females (n=8), virgin males (n=6), and postpartum females (n=7) were coronally sectioned from 5 sections anterior and 20 sections posterior to the level of the AC. The AVPV was defined as the area within 130 µm from the edge of the third ventricle, where sexually dimorphic population of OXTR neurons are found (Sharma, K. et al., 2019), indicated by the dashed line in Fig 3.1Ai-ii, Bi-ii, and Ci-ii. Confocal images of OXTR-Venus (Fig 3.1 Ai) and TH⁺ cells (Fig 3.1 Aii) in the AVPV were observed in the brain sections from a virgin female. Merged image (Fig 3.1 Aiii) shows that some of the OXTR-Venus cells in the AVPV were also immunoreactive to TH. The number of OXTR-Venus cells and TH⁺ cells were also observed in sections from a postpartum female (Fig 3.1 Bi, Bii). Consistent with our previous study, OXTR-Venus expression is sexually dimorphic and few to none OXTR-Venus cells were found in the AVPV of virgin males (Fig 3.1 Ci). TH⁺ cells were also observed in the AVPV of male mice (Fig 3.1 Cii); however, merged images (Fig 3.1 C iii) showed that none of the OXTR-Venus cells were TH⁺ in the AVPV of male mice.

To compare the overall distribution and number of OXTR-Venus cells between the groups, the number of OXTR-Venus cells in the AVPV of each brain section was counted and plotted (Fig 3.1Di). Significantly more OXTR-Venus cells were observed on sections #5 to #20 of postpartum females than that of virgin females or males (Fig 3.1Di). The total number of OXTR-Venus cells in the AVPV was significantly greater in postpartum females (1371 ± 89 cells) than in virgin females (644 ± 23 cells; t(18) = 9.89, p<0.0001; Fig 1Dii) and virgin males (25 ± 2 cells; t(18) = 17.02, p<0.0001; Fig 3.1Dii). Similar to our previous finding, the total number of OXTR-Venus cells was also significantly greater in virgin females than that of virgin
males ($t_{(18)} = 8.06$, p<0.0001; Fig 3.1Dii).

Figure 3.1. Sexually dimorphic OXTR-Venus and TH$^+$ cell expression in the AVPV is enhanced in postpartum females. Confocal fluorescent images of coronal section showing OXTR-Venus cells (panels i), TH$^+$ cells (panels ii), and merged image (panels iii) from a virgin female (A), a postpartum female (B), and a virgin male (C). Rectangular dashed box delineates the AVPV area. Arrows indicate OXTR-Venus cells that were also immunoreactive to TH. Solid square box represents the zoomed area in panels iv. Plot of the average number of OXTR-Venus cells (Di), TH$^+$ cells (E i), and OXTR-Venus$^+$/TH$^+$ cells (Fi) in the AVPV/section in the serial section from 5 sections anterior to 20 sections posterior of the AC. Asterisks represent the brain sections in postpartum females where significantly more OXTR-Venus immunoreactive to TH were observed compared to virgin female. The mean total number of OXTR-Venus cells (Dii), TH$^+$ cells (E ii), and OXTR-Venus$^+$/TH$^+$ cells (F ii), in the AVPV among postpartum females was greater than among virgin females and virgin males. All numerical data are expressed as the mean ± SEM. Scale bar = 100 µm. 3V: third ventricle.

(fig. cont’d)
To compare the overall distribution and number of TH$^+$ cells and OXTR-Venus cells also immunoreactive to TH (OXTR-Venus$^\dagger$/TH$^+$) in the AVPV, the number of TH$^+$ cells and OXTR-Venus$^\dagger$/TH$^+$ cells (Fig 3.1Ei and 3.1Fi respectively) was counted and plotted. The total number of TH$^+$ cells was significantly higher in virgin females (953 ± 34 cells) than that in virgin males (622 ± 18 cells; t$_{(18)} = 3.8$, p=0.0013; Fig 3.1Eii). The total number of TH$^+$ cells is enhanced in postpartum females and significantly more TH$^+$ cells (1316 ± 97 cells) are present in postpartum females compared to virgin females (t$_{(18)} = 4.36$, p=0.0004; Fig 3.1Eii) and males (t$_{(18)} = 7.75$, p<0.0001; Fig 3.1Eii). Similarly, significantly more OXTR-Venus$^\dagger$/TH$^+$ cells were found in virgin females (198 ± 7 cells) than that in males (4 ± 1 cells; t$_{(18)} = 5.65$, p<0.0001; Fig 3.1Fii). The number of OXTR-Venus$^\dagger$/TH$^+$ cells are also enhanced in postpartum females (339 ± 41)
and was significantly higher than that of virgin female ($t_{(18)} = 4.30$, $p=0.0004$; Fig 3.1Fii) and male mice ($t_{(18)} = 9.49$, $p<0.0001$; Fig 3.1Fii).

**Validation of OXTR-expressing cells and TH$^+$ cells in the AVPV of OXTR-Cre male and female mouse.**

To validate the expression Cre recombinase in OXTR-expressing cells of the OXTR-Cre mouse, OXTR-Cre mice were crossed with homozygous Ai14 mice. Ai14 is a Cre reporter allele designed to have a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant (tdTomato) inserted into the $Gt(ROSA)26Sor$ locus. The resulting offspring (OXTR-Cre:Ai14) mice express robust tdTomato fluorescence following Cre-mediated recombination in tissues that express Cre (Madisen, L. et al., 2010). Brains from age matched (10 week old) OXTR-Cre:Ai14 virgin female ($n=5$) and virgin male ($n=5$) siblings were coronally sectioned and the expression of OXTR-Cre$^{+/tdomato}$ cells were observed. Immunocytochemistry using anti-TH antibody was also conducted to validate the presence of TH$^+$ cell in these mice.

At the level of anterior commissure (AC; Fig 3.2A), OXTR-Cre$^{+/tdomato}$ cells were sparsely distributed in the dorsolateral part of the cortex including the cingulate (Cg1 & 2), motor (M1 & 2), and somatosensory (S1 & 2) cortex. In contrast, clusters of OXTR-Cre$^{+/tdomato}$ cells were found in the dorsal (DEn) and intermediate (IEn) endopiriform claustrum of piriform cortex, and dorsal agranular insular cortex (AID). Dense clusters of OXTR-Cre$^{+/tdomato}$ cells were also found in the intermediate (LSI) and ventral (LSV) lateral septum along with the bed nucleus of stria terminalis (BNST). In the preoptic area OXTR-Cre$^{+/tdomato}$ cells were found in cluster in the magnocellular preoptic area (MCPO), whereas a sparse population was observed in the medial preoptic area (MPOA). OXTR-Cre$^{+/tdomato}$ cell expression in other regions of the brain was similar to our previous report of OXTR-Venus distribution (Sharma, K. et al., 2019).
Figure 3.2. Fluorescent photomicrographs of coronal sections from OXTR-Cre:Ai14 a male and a female mouse. A. Numerous OXTR-Cre<sup>+/tdTomato</sup> cells were observed in various regions in the coronal sections from both male and female mouse brain. Scale bar = 1 mm. B. Fluorescent photomicrographs showing OXTR-Cre<sup>+/tdTomato</sup> cells (i), TH<sup>+</sup> cells (ii), and merged image (iii) in the AVPV of a male OXTR-Cre:Ai14 mouse. C. Fluorescent photomicrographs showing OXTR-Cre<sup>+/tdTomato</sup> cells (i), TH<sup>+</sup> cells (ii), and merged image (iii) in the AVPV of a female OXTR-
Cre:Ai14 mouse. OXTR-Cre<sup>+/tdTomato</sup> cells in the AVPV were sexually dimorphic and present in females (Ci) but not in males. Merged images showed OXTR-Cre<sup>+/tdTomato</sup> cells are also immunoreactive to TH in females (Ciii, indicated by arrows) but not in males (Biii). Dashed rectangular box indicates the AVPV region. Scale bar = 100 µm. 3V: third ventricle; AC: anterior commissure; AID: agranular insular cortex; BNST: the bed nucleus of stria terminalis; CC: corpus callosum; Cg1 and Cg2: cingulate cortex; Den: dorsal endopiriform claustrum; IEn: intermediate endopiriform claustrum; LSI and LSV: intermediate and ventral parts of lateral septum; MCPO: magnocellular preoptic nucleus; M1, M2: motor cortex; S1, and S2: somatosensory cortex;

A sexually dimorphic distribution of OXTR-expressing cells occurred in the AVPV where the presence of OXTR-Cre<sup>+/tdTomato</sup> cells was observed in females (Fig 3.2Ci) but not in males (Fig 3.2Bi). TH<sup>+</sup> cells were also identified in the AVPV of OXTR-Cre:Ai14 male (Fig 3.2Bii) and female (Fig 3.2Cii) mouse. The distribution of TH<sup>+</sup> cells were also sexually dimorphic and considerably more cells were observed in females. In addition, some of OXTR-Cre<sup>+/tdTomato</sup> cells were also immunoreactive to TH in females (Fig 2Ciii) but not in males (Fig 3.2Biii).

**The assessment of lactation, fostering, and maternal behavior exhibited by OXTR-Cre mice.**

To validate that the **OXTR-Cre** allele elicits no defects in lactation and fostering, offspring from wild-type (WT, n=20 litters), heterozygous OXTR-Venus (Ven<sub>u</sub>+/−, n=20 litters), homozygous OXTR-Venus (Ven<sub>u</sub>+/<sup>+</sup>, n=20 litters), and OXTR-Cre mice (n=20 litters) were examined from one day postpartum (PPD1) to the day of weaning (postpartum day 21). Stored milk was detected in the digestive tracts of pups born from WT, Ven<sub>u</sub>+/−, and OXTR-Cre mice (Fig 3.3A) on PPD1. There was no difference in the average number of pups born per litter from WT (7.4 ± 0.37 pups), Ven<sub>u</sub>+/− (7.7 ± 0.36 pups), Ven<sub>u</sub>+/<sup>+</sup> (7.25 ± 0.33 pups), and OXTR-Cre (7.1 ± 0.37 pups) dams (Fig 3.3B; F<sub>3,79</sub>=0.513, p=0.675). Similarly, no significant difference was observed in the average number of pups that survived at weaning from WT (6.6 ± 0.41 pups), Ven<sub>u</sub>+/− (7.25 ± 0.41 pups), and OXTR-Cre (6.35 ± 0.29 pups) dams (Fig 3.3C;
Figure 3.3. **OXTR-Cre** genotype does not alter reproductive function and maternal behavior. A. Images of PPD1 pups born from wild-type (WT), heterozygous OXTR-Venus (Venus +/-), and OXTR-Cre mice. Circle area indicates milk spots. B. Average litter size of WT, Venus +/-, homozygous OXTR-Venus (Venus +/+), and OXTR-Cre mice. C. Average number of pups weaned 21 days after birth. D. Percentage of pups that survived at the day of weaning. E. Body growth chart from PPD1 to the day of weaning. F. Latency to retrieve 3 pups in a 10 min period of WT, Venus +/-, and OXTR-Cre mice. G. Total time spent inside the nest in an hour-long period. H. Total time spent building nest in an hour. I. Total time spent crouching over pups in an hour-long period. J. Total time spent grooming pups. K. Total time spent grooming self. L. Total time spent eating or drinking within an hour. Female were selected regardless of their ovulatory cycle. OXTR-Cre mice successfully fostered all pups and behaved similar to the WT mice. All numerical data are expressed as the mean ± SEM. Scale bar = 1 cm.

( fig. cont’d)
F_{(2,59)}\text{= }p=0.23). Hence, the survival rate of pups at weaning by OXTR-Cre (90.5 ± 2.5 %) dams was not significantly different among OXTR-Cre, WT (88.8 ± 2.9 %), and Venus +/- (94 ± 2.4%) dams (Fig 3.3D; F_{(2,59)}\text{= }p=0.37). Venus +/- dams have a bi-allelic expression of Venus fluorescent protein in place of OXTR, thereby, serving as an OXTR knock out model (Yoshida, M. et al., 2009). Offspring from Venus +/- mice died within 24 hr after birth; therefore, stored milk could not be detected on pups from Venus +/- mice. The OXTR-Cre and Venus +/- mothers successfully fostered all pups and the body weight of pups born of OXTR-Cre and Venus +/- dams were similar to that of pups from WT (Fig 3.3E). This indicates that lactation and fostering behavior of OXTR-Cre mothers is normal, and the OXTR-Cre allele maintained the functions of WT OXTR gene.

To confirm that the maternal behavior exhibited by OXTR-Cre (n=5) mothers were similar to WT (n=6) and Venus +/- (n=7) mice, the three groups were subjected to maternal behavior tests. All three groups successfully retrieved 3 pups scattered in the opposite site of the nest. No significant difference was found in the cumulative latency between WT (94.5 ± 42.6 s), Venus +/- (94.4 ± 34.5 s), and OXTR-Cre (59.8 ± 4.5 s) to retrieve all 3 pups (Fig 3.3F; F_{(2,17)}\text{= }0.31, p=0.74). In an hour long home cage tests, the time spent inside the nest by OXTR-Cre mothers (1345.2 ± 349.5 s) were comparatively similar to the WT mothers (Fig 3.3G; 1995.7 ± 459.4 s; t_{(15)}\text{= -1.26, }p=0.22); however, OXTR-Cre mothers spent less time inside the nest as compared to Venus +/- mothers (Fig 3.3G; 2685.7 ± 145.9 s; t_{(15)}\text{=2.85, }p=0.01). The total time
spent building the nest by OXTR-Cre mothers (586.3 ± 213.5 s) was similar to the time spent by WT (465.3 ± 173.6 s) and Venus +/- (684.3 ± 229.4 s) mothers (Fig 3.3H; F(2,17)=0.29, p=0.75). Time spent crouching over the pups by OXTR-Cre mothers (1082.6 ± 374.5 s) was similar to the WT mothers (Fig 3.3I; 1435.7 ± 452.9 s; t(15) = -0.71, p=0.49); however, Venus +/- mothers spent more time crouching over pups (2357.7 ± 175 s) compared to OXTR-Cre mothers (Fig 3.3I; t(15)=2.63, p=0.019). OXTR-Cre mothers spent similar amount of time grooming pups (1037.4 ± 381.1 s) as the WT mothers (958.7 ± 164.6 s; t(15)=0.186, p=0.855) and Venus +/- mothers (1844.9 ± 294.5 s; t(15)=1.97, p=0.068) while Venus +/- mothers spent more time grooming pups than WT mothers (Fig 3.3J; t(15)=2.27, p=0.038). The total time spent by OXTR-Cre mothers grooming themselves (125.6 ± 42.6 s) was similar to the time spent by WT (170.3 ± 46.3 s) and Venus +/- (153.1 ± 48.2 s) mothers (Fig 3.3K; F(2,17)=0.21, p=0.81). OXTR-Cre mothers spent significantly more time eating and drinking (1181± 2111.6 s) compared to WT (386.3 ± 180.4 s; t(15)=3.64, p=0.002) and Venus +/- mothers (Fig 3.3L; 120.6 ± 44 s; t(15)=-5.02, p=0.0002). Taken together, these data suggest that OXTR-Cre mothers exhibit similar maternal behavior to that of WT mothers.

**Activity of OXTR-expressing cells in AVPV is necessary for maternal behavior.**

To assess whether the activity of OXTR-expressing cells in the AVPV is necessary for various maternal behaviors, viral expression of DREADDs was achieved by injecting DIO-mCherry (DIO: control, n=3) or hM4D(Gi)-mCherry (Gi, n=6) in the AVPV of OXTR-Cre female mice (Fig 3.4A). A total of 38 mice were bilaterally injected, however only unilateral injection into the AVPV was achieved in 9 mice. Histological analysis performed post-behavior tests revealed that DIO or Gi-mCherry was largely confined in neurons within the AVPV (Fig
Figure 3.4. Unilateral inactivation of OXTR-Cre cells in the AVPV impaired pup retrieval. A. Schematic illustration (left panel) and validation of injection site. Dashed lines represent the injection track and rectangular box (middle panel) is the area shown in higher magnification (right panel). Scale bar = 100 µm. B. Key time points leading up to the behavioral test day. C. Timeline illustrating the behavior tests procedure. D. Latency to retrieve 3 pups in a 10 min period between DREADD-DIO (DIO, control) injected with CNO, DREADD-Gi (Gi) injected
with saline, and Gi with CNO injected mothers. E. Total time spent inside the nest in an hour-long period. F. Total time spent building nest in an hour. G. Total time spent crouching over pups. H. Total time spent grooming pups. I. Total time spent grooming self. J. Total time spent eating or drinking within an hour. K. Photomicrographs showing the patched cell in the AVPV. Cells were identified with mCherry expression (Red) under the microscope. Scale bar = 25 µm. L. Example of the effect of CNO in the membrane potential and firing pattern of Gi injected OXTR-Cre cells. M. The changes in mean membrane potential in the presence of ASCF, OT, and CNO. All numerical data are expressed as the mean ± SEM. 3V: third ventricle.

3.4A). Since the number of OXTR-expressing cells immunoreactive to TH was significantly more at the level of AC (section -3 to 2, Fig 3.1Fi), the injections were targeted for that particular stereotaxic coordinate (see materials and methods). Stereotaxic injections of DREADDs were performed on 8 week-old female mice, and behavior tests were performed one day postpartum (PPD1, Fig 3.4B). Behavior tests were performed during the dark cycle for the mice. I.p. injection of saline was performed 30 min before the pup retrieval test (Fig 3.4C). During testing, 3 pups were introduced into the mother’s home cage, and pup retrieval was observed for 10 min. Following pup retrieval test, the mother’s home cage behavior was observed for an additional hour (Fig 3.4C). The pup retrieval and home cage tests were repeated with i.p injection of clozapine-N-oxide (CNO, engineered ligand of Gi) on the same mothers 30 min after the end of first home cage test (Fig 3.4C). Significant differences were observed in the latency to retrieve first pup ($F_{(2,14)}=17.47, p=0.0003$), second pup ($F_{(2,14)}=12.86, p=0.001$), and third pup ($F_{(2,14)}=5.89, p=0.0165$) following injection of CNO in Gi injected mothers (Fig 3.4D). The total time taken by control mothers, injected with CNO (49.7 ± 12.9 s), to retrieve first pup was similar to the Gi injected mothers injected with saline (Fig 3.4D; 53.2 ± 8.9 s; $t_{(12)}=0.29, p=0.77$). The total time taken by control mothers, injected with CNO (60.7 ± 12.9 s), to retrieve second pup was similar to the Gi injected mothers injected with saline (Fig 3.4D; 71.2 ± 15.1 s; $t_{(12)}=0.61, p=0.56$). Finally, The total time taken by control mothers, injected with CNO (71 ±
14.9 s), to retrieve all three pups was similar to the Gi injected mothers injected with saline (Fig 3.4D; 107 ± 33.9 s; t(12)=0.9, p=0.37). Following injections of CNO to the same Gi injected mothers, pup retrieval was impaired and no pups were retrieved (Fig 3.4D; reported as not retrieved: N.R). No significant differences were observed in maternal behaviors such as total time spent inside the nest (Fig 3.4E; F(2,14)=15.13, p=0.2594), total time spent building nest (Fig 3.4F; F(2,14)=1.85, p=0.2), total time spent grooming pups (Fig 3.4H; F(2,14)=2.60, p=0.112), time spent by mothers grooming themselves (Fig 3.4I; F(2,14)=2.34, p=0.14), and time spent eating or drinking (Fig 3.4J; F(2,14)=0.47, p=0.64) across the group. However, there was a significant difference in the amount of time spent by mothers crouching over the pups (Fig 3.4G; F(2,14)=5.17, p=0.024). Gi injected mothers spent significantly less amount of time (915.3 ± 285.9 s) crouching over the pups following injection with CNO compared to injection of saline (Fig 3.4G; 1993.5 ± 86.08 s; t(12)=3.19, p=0.008). However, this difference was not observed with the control injected mother (1596.3 ± 487.7 s, t(12)=1.65, p=0.13).

To validate the effect of CNO on the neurons in the AVPV of Gi injected mothers, we performed whole cell patch-clamp recordings in brain slices (Fig 3.4K). Virally infected cells were identified by the expression of mCherry under fluorescent microscope fitted with water-immersion lens. Electrophysiological recording were performed in ASCF containing DNQX and picrotoxin that blocked the synaptic activity. A bath application of OT did not cause measurable changes in the membrane potential (-59.35 ± 4.2 mV; t(3)=0.43, p=0.6), however, OT induced a repetitive firing of action potentials (Fig 3.4L and 3.4M). Bath application of CNO caused a significant hyperpolarization in all cells expressing Gi-mCherry (-80.6 ± 0.2 mV) compared to the application of OT (t(3)=4.51, p=0.02) and ACSF (Fig 3.4M; -61.4 ± 3.9 mV; t(3)=4.04, p=0.027).
The assessment of neuronal projections of OXTR-Cre cells in the AVPV using channelrhodopsin-mediated circuit mapping.

To assess efferent projections of the OXTR cells in the AVPV, ChR2-tdTomato was unilaterally injected into the AVPV (Fig 3.5B) of female OXTR-Cre mice (n=3). Histological analysis, following a 3-week period to allow viral expression, showed a robust tdTomato expression in the OXTR-Cre cells and their projections. At the level of the organism vasculosum of the lamina terminalis (Fig 5A; OVLT; Bregma 0.62 mm), a dense group of ascending fibers were observed in the OVLT and the surrounding medial preoptic area (Fig 3.5Ai; MPOA). A few sparse fibers were also found in the ventral pallidum (Fig 3.5Ai; VP) and the nucleus accumbens (Fig 3.5Aii; NAc). At the level of AC (Fig 3.5B; the region where AC is “connected”; Bregma 0.14 mm), projection from the OXTR cells were mostly restricted within the AVPV (Fig 3.5Bi). However, a few sparsely distributed fibers were observed in the MPOA (Fig 3.5Bii), posterior, and ventral bed nucleus of the stria terminalis (Fig 3.5; BNST). Some projections were also observed in the AVPV and MPOA on the contralateral side (Fig 3.5 Bi and Bii). The majority of labeled fibers that arose from the injected site in the AVPV coursed caudally through the periventricular zone of the hypothalamus. At the level of the supraoptic nucleus (Fig 3.5C; SON; Bregma -0.58 mm), labeled axons were identified coursing parallel to the optic tract, projecting towards the SON (Fig 3.5Ci). Dense cluster of fibers were observed in the anterior hypothalamic area (AHA), dorsolateral (SChDl), and ventromedial suprachiasmatic nucleus (Fig 3.5Cii; SChVm). Projections from the suprachiasmatic nucleus were found to cross over to the contralateral side at the ventral end (Fig 3.5Cii). At the level of the retrochiasmatic area (Fig 3.5D; RCA; Bregma -0.94 mm), a robust tdTomato expression was observed at the ventral edge of the 3V in the RCA (Fig 3.5Dii). Labeled projections passing through this region was found to travel dorso-laterally towards the magnocellular preoptic nucleus (Fig 3.5Dii;
Figure 3.5. Optogenetic mapping of molecularly defined AVPV OXTR-Cre circuit.
A-F (left panels). Fluorescent photomicrographs of coronal sections from a ChR2-tdTomato injected female OXTR-Cre mouse. Whole brain section depicts major site of neuronal projections from the AVPV with co-ordinates from bregma. B. Dashed line represents injection track. Scale bar = 1 mm. Ai and Aii – Fi and Fii (middle and right panels). High magnification fluorescent images of regions of the brain that show ChR2-tdTomato projections Scale bar = 100 µm. 3V: third ventricle; AC: Anterior commissure; Aq: Aqueduct; RM: Retromammillary nucleus; OC: Optic chaism; Ot: Optic tract  

(fig. cont’d)
MCPO) on the ipsilateral as well as contralateral side of the injection site. At the level of median eminence (Fig 3.5E; ME; Bregma -1.58 mm), robust tdTomato expressing projections were observed in the ME, arcuate nucleus (Arc), and in the ventrolateral part of the ventromedial hypothalamus (Fig 3.5Ei; VMH). Sparsely distributed projections were observed coursing dorso-laterally along the cerebral peduncle towards stria terminalis (st) and ventral posterolateral thalamic nuclei (Fig 3.5Eii; VPL). Finally, projections from the AVPV OXTR cells were observed in the midbrain regions. At the level of the ventral tegmental area (Fig 3.5F; VTA; Bregma -3.28 mm), sparsely distributed fibers were observed in the periaqueductal gray (Fig 3.5Fi; PAG) and the VTA (Fig 3.5Fii).

3.4. Discussion

Our previous study identified estrogen-dependent sexually dimorphic population of OXTR-expressing cells in the AVPV in female mice. The present study identified OXTR-expressing cells in the AVPV as an essential node of circuitry for mediating maternal behaviors, especially pup retrieval. Inactivation of OXTR-expressing cells in the AVPV specifically impaired the retrieval behavior of female mice.

Intersection of OXTR-expressing and TH+ cells in the AVPV

Dopamine (DA) is most studied in the context of reward-associated maternal behavior. Studies with DA antagonists administered to female rats showed impairments in maternal behavior (Byrnes, E.M. et al., 2002; Hansen, S. et al., 1991a, 1991b), while DA agonist reversed
this effect (Giordano, A.L. et al., 1990). Activating DA D1 receptor, via selective agonist, in the MPOA reduced the latency to demonstrate maternal behavior in female rats (Stolzenberg, D.S. et al., 2007) while selective antagonism impaired pup retrieval (Miller, S.M. & Lonstein, J.S., 2005). A recent study showed that specific ablation of TH+ cells in the AVPV significantly impaired pup retrieval, reduced the latency of maternal care, and reduced plasma OT levels in female mice (Scott, N. et al., 2015). In contrast, optogenetic activation of these TH+ cells enhanced maternal behavior and OT section from the PVN. Interestingly, manipulating TH+ cells in the AVPV of male mice did not affect paternal behavior, but rather increased intermale aggression (Scott, N. et al., 2015). These finding not only suggests a sexually dimorphic role of DA in relation to parental behavior, but also an influence of DA in the OT system. Indeed, the vice-versa is also true. Our tracing study identified projections of OXTR cells from the AVPV to the VTA which suggests a role of OT system to influence dopaminergic neuron of the VTA. A body of evidence also suggests that OT can influence reward-associated behaviors via OXTRs located throughout the mesocorticolimbic (extending from the VTA to the NAc and prefrontal cortex) dopamine system (Bartz, J.A. et al., 2011; Burkett, J.P. & Young, L.J., 2012; Gordon, I. et al., 2011; Love, T.M., 2014). Infusion of OT into the ventral tegmental area (VTA), an area containing dopaminergic neurons, increased dopaminergic concentrations in the nucleus accumbens (Shahrokh, D.K. et al., 2010). This increase in dopaminergic concentrations is induced by pup grooming behavior in rats and could be inhibited via administration of OXTR antagonists into the VTA. Furthermore, OXTRs in the ventral and dorsal striatum can occur as heterodimers coupled to a dopamine D2 receptor, which is thought to produce the rewarding effect of a social stimulus during pair bonding (Romero-Fernandez, W. et al., 2013).
This study identified a group sexually dimorphic OXTR-expressing cells that are also immunoreactive to TH (OXTR-Venus⁺/TH⁺) in the AVPV of female mice. Both the expression of OXTR-expressing cells and TH⁺ cells are sexually biased and increased substantially in lactating females. Although the TH⁺ cells were also present in the AVPV of male mice, OXTR-expressing neurons are almost exclusively present only in the females. Our results indicate that there may exist two separate populations of OXTR-Venus⁺/TH⁺ cells (Fig 3.1). First, at the level of anterior commissure, significantly higher population of OXTR-Venus⁺/TH⁺ cells were observed in PPD1 mothers compared to virgin females. This difference was only observed between 5 sections anterior and 1 section posterior to the anterior commissure (280 µm). A second population of OXTR-Venus⁺/TH⁺ cells appeared 12 sections posterior and extended to 19 sections posterior of the anterior commissure (320 µm). Since the latter is considered to be periventricular hypothalamic nuclei rather than the AVPV (Franklin, K.B.J. & Paxinos, G., 2013), our subsequent chemogenetic experiment targeted the AVPV where AC meets. Unilateral inhibition of OXTR-expressing cells in the AVPV significantly impaired the latency to retrieve pups and crouching behavior. No pups were retrieved by the mothers injected with inhibitory DREADD within the 10 min test period following i.p injections of CNO. Scott et al. showed that upon receiving signals from the pup, the AVPV of the maternal brain increased dopamine release to the PVN through a monosynaptic input; resulting in increased OT secretion that leads to an enhanced maternal behavior (Scott, N. et al., 2015). Our results showed that the total percentage of OXTR-Venus⁺/TH⁺ cells did not change between reproductive states (25.1% in virgin females and 25.9% in PPD1 mothers); suggesting only a partial influence of TH⁺ cells on the OT circuitry of the AVPV that regulates maternal behavior. Also, the OXTR-expressing cells in the AVPV not only project to the PVN, but also send efferents into the SON. This implies that OT
itself regulates the OT secretion, which ultimately enhances maternal behavior. The positive feedback mechanism of OT secretion has been well documented (Ivell, R. & Russell, J.A., 1996; Jourdain, P. et al., 1998; Neumann, I. et al., 1996), and such a case may be regulated through OXTR-expressing cells and, in part, by OXTR / TH+ cells in the AVPV. In addition, the lack of parental care in male mice (Brooks, R.J. & Schwarzkopf, L., 1983; Lonstein, J.S. & De Vries, G.J., 2000b) maybe due to the absence of OXTR-expressing cells in the AVPV.

**Inhibition of neuronal population versus global knockout**

Oxytocin acting via its receptor, the OXTR, is vital for the physiology and behavior associated with parturition and maternal behavior. The use of OT and OXTR knockout (KO) mice made significant contribution to understanding the importance of OT signaling. Impairments in retrieval behaviors have been established for OT-KO mice (Pedersen, C.A. et al., 2006), although the mice tested were very old virgins and the pups they retrieved were from other females. However, OT-KO mice displayed normal parturition and maternal behavior, despite being unable to eject milk (Nishimori, K. et al., 1996). This maybe so as a similar peptide, arginine vasopressin (AVP), is able to signal through OXTR (Ragnauth, A.K. et al., 2004). To avoid these discrepancies and provide a complete disruption of OT signaling, OXTR-KO mice were created (Lee, H.J. et al., 2008; Takayanagi, Y. et al., 2005). Young virgin and primiparous OXTR-KO mice show impairments in foster pup retrievals (Takayanagi, Y. et al., 2005). In the same report, the authors mention that they observed no effects of OT-KO on maternal behavior in young virgin mice, although the data are not shown. A forebrain specific deletion of OXTR from cells expressing calcium/calmodulin dependent protein kinase (CamK2a) showed very modest impairment in pup retrieval (Macbeth, A.H. et al., 2010). The results of this study using OXTR-Venus +/+ mice also support this notion. OXTR-Venus +/+ mice have a bi-
allelic expression of Venus fluorescent protein instead of a functional OXTR (Sharma, K. et al., 2019). OXTR-Venus +/+ mice successfully gave birth, but the pups were either cannibalized or died because of lack of maternal care (Fig 3). However, these findings are a result of a global KO of OXTRs and not a specific population of OXTR.

To overcome the barrier to understand the specific role of OXTR-expressing cells in the AVPV, we employed a chemogenetic tool that can specifically inhibit OXTR cells in a specific area. The DREADD technology use CNO, which is a small molecule that can penetrate the blood-brain barrier (Bender, D. et al., 1994), has favorable pharmacokinetics in (Bender, D. et al., 1994), and is pharmacologically inert (Armbruster, B.N. et al., 2007). The initial study reported that HM4D(Gi)-DREADD induces neuronal silencing via Gαi mediated activation of inwardly rectifying potassium channels (Armbruster, B.N. et al., 2007). Subsequent studies have also reported that CNO-mediated activation of HM4D(Gi) attenuated neuronal firing (Atasoy, D. et al., 2012; Ferguson, S.M. et al., 2011; Krashes, M.J. et al., 2011). Electrophysiology of HM4D(Gi) injected brain slice showed CNO induced neuronal silencing in this study (Fig 4).

Bath application of CNO hyperpolarized the cell to ~-80 mV, bringing the membrane potential closer to the equilibrium potential of potassium. Unilateral injection of HM4D(Gi)-DREADD directly into the AVPV showed profound effects on pup retrieval and crouching upon activation by CNO (Fig 3.4). These findings suggest that OXTR-expressing cells in the AVPV are essential in regulating maternal behavior, especially pup retrieval.

**Role of estrogen dependent OXTR-expressing cells in the AVPV**

Estrogen increases OXTR transcription in the brain (Franczak, A. et al., 2002). The surge of estrogen during late pregnancy and parturition is essential for the onset of maternal behavior (Bridges, R.S., 1984; Rosenblatt, J.S. & Siegel, H.I., 1975; Siegel, H.I. & Rosenblatt, J.S., 1975).
Early studies showed that the lack of estrogen reduces the latency to initiate maternal behavior in ovariectomized female rats (Bridges, R.S., 1984; Doerr, H.K. et al., 1981). A study also showed that the treatment with estrogen resulted in significant increase in OXTR binding in the MPOA and lateral septum of virgin female rats (Champagne, F. et al., 2001). Indeed, the MPOA is known to express one of the highest levels of estrogen receptor alpha (ERα; Mitra, S.W. et al., 2003). Estradiol implanted into the MPOA shortened the latency for the onset of maternal behavior (Fahrbach, S.E. & Pfaff, D.W., 1986). All together, these studies suggest that estrogen facilitates the onset of maternal behavior through MPOA cells that express ERα.

A recent study identified MPOA cells that express ERα as an essential population for mediating maternal behavior in female mice (Fang, Y.Y. et al., 2018). These cells were found to be active prior to and during pup retrieval. Inactivating ERα cells in the MPOA, via chemogenetic approach, impaired pup approach and retrieval behavior, whereas activation via optogenetic approach induced pup retrieval (Fang, Y.Y. et al., 2018). Tracing as well as electrophysiological experiments further showed that the efferents of MPOA ERα cells to the VTA facilitate this maternal behavior. However, this study did not differentiate the AVPV as a sub-population of the MPOA per se. Our previous study showed that the OXTR-expressing cells are sexually dimorphic only in the AVPV, and no difference in the number of OXTR-Venus cells was found in the MPOA (Sharma, K. et al., 2019). Furthermore, all OXTR-expressing neurons in the AVPV were immunoreactive to ERα and ovariectomy resulted in the absence of these neurons. Therefore, OXTR-expressing cells in the AVPV express ERα, project to the VTA, and chemogenetic inhibition of these cells resulted in impairments in pup retrieval and crouching behavior. Together, these results suggests a role of AVPV OXTR-expressing cells in mediating...
maternal behavior. However, whether these cells provide inhibitory or excitatory inputs to the
VTA needs to be further tested.
CHAPTER 4
CONCLUSIONS

Postpartum depression (PPD) is a widespread disorder that affects approximately 15% of births (Gaynes, B.N. et al., 2005; Pearlstein, T. et al., 2009). PPD is a major health concern that has adverse effects on both the mother and child (Field, T., 2011). Females suffering from PPD exhibit poor maternal care in the postnatal period that may be harmful to child development (Campbell, S.B. et al., 2007; Field, T., 2010). In recent years, the oxytocin (OT) system in the brain has received tremendous attention as a potential pharmacological target for the treatment of many psychiatric disorders, that includes autism spectrum disorders, anxiety, and sex-specific psychiatric disorders, such as PPD. Clinical trials for intranasal application of OT are currently underway to investigate its effects in a variety of psychiatric disorders (Walum, H. et al., 2016).

A growing body of evidence suggests an altered OT system to be involved in the development of PPD (Kirsch, P., 2015; Stuebe, A.M. et al., 2012). Methylation of the oxytocin receptor (OXTR) gene has been linked to PPD in humans (Kimmel, M. et al., 2016). Additionally, estrogen-induced DNA methylation changes is significantly associated with PPD (Mehta, D. et al., 2014). Estrogen has been known to increase gene transcription in the uterus and the brain (Mamrut, S. et al., 2013; Quinones-Jenab, V. et al., 1997). In fact, it can regulate the transcription of the OXTR gene through CG-rich transcription factor binding sites (Fleming, J.G. et al., 2006). In the female brain, OXTR expression changes significantly in various brain areas during reproductive states (Bealer, S.L. et al., 2006; Young, L.J. et al., 1997). Together, this suggests a role estrogen-dependent OXTRs in the manifestation of PPD. However, the cellular characterization and regulatory mechanism of OXTR-expressing neurons in the brain are still largely unknown. Therefore, with this dissertation research, I sought to identify the estrogen-
dependent population of OXTR neurons in the brain and how manipulating such population may affect maternal behavior.

In the second chapter, we were the first to demonstrate the sexually dimorphic distribution of OXTR neurons in the AVPV in the mouse brain. The presence of OXTR-Venus neurons in the AVPV was nearly exclusive to females. Additionally, we showed that the expression of OXTR in the AVPV is estrogen dependent, as ovariectomy resulted in the absence of OXTR-Venus, whereas estrogen replacement therapy restored the expression of OXTR-Venus. The estrogen-dependent expression of OXTR may be mediated by ERα as ERα immunoreactivity was observed in all OXTR-Venus cells in the AVPV; however, this notion must be confirmed by specific manipulation of ERα activity in these OXTR neurons. The functional significance of sexually dimorphic OXTR neurons in the AVPV is currently unknown; however, because the onset of proper maternal behavior at parturition requires activation of OXTR in the MPOA, the female specific expression of OXTR in neurons of the AVPV implies that these neurons are involved in the induction of maternal behavior.

In the third chapter, the role of OXTR neurons, of the AVPV, in the regulation of various maternal behaviors was assessed. Chemogenetic inactivation of OXTR-expressing cells in the AVPV resulted in severe impairment in maternal behavior, specifically pup approach, retrieval, and crouching behavior. Additionally, whole-cell patch clamp electrophysiological experiments validated that only the OXTR neurons infected with the DREADD-Gi were affected by chemogenetic inactivation. Finally, using neural tracing technique, I identified several projection sites of the OXTR neurons in the AVPV. Important brain areas that are known to play a role in regulating maternal behavior, such as NAc, BNST, MPOA, PVN, SON, and VTA showed robust innervations from the OXTR neuron in the AVPV (Fig 4.1). This is the first time that the
sexually dimorphic OXTR neurons in the AVPV have been demonstrated as an important node of circuitry that regulates maternal behavior.

Figure 4.1. Schematic illustration showing projections of AVPV OXTR neurons.

As the exact mechanism of OXTR mediated maternal behavior remains unknown, results from the above-mentioned studies provide insights into the regulatory role of OXTR neurons in the AVPV in mediating maternal behavior. However, with the overall goal to elucidate the physiological and behavioral significance of sexually dimorphic OXTR neurons in the AVPV, further studies are required.

4.1. Remaining Questions and Future Directions

While the results of my doctoral research provide valuable insight into the role of OXTR neurons in the AVPV in regulating maternal behavior, several important questions still remain. In Chapter 2 I provide insight into the sexually dimorphic expression of OXTR-expressing neurons and their dependency on estrogen. However, additional aspects of the expression and
dependency on steroid hormones have yet to be resolved. For example, could OXTR expression in the AVPV be dependent on progesterone? Several studies showed the presence of progesterone receptors in the AVPV (Chakraborty, T.R. et al., 2003; He, W. et al., 2017; Simerly, R.B. et al., 1996). Serum progesterone levels increase with the progression of pregnancy and reach a peak at gestation day 16 (Piekorz, R.P. et al., 2005; Virgo, B.B. & Bellward, G.D., 1974). In Chapter 3 I observed a significant increase in the total number of OXTR neurons in the AVPV of PPD1 mothers. Although the progesterone levels rapidly decrease before parturition, there is a possibility that progesterone may play a significant role in the increased expression of OXTR neurons in the AVPV. Such a possibility needs to be examined to better understand the sexually dimorphic expression of OXTR in the AVPV. Our study assessed the dependency on estrogen by examining ERα immunoreactivity first. While more ERα immunoreactive cells are present in the mouse MPOA (Kudwa, A.E. et al., 2004), ERβ immunoreactive cells are also present. Furthermore, expression of both ERs in the same neurons, could alter transcription by forming heterodimers (Ogawa, S. et al., 1998), and cause a differential response to estrogen (Hall, J.M. & McDonnell, D.P., 1999; Patrone, C. et al., 2000). Disruption of the ERβ gene has also been shown to significantly impair spatial learning in female mice (Rissman, E.F. et al., 2002) which could be important for pup retrieval behavior. Therefore, the function of ERβ on OXTR neurons in the AVPV needs to be further examined.

In Chapter 3 we observed a group of OXTR-expressing neurons also immunoreactive to TH (OXTR+/TH+) in the AVPV. Oxytocin and dopamine signaling is known to enhance maternal behavior (Kuroda, K.O. et al., 2011; Love, T.M., 2014). The presence of OXTR+/TH+ cells in the AVPV suggests that dopaminergic-OXTR cells are involved in the regulation of maternal behavior. However, our findings showed that only 25% of the OXTR neurons in the
AVPV were TH⁺ in virgin female mice. Although the expression of dopaminergic-OXTR cells increased significantly in PPD1 mothers, the total percentage did not change. Our study did not distinguish between dopaminergic-OXTR and non-dopaminergic-OXTR neurons in the AVPV. Therefore, to understand the role of OXTR-expressing neurons in the AVPV, we first have to assess the cell types that are non-dopaminergic.

The AVPV is also known to contain a group of estrogen-sensitive kisspeptin neurons (Mayer, C. et al., 2010; Smith, J.T. et al., 2005). Kisspeptin is a neuropeptide critical for luteinizing hormone (LH) surge during ovulation (Oakley, A.E. et al., 2009). In contrast, the synthesis and release of LH are inhibited during pregnancy (Cheng, K.W., 1976; Hirano, M. et al., 1976). A study has suggested the antagonistic action of OT in the regulation of gonadotropin secretion in humans (Chiodera, P. et al., 2003). Since OXTR neurons in the AVPV project to the areas where gonadotropin-releasing hormone expressing (GnRH) neurons are located, could OXTR neurons play a role in gonadotropin release? A possible interaction and opposing function to the kisspeptin neurons should also be examined.

Although, the AVPV neurons are known to synapse onto GnRH neurons (Simonian, S.X. et al., 1999), these are not the only neuron they communicate with. The AVPV also receives inputs from the ventral premammillary nucleus which contains cells that express leptin receptors. These inputs are suggested to mediate adiposity signaling to control metabolism and fertility (Donato, J., Jr. et al., 2011). The AVPV also receives input from the SCN (Watson, R.E., Jr. et al., 1995), suggesting AVPV as a site for the integration of daylight signals which is important for reproductive function. Furthermore, projections from the AVPV have been identified in the region surrounding the OVLT and the arcuate nucleus (Gu, G.B. & Simerly, R.B., 1997), suggesting a role thirst management and feeding respectively. A recent study identified
projections from ERα+ cells in the MPOA to the VTA (Fang, Y.Y. et al., 2018). This circuitry was found to be essential in driving maternal behaviors. Optogenetic activation of the projections revealed both excitatory and inhibitory postsynaptic currents, indicating the presence of both GABAergic and glutamatergic neurons. Most neurons in the AVPV are multipeptidergic. During ovulation GABAergic vesicles decline, while glutamatergic vesicles increase in the terminals (Ottem, E.N. et al., 2004). This suggests differential regulation of a particular neuron of the AVPV during different reproductive states. In Chapter 3, I showed projections from the OXTR neurons in the AVPV to various regions of the brain. However, we have not yet investigated the role of OXTR neurons in the areas that they project to. The maternal behavior is not just driven by a single population of neurons, but rather through a complex neural circuitry between various regions of the brain (Fig 4.1). Thus, the role of AVPV OXTR neurons in all the projected areas must be elucidated to understand the behavioral and physiological significance of sexually dimorphic OXTR neurons in the AVPV. The outcome from such studies will provide useful insight into sex-specific pharmacological interventions that may treat sex-typical psychiatric disorders, such as postpartum depression.
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VITA

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