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YEDİTEPE UNIVERSITY
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THE EFFECTS Of HYPOTHALAMIC PARAVENTRICULAR OXYTOCIN NEURONS On The HYPOFRONTALITY In TRANSGENIC MALE MICE

MASTER THESIS

Deniz Öykü ÖZEN

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SUPERVISOR

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Prof. Dr. Bayram YILMAZ
Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

27.07.2021

Deniz Öykü Özen

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LIST OF SYMBOLS AND ABBREVIATIONS

AAV	Adeno-Associated Virus
AN	Accessory Nuclei
BNST	Bed Nucleus of the Stria Terminalis
CNO	Clozopine-N-oxide
CNS	Central Nervous System
EPM	Elevated Plus Maze
HPA	Hypothalamic-Pituitary-Adrenal Axis
MNCs	Magnocellular Neurosecretory Cells
NA	Nucleus Accumbens
OF	Open Field
OTR	Oxytocin Receptor
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PFC	Prefrontal Cortex
PVN	Paraventricular Nuclei
SI	Social Index
SN	Substantia Nigra
SON	Supraoptic Nuclei
SP	Side Preference
SPI	Social Preference Index
VTA	Ventral Tegmental Area

ABSTRACT

Özen, D. (2021). The Effects of Hypothalamic Paraventricular Oxytocin Neurons on the Hypofrontality in Transgenic Male Mice. Yeditepe University, Institute of Health Science, Department of Physiology, MSc Thesis, Istanbul

Oxytocin is a neuropeptide synthesized in the neurons of hypothalamic paraventricular (PVN) and supraoptic nuclei (SON) and has a modulatory effect on various social behaviors. There is only one oxytocin receptor (OTR) known to date, and oxytocin exerts its neuromodulatory effects through this receptor. Oxytocin receptor is found in many areas of the brain, including the prefrontal cortex (PFC). In addition, several studies have shown that hypothalamic oxytocin neurons project to the PFC. Dopamine has an important role in regulating the cognitive function of the PFC and it is thought that oxytocin and dopamine systems work together and have similarities in the areas of organization and projection. In this study, it was aimed to investigate effects of oxytocin on hypofrontality-induced behavioral disturbances and the interaction of oxytocin and dopamine neurons in PFC. Adult male oxytocin-cre mice were used in this study. Recombinant adeno-associated virus with stimulatory hM3D subunit or inhibitory hM4D subunit were injected to paraventricular area in order to activate and inhibit oxytocin neurons. Then, effects of manipulation of oxytocin neurons on several behaviours were evaluated. Anxiety, locomotor activity, social interaction and short-term memory were examined by using elevated plus maze, open field test, social interaction test and T-maze test, respectively. Fiber photometry Ca^{2+} imaging was used to investigate interaction of oxytocin and dopamine neurons in the PFC. Oxytocin did not significantly change anxiety and locomotor activity. Inhibition of paraventricular oxytocin neurons significantly decreased the social novelty preference of oxytocin-cre mice and could cause short-term memory impairment. Further, Ca^{2+} signal recordings from prefrontal dopamine neurons were taken before and during acute activation of paraventricular oxytocin neurons, but no significant effect of acute activation of paraventricular oxytocin neurons on the activity of prefrontal dopamine neurons was found. In this study, the effects of manipulation of oxytocin neurons on various behaviors was revealed. Thus, modulation of prefrontal dopaminergic neuronal activity by chemogenetic activation and inhibition of oxytocin neurons was examined for the first time.

Key words: Oxytocin, Dopamine, Prefrontal Cortex

ÖZET

Özen, D. (2021). Erkek Transgenik Farelerde Hipotalamik Paraventriküler Çekirdekte Bulunan Oksitosin Nöronlarının, Prefrontal Korteksteki Hipofrontalite Üzerine Etkilerinin Araştırılması. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Fizyoloji ABD, Master Tezi. İstanbul

Oksitosin hipotalamik paraventriküler ve supraoptik çekirdekte üretilen ve birçok sosyal davranışta modülatör etkisi olan bir nöropeptitdir. Bugüne kadar bilinen tek bir oksitosin reseptörü vardır ve oksitosin nöromodulatör etkilerini bu reseptör üzerinden gerçekleştirmektedir. Oksitosin reseptörleri prefrontal korteks dahil olmak üzere beynin birçok bölgesinde bulunur ve birçok çalışmada hipotalamik oksitosin nöronlarının prefrontal kortekse projeksiyon yaptığı da gösterilmiştir. Dopamin, prefrontal korteks'in bilişsel işlevini düzenlemede önemli bir role sahiptir ve oksitosin ve dopamin sistemlerinin birlikte çalıştığı, organizasyon ve projeksiyon alanlarında benzerlikler olduğu bulunmuştur. Bu çalışmada, oksitosinin hipofrontalite kaynaklı davranış bozuklukları üzerindeki etkisini, oksitosin ve dopamin nöronlarının prefrontal korteksteki etkileşimini ortaya koymak amaçlanmıştır. Bu çalışmada yetişkin erkek oksitosin-cre fareler kullanıldı. HM3D ve hM4D alt birimli rekombinant adeno-ilişkili virüs, oksitosin nöronlarını aktive etmek ve inhibe etmek için paraventriküler alana enjekte edildi. Daha sonra oksitosin nöronlarının manipülasyonunun çeşitli davranışlar üzerindeki etkileri değerlendirildi.Sırasıyla yükseltilmiş artı labirent testi, açık alan testi, sosyal etkileşim testi ve T-labirent testi kullanılarak anksiyete, lokomotor aktivite, sosyal etkileşim ve kısa süreli bellek incelendi. PFC'deki oksitosin ve dopamin nöronlarının etkileşimini araştırmak için fiber fotometri Ca^{2+} görüntüleme kullanıldı.Oksitosin, anksiyete ve lokomotor aktiviteyi anlamlı ölçüde etkilemese de paraventriküler oksitosin nöronlarının inhibisyonu, oksitosin-cre farelerin sosyal yenilik tercihini anlamlı bir şekilde azalttığı ve kısa süreli hafıza bozukluğuna neden olabileceği gözlemlendi. Ayrıca, paraventriküler oksitosin nöronlarının aktivasyon öncesi ve aktivasyon durumunda prefrontal dopamin nöronlarından alınan Ca^{2+} sinyal kayıtları sonucunda paraventriküler oksitosin nöronlarının akut aktivasyonun prefrontal dopamin nöron aktivitesi üzerine anlamlı bir etkisi bulunamamıştır. Bu çalışmada oksitosin nöronlarının manipülasyonunun çeşitli davranışlar üzerindeki etkileri ortaya konmuştur. Böylece, oksitosin nöronlarının kemogenetik aktivasyonu ve inhibisyonu ile prefrontal dopaminerjik nöronal aktivite üzerine modülasyonu ilk kez incelenmiştir.

Anahtar Kelimeler: Oksitosin, Dopamin, Prefrontal Korteks

1. INTRODUCTION and PURPOSE

Oxytocin is a neuropeptide consisting of nine amino acids synthesized by magnocellular neurons in the supraoptic (SON) and paraventricular nuclei (PVN) and parvocellular neurons in the PVN of the hypothalamus[1]. There is only one oxytocin receptor that has been identified to date[2]. After oxytocin binds to its receptor, different intracellular processes become involved depending on the location of the receptor in the body[3]. Oxytocin receptors (OTRs) are most concentrated in the brain including cortex, hypothalamus, hippocampus, limbic system, nucleus accumbens, olfactory bulbus, basal ganglia and medial preoptic area[3].

Many studies in humans and animals show that oxytocin plays a role in bonding and social behavior[4]. Oxytocin levels have been found to be different from healthy controls in many psychiatric disorders such as autism and schizophrenia, which are known for their social difficulties. These findings pioneered studies investigating the therapeutic effect of oxytocin in psychiatric disorders. It has been found that intranasal oxytocin administration is effective in understanding the emotions of others and recognizing faces[5]. Another important effect of oxytocin is that it suppresses cortisol release triggered by stress and creates a sense of trust by reducing anxiety.

Oxytocin and dopamine systems are two important neuromodulators of brain functions that show similarities in organization and projection[6]. Oxytocin and dopamine neurons project to similar forebrain regions such as the prefrontal cortex, nucleus accumbens, and striatum to control social and affiliative behavior[6]. The presence of dopamine receptors in oxytocin neurons and the detection of oxytocin receptors in both the ventral tegmental area and the substantia nigra is one of the important findings showing that these two systems work together[7].

The prefrontal cortex (PFC) plays an important role in the personality and behavior of the individual[8]. The decrease in the activity of the frontal lobes is called hypofrontality. One of the reasons of the hypofrontality is thought to be the decreased secretion of dopamine, which plays an important role in the normal functioning of the PFC[9]. PVN^{OT} neurons are thought to have an impact on hypofrontality and dopamine activity in the PFC. In this study, behavioral tests are employed to elucidate whether the activation and inhibition of PVN^{OT} neurons causes hypofrontality-induced behavioral disturbances and the interaction of oxytocin and dopamine neurons.

2. LITERATURE REVIEW

2.1. Oxytocin System

Oxytocin is a nine amino acid neuropeptide discovered in 1906 as a first peptide reproductive hormone. Oxytocin is synthesized in the paraventricular nucleus (PVN), supraoptic nucleus (SON), and accessory nuclei (AN) of the hypothalamus[10]. Large amounts of oxytocin is synthesized in magnocellular neurons of the PVN, SON and AN of the hypothlamus and also lesser amounts of oxytocin is sythesized in parvocellular neurons of PVN[11].

Magnocellular oxytocin neurons have long been thought to project their axons only to the posterior pituitary from here oxytocin entering peripheral circulation to stimulate uterine contraction and milk ejection, but recently researches with leading tracking techniques have indicated that some magnocellular oxytocin neurons also project to limbic brain regions to regulate mood and behavior[10].

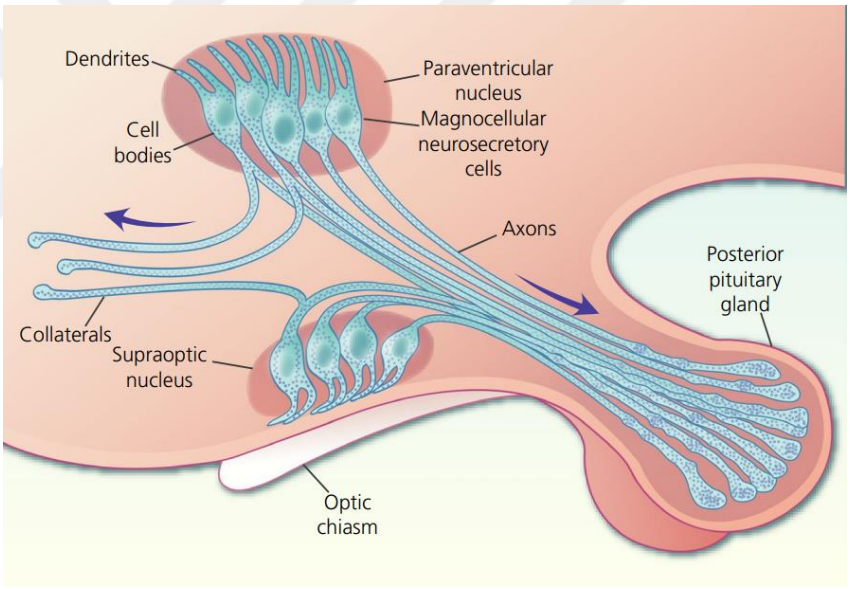

Figure 1. Magnocellular neurosecretory cells (MNCs) of PVN and SON of the hypothalamus[11].

Parvocellular neurons of the PVN projecting to various brain regions release oxytocin centrally to regulate behaviors[12]. These neurons contain much less oxytocin levels than magnocellular neurons[13]. PVN oxytocin neurons project to prosocial-related brain areas such as nucleus accumbens, medial prefrontal cortex, amgdala, hippocampus and BNST (Bed Nucleus of the Stria Terminalis) to modulate social behaviors[11].

The release of oxytocin has been defined from dendritic, somatic and axonal neuronal compartments[14]. In the brain, oxytocin is released synaptically by parvocellular neurons of PVN and dendritically by magnocellular neurons of PVN and

SON. A large number of oxytocin-containing vesicles are found on the dendrites of magnocellular neurons and can be released via exocytosis independently of peripheral release. Somatodentritic oxytocin release is not particularly targeted at synapses and the long half-life of oxytocin in the central nervous system (CNS) and abundance of oxytocin in the extracellular fluid indicate that it is likely to diffuse to distant target areas after release and have prolonged behavioral effects.

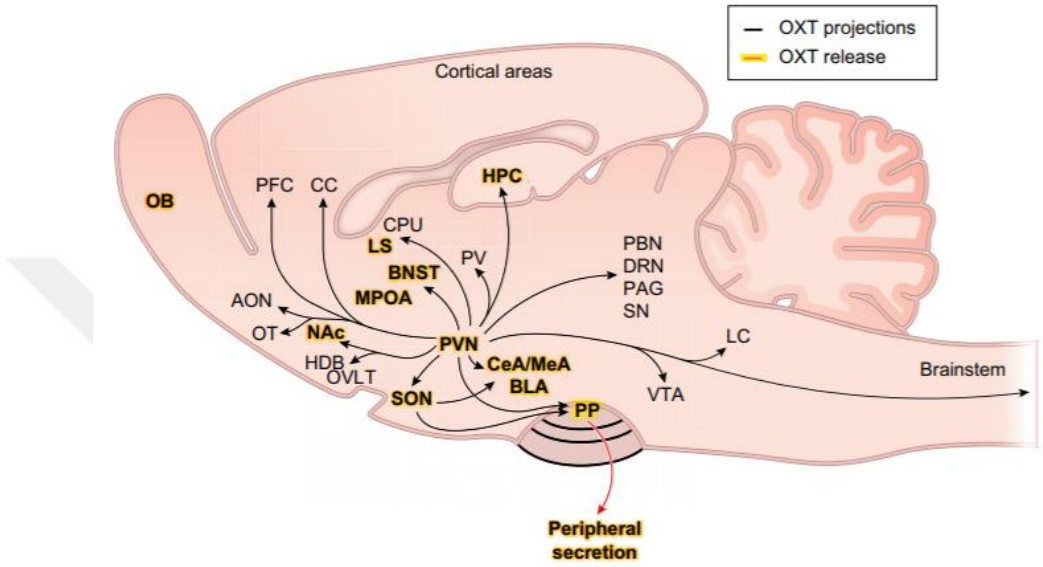

Figure 2. Representative anatomical scheme of a rodent brain. Black lines represents oxytocinergic projections from the PVN to various brain regions. Red halo highlightes the brain regions where oxytocin release has directly been indicated by microdialysis[15].

2.1.1. Oxytocin Receptor (OTR) System

It is well recognized that receptors for neuropeptides are distributed heterogeneously throughout the brain and may be expressed on axon terminals, dendrites and cell bodies. Oxytocin receptors are found in almost all brain areas and oxytocin-producing neurons project from the hypothalamus to several brain regions containing the brain cortex and limbic regions. There is a single OTR gene that has been identified to date. OTR is among the G protein-coupled receptor family. OTR is coupled to specific G proteins, $G_{\alpha i}$ and $G_{\alpha q}$, and activates different intracellular pathways with protein kinase C/phospholipase Cβ as downstream effectors[14,16]. The pattern of oxytocin secretion and the tissue-selective expression of the receptor determine the particular effects of oxytocin, which are dependent on its comparatively high affinity for the OTR[15].

OTR is a key receptor system for birth and breastfeeding, but also it is important for social behaviors[16]. The expression of OTR across species and tissues have

elucidated by autoradiography and mRNA studies using specific OTR ligands. OTRs are expressed in various tissues including uterus, heart, kidney, pancreas, thymus, adipocytes and brain[17]. The OTR gene sequence has been identified in mice, rats, pigs, rhesus monkey and sheep as well as humans. The OTR expressing in neurons is widely distributed in brain regions including the hypothalamus (dorsomedial, ventromedial, magnocellular nuclei), limbic brain structures (amygdaloid nuclei; medial preoptic nucleus, septal nuclei), hippocampus, olfactory processing structures (priform cortex, olfactory nuclei), brainstem (sensory and motor nerve nuclei regulating mouth and face). The OTR distribution among brain regions is highly species specific. OTRs are present in the CNS of rodents, and are generally located in the same areas as oxytocin fibers. In the rodent brain, OTRs have been found in the limbic system, hippocampus, amgdala, cortex, olfactory bulbs, medial preoptic region, basal ganglia, and the brain stem, in addition to PVN and SON[18].

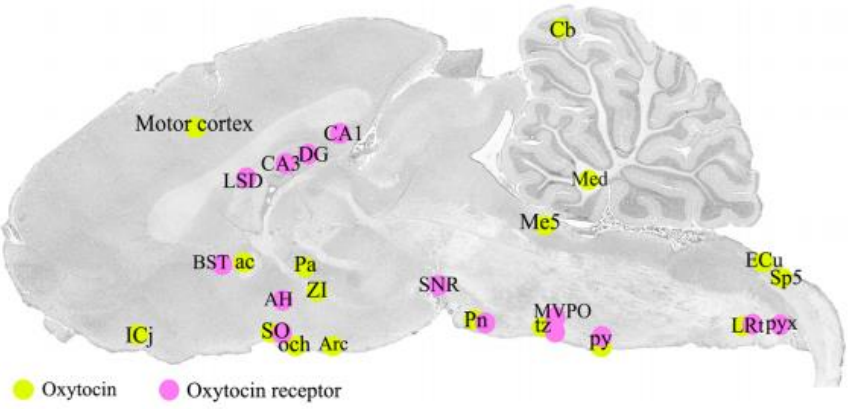

Figure 3. Oxytocin and OTR expression in rodent brain. Yellow color represents oxytocin and pink color represents OTR immunoreactivity[13]

2.1.2. Role of oxytocin in social behavior

It has been suggested that without the physiological and behavioral functions of oxytocin, complex social interactions, social bonds and high levels of social cognition could not be evolved in humans and animals[19]. Many of the brain regions are thought to have crucial roles in several social behaviors, also be included in anxiety and stress; release of oxytocin in these brain regions might then modulate social behaviors. Thus, it appears that genetic variation in OTR gene and oxytocin secretion have an impact on empathy, trust, pair bonding, stress reactivity, and emotion recognition[10].

Animal studies have demonstrated that anxious and stressful stimuli can trigger release of oxytocin within the PVN, indicating that oxytocin may play a important modulatory role in anxiety- and stress-related behaviors[20]. Oxytocin inhibits

hypothalamic-pituitary-adrenal axis behavioral and secretory responses to stress and inhibits corticotrophin-releasing hormone neurons via primarily acting within the PVN[21]. Mice with deleted oxytocin gene provide unique approach to understand the function of oxytocin in stress and anxiety-related behavior[22]. It has been reported that oxytocin-null mice demonstrated enhanced anxietic behavior[23]. Furthermore, magnocellular oxytocin neurons are inhibited by glucocorticoids such as corticosterone, therefore, the antistress and anxiolytic effects of oxytocin are decreased during depression and chronic stress. Thus, lack of central oxytocin is linked with depression[24]. Moreover, clinical trials indicated that intranasal oxytocin treatment enhanced social and cognitive abilities which are disturbed in major depressive disorder patients[24].

Genetic studies showed that OTR and oxytocin knockout mice demonstrate disturbances in memory impairment in addition to aggression, stress and anxiety. Recent studies also indicated that oxytocin demostrate a modulatory effect on emotional memories both in human and animal models. The social recognition memory is the ability to recognize individual animals of the conspecifics and to distinguish them from other ones and it is essential for social interaction[25,26]. Studies have shown that mice with the OTR gene knocked out have difficulty in recognizing familiar mice, which can be restored by administration of oxytocin before being exposed to the familiar animal[27]. On the other hand, oxytocin administration enhances sociability in human and experimental models[28]. Acute and chronic administration of oxytocin has been demonstrated to influence social behavior in rodent models, enhancing social memory and increasing social interaction time[28].

Besides from the effects of oxytocin on anxiety and social interaction, the effects of oxytocin on locomotor activity have been also investigated. Oxytocin has dual effects on locomotor activity: at lower dosages, it slightly increases locomotor activity or has no significant impact while the higher doses of oxytocin indicates certain signs of sedative effects as seen by a decrease of locomotor activity and rearing (1 and 3 mg)[29].

Disturbance of the oxytocin system including alterations in structure and release of the peptide, and genetic variations of the OTR have been associated with several neuropsychiatric disorders including anxiety, autism spectrum disorder, schizophrenia, mood disorders[30]. Autism spectrum disorder is defined as impaired social communication and interaction, as well as repetitive, stereotyped behaviors. In autism spectrum disorder, reduced activity of oxytocin system and polymorphisms in OTR gene have been implicated. Since intranasal and intravenous oxytocin infusions have

been demonstrated to improve social cognition and improve social information processing and retention and decrease repetitive behaviors in patients with autism spectrum disorder[31].

There is limited evidence indicating oxytocin and/or OTR gene polymorphisms may be associated with pathology of schizophrenia and intranasal, intramuscular and intravenous oxytocin treatment emerges improvement of some symptoms of schizophrenia[32]. Abnormal dopaminergic transmission and prefrontal cortical dysfunction have been linked to a dopaminergic mechanism of action for oxytocin in schizophrenia[26]. The central dopaminergic systems and oxytocin have an extensive interactions. As such, excessive dopamine transmission in the mesolimbic system is closely linked to positive symptoms of schizophrenia patients, the findings suggest that oxytocin's inhibitory control of mesolimbic dopamine may be a component of its antipsychotic action mechanism[26].

Some researches report decreased plasma oxytocin levels in depressed patients compared with healthy control group. It has been reported that dysregulation of the oxytocin system is associated with major depressive disorder[10]. Involvement of oxytocin in the pathophysiology and treatment of the abovementioned diseases shows that oxytocin plays a critical role in the regulation of a wide-range of social behaviors.

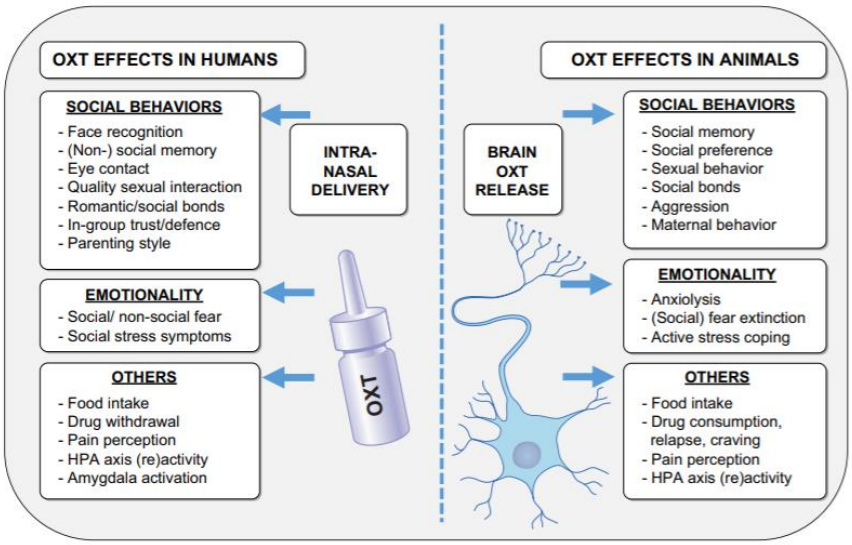

Figure 4. Summary of synthetic or endogenous oxytocin effects on social behaviors, and other functions[15].

2.2. Dopamine System and Its Relation with Oxytocin System

Dopamine and oxytocin systems are two important neuromodulators of the brain functions displaying similarity in organization and projection sites. In the brain, dopamine neurons are exclusively present in the substantia nigra (SN) and ventral tegmental area (VTA), while oxytocin neurons are primarily found in the hypothalamus. Oxytocin and dopaminergic neurons that control various social and affiliative behaviors project to similar forebrain regions contaning nucleus accumbens, prefrontal cortex and striatum. It is tempting to assume that both oxytocin and dopaminergic systems may modulate each other, given that OTRs are present in both SN and VTA and dopamin receptors are detected on PVN^{OT} neurons[33].

There are two significant dopaminergic pathways that regulate social behaviors; mesolimbic pathway and mesocortical pathway. In mesolimbic pathway, cell bodies of dopamin neurons are organized in VTA and terminate in various limbic regions including amygdala and nucleus accumbens (NA) to involve in reinforcement, desire and reward behaviors. In mesocortical dopamine pathway, dopamin fibers in VTA project to the cortex and mediate motivational and emotional response[24]. Recent studies indicate that oxytocinergic neurons projecting to amygdala, hippocampus and VTA stimulate mesocortical/mesolimbic dopaminergic neurons and that the activation of these dopaminergic neurons in turn stimulate PVN^{OT} neurons[34]. Moreover, electrophysiological recordings from dopamin neurons indicate that both optogenetic stimulation of oxytocinergic terminals and the oxytocin application to the bath suffice to increase activity of dopamin neurons in the VTA, but downregulate the dopamin neuron activity in the SNc[35]. Release of oxytocin directly activates dopamin neurons in the VTA, but indirectly inhibits dopaminergic neurons in the SNc through local GABA neurons[35].

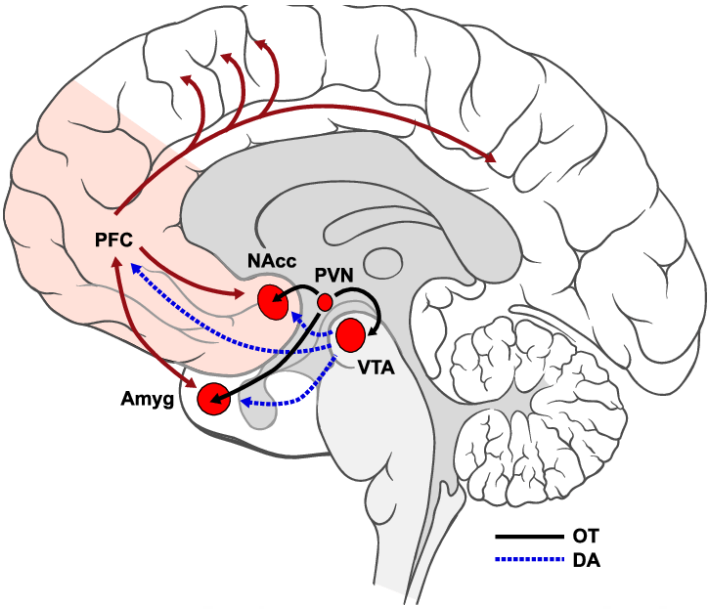

Figure 5. Schematic representation of oxytocinergic (OT; solid black) and mesocorticolimbic dopaminergic (DA; dashed blue) pathways[31]

OTRs localizing to both Nac and VTA have detected by receptor autoradiography in rats and other species[36]. Also oxytocin injection into the VTA of rats increases dopamin release in the Nac. These findings also show that OTR fuctionally stimulate firing of dopamin neurons in the mesolimbic pathways. Oxytocin administration in the VTA enhances release of dopamin in the Nac in response to sociality thereby prosocial behaviors are increased due to activation of this circuit.

Disturbances in the levels of central and peripheral oxytocin have recently been reported in certain individuals with dopamine-dependent disorders[24]. Dopamine neurons in the VTA that are sensitive to stress project to cortical areas, thus release of dopamine increases in the PFC in response to the stressors. Microarray analysis of the human brain indicates abnormal regulation of dopamine in the PFC in several psychiatric disorders including bipolar disorder, schizophrenia and depression[37].

Thus, it is not inconceivable that oxytocin might be implicated in a variety of dopamine-dependent behavioral disorders. Hence, oxytocin could be used as a therapeutic target in the clinical management of these diseases.

2.3. The Roles of Dopamine and Oxytocin in the Prefrontal Cortex

Prefrontal cortex, the most anterior part of the frontal cortex, contains several subregions that promote various activities ranging from motivation to cognition, emotion to decision making. PFC is well located to coordinate a wide range of neural

processes. It sends and receives projections from many subcortical regions, motor systems and virtually all cortical sensory regions[38].

Hypofrontality refers to decreased activity of PFC than normal, and it can be detected in several neuroimaging investigations in various neurological disorders including, schizophrenia, attention deficit hyperactivity disorder (ADHD), bipolar disorder, and major depressive disorder[39]. It is proposed that neurological disorders implicate changes in the behavioral control system provided by the PFC and its connections with other brain regions. In support of this idea, it has been demonstrated that patients suffering from neuropsychiatric disorders show changes in PFC-dependent cognitive functions including attention, decision making, fear extinction, working memory and cognitive flexibility[40]. Also, recent studies showed that mPFC lesions in rodents impair working memory, set-shifting and recall of conditioned fear extinction[27]. Therefore, rodent models have influential features for examining the neural and behavioral properties associated with the control of behavior[41].

Dopamine is a monoaminergic neurotransmitter that regulates PFC function particularly in attention and working memory[42]. Derangement signaling of dopamine is implicated in the pathogenesis of various neuropsychiatric disorders such as schizophrenia, post-traumatic stress disorder (PTSD), ADHD and drug addiction. It has been suggested that either too much or too little stimulation of dopamine receptor (D1R) impairs the function of PFC[43].

The PFC expresses OTRs in high numbers and innervated by oxytocin synthesizing neurons contributing to a neural circuit that regulates social behavior[44]. Electrophysiological and neuroanatomical experiments using mice with direct expression of Cre-recombinase to cells producing OTR indicated that neurons synthesizing functional OTR(s) in PFC are glutamatergic or GABAergic, with the following long-range projections to subcortical brain regions. Optogenetic stimulation of OTR-expressing neurons in the PFC enhanced neuronal activity in mesolimbic nuclei associated with social, rewarding and anxiety-like behaviors[45]. *In vivo* optogenetic stimulation of OTR-expressing neurons in the PFC attenuates social recognition, but locomotor activity, anxiety-like behavior and sociability is not affected resulting of activation of these selective neurons[45].

Oxytocin can be administered subcutaneously, intranasally or via infusion into the PFC. These particular routes exert different consequences in the behavioral tests depend on the dosage, so oxytocin may produce some biphasic effects. Anxiolytic

effects of oxytocin presumably are regulated via the dopaminergic system of the PFC-amygdala network. Dopaminergic emotion processing systems and oxytocinergic input to the amygdala are fundamental for emotional stimuli processing in a social context. These two systems together promote signaling between the PFC and amygdala thus increases social awareness and attention[46].

The most evolved brain area, mPFC, plays an essential role in regulating working memory, thinking, decision making and emotion via interactions with other brain areas[47]. The mesocortical circuit, which is important for encoding emotional and reward regulation is constructed via mPFC receiving dopamine neurons from the VTA[47]. According to experimental studies, repeated social-defeat stressors dramatically reduce VTA-mPFC dopaminergic neuron firing patterns and impede presynaptic release of dopamine in the PFC. Reduced dopaminergic transmission in the mPFC might be a reason of cognitive impairment of neuropsychiatric disorders including schizophrenia and depression. More recently, reduced dopaminergic transmission in the mPFC is associated with the pathophysiology of depression[48]. Previous studies investigated the antidepressant effect of oxytocin via local administration to the mPFC[48]. In both naive and social defeat stress depressive animal model, local oxytocin administration into the mPFC resulted in antidepressant-like effects through elevation of dopamine concentrations depending on activation of OTR[48]. Increased dopamine levels in mPFC might further enhance excitatory synaptic transmission[48].

3. MATERIALS and METHODS

3.1. Animals

In the present study, adult male Oxytocin-Ires-Cre mice were used. The transgenic Oxy-Ires-Cre line (Code: 024234) was obtained from Jackson Laboratories (USA). Mice were housed as 4 animals/cage under standard housing conditions (12:12 light-dark cycle, room temperature at 21 ± 2°C). The animals had *ad libitum* access to food and water. Tissue samples were collected from 30 days old offsprings and the DNA was isolated. After DNA isolation, polymerase chain reaction (PCR) was performed to determine the locus of Cre-gene.

3.2. Breeding

Transgenic male mice were mated with the inbred substrain C57BL/6 wild type female mice. Breeding cages contained 1:2 ratio of male-female mice. Male animals were separated from the breeding cages after the 14^{th} day of pregnancy. The cages were cleaned before the birth of offsprings. After the lactation period, tissue samples were taken from 25-30 days old offsprings for genotyping.

3.3. Genotyping

Ear tissues with 2 cm in diameter were taken from anesthetized offsprings. Tissues were taken at room temperature and transferred to the PCR tubes. 100 µL alkaline buffer (25 mM NaOH -Sigma, 306576 and 0.2 mM EDTA) was added to each PCR tube containing tissue samples and the tubes were placed in a PCR device (Labnet International, TC9610). Tissue samples in alkaline buffer were boiled at 98 °C for 1 hour. After 1 hour, 100 µl of neutralization buffer (40mM Tris HCl, Sigma-T5941) was added to each tube and the tubes were placed in a centrifuge (SIGMA 1-14 microcentrifuge) at 4000 rpm for 4 minutes. The supernatant of each mixture was transferred to the separate tube and stored at 20^{o}C. The separated supernatants including DNA samples were used for polymerase chain reaction.

3.4. Polimerase Chain Reaction (PCR)

DNA samples, 2X master mix containing Taq polymerase enzyme (New England Biolabs, MO270S), primers and double distilled water were used for polymerase chain reaction. The mixture was prepared on ice at 4^{o}C and divided to tubes. Finally, DNA samples were added into the tubes and the negative and positive controls were prepared in addition to DNA samples. The negative control did not contain DNA and it included only the PCR mixture. The positive control included DNA from father or mother known to be Oxytocin-Ires-Cre.

Table 1. Primer Sequences

Primers	Primer Sequences (5' ➔ 3')
oIMR1084	GCG GTC TGG CAG TAA AAA CTA TC (Cre F)
oIMR1085	GTG AAA CAG CAT TGC TGT CAC TT (Cre R)

Table 2. Standard PCR Protocol (12.5 µl per tube)

Material	Concentration	Volume
Taq polymerase enzyme	2X	6 µl
5' Primer	20µM	0,125 µl
3' Primer	20 µM	0,125 µl
Double distilled water (ddH_2O)		5,75 µl
Template DNA		0,5 µl

Table 3. Standard PCR Program

Steps	Temperature	Time
1	94°C	2 min
2	94°C	20 sec
3	65°C	15 sec [-0.5°C per cycle decrease]
4	68°C	10 sec
5	Repeat steps 2-4 for 10 cycles (Touchdown)	
6	94°C	15 sec
7	60°C	15 sec
8	72.0°C	10 sec
9	Repeat steps 6-8 for 28 cycles	
10	72°C	2 min
11	10.0°C	∞

3.5. Agarose Gel Imaging

To prepare %2 agarose gel mixture, 2 g agarose (Bioshop, AGA001.1) was added to 100 ml 1X TBE buffer and the mixture was placed in a microwave oven. The mixture was heated until it became completely homogeneous. Ethidium Bromide (5µl) was added into the 2% agarose gel mixture. The mixture was poured into the electrophoresis cassette and combs were placed. 20 minutes were waited for freezing of gel. After freezing of gel, combs were removed and the cassette was placed in the electrophoresis tank. Running buffer (1X TBE buffer) was poured to the electrophoresis tank until the gel would sink completely into the buffer. 1 µl 5X loading dye (Sigma-G7654) was added into the PCR products by using micropipette. HyperLadder (3 µl; Bioline, H4-215108) was loaded into the first well of the agarose gel. PCR products containg 1 µl 5X loading dye were loaded into wells, respectively. DNA samples were run toward the positively charged end of the gel at 200 Volts for 15 minutes. After 15 minutes, the gel was displayed by using BioRAD ChemiDoc imaging system. Samples having bands at 727 basepairs were counted as Cre positive. The mice containing Cre-gene were separated from the mice that were negative for Cre-gene.

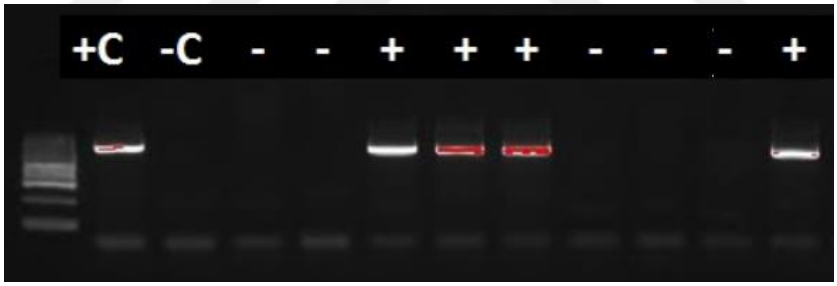

Figure 6. Representative agarose gel image for PCR results**.** +C represents positive control (750bp); -C represents negative control; (+) represents Cre positive results; (-) represents Wild-type results.

3.6. Stereotactic Intracranial Surgery

Mice were anesthetized by using isoflurane and placed to the stereotactic frame through their upper teeth. The tongue was putted out of the mouth to open the breath path. The mice were inhaled with air and isoflurane mixture during the operation. The foot was tightened by using forceps for controlling the reflex. The skull was placed in the stereotactic frame and fixed with ear bars. The skin was cutted vertically by using scalpel. The bregma and lambda points were detected and the point where the glass micropipette was touched to the Bregma point was assumed to be zero for x, y and z

coordinates. The mineral oil filled to the glass micropipette to form a phase difference with the virus. The glass micropipette was also used for the determination of the coordinates on the mice skull. The X- and Y- coordinates was straightened that was important for successful injection and preventing the entry to a brain region other than the targeted region. After the coordinates of the targeted brain region was determined, the skull was drilled by using a dental drill. The glass pipette containing mineral oil was filled with 800 µl virus. pAAV-EF1a-DIO-hM3D (Gq) –mCherry and pAAV-EF1a-DIO-hM4D(Gi)-mCherry viruses were used for the activation and inhibition group respectively and pAAV-CAG-flex-rev-tdTomato was used for the control group. The Z-coordinate was zeroed when the glass micropipette was touched to the cortex and the micropipette reached to PVN. 300 nl of virus was injected to both sides of PVN at 80nl/min speed. After the each injection of 300 nl virus, 15 minutes were waited to prevent negative pressure before leaving the tissue. The wound lips were brought together when the injection was completed and sutured with 5.0 sterilized surgical suture. Betadine was applied to the sutured area. After 20 minutes, the anesthetized mouse awake. The PVN coordinates were determined as 0.750 mm posterior, ± 0.350 mm lateral and 4.70 mm vertical. Anterior/posterior coordinate shoud be calculated depending the distance between Bregma and lambda points for each mouse. The distance between bregma and lambda is 4,2 mm in the Allen Brain Atlas so the Y coordinate will be calculated as 'Distance between Bregma-Lambda= (Y*0,82) /4,2'. 0,82 is the coordinate of PVN in the Paxinos & Watson Brain Atlas. After the two-week infectious period, the virus was expressed in the PVN^{OT} neurons.

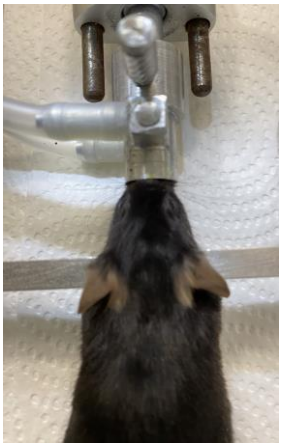

Figure 7. Placement of mouse in the stereotaxic frame.

3.7. Behavioral Tests

The PVN regions of Oxytocin-Ires Cre mice were targeted by expression of ligand sensitive G protein-coupled channels (hM3D and hM4D) as a result of infection by adeno-associated virus (AAV). After the two-week infection period, oxytocin neurons would have channel proteins that could be activated by Clozopine-N-oxide (CNO). CNO, a muscarinic receptor type 3 agonist, stimulate the target the brain region acutely (15-120 minutes). The only variable in the control group was the virus type (pAAV-CAG-Flex-rev-tdTomato), and no change would be observed in the oxytocin neuron group with intraperitoneal (i.p.) administration of CNO. A behavioral test was performed 30 minutes after the i.p. CNO administration (3mg/kg) to mice. One to three days intervals were allowed between behavior tests to avoid the CNO effects in the tissues. Behavioral tests were performed during the light phase. Elevated plus maze, open field, social interaction test and T maze test were performed, respectively. Mice were brought to the behavioral laboratory at least 30 minutes before the experiment.

Table 4. The experimental groups of OXY-Ires-Cre mice

	Virus-infected in PVN^{OT} Neurons
Control Group	pAAV-CAG-flex-rev-tdTomato
Activation Group	pAAV-EF1a-DIO-hM3D (Gq) –mCherry
Inhibition Group	pAAV-EF1a-DIO-hM4D(Gi)-mCherry

3.7.1. Elevated Plus Maze (EPM) Test

The elevated plus maze was used to evaluate the anxiety behavior in the mice. Total arm entries, the percentage ratio of time spent on the open arms relative to time spent on total arms and the percentage ratio of open arm entries relative to total entries are the parameters of elevated plus maze test[49].

Equipment: The maze, a plexiglass platform with lenght of 110x110cm, consisted of four arms, two vertical open arms and two closed arms whose sides are closed by plexiglass walls.

Procedure: Before the experiment started, the light intake of the center and the end points of the platform were measured and the center was set to 100 lux. Room temperature was recorded as 22^{o}C. Each animal was placed to the center of the platform as their faces toward to the same open arm. Mice were allowed to move freely for 5 minutes and their movements were recorded with EthoVision XT video tracking system. After each trial, the platform was cleaned with 70% ethanol to prevent a bias

based on olfactory sense. The time spent on the closed arms and the open arms were analyzed for each animal.

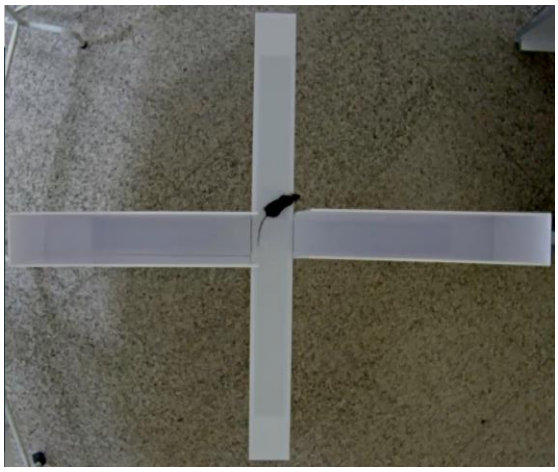

Figure 8. Elevated Plus Maze apparatus.

3.7.2. Open Field (OF) Test

The open field test is used to analyze overall locomotor activity, anxiety and stereotypical behaviors such as rearing and grooming in rodents. Changes in locomotor activity can indicate altered neurological processes therefore may demonstrate abnormal brain function. Mice showing anxiety-like behaviors prefer to stay close to the walls, spend more time on the corners and sidelines and show less locomotor activity. Mice with lower anxiety prefer to spend more time in the center of the open field[50].

Equipment: The test area is a large square made up of plexiglass with lenght of 80x80cm and high of 45cm.

Procedure: Before the experiment began, the light reception of the center and the endpoints of the platform were measured and the center was set to 125 lux. Room temperature was recorded as 22ºC. Each animal was placed on the center of the open field and allowed to freely explore the platform for 10 minutes. Each trial was recorded with EthoVision video tracking system. The platform was cleaned with 70% ethanol after each trial. The locomotion and anxiety-like behaviors of each animal were analyzed.

Figure 9. Open field apparatus

3.7.3 Social Interaction Test (Three-Chamber Test)

The three-chamber test has been used to study social affiliation and social memory in rodents. The main principle of the three-chamber test is based on the free choice of experimantal mouse to spend time in any of the three compartments of the box during two experimental sessions, containing contact indirectly with one or two unfamiliar mice. To evaluate social tendencies of the subject mouse; preference for a familiar vs. a novel conspecific and the time spent with a novel conspecific are measured. Social motivation/affiliation and social novelty and memory are critical aspects of social behavior and the experimental design of the three-chamber test allows to evaluate these aspects. In this case, "Sociability" is defined as the tendency to spend more time with another mouse rather than spend time alone in the same but empty chamber. "Social novelty preference" is defined as the tendency to spend more time with an unfamiliar mouse compared to time spent with already-investigated mouse[51].

Equipment: The Plexiglas platform (63x41x30cm) is divided into three compartments with small rectangular openings (5cm widex3cm tall). Each side compartment has triangular sections where another mouse is placed.

Procedure: Three-chamber social interaction test was consisted of 3x10 min sessions. Each animal was placed to the center area for 10 minutes habituation, in which no stimulus was present. The second session was sociability, a novel mouse was put to the right chamber of the platform (stranger zone 1) while the other chamber cage was empty and the experimental mouse was allowed to freely explore all of the chambers for 10 min. The last session measured social novelty preference in which unfamiliar mouse was placed to the left chamber (stranger zone 2) while already-investigated mouse was

still in the right chamber and the experimental mouse was allowed to freely explore all chambers for 10 min. The behaviors of the experimental mouse were recorded with EthoVision video tracking system. The social index and social preference were measured for each animal as follows: (Social interaction test procedure) (social interaction gl)

a) Social Index[51] = Time in strange zone 1/ Time in empty area
b) Social Preference Index[51] = Time in strange zone 2/ Time in strange zone 1

3.7.4. T Maze Test

T maze test is an useful method for evaluating the cognitive ability of rodents. The T-maze is an enclosed platform in the form of T placed horizontally. Each animal is started from the base of the T-maze and allowed to select one of the two goal arms. If two trials are made in a rapid succession, the rodent tends to select the non-visited arm before, on the second trial. That reflects the memory of the first choice and the process is called 'spontaneous alternation'. Thus, T-maze sponteneous alternation test can be used to assess the working (short-term) memory[52].

Equipment: The T-maze is a T-shaped platform consisting of a base (36.8x6.4x10.2cm) and two goal arms (30.5x6.4x10.2cm).

Procedure: Each animal was placed to the start arm and allowed to choose one of the goal arms. Once all four paws of the mouse were entered the particular goal arm, T junction between the opposing goal arm and start arm was blocked with the door. The mouse was left in the choosen goal arm for 30 seconds then the mouse was placed back in its cage for 30 seconds. Then the door was removed from the maze and mouse was replaced to start arm and allowed to choose between two open goal arms for 2 minutes. Each trial was recorded with EthoVision video tracking system. The % Side Preference was calculated for each animal.

a) % Side Preference[52] = [The number of preferred side that the mouse has choosen/ Total runs performed]*100

3.8. Virus injection and fiberoptic insertion to PFC

As the coordinate determination was explained in detail in the stereotactic intracranial surgery part, the Ca^{2+} signal-sensitive Cre-independent pAAV-hSyn-GRAB_DA1h virus was injected into the prefrontal cortex region of OXY-Cre mice. The PFC coordinates were determined as 1.90 mm anterior, ±0.30 mm lateral and 1.80 mm vertical. A fiber optic cable (200 µm diameter aperture; BFH37-200 Multimode;

NA 0.37 Thor labs) was placed over the PFC of Oxytocin-Ires-Cre mice for in-vivo recording. Fiber cables with a light transmittance of more than 50% were used. C&B Metabond Quick Cement and dental acrylic were used to fix the ceramic part of the fiber cable on the skull.

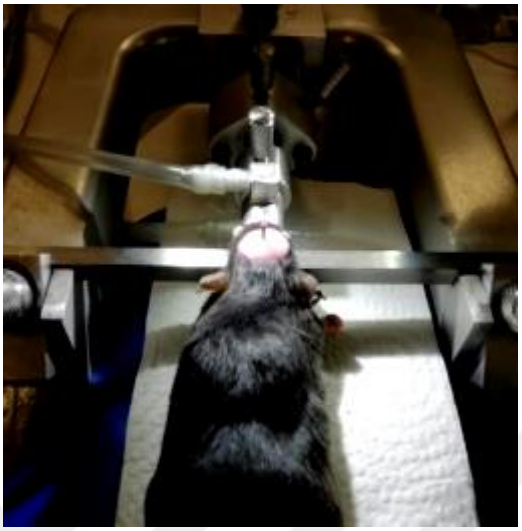

Figure 10. Ferrules inserted into the mouse brain for *in-vivo* recordings.

3.9. Fiber Photometry (Ca^{2+} imaging)

For *in vivo* fiber photometry, OXY-Cre mice were first injected with intracranial pAAV-hSyn-GRAB_DA1h virus to the PFC. After virus injection, ferrule was inserted into the brain (200 µm diameter aperture; BFH37-200 Multimode; NA 0.37 Thor labs) using the same coordinates and attached to the skull with dental acrylic. The virus infection period was waited for two weeks. Fiber photometry Mini Cube (Doric Lenses) equipped with two excitation and one emission ports was used. The first excitation port was 400-420 nm, the second excitation port was 460-490 nm and the emission port was 500-550 nm. During all experiments, the light intensity emanating from the fiber tip was kept constant in the range 10-15 µW. The signals that did not belong to the Ca^{2+} signal were filtered out in the received Ca^{+2} signal data. The received signals were calculated using the ΔF / F formula. ΔF / F= (473 nm-405 nm)/405 nm. *In vivo* recording was analyzed using Doricstudio program. The results were transferred to the Graphpad Prism®6 software and graph drawing was performed.

3.10. Cardiac Perfusion and Brain Slicing

Mice were anesthetized by isoflurane 30 minutes after i.p. CNO administration (3mg/kg). In the right atrium a small incision was made and circulation of blood was stopped. 0.9% isotonic sodium chloride solution and 4% paraformaldehyde (PFA) were given, respectively, to the left ventricular circulation. The extracted brain was

transferred into the 4% PFA solution and left for 4 hours. Brains were cross sectioned at a thickness of 70 μm by using a vibrotome system.

3.11. Imaging

20X images of control group expressing pAAV-CAG-flex-rev-tdTomato virus in their PVN^{OT} neurons; activation group expressing pAAV-EF1a-DIO-hM3D(Gq)–mCherry virus in PVN^{OT} neurons and inhibition group expressing pAAV-EF1a-DIO-hM4D(Gi)-mCherry virus in PVN^{OT} neurons were taken by using Zen Confocal microscopy.

3.12. Analysis

GraphPad Prism®6 was used for the statistical analyses. All data were analyzed as mean ± Standard Deviation (STD). Shapiro-Wilk normality test was used to determine normal distribution of data. $P<0.05$ was considered to be statistically significant.

4. RESULTS

4.1. Elevated Plus Maze (EPM) Test

Oxytocin-Ires-Cre mice with tdTOM, hM3D and hM4D virus-infected in PVN^{OT} neurons of control, activation and inhibition groups respectively were subjected to the EPM test for anxiety measurement. The time spent in the open arm and the closed arm by the animals taken from the EPM test was put into the Shapiro-Wilk normality test with the GraphPadPrism6 software, and since they could not pass this test, the non-parametric test, the Kruskal-Wallis test was used for statistical analysis. When the time spent in the open arm of the control, activation (hM3D) and inhibition (hM4D) groups were compared, no significant differences were found between the groups (Figure 11). When the time spent in the open arm and closed arm was compared within the group, it was found that the activation (hM3D) and inhibition (hM4D) groups spent significantly more time in the closed arm than open arm, although no significant change was observed in the control group (Figure 12).

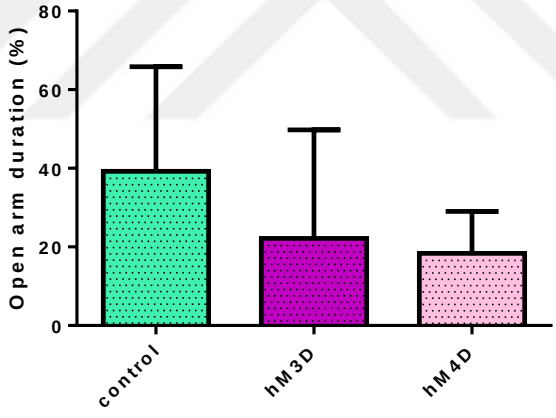

Figure 11. Duration time of groups in open arm of EPM (n=8 for control group; n=7 for hM3D group; n=5 for hM4D group). Kruskal-Wallis test.

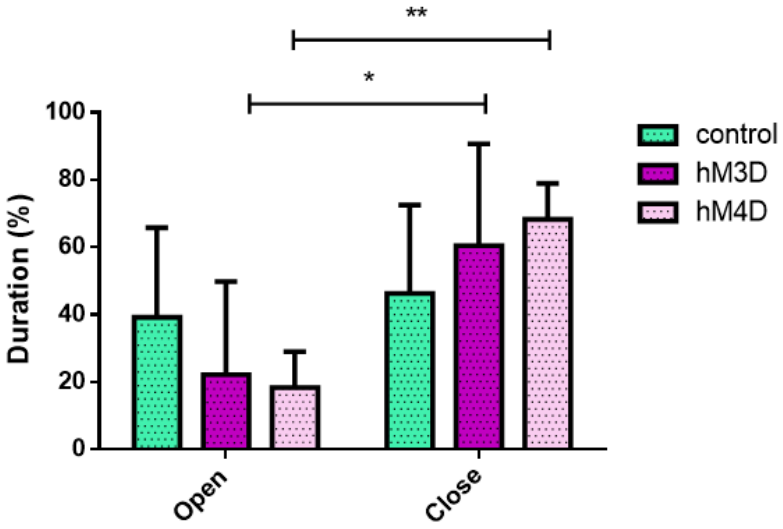

Figure 12. Duration time of groups in both open and closed arms of EPM (n=8 for control group; n=7 for hM3D group; n=5 for hM4D group). Two-way ANOVA, $p<0.05$ for control group, Sidak's multiple comparisons test.

4.2. Open Field Test

Oxytocin-Ires-Cre mice with tdTOM, hM3D and hM4D virus-infected in PVN^{OT} neurons of control, activation and inhibition groups respectively were subjected to the open field test for evaluation of anxiety and locomotor activity. The time spent in the center zone of open field platform was analyzed for anxiety behavior. Kruskal-Wallis analysis showed that there was no significant difference in center zone duration between groups (Figure 13). Velocity (cm/s) of the groups in open field was analyzed for the measurement of locomotor activity. According to the Kruskal-Wallis test, there was no significant difference in the locomotor activity between groups (Figure 14).

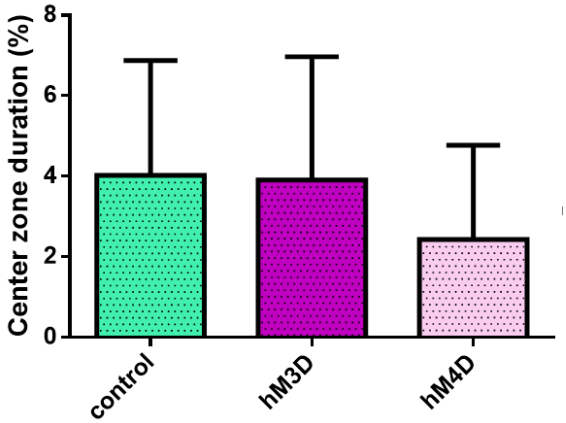

Figure 13. Center zone duration (%) of groups in open field test. (n=6 for control group; n=7 for hM3D group; n=5 for hM4D group). Kruskal-Wallis test.

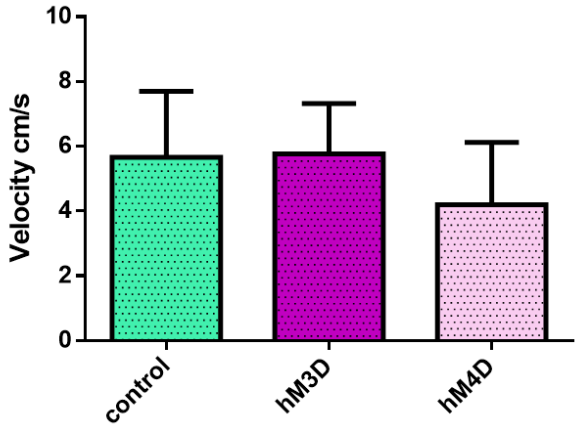

Figure 14. The velocity (cm/s) of groups in open field test. (n=6 for control group; n=7 for hM3D group; n=5 for hM4D group). Kruskal-Wallis test

4.3. Social Interaction Test (Three-Chambered Test)

Oxytocin-Ires-Cre mice with tdTOM, hM3D and hM4D virus-infected in PVN^{OT} neurons of control, activation and inhibition groups respectively were subjected to the social interaction test for measurement of sociability and social novelty preference. In the sociability session of the test, the time spent in the empty chamber and stranger mouse including chamber was analyzed with Two-way ANOVA, not RM test. More time spent in the chamber with stranger mouse compared to the empty chamber indicates normal sociability. According to Sidak's multiple comparision test, it was found that the time spent in the chamber with stranger mouse was significantly higher than the time spent in the empty chamber within groups (Figure 15). According to Tukey's multiple comparison test, no significant difference was found when the time spent in the chamber with the stranger mouse and in the empty chamber were compared between the groups. In the social novelty preference session of the experiment, the experimental mouse is expected to freely choose between the already-investigated mouse and the novel unfamiliar mouse, and spending more time with the novel mouse indicating intact social memory[51]. When the time spent in the chamber with the novel mouse and the chamber with the already-investigated mouse was compared no significant difference was found between and within groups except inhibition (hM4D) group according to Sidak's multiple comparison test (Figure 16). There was a significant decrease in social novelty preference of inhibition (hM4D) group. Social Index and Social Preference Index were analyzed with Kruskal-Wallis test, yet there was no significant change between the groups (Figure 17&18).

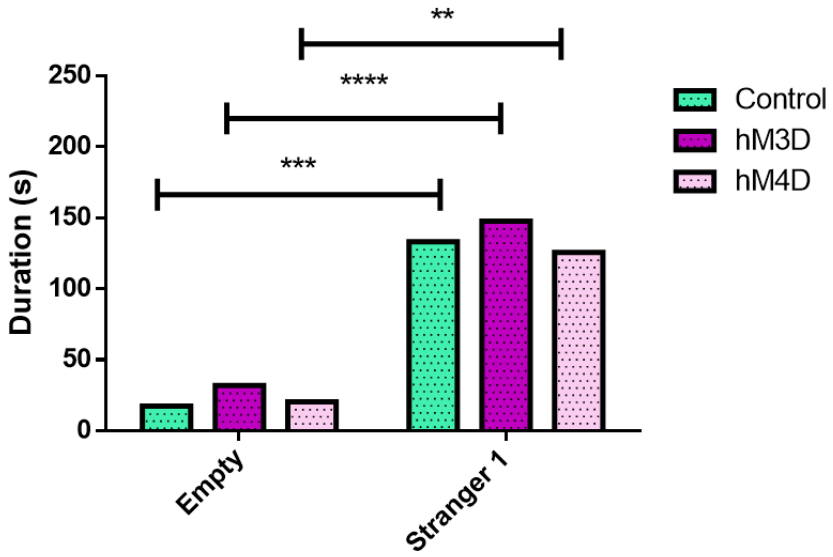

Figure 15. Duration time (s) of groups in three chamber test compartments (n=6 for control group; n=7 for hM3D group; n=5 for hM4D group). Two-way ANOVA, $p<0.05$, Sidak's multiple comparisons test.

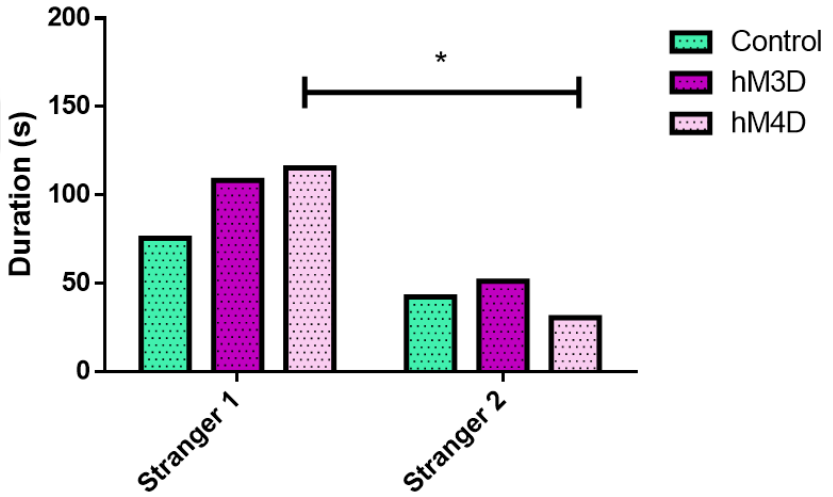

Figure 16. Duration time (s) of groups in three chamber test compartments (n=6 for control group; n=7 for hM3D group; n=5 for hM4D group). Two-way ANOVA, $p<0.05$ for hM4D group, Sidak's multiple comparisons test.

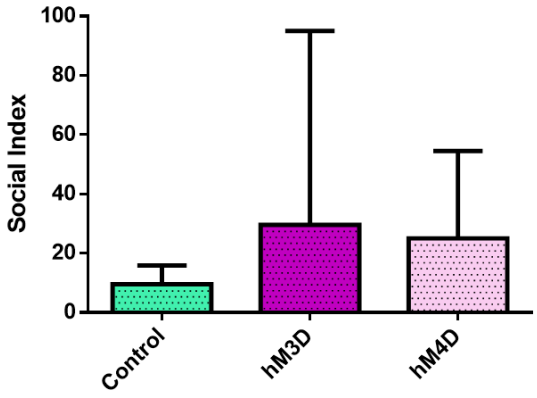

Figure 17. The social index of groups. (n=6 for control group; n=7 for hM3D group; n=5 for hM4D group). Kruskal-Wallis test.

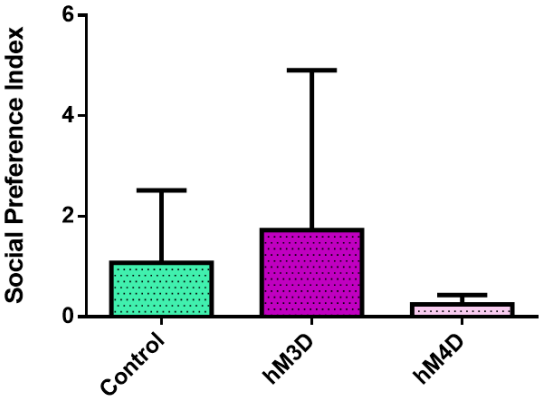

Figure 18. The social preference index of groups. (n=6 for control group; n=7 for hM3D group; n=5 for hM4D group). Kruskal-Wallis test.

4.4. T-Maze Test

Oxytocin-Ires-Cre mice with tdTOM, hM3D and hM4D virus-infected in PVN^{OT} neurons control, activation and inhibition groups respectively were subjected to the T-maze test to evaluate spatial learning and short-term memory. The T-maze spontaneous alternation test is an effective behavioral test that may effectively detect and assess the short-term memory loss. The side preference rate which is the ratio of the number of preferred side that the mouse has choosen to total runs performed was analyzed. According to Tukey's multiple comparision test, there was a significant increase in social preference rate of inhibition (hM4D) group compared to control and activation (hM3D) group (Figure 19). However, there was no significant difference in side preference between control and activation group (Figure 19).

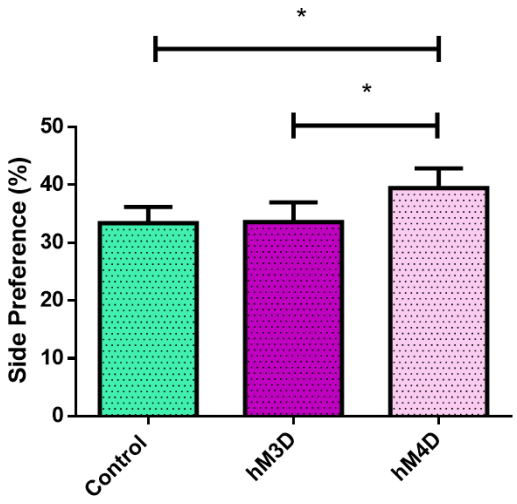

Figure 19. The side preference rate of groups. (n=6 for control group; n=7 for hM3D group; n=5 for hM4D group). One-way ANOVA, $p<0.05$, Tukey's multiple comparision test.

4.5. In vivo PFC^{DA} neuron recordings and the effect of acute activation of PVN^{OT} neuron via CNO administration

Ca^{2+} imaging by fiber photometry provides an opportunity to monitor the spontaneous firing of the desired neuron group or to instantly follow the electrical changes of specific neuron group[53]. In the present study, the effect of PVN^{OT} neuron's acute activation via CNO administration on the electrical properties of PFC^{DA} neurons was examined using a fiber photometry. pAAV-hSyn-GRAB_DA1h virus was injected into the prefrontal cortex of Oxytocin-Ires-Cre mice, which were first injected with pAAV-EF1a-DIO-hM3D(Gq)–mCherry virus in their PVN regions. At the same time, after the virus injection, a fiber cable with a diameter of 200µm was placed in the prefrontal region of the brain. After waiting for the virus infection period, Ca^{2+} signal was started to record. 20 minutes after recording began, mice were given i.p. CNO and acute activation of PVN^{OT} neurons was achieved. When the results were analyzed, no significant effect of acute activation of oxytocin on prefrontal dopamine activity was found (Figure 20).

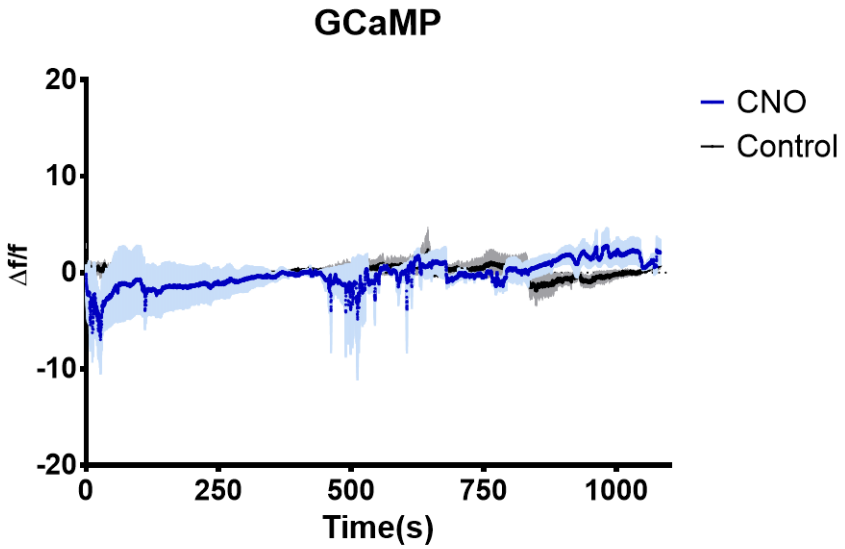

Figure 20. The graph representing Ca^{2+} signal recordings of PFC^{DA} neurons.

4.6. Imaging

20X images of control group expressing pAAV-CAG-flex-rev-tdTomato virus in their PVN oxytocin neurons; activation group expressing pAAV-EF1a-DIO-hM3D(Gq)–mCherry virus in the PVN^{OT} neurons, inhibition group expressing pAAV-EF1a-DIO-hM4D(Gi)-mCherry virus in the PVN^{OT} neurons were taken by using Zen Confocal microscopy.

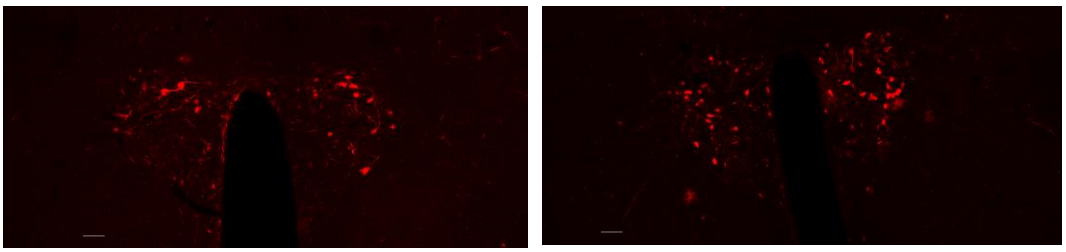

Figure 21. 20X Confocal images of tdTOM infected PVN^{OT} neurons of control group. Scale bar, 50μm.

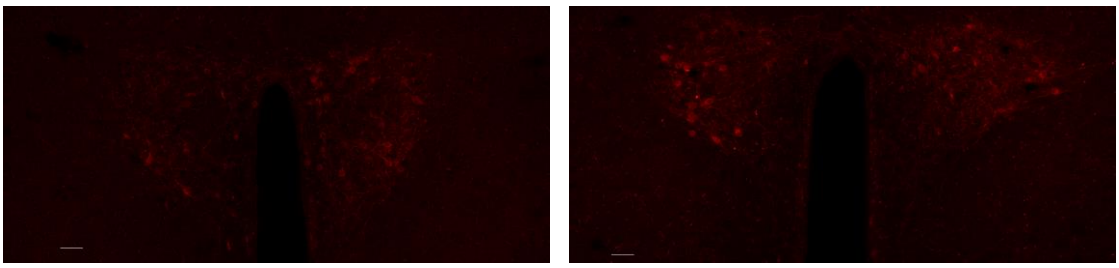

Figure 22. 20X Confocal images of mCherry infected PVN^{OT} neurons of hM3D group. Scale bar, 50μm.

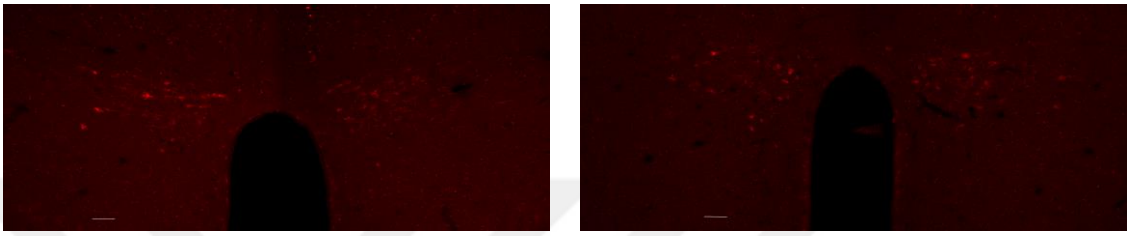

Figure 23. 20X Confocal images of mCherry infected PVN^{OT} neurons of hM4D group. Scale bar, 50μm.

5. DISCUSSION

Recent studies have indicated that genetic variations in OTR gene and oxytocin secretion have a crucial impact on various social behaviors including empathy, trust, pair bonding, anxiety, social interaction, emotion recognition and also locomotor activity.

Animal studies had demonstrated that anxious and stressful stimuli could trigger release of oxytocin within the PVN, indicating that it might play an important modulatory role in anxiety- and stress-related behaviors[23]. Mice with deleted oxytocin gene provide unique approach to understand the function of oxytocin in stress and anxiety-related behaviours and studies indicated that oxytocin -null mice demonstrated enhanced anxiety behavior. In this study, EPM test was used to evaluate anxiety-related behaviors of oxytocin-cre mice with tdTOM, hM3D and hM4D virus-infected PVN^{OT} neurons of control, activation and inhibition groups respectively. 30 minutes before all behavioral tests used in this study, i.p CNO (3mg/kg) was administered to oxytocin-cre mice and allowed acute (up to 2 hours) activation and inhibition of PVN^{OT} neurons. The EPM test was performed to investigate whether acute activation and inhibition of PVN^{OT} neurons resulted in anxiety-like behavior. The mice spend more time in closed arms of EPM rather than open arms indicated that the mice might be anxious. When the time spent in the open arm of the control, activation (hM3D) and inhibition (hM4D) groups were compared, no significant differences were found between the groups. When the time spent in the open arm and closed arm was compared within the group, it was found that the activation (hM3D) and inhibition (hM4D) groups spent significantly more time in the closed arms of EPM indicating that hM3D and hM4D groups showed anxiety-like behaviors. The same results in both hM3D and hM4D groups suggested that the acute activation and inhibition of PVN^{OT} neurons did not have indicative or sufficient effect on anxiety. On the other hand, no significant difference between open and closed arm durations was found in the control group indicating the mice in control group did not show anxiety-like behaviors.

The effect of endogenous oxytocin on locomotor activity was also investigated in this study. It had been suggested that oxytocin had dual effects on locomotor activity: at lower dosages, oxytocin slightly enhanced locomotor activity or had no significant impact while the higher doses of oxytocin indicated certain signs of sedative effects as seen by a decrease of locomotor activity and rearing (doses 1 and 3 mg)[29]. Open field test was used to measure locomotor activity and also anxiety-related behaviors of

oxytocin-cre mice with tdTOM, hM3D and hM4D virus-infected PVN^{OT} neurons of control, activation and inhibition groups respectively. When velocity indicating the locomotor activity of mouse was analyzed, no significant difference was found in the locomotor activity between groups. In addition, the time spent in the center zone of open field platform was also analyzed for the anxiety measurement and there was no significant difference in center zone duration between groups.

In this study, sociability and social nolvelty preference were evaluated in oxytocin-cre mice with tdTOM, hM3D and hM4D virus-infected PVN^{OT} neurons of control, activation and inhibition groups respectively. In the sociability session of the social interaction test, the time spent in the empty chamber and the chamber with stranger mouse was analyzed. More time spent in the chamber with stranger mouse compared to the empty chamber indicates normal sociability[51]. According to statistical analysis, it was found that the time spent in the chamber with stranger mouse was significantly higher than the time spent in the empty chamber within groups but not between groups. In the social novelty preference session of the experiment, the experimental mouse is expected to freely choose between the already-investigated mouse and the novel unfamiliar mouse, and spending more time with the novel mouse indicating intact social memory[51]. According to Sidak's multiple comparison test, no significant difference was found between and within groups except inhibition (hM4D) group which spend more time in the chamber with the already-investigated mouse compared to the time spent in the chamber with the novel mouse. Recent studies had shown that acute and chronic administration of oxytocin had been demonstrated to influence social behavior in rodent models, enhancing social memory and increasing social interaction time[28]. In this study, it was observed that acute inhibition of PVN^{OT} neurons caused a significant decrease in the social novelty preference. Thus, acute inhibition of PVN^{OT} neurons might affect and attenuate the social novelty preference.

In this study, the T-maze test was used to investigate the effects of acute activation and inhibition of PVN^{OT} neurons on short-term memory. The T-maze spontaneous alternation test is an effective behavioral test that may effectively detect and assess the short-term memory loss[52]. The side preference rate which is the ratio of the number of preferred side that the mouse has choosen to total runs performed was analyzed. According to Tukey’s multiple comparision test, there was a significant increase in social preference rate of inhibition (hM4D) group compared to control and activation (hM3D) group. However, there was no significant difference in side

preference between control and activation group. Thus, acute inhibition of PVN^{OT} neurons might reduce spatial learning and cause short-term memory loss.

The PFC consists of the dorsolateral prefrontal cortex (DLPFC) which is responsible for executive functions, the orbitofrontal cortex (OFC) which is involved in the regulation of impulses and emotions, and the anterior cingulate regions which are involved in processes such as attention, motivation, and memory. Hypofrontality refers to decreased activity of prefrontal cortex than normal. Hypofrontality can be detected in several neuroimaging investigations in various neurological disorders including, schizophrenia, attention deficit hyperactivity disorder (ADHD), bipolar disorder, and major depressive disorder.

Dopamine is produced by specialized neurons in the midbrain, which transmit their axons to a variety of brain areas, including the PFC via mesocortical dopamine pathway where it influences processess including attention, working memory and flexible behavior depending on the PFC. Thus, dopamine is crucial for cognitive control functions of the PFC. Once these neurons fire, dopamine is released widely into the neural tissue through terminal ends and varicosities affecting numerous PFC neurons.

Since dopamine is important and necessary for the control of the cognitive function of the PFC, low dopamine activity in the PFC may be associated with hypofrontality. In this study, hypofrontality was discussed and examined through the low dopamine activity in the PFC[9].

Dopamine and oxytocin systems are two important neuromodulators of brain functions displaying similarity in organization and projection sites. Oxytocin and dopamine neurons that control various social and affiliative behaviors project to similar forebrain regions contaning PFC.

Electrophysiological dopamine neuron recordings indicate that both optogenetic stimulation of oxytocinergic terminals and the oxytocin application to the bath suffice to enhance the activity of dopamine neurons in VTA but downregulate the dopamine neuron activity in the SNc. Release of oxytocin directly activates dopamine neurons in VTA but indirectly inhibits dopamine neurons in SNc through local GABA neurons[35]. Based on these findings, this study aimed to reveal the interaction of these two important neuromodulators in the PFC.

In this study, fiber photometry was used to investigate the effect of PVN^{OT} neuron's acute activation via CNO administration on the electrical properties of PFC^{DA} neurons. pAAV-hSyn-GRAB_DA1h virus was injected into the PFC of oxytocin-cre

mice, which were first injected with pAAV-EF1a-DIO-hM3D(Gq)–mCherry virus in their PVN regions. When the results were analyzed, no significant effect of acute activation of oxytocin on PFC^{DA} neuron activity was found. It was suggested that acute activation of PVN^{OT} neurons was not sufficient to alter the activity of PFC^{DA} neurons. It was suggested that oxytocin might have a regulatory role on dopamine activity by effecting indirect pathways such as glutamate and GABA pathways.

In this study, the effects of manipulation of oxytocin neurons on various behaviors was revealed. Thus, modulation of prefrontal dopaminergic neuronal activity by chemogenetic activation and inhibition of oxytocin neurons was examined for the first time.

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