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Kara A. Kittelberger Date

Novel Approaches to Enhance Social Cognition by Stimulating Central Oxytocin Release using a Melanocortin 4 Receptor Agonist

By

Kara A. Kittelberger

Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Neuroscience

Larry J. Young
Advisor

Shannon Gourley
Committee Member

Donald Rainnie
Committee Member

Sam Sober
Committee Member

David Weinshenker
Committee Member

Accepted:

Lisa A. Tedesco, Ph.D. Dean of the James T. Laney School of Graduate Studies

Date

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By

Kara A. Kittelberger

B.S., Northeastern University, 2009

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ABSTRACT

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The evolutionarily conserved neuropeptide oxytocin (OT) modulates sociocognitive functioning by coordinating activity across brain regions responsible for integrating the physiological, motivational, and associative properties of social stimuli. Recently, there has been strong clinical interest in pharmacological targeting of the OT system as a potential treatment of social impairments in psychiatric or neurodevelopmental disorders, including autism spectrum disorder (ASD). To date, effective manipulation of the central OT system in a behaviorally- and ecologically-relevant manner has remained elusive. Social attachment formation in the monogamous prairie vole (*Microtus ochrogaster*) is an ideal model for the study of OT-dependent sociocognitive behaviors with face, construct, and predictive validity for functional human social cognition. We propose here a novel method of enhancing OT-dependent social cognition and neuronal network processing through the use of a melanocortin 4 receptor (MC4R) agonist, Melanotan II (MTII). Stimulation of MC4Rs enhances stimulus-evoked endogenous central OT release. It is hypothesized that MTII could mirror the behavioral effects of direct exogenous OT, and previous studies in female prairie voles report enhancements in social attachment formation following MTII administration. To demonstrate equivalent efficacy of this novel potential treatment in both sexes, we first successfully replicated these published findings using male subjects. Quantitative analysis of reciprocal social interactions did not reveal any MTII-mediated positive shifts in affiliative social behaviors. We therefore hypothesized MTII administration, and consequent elevations in central OT release, may instead modulate neuronal network processing to affect the perception of social stimuli rather than social interactive behavior itself. In a social exposure context, but not in the homecage, MTII treatment robustly increases neuronal activation in brain regions known to mediate social reward and social motivation, the nucleus accumbens shell (NAcc shell) and prelimbic cortex (PLC). Context-dependent increases in NAcc shell and PLC activation following MTII treatment were significantly attenuated by selective blockade of OT receptors. These results support the hypothesis that MTII administration elevates endogenous central OT release in social contexts and positively modulates social salience processing. Enhancing endogenous OT release with MTII could have significant clinical utility as a pharmacological adjuvant to behavioral therapies, particularly those targeting social reward or social motivation.

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ABSTRACT

The central oxytocin (OT) system modulates neuronal activity across distributed cortical, midbrain, and basal forebrain circuits to affect multiple domains of sociocognitive processing. Central OT has been shown in multiple mammalian species, including humans, to modulate sociosensory information processing, social salience, social reward and motivation, and learning and memory. Many early seminal studies on the role of central OT in complex sociocognitive processes were performed in the socially monogamous prairie vole. Prairie voles are a unique model animal with high levels of affiliative social behavior, and have shown significant translational utility in the study of OT-dependent social cognition. The diverse neuromodulatory function of OT as it pertains to social functioning has generated considerable interest as a potential target for novel pharmacotherapies designed to ameliorate social deficits in psychiatric or neurodevelopmental disorders, including Autism Spectrum Disorder. Unfortunately, the physiological properties of the OT system have hindered the development of potent and stable oxytocinergic pharmacological treatment strategies. We propose that focusing on targets upstream of the central OT system is a viable and effective method of manipulating central OT levels to enhance social cognition. Stimulation of melanocortin 4 receptors (MC4Rs) located on OT neurons could enhance endogenously-evoked OT release in a behaviorally- and ethologically-relevant manner. Further study of the behavioral and physiological effects of MC4R agonists in the prairie vole model will support translation of this novel potential treatment of social deficits in human patient populations.

Introduction

Social interactions are an important component of animal life, critical for an individual's survival as well as reproduction and propagation of species. A wide range of diversity exists in the level of sociality displayed by various species. Some animals are relatively solitary, coming together only for the purposes of mating, while other species show high levels of sociality, including biparental care of young, group living, and cooperative behaviors [1]. Sociality is comprised of multiple subdomains of behavior, including correct detection of incoming sociosensory information, social recognition and memory, motivation to approach or avoid conspecifics, social reward, and attachment [1]. These complex sociocognitive processes require appropriate processing of social cues, encoding of rewarding or aversive valences, and learning and synaptic plasticity [2, 3]. Translating contextual stimuli into a behavioral response requires coordinated processing across distributed cortical, midbrain, and basal forebrain circuits [4]. Furthermore, it has been proposed that disrupted or aberrant processing within these networks could underlie pathological social deficits observed in some psychiatric or neurodevelopmental disorders, including most notably Autism Spectrum Disorder (ASD) [2, 5]. ASD is a neurodevelopmental disorder commonly characterized by disruptions in sociocognitive functioning, including impairments in social interaction, communication, and stereotyped or restricted behaviors and interests [6]. Deficits in the ability to engage in normal reciprocal social interactions can severely impact the quality of life for ASD patients, including decreased ability to form meaningful relationships with peers or family members, or limiting opportunities for workplace advancement [7-9]. While there are

currently no FDA-approved pharmacological treatments targeting the core social deficits of ASD, the neuropeptide oxytocin (OT) has recently emerged as an attractive potential therapeutic target. OT has been proposed to broadly modulate and coordinate responses within the distributed neural networks responsible for integrating the physiological, motivational, and associative properties of social stimuli in multiple species, including humans [10]. This ability to affect whole-brain network processing of social stimuli has made OT the subject of considerable study in the field of social behavior, and a prime candidate for the targeted development of pharmacological treatments able to ameliorate social deficits in disease states.

Oxytocin and Social Cognition

OT is a nine-amino acid peptide hormone produced primarily in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus [11]. OT, or its ancestral homologues, can be found in both invertebrate and vertebrate lineages, where it plays an important and canonical role in reproduction [12]. Over evolutionary time, and particularly in mammalian species, it appears the role of OT has expanded to incorporate not only the peripheral and physiological processes of reproduction but also maternal behavior and social behavior more generally [12].

Magnocellular OT-producing neurons in the SON and PVN send dense projection fibers to the posterior pituitary, where OT is released onto fenestrated capillaries to enter peripheral blood circulation [13]. Peripheral OT is important for coordinating the contractions of uterine muscles during parturition, and is essential for milk let-down during lactation [14]. In addition to these peripheral reproductive roles, central OT is now known to affect multiple aspects of social information processing to aid in

coordinating behavioral response to a diverse array of social stimuli. Sophisticated tract-tracing experiments have documented large caliber, unmyelinated axon collateral projections from magnocellular OT neurons of the PVN to numerous cortical, sensory, striatal, and limbic regions [15]. Complimentary electron microscopy examinations reveal high densities of OT-containing large dense core vesicles (LDCVs) near the cellular membrane of essentially all parts of magnocellular OT neurons, including the soma, dendrites, and the entire length of the unmyelinated axonal fibers. Interestingly, the central axon terminals themselves show little evidence of OT-containing LDCVs, and primarily form canonical asymmetrical glutamatergic synapses [16]. Central OT-release via exocytosis of LDCVs is stimulated by a rise in intracellular concentrations of Ca^{2+}, and this has been shown to occur semi-independently of axonal release within the posterior pituitary, in a context-dependent manner [13]. Local somatodendritic OT released within the PVN itself can travel through the extracellular space to distal target brain regions at distances of up to 4-5mm away via volume transmission [17]. Distal targeting can also be achieved through dendritic innervation of the third ventricle, allowing OT to be released directly into ventricular circulation [10]. Semi-localized OT release in a more region-specific manner has also been proposed, including release from axon collateral fibers, or dendrites. Given its relatively long half-life within the brain (approximately 20 minutes) and multimodal central release mechanisms, OT is exquisitely poised to act as a broad neuromodulator capable of coordinating neuronal responses across the brain with unique spatial and temporal characteristics distinct from classical neurotransmission at discrete synapses [13].

OT acts through a single G-protein coupled receptor subtype (OXTR) which can be found in multiple brain regions responsible for sensory information processing, attention orienting, motivation, reward and reinforcement, and learning and memory [18]. OT is a highly efficacious signaling molecule, able to stimulate its receptor with low nanomolar affinity. It has been suggested that a single OT-containing LDCV contains enough OT molecules to activate all the OXTRs within the immediate area of local release [17]. While there is a high degree of evolutionary conservation within the OT-producing system itself, species differences exist in the relative central distribution patterns of OXTR. Inter- and intraspecies OXTR distribution patterns reflect differences in social organization of the species, the primary sensory modalities engaged during species-specific social behaviors, and have in some instances been directly correlated with social behavioral diversity within species [19]. Thus, while there is a high degree of conservation of function for OT and its associated receptor, divergence in specific aspects of sociality can also be traced back to variations in the central OT system.

One particularly striking example of the interplay between central OXTR distribution and inter- and intraspecies differences in social behavior exists in comparative studies of Microtine rodents. The prairie vole (*Microtus ochrogaster*) is one of the ~3-5% of mammalian species known to display a socially monogamous mating strategy. Prairie voles form life-long selective social attachments (pair bonds) with opposite sex mating partners, displaying a strong preference for their pair-bonded partner over other novel opposite-sex individuals, mate-guarding behaviors, and biparental care of offspring [20]. Comparative studies of OXTR distribution in prairie voles and their closely related but promiscuous cousin species, montane voles (*Microtus montanus*) and

meadow voles (*Microtus pennslyvanicus*), suggest that differences in OXTR expression underlie species differences in mating strategies. Monogamous prairie voles, but not promiscuous montane or meadow voles, show high levels of OXTR in several mesocorticolimbic regions known to underlie social motivation and reward, including the nucleus accumbens (NAcc) and medial prefrontal cortex (mPFC) [21]. Pair bond formation in the laboratory is assessed using a partner preference test, in which a subject animal is first cohabitated with a partner under varying conditions and then subsequently tested in a choice assay. Time spent in direct social contact with the cohabitation partner or a novel opposite sex stimulus animal is then quantified. A pair bond, or partner preference, is said to be present when the subject animal chooses to spend a majority of test time in social contact with their cohabitation partner compared to a novel stranger [22, 23]. Partner preference formation has been well-characterized as a model of a complex sociocognitive process which requires social information processing, reward learning and social motivation [3]. Foundational studies using the partner preference testing paradigm reveal that blockade of OXTR signaling in either NAcc or mPFC, using site-specific injections of a highly selective OXTR antagonist prior to cohabitation and mating, is sufficient to prevent pair bond formation [21]. Furthermore, within the prairie vole species, genetic variation in *Oxtr* predicts relative expression levels of OXTR in the NAcc, and this intraspecies individual variation in *Oxtr* genotype is also positively correlated with pair bonding behavior [24]. Decades of extensive research have established pair bond formation in prairie voles as an ideal animal model of a complex sociocognitive process which relies on OT-dependent social information processing and the linkage of social cues with appetitive and motivational valences. Even beyond

exploring the discrete mechanisms of pair bond formation or maintenance itself, prairie voles as a highly social and affiliative model species are invaluable for the study of social cognition in general. Compared to rats or mice, prairie voles in the lab can display social memory which lasts weeks instead of hours, show overall higher levels of affiliative social behavior or alloparental care of young, and have even been show to engage in empathy-like consolation behaviors towards stressed mates [25]. As we will describe in greater detail in subsequent sections, studies in prairie voles have repeatedly complimented and expanded upon social behavior research from traditional rodent models. Furthermore, the prairie vole model has shown itself to have tremendous translational value for the study of behaviorally- and ethologically-relevant pharmacological manipulations of the OT system as potential treatments of social deficits in psychiatric or neurodevelopmental disorders.

OT modulates multiple levels of social information processing, social motivation, social memory, and functional connectivity across distributed brain loci and through evolutionarily conserved mechanisms [10]. This distributed and wide-ranging effect positions OT to act as the ultimate coordinator of social perception and behavior, binding together and integrating activity across cortical, limbic, and basal forebrain networks. In every social context an individual may find themselves in, broad neuromodulation, via OT, permits the emergence of discrete patterns of activity across neural networks. Thus, OT neuromodulation within distinct environmental and "neural contexts" functions to facilitate diverse, or even oppositional, effects; giving rise to exquisite variations and subtleties in social behavioral outputs.

Social Information Detection and Processing

Olfaction is the primary sensory modality engaged by rodent species during social interactions. Olfactory cues from conspecifics provide rodents with a variety of highly relevant social information, including the identity of an individual as known versus unknown, the potential for mating opportunities, and even relative health or potential parasitic load of others [1, 26, 27]. These cues can help orient the individual and then select an appropriate behavioral response of approach or avoidance. OT has been repeatedly shown to positively modulate the processing of rodent olfactory social cues across multiple brain nuclei, beginning in primary sensory processing regions and continuing throughout the duration of processing and ultimate behavioral output selection. The anterior olfactory nucleus (AON) provides top-down cortical control, via projections to granule cell interneurons, of activity within the mitral and tufted cells of the main olfactory bulb. These mitral and tufted cells are responsible for primary processing of odorant stimuli [28]. The rodent AON is innervated by OT afferents from the PVN, and in turn expresses high levels of OXTR [15, 28]. OT acting in the AON facilitates top-down recruitment of interneurons within the main olfactory bulb to increase overall inhibitory tone, enhancing odorant coding by improving the signal-to-noise ratio. Pharmacological blockade or selective ablation of OXTRs in the AON impairs social recognition in mice [28]. Interestingly, oxytocinergic modulation of socially-relevant odorant processing does not only occur in positive social contexts. Instead, OT functions to encode socially salient sensory cues regardless of valence. In an elegant series of experiments by Choe et al., male mice were first trained to associate a neutral odor with exposure to an estrus female. In this paradigm, mice will later significantly prefer that odor over another neutral odor in a probe trial. Blockade of

OXTR signaling during the initial odorant training session impairs the development of this positive odorant preference. This effect is specific to social-odorant pairings, as OXTR blockade does not affect the development of odorant preferences associated with non-social (sucrose) reward. In a complimentary experiment using an odor-driven aversion paradigm, a neutral odorant was paired with exposure to a previously aggressive mouse during training. This facilitates avoidance of the aggressor-associated odor in a probe trial. This effect is abolished by OXTR antagonist administration prior to training, even though it did not affect specific avoidance of the aggressive mouse during the training sessions themselves. Rather, it appears OT is necessary for the integrative encoding of a socially-relevant odor with salience regardless of appetitive or aversive valence, and this salience encoding in turn can facilitate appropriate selection of future behavioral outputs [29].

In primate species, including both non-human primates (NHP) and humans, vision is the primary sensory modality used in the transmission and detection of sociosensory information [19]. Vision is important not just for observing the environment around an individual or detecting the location of conspecifics, but also for discrete aspects of social communication such as gaze following or eye contact. Recent advances in the development of highly sensitive and selective radiolabeled OXTR ligands have enabled detailed mapping of OXTR distribution in NHPs, and are currently being applied to human brain tissue as well. In four species of NHP, there is a striking and conserved pattern of OXTR expression in brain regions critical for primary visual processing, control of gaze direction or saccadic eye movements, and attentional orienting to visual stimuli [30]. Additionally, strong OXTR expression is observed in the nucleus basalis of

Meynert (NBM) [30]. The NBM is one of the primary sources of acetylcholinergic inputs to the rest of the brain, including the amygdala, and provides important coordinated modulation of motivation and selective attention. In humans, intranasal administration of exogenous OT (IN-OT) has repeatedly been reported to increase attention and focus towards the eye region of faces [31, 32]. It has further been suggested OT may modulate both overt and covert visual-attentional shifts at even the very earliest stages of attentional processing [33].

Eye tracking experiments across the lifespan of persons with ASD reveal abnormal patterns of eye movements in response to both images and videos of human faces [34]. These abnormalities can include avoidance of the eye region, decreased attentional bias toward faces, or increased fixation on the nonsocial aspects of a presented social scene image. In children as young as 12 months of age, reductions in eye gaze or joint attention have recently been reported as one of the earliest markers of ASD phenotypes. These deficits are predictive of both later ASD diagnosis and ultimate symptom severity of social deficits later in life [35]. In youths, adolescents, and adults with ASD, acute experimental IN-OT treatment increases eye contact and reduces eye gaze aversions [36, 37]. While there are caveats associated with the interpretation and ultimate applicability of IN-OT as a clinical treatment strategy, which we will discuss in detail in a later section, these preliminary findings nonetheless suggest proof-of-principle efficacy of targeting the OT system to ameliorate social deficits in ASD.

Social Salience

Sociosensory information initially processed in primary sensory areas is later relayed to basal forebrain regions, including the amygdala. The capacity of the amygdala to

integrate sensory information with emotional salience encoding has been recognized for well over 100 years. In 1888, Schäfer and Brown performed a bilateral ablation of the temporal lobe of a rhesus macaque. In reporting their findings, they describe, "A remarkable change…is manifested in the disposition of the Monkey…He gives evidence of hearing, seeing, and of the possession of his senses generally, but it is clear that he no longer understands the meaning of the sounds, sights, or other impressions that reach him."[38] Today, a large body of literature converges on a general understanding of the amygdala as a collection of parallel circuits capable of processing and responding to multiple and even divergent aspects of emotionally salient stimuli [39]. The subregions of the amygdala are heavily interconnected and function together to process and encode emotionally and behaviorally salient stimuli, before ultimately sending outputs to cortical, striatal and limbic brain regions. The basolateral complex of the amygdala (BLA) shows heavy reciprocal connections with midline and orbital prefrontal cortices, as well as the hippocampus and sensory association areas [40]. The predominantly unidirectional output targets of the BLA include the NAcc, a critical integration site at the interface of emotion, motivation, and behavioral output [41]. The medial amygdala (MeA) receives sensory information inputs and ultimately sends outputs to the NAcc by way of local connections to the BLA [14]. In rodents, the MeA receives direct input from the vomeronasal system via the accessory olfactory bulb, and indirect input from the main olfactory system [14]. In this way, the MeA is positioned to facilitate the integration of sensory information with emotional salience, and ultimately engages attentional processes to further enhance encoding of incoming sensory information [39]. Thus, the integrational processing functions of the MeA aid in the final selection of

appropriate behavioral responses. Linkage of sociosensory information with salience coding is a critical component of social cognition, as the salience of a given stimulus can function to either recruit additional attentional mechanisms to reorient an individual and promote social engagement, or alternatively signal a stimulus is irrelevant and may be filtered out.

The MeA shows strong OXTR expression in multiple rodent species, and receives dense OT innervation from the PVN [16, 21, 42]. In transgenic knockout mice lacking the gene which codes for OT (OTKO), the MeA shows relatively sparse expression of the immediate early gene product Fos, a proxy marker of neuronal activation, following a non-sexual social encounter [42]. OTKO mice display profound deficits in social recognition, despite retaining full functioning of odorant discrimination abilities when tested with non-social odorant cues [43]. This selective social recognition deficit can be restored by site-specific infusion of OT into the MeA [42]. In both male and female prairie voles, cohabitation and social interaction with a mating partner activates the MeA, as visualized by elevated Fos expression compared to animals not exposed to social stimuli [44-46]. Lesion of the MeA in male prairie voles disrupts social recognition of a mating partner, as well as the expression of affiliative or parental behaviors [47]. Interestingly, a large and novel population of dopaminergic neurons within the MeA has been described in prairie voles which are not predominantly found in non-monogamous mice or rats. There are 3-5 times as many of these dopaminergic MeA neurons in male prairie voles as compared to females, however female prairie voles still show greater abundance when compared to non-monogamous rodents [48]. These neurons show elevated levels of Fos expression following social interaction [46]. It has been proposed

that one of the key mechanisms by which OT acts to enhance social cognition is via regulation of the salience coding and attention reorienting signals of dopamine. The positive and synergistic effect of convergent OT and dopamine signaling occurs in multiple mesocorticolimbic loci, however the specific interaction effect as it relates to attention reorienting has been suggested to primarily be attributable to processing within the amygdala [1]. Thus, it can be hypothesized that OXTR activation in the MeA of prairie voles could recruit engagement of these novel dopaminergic neurons, either directly or indirectly, to markedly enhance the salience encoding and attention-orienting capacity of partner-specific odorant cues in a manner unique to social attachment formation in a monogamous species. This now highly salient sensory information could then be relayed, via glutamatergic projections of the BLA, to other mesocorticolimbic targets known to serve as sites of convergence for OT and dopamine in the formation of selective social attachments, including the NAcc and mPFC.

In humans, a great deal of study has been conducted investigating the effect of IN-OT administration on amygdalar activation patterns in a variety of paradigms. In an early fMRI study, IN-OT was reported to suppress amygdala activation in response to social threats in healthy subjects [49]. Since then, it has been reported that IN-OT in humans reduces amygdala activation in response to negative emotional expression (images of angry, fearful, or sad faces), imagining partner infidelity, listening to an infant cry, seeing others in pain, and negative social interactions (experiencing betrayal, social evaluative threats, unreciprocated cooperation attempts) [50-62]. Further, functional connectivity between amygdala and brainstem is reduced in healthy subjects receiving IN-OT [49]. Other studies report IN-OT decreases functional connectivity between

amygdala and areas in the occipital and inferior temporal cortex during social tasks [50, 63, 64]. In this way, OT is perhaps modulating the social fear inducing effects of negative social interactions by turning down the intensity of aversive salience coding within the amygdala. These results could also be reflective of IN-OT acting to suppress amygdalar output to modulate the downstream impact of aversively-encoded social stimuli on future social behavior. In positive social contexts, IN-OT can increase functional connectivity between amygdala and other regions of the social salience network, including the caudate and insula, and the intensity of this coupling is associated with improved social learning [65]. These apparent context-dependent effects of IN-OT on patterns of human amygdala activation and functional connectivity further support the social salience hypothesis of OT. In humans, as in rodents, it appears that OT is capable of modulating the processing of socially-relevant information regardless of valence. It is important to note that while the canonical terminology of fMRI literature uses language which describe regions that are "activated" or "inhibited", it is not possible to determine which neuronal subtypes or local processing mechanisms are engaged just by viewing the BOLD response, nor can we say with absolute certainty if BOLD responses are reflective of a brain regions' ultimate output intensity. Some contemporary theories have suggested the localized field potential and local neuronal processing are the best correlates of the fMRI BOLD response in a given brain region. This local processing would be reflective of both subthreshold local neuronal activity and the magnitude of converging synaptic inputs to the area [66, 67]. Following this theory, it would perhaps be more appropriate to interpret BOLD responses as a reflection not of downstream outputs but instead processing of input signals. Put more simply, it may reflect what a given brain region is

"hearing" rather than what it is "saying". Regardless of the ultimate or proximate causes of alterations in BOLD response following IN-OT administration, it remains clear that OT modulates the processing of socially salient cues in a manner that influences neuronal activity within the amygdala, as well as its functional connectivity with cortical, limbic, and brainstem regions. These IN-OT evoked changes in either regionally-restricted or functionally correlated activity patterns of the amygdala, and its associated inputs and outputs, could explain some of the observed behavioral enhancements in social cognition following acute administration. IN-OT enhances the ability to accurately detect and identify emotional expression in faces, including during paradigms where the amount of social information is restricted in some manner. In the Reading the Mind in the Eyes Task (RMET), participants are asked to identify the primary emotion of another individual by viewing images which exclusively contain the eye region. This task engages emotion recognition skills but is also thought to involve mentalizing or Theory of Mind, and IN-OT has repeatedly been shown to enhance accuracy and performance in RMET in healthy subjects [5, 33, 68].

In patients with ASD, abnormal BOLD responses in the amygdala are observed during tasks requiring social perception of faces and eyes, including RMET [69, 70]. Acute IN-OT improves behavioral and neural responding, bringing the ASD patients closer to the brain activation patterns and behavioral performance levels observed in healthy subjects [71]. Altered amygdala responding could suggest social interactions are perceived as more stressful or intense for persons with ASD. Exogenous OT treatment could attenuate this aversive encoding, potentially acting to positively shift the valence of social interactions in general.

Social Motivation and Social Reward

Motivation can generally be defined as "the set of processes through which organisms regulate the probability, proximity, and availability of stimuli."[72] Behavioral motivation to engage or avoid a given stimuli is inherently linked to positive (hedonic) or negative (aversive) emotional elements. The generation of a motivated behavior emerges through coordinated processing across reciprocally connected regions which encode salience, assign valence, and initiate appropriate behavioral output selection mechanisms [4]. Behaviors which are critically important for a species, such as feeding or mating, act as natural (primary) rewards, and are thus generally associated with cues that are highly salient, positively-valenced, and motivating [1]. Social interactions with conspecifics can serve as natural rewards, and therefore engage several brain regions responsible for mediating behavioral motivation. Furthermore, plasticity within these networks can generate behavioral flexibility, permitting the incorporation of new and salient social information into decision-making processes which guide future behaviors. Social salience encoding is directly complimented by valence encoding, and oxytocinergic modulation is integral to both processes [33]. As we have noted in the previous section, sociosensory cues endowed with salience encoding during processing in the amygdala are in turn transmitted, via glutamatergic projections, to the NAcc. The NAcc also receives glutamatergic projections from the hippocampus and mPFC, dopaminergic inputs from the VTA, and OT innervation emanating from the PVN [15, 16, 41, 73]. Downstream projections to motor output systems position the NAcc as the limbic-motor interface. The NAcc consolidates saliency from the amygdala, context from the hippocampus, and goal-

directed information from the mPFC to ultimately influence behavioral output, "translating the will into action" [72] (Figure 1.1).

Much of what we now know about the role of OT in social reward comes from work in prairie voles. As we have noted previously, individual variability in *Oxtr* genotype predicts both NAcc OXTR expression and individual differences in pair bonding behavior [24]. Pharmacological blockade of either OXTRs or D2 dopamine receptors in the NAcc shell prevents mating-induced pair bond formation. Furthermore, OT microinjection into NAcc shell facilitates pair bond formation, and this effect is blocked when OT is co-injected with a selective D2 receptor antagonist. Alternatively, pair bond formation can be induced by microinjection of a D2 receptor agonist into NAcc shell, but this effect is blocked by co-injection of a selective OXTR antagonist (OTA) [74]. Thus, pair bond formation requires simultaneous and coordinated activation of both OXTRs and D2 dopamine receptors in the NAcc shell. It has been suggested that the interactive effect of OT and dopamine in the prairie vole NAcc shell engages synaptic plasticity mechanisms. Modifications of synaptic inputs onto NAcc shell would ensure that future exposure to the olfactory signature of the partner would be accompanied by enhanced activation of the mesocorticolimbic reward system, motivating an individual to remain in close proximity and continue engaging socially with their partner [14]. In support of this hypothesis, it was recently shown that mating in female prairie voles, which precipitates pair bond formation, enhances functional connectivity between the mPFC and NAcc shell. This mPFC modulation of NAcc shell activity was shown to causally predict the emergence of affiliative huddling behavior between mates. Females which showed greater strengthening of mPFC-NAcc shell net modulation after mating

ultimately went on to display more affiliative huddling behavior throughout the cumulative duration of a 6-hour cohabitation. This mating-enhanced rhythmic action of mPFC on NAcc shell is suggested to engage plasticity mechanisms that alter how the NAcc responds to partner cues in the future [75]. It is unlikely that these mechanisms of NAcc synaptic plasticity are not applicable to other types of social rewards in a more general sense, and there is some evidence they do occur in other species. Based on early comparative studies using receptor autoradiography to map central OXTR distribution, sparse NAcc OXTR expression in mice was interpreted to mean that accumbal OT played relatively little role in non-monogamous murine social behavior. Contemporary studies have updated this view and we now know that activation of OXTRs in the NAcc shell of mice is necessary for social reward. Site-specific infusion of OTA into the NAcc shell blocks the development of social conditioned place preferences. It was further shown that OT acting in the NAcc shell engages synaptic plasticity mechanisms, specifically long-term depression, and that these specific synaptic modifications are necessary for social experiences to be perceived as rewarding [76].

IN-OT in healthy human subjects increases activity in multiple dopaminergic reward regions while viewing positive social stimuli (e.g. own child or partner's face, happy faces), including NAcc, dorsal striatum, insula, and VTA [56, 77-79]. These same dopaminergic regions are activated in anticipation of social reward, or engaging in cooperation with others [68, 77, 80]. When heterosexual men are given IN-OT and presented with images of women's faces, including their own partner, men rated their partners as more attractive and showed increased activation in NAcc while viewing their partner's image [81]. Interestingly, it has been reported that polymorphisms in the *Oxtr*

gene in youths with ASD are associated with abnormalities in resting state functional connectivity between the NAcc and PFC, potentially reflecting a genetic predisposition towards a more inefficient modulation of PFC-to-NAcc inputs important for the processing of social reward [82]. In youths with ASD, IN-OT followed by video presentations of biological motion increased functional connectivity between NAcc and PFC [83]. Additional IN-OT evoked enhancements in functional connectivity between NAcc and cortical regions are observed during auditory presentation of happy voices. This enhanced NAcc-seeded functional connectivity is not observed when participants listen to angry voices [83]. As we have seen in prairie voles, enhanced connectivity within this pathway is associated with appetitive modifications in the representation of social reward value and is positively associated with increased social motivation. Taken together, these results suggest that the strength of NAcc connectivity with associated mesocorticolimbic reward regions can be modulated by OT in humans in a manner similar to what is observed in rodent models. Thus, in humans, as in other species, OT signaling participates in the assignment of valence to social stimuli. In positive social contexts, encoding of social stimuli with a hedonic valence would motivate continued social engagement. In persons with ASD, pharmacologically enhancing the perception of social interactions as rewarding by targeting OT could ultimately increase their level of social motivation, leading to positive shifts in an individual's propensity to initiate or sustain social interactions.

Social Memory

The process of conferring salience and valence onto sociosensory cues would be of little long-term use to an individual without simultaneous recruitment of memory encoding

mechanisms. The hippocampus has long been recognized as a primary site of memory processing. In both rodents and NHPs OXTR expression has been reported in several subregions of the hippocampus [19]. Furthermore, the modulation of hippocampal synaptic plasticity mechanisms relevant for social memory storage are strongly regulated by activation of hippocampal OXTRs. The fidelity of social information transfer in the hippocampus is improved by OT, which enhances of the firing rate of fast-spiking interneurons [84]. This modulation functions to suppress background firing rate of CA1 pyramidal neurons, and ultimately increase the signal-to-noise ratio. The hippocampus shares reciprocal connections with the mPFC, which likely serves to integrate contextual or environmental information to coordinate and select appropriate behavioral responses [72]. In mice, social recognition and social discrimination are dependent on CA1 pyramidal cells of the ventral hippocampus which project to the NAcc shell. Inhibition of these pyramidal cells, or their terminals within the NAcc shell, disrupts performance in a social discrimination task. Ventral CA1 pyramidal neurons activated during social exposure to a specific stimulus mouse are preferentially reactivated by re-exposure to that same mouse, as compared to exposure to a second novel mouse. While the ventral CA1 also shows strong projections to the olfactory bulb and the BLA, it is the specific projections to the NAcc shell that are both necessary and sufficient for social discrimination performance in mice [85].

In both NHPs and humans, electrophysiological recordings from the temporal lobe, including the hippocampus, reveal large populations of neurons that preferentially respond to socially relevant cues such as faces or voices [86-88]. In humans, these recording experiments have further shown some of these socially-responsive

hippocampal neurons can be preferentially activated by viewing famous or personally relevant faces as compared to unfamiliar individuals [89]. IN-OT enhances memory for faces, and it has been proposed that this effect may be mediated by oxytocinergic enhancements in social memory encoding [90, 91]. In healthy human populations, single nucleotide polymorphisms in the *Oxtr* gene have been associated with face-recognition skills, and carriers of the specific *Oxtr* SNP rs53576 GG allele are likely to show increased local volume of the left hippocampus [92-94]. Furthermore, GG homozygotes of the rs53576 SNP are more responsive to the behavioral effects of IN-OT in some social tasks [95, 96].

Oxytocin and modulation of the social salience network

Successful adaptation to complex social environments requires flexible behavioral strategies. Across mammalian species, OT recruits synaptic plasticity mechanisms in the mesocorticolimbic reward pathway and the social salience network in a diverse array of social contexts. Oxytocinergic modulation of these networks and pathways facilitates the integration of an individual's internal physiological state with socially-relevant sensory, contextual and motivational information. Thus, social behavioral flexibility is achieved through coordinated whole-brain neuronal network modulation to support the emergence of a highly adaptable repertoire of social behavioral responding. The remarkable evolutionary conservation of OT function supports the use of animal models in translational studies which have direct relevance to human social cognition. As we have seen, much of what we know about OT from studies of rodent social behavior can be recapitulated when those same domains of social cognition are probed in human studies. Furthermore, the mechanisms underlying healthy functional social cognition provide an

important starting point for the development of targeted pharmacological treatments of social deficits in psychiatric or neurodevelopmental disorders, including ASD. While our current understanding of the true etiology of ASD is relatively limited, this does not prevent the exploration of potential novel treatments. In fact, treatment targets need not inherently be tied to the ultimate or proximate disease mechanisms themselves, and perhaps this disconnect may be a benefit rather than a detriment. Interestingly, despite the clear importance of OT in social behavior, there is relatively limited evidence for the existence of widespread pathological genetic polymorphisms in *Oxt* or *Oxtr* in ASD patients, and the findings that do exist often do not replicate in different ethnic populations or when statistical corrections for multiple comparisons are appropriately applied [6, 97-106]. Although perhaps surprising, from a treatment standpoint these findings can be interpreted in a positive light. If instead there was, for example, very strong evidence of polymorphisms in *Oxtr* which significantly affected the efficacy of OXTR signaling in response to ligand binding, it would be inherently much more difficult to target treatments to the OT system. Fortunately, the apparent lack of direct connections between aberrant OT signaling and ASD etiology has not deterred pre-clinical investigations of OT manipulations as a method of augmenting sociocognitive functioning. Instead, a growing body of literature strongly supports the notion that OT-targeted pharmacotherapies have enormous potential in the treatment of social deficit symptoms in multiple psychiatric or neurodevelopmental disorders.

Pharmacological Targeting of the Oxytocin System

Despite the immense theoretical promise of manipulating the OT system in the treatment of social deficits, significant barriers exist to the practical application of this approach.

While biologically stable preparations of OT, such as pitocin, have long been approved for clinical use in accelerating labor, the biochemical properties (pharmacodynamics) of OT prevent successful repurposing of these peripherally-acting compounds [107]. OT is a large, charged molecule whose polarity and size itself significantly impede its ability to cross the blood-brain barrier (BBB) [108]. In early calculations derived from experiments using subcutaneous OT injection in rats it was estimated that just 0.002% of injected OT is able to penetrate the central nervous system [109]. Furthermore, because of ubiquitous aminopeptidases, OT is relatively unstable over longer timespans, showing a half-life of approximately 20 minutes in the brain and only 3-8 minutes in peripheral circulation [110]. These unfavorable biochemical properties of OT itself are limiting factors for peripheral administration if the aim is to ultimately affect central OT signaling. Multiple alternative strategies have been developed to attempt to circumvent these physiological hurdles. These include alternative routes of administration, novel synthetic OT receptor agonists or positive allosteric modulators, and ligands targeting receptors upstream of OT to facilitate or enhance endogenous release.

Intranasal Oxytocin

Previous sections have highlighted just a small fraction of currently existing IN-OT studies seemingly demonstrating effectiveness in nearly every subdomain of social cognition, in both healthy and patient populations. While we have touched upon many of the seminal and relevant IN-OT findings above, it is necessary to discuss some of the major caveats associated with this route of administration, and the ways in which research in this field is conducted which could contribute to sentiments which have been described by some as, “irrational exuberance” [111].

Due to the limitations of traditional peripheral routes of administration, IN-OT was initially proposed as a way to target an area thought to be a "weak spot" in the BBB, the nasal epithelium. There are two proposed primary mechanisms by which IN-OT could bypass the BBB to gain access to central OXTRs. The first hypothesis suggests IN-OT may become internalized into olfactory or trigeminal neurons. Following internalization, IN-OT molecules could be subject to axonal transport and subsequent exocytosis into the brain. This explanation has been criticized based upon doubts as to whether IN-OT molecules could survive internalization without being degraded, and the proposed time course which suggests it would take several hours for substances to enter the brain via axonal transport [112]. Another potential route of entry could be passage through intracellular clefts within the subarachnoid space, however transport across this membrane is not widely acknowledged as a significant point of entry for solutes into the brain [113]. Despite the anatomical features of the arachnoid membrane which create an effective barrier to entry, it is possible that accumulation of supraphysiological levels of OT in the subarachnoid space could create a strong enough concentration gradient to support a type of non-specific passage across the BBB. Currently established dosages for IN-OT in human and animal research far exceed physiological levels, often surpassing the average total pituitary content in one single bolus dose. This could, in theory, create a strong concentration gradient as referenced above, and promote central penetrance [110].

Several studies have attempted to demonstrate central penetrance by combining IN-OT administration with subsequent sampling of the cerebrospinal fluid (CSF) at varying timepoints in several species, including rats, mice, humans, and macaques. While some of these studies report no significant increases in CSF OT concentration, of

those that do report significant increases the findings can be summarized to estimate that only 0.005% of IN-OT enters CSF within 1 hour [110, 114-118]. One alternative explanation of how IN-OT could affect behavioral changes despite poor central penetrance is a feed-forward mechanism whereby activation of peripheral OXTRs, via some unspecified mechanism, promotes activation of hypothalamic OT neurons to ultimately stimulate endogenous central release. In a recent study using d5-deuterated OT and a sensitive quantitative mass spectrometric assay designed to distinguish between d5-OT and endogenous (d0) OT, Lee et al. compared the efficacy of IN and intravenous (IV) routes of OT administration on central penetrance and ability to evoke endogenous central OT release. After both IN and IV d5-OT administration, d5-OT was identified in plasma and CSF. Surprisingly, subjects receiving IV-OT showed significantly higher levels of d5-OT in CSF compared to IN-OT subjects, seemingly dispelling the myth of a "privileged" nose-to-brain route of central entry. Furthermore, there was no evidence of a feed-forward effect on endogenous d0-OT, as neither plasma nor CSF concentrations of d0-OT were increased up to 60 minutes after d5-OT administration [116].

Finally, methodological and statistical caveats exist within the IN-OT literature (see Walum et al. for a thorough analysis and review). Briefly, many of IN-OT studies appear to be woefully underpowered, employ questionable statistical methods, and are likely subject to significant publication bias [111]. Taken together, this leads to an increased chance that published positive findings do not, in reality, represent true effects. Despite these issues, it is unlikely that *all* published IN-OT findings are false positives or the result of "tortured data". Thus, while caution should be exercised in interpretation of IN-OT studies, there is still justified excitement at the prospect of manipulating OT

circuitry in the treatment of psychiatric or neurodevelopmental disease. Furthermore, multiple alternative methods of stimulating the OT system currently exist, and still more are being actively developed and investigated.

Melanocortin 4 Receptor Agonists and Endogenous Central Oxytocin Release

One particularly promising method of manipulating central OT is to use ligands which activate receptors expressed on OT neurons which then promote the release of endogenous central OT. Melanocortin 4 receptors (MC4Rs) are expressed on magnocellular OT neurons in the PVN and SON, and stimulation of MC4Rs has been shown to enhance somatodendritic OT release both *in vitro* and *in vivo* [119]. This novel method of stimulating central OT release is particularly attractive because selective, brain-penetrant MC4R agonists already exist. In the prairie vole model, peripheral administration of Melanotan II (MTII), a synthetic and brain-penetrant selective MC4R agonist, prior to cohabitation facilitates the OT-dependent sociocognitive process of pair bond formation in females, as shown by partner preference testing. Importantly, this behavioral effect is long-lasting. Females injected peripherally with MTII prior to cohabitation but then separated from their mate still show a preference for their cohabitation partner when tested 7 days later. Females treated with saline show no preference for their cohabitation partner either directly after cohabitation or in the 7-day separation paradigm. Additional experiments reveal that peripheral MTII in female prairie voles does not enhance NAcc OT release on its own. Instead, MTII potentiates OT release in response to osmotic challenge with hypertonic saline, a physiological stimulus that in and of itself evokes central OT release [120]. This suggests that administration of MTII, or other MC4R agonists, can increase central OT levels in a

context-dependent manner. Furthermore, this enhanced endogenous OT release is targeted to brain regions which are responsive to OT, as it harnesses existing OT circuitry. Context-dependent enhancement of endogenous OT release is far superior to IN-OT, which provides a bolus dose of OT that is completely non-specific or centrally targeted, and not directly associated with a context. While these early behavioral and neurophysiological results in prairie voles are incredibly promising, stimulation of MC4Rs can also affect central signaling in other systems known to modulate pair bond formation, including dopamine, opioids, and CRF [121]. Furthermore, it is important to investigate the behavioral and neurophysiological effects of MC4R agonists in males as well as females. There is a strong sex difference in ASD prevalence; the current diagnosis rate for males is 1 in 42, for females the rate is 1 in 189 [5]. Studies which combine behavioral testing in the prairie vole model with a thorough analysis of neuronal network activation patterns will provide the best information to inform potential future applications of MC4R agonists to clinical treatment of social deficits in ASD or other psychiatric or neurodevelopmental disorders.

Conclusions

The OT system acts to broadly modulate multiple sociocognitive domains of neuronal network processing. Coordinating activity across nodes of the Social Salience Network and the mesocorticolimbic reward pathway enhances an individual's adaptability to a variety of social contexts. Of particular interest to the field of psychiatry is OT's ability to enhance social motivation and social reward in positive social contexts and dampen social fear processes which might prevent future social engagement. Furthermore, it has been suggested that OT pretreatment could amplify the benefits of behavioral therapies

currently used with children with ASD. Behavioral-based therapies show efficacy in improving social responding and reciprocity in children with ASD however they often require intensive weekly sessions sometimes in excess of 30 hours per week over many years [5]. Integration of OT pretreatment could accelerate the benefits of behavioral-based therapies such as the Early Start Denver Model, which has the explicit target of increasing a child's interest in affective social engagement and social reward sensitivity, both of which are sociocognitive domains sensitive to OT [122]. While early pilot studies provide strong evidence for the use of OT-based treatment strategies in ASD patient populations, further study is needed to determine the best method of manipulating central OT. IN-OT in both healthy and patient populations seems to affect multiple aspects of social cognition and behavior, however it is unclear if this is directly related to central penetrance of exogenous OT. To circumvent the potential limitations of IN-OT, brain-penetrant MC4R agonists could prove to be an alternative and superior method of manipulating central OT release in a context-dependent and behaviorally-relevant manner. The prairie vole model provides unique opportunities to study a complex OT-dependent sociocognitive process using invasive techniques with cellular resolution only possible in animal models.

The experiments presented in this dissertation will outline behavioral and neuronal effects of MC4R agonists in the prairie vole model. The results described shed light on the cellular mechanisms by which MC4R agonists engage the Social Salience Network to facilitate enhancements in social cognition. These data support the translational utility of the prairie vole model in the study of social cognition, and provide strong evidence that MC4R agonists increase central OT release in a behaviorally- and

ecologically-relevant manner. Based on these results, we propose MC4R agonists should be investigated in human ASD patient populations for safety and efficacy in the treatment of social deficits in disease states.

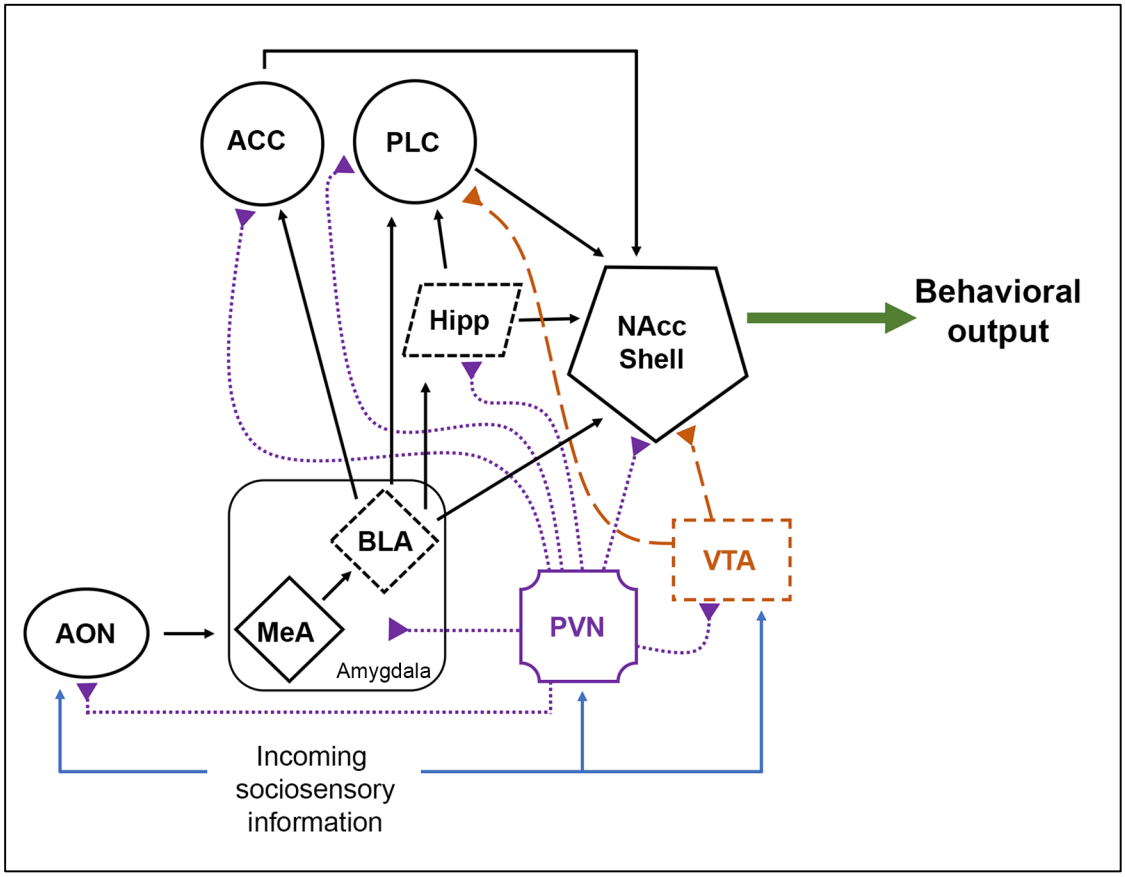

Figure 1.1. Oxytocin modulates neuronal activity across multiple regions of the Social Salience Network. Magnocellular OT-producing neurons in the paraventricular nucleus of the hypothalamus (PVN, purple) send oxytocinergic projections to multiple brain loci which comprise the Social Salience Network. Additional neuromodulation in this circuit can be facilitated via dopaminergic projections from ventral tegmental area (VTA, orange) to mesocorticolimbic regions of the nucleus accumbens shell (NAcc shell) and the prelimbic cortex (PLC), a subregion of the medial prefrontal cortex (mPFC). Brain regions outlined with a solid line represent loci analyzed for neuronal activation patterns in Chapter 3, loci outlined with a dashed line represent important nodes in the Social Salience Network that were not analyzed for neuronal activation. Note that not all known connections within this network are represented in this figure. Abbreviations: anterior olfactory nucleus (AON), anterior cingulate cortex (ACC), basolateral amygdala (BLA), hippocampus (Hipp), medial amygdala (MeA).

CHAPTER 2:

Behavioral effects of melanocortin 4 receptor agonists in prairie voles

ABSTRACT

Central oxytocin (OT) is an evolutionarily conserved neuromodulator capable of broadly coordinating social responses in both animal models and humans. This has led to strong clinical interest in the development of OT-targeted pharmacotherapies for the treatment of social deficits symptomatic of psychiatric or neurodevelopmental disorders, including autism spectrum disorder (ASD). Effective targeting of the central OT system is difficult using currently available pharmacological agents, however recent work has identified melanocortin 4 receptor (MC4R) agonists as a novel method of enhancing endogenous central OT release in a behaviorally- and ecologically-relevant manner. Social attachment formation in the socially monogamous prairie vole (*Microtus ochrogaster*) is proposed as an animal model of a OT-dependent complex sociocognitive process with face, construct, and predictive validity for human social cognition. In this chapter, we present data showing that MC4R agonists enhance social cognition and social attachment formation in male prairie voles, replicating previously published behavioral effects in female prairie voles. These results are consistent with the known behavioral effects of direct central administration of exogenous OT in prairie voles. MC4R agonist-mediated enhancements in social attachment formation in males or females do not appear to be the result of positive shifts in the quality or quantity of reciprocal social interactions with an opposite sex stimulus animal. This suggests MC4R agonist evoked OT-release could instead modulate the perception of social stimuli rather than social interactive behavior itself. This novel application of MC4R agonists in a social behavioral paradigm represents a new strategy for enhancing central OT signaling in the clinical treatment of social impairments.

Introduction

Humans have a biological imperative to form social bonds throughout their lifespan, including selective social bonds between family members, friends, and spouses. When the ability to form and maintain stable long-term social relationships is impaired there are profound negative consequences in many aspects of life – for example by limiting opportunities for workplace or economic advancement and increasing the risk for physical or mental illnesses following a traumatic event [7, 8]. Psychiatric and neurodevelopmental disorders that affect social functioning represent a tremendous social, economic, and healthcare burden. Currently the functional outcomes and quality of life for individuals with impaired social functioning are poor, in part due to the lack of effective pharmaceutical treatments. Impairments in social cognition and social interaction can manifest in depression, social anxiety, schizophrenia, and, most notably, autism spectrum disorder (ASD) [68]. ASD is a neurodevelopmental disorder characterized by deficits in social functioning, including difficulties with forming lasting social relationships, understanding and responding to social cues, and stereotyped or repetitive behaviors or interests [9]. ASD is highly prevalent, and is currently diagnosed at a rate of 1 out of every 68 children, although there is a significant sex difference, with males being diagnosed at a rate 3-4 times higher than females [5]. The lack of effective pharmaceutical treatments for social deficits in ASD and other psychiatric or neurodevelopmental disorders represents a critical unmet clinical need.

Interestingly, despite overwhelming data demonstrating the importance of oxytocin (OT) in sociocognitive functioning, there is no clear evidence that aberrant central OT signaling is a major contributor to social deficits in ASD [100]. Nevertheless,

it is still possible that ASD symptoms could be ameliorated through the use of OT-targeted therapeutics. Indeed, as we have seen in the previous chapter, IN-OT administered to ASD patient populations is reported to positively modulate several aspects of social cognition and social functioning, including increased reciprocal play, emotion recognition, and feelings of trust [5, 36, 37, 69, 70, 79, 83, 123-127]. These findings provide strong proof-of-principle for the utility of OT-based therapeutics in the treatment of social deficits, but further study is needed to determine the best method of manipulating central OT signaling in a behaviorally-relevant manner. While studies in humans have catalogued many parameters of social behaviors potentially responsive to OT treatment, such work cannot achieve the same degree of cellular resolution, experimental control, or invasive manipulations possible in rodents, making animal models a critical asset for the discovery of novel drug targets and treatments.

ASD shows high heterogeneity of both etiology and symptom presentation. Although some monogenic forms have been identified, including deletions or mutations of *Fmr1*, *Cntnap2*, *MeCP2*, *Dhcr7* and *Ube3A*, together these account for only approximately 3% of total ASD cases and are thus relatively rare [3, 6, 98, 128, 129]. Animal models which recapitulate specific mutations or deletions found in monogenic forms of ASD have provided solid entry points to begin to understand the underlying disease mechanisms, however it is not entirely clear to what extent these findings are broadly translatable to other forms of ASD. Animal models with the greatest utility for the development of novel treatments targeted to sociocognitive functioning should ideally have three key features: 1) face validity, meaning the model exhibits phenotypic behavior also present in the disorder, 2) construct validity, a common biological mechanism is

shared between the model and the disorder and 3) predictive validity, pharmacological treatments that have a given effect in the model should have a similar effect in humans [3]. Strong animal models are best used in combination with sensitive behavioral paradigms relevant to the social impairments observed in ASD. These behavioral assays should ideally have face validity and construct validity (i.e. the assay should quantify some aspect of social cognition which is based on shared evolutionary neurobiological mechanisms) [3]. Our current understanding of ASD in general suggests a variety of environmental and polygenic factors likely contribute to the etiology of this disorder in the majority of patients [129]. Given the complex nature of ASD etiology, successful recapitulation of multifactorial disease mechanisms in strong animal models, as described above, is difficult.

One alternative approach to identifying potential therapeutic targets is to look not at models of social deficits or ASD specifically but instead at models of healthy, functional social cognition. The socially monogamous prairie vole (*Microtus ochrogaster*) has face and construct validity for functional social cognition [3]. Furthermore, we will argue that the prairie vole, and in particular the formation and expression of pair bonds which can be probed using the Partner Preference Test, has predictive validity for the identification of drugs that may enhance social learning or social cognition more generally. The Partner Preference Test (PPT) is a two-component assay. First, a period of social learning occurs during which an experimental subject cohabitates with a novel opposite sex stimulus animal for a set duration of time. If the experimental question is predicated upon pair bond formation under naturalistic conditions, the two animals are allowed to mate freely and will cohabitate together for

longer periods of time, often at least 24 hours. Under conditions with unrestricted mating, the opposite sex stimulus animal will have a high social value for the experimental animal, as mating is highly socially salient and rewarding, and necessary for pair bond formation in the wild. In these conditions, a vast majority of experimental animals will form selective social attachments which can be observed during the second half of PPT. The second component of PPT is a choice assay designed to probe for social motivation and expression of a selective social attachment or 'partner preference'. A 3-chambered arena is used wherein the cohabitation partner is tethered and therefore restricted to move only within one chamber, while a novel opposite sex stranger of equivalent stimulus value is similarly tethered in a separate chamber on the opposite side of the arena. The center chamber is left unoccupied as a neutral 'non-social' zone. At the start of the 3-hour test period, the experimental animal is placed in the center non-social zone and allowed to move freely throughout the arena for the duration of the test period. At the end of the test, time spent in immobile social contact (huddling) with either the cohabitation partner or the novel stranger is quantified [22]. While non-monogamous rats and mice tested in this paradigm would invariably prefer to engage socially with a novel stimulus animal compared to a familiar one, pair-bonded prairie voles will show a strong and distinct preference for social contact with their cohabitation partner ('partner preference') [22, 23].

Alternatively, cohabitation conditions can be modified such that the initial phase of social learning occurs under suboptimal conditions. The most common manipulations include shortened duration of cohabitation and prevention of mating. Under these suboptimal conditions, a control group of animals will not typically display a preference

for social contact with their cohabitation partner, and will instead spend an equivalent amount of time engaging socially with both the partner and the stranger during the probe trial. In this paradigm, experimental manipulations can be made to attempt to enhance social cognition and social learning. Effective manipulations of social cognition will promote the emergence of partner preferences under these conditions in which they do not normally form. This suboptimal paradigm can be considered to have predictive validity for human social cognition. Indeed, PPT under suboptimal conditions was first used to identify the important role of OT in pair bonding, as intracerebroventricular (ICV) injection of OT induced partner preference formation following a brief cohabitation period and in the absence of mating [130]. As we now know, OT also plays an important role in human social cognition, demonstrating the predictive utility of PPT for screening drugs that can enhance social learning, social motivation, or social cognition more generally. This suboptimal social learning paradigm has been subsequently tested with a variety of putative compounds suggested to have clinical efficacy in enhancing social cognition in humans.

While OT itself appears to have some measure of viability based on early human studies and preclinical data from animal models, its unfavorable biochemical properties and poor central penetrance raise questions about its ultimate utility in larger patient populations. To circumvent the problems associated with the delivery of exogenous OT itself, alternative methods of stimulating endogenous central OT release have been tested in animal models of sociability, including prairie vole partner preference formation. The hypothalamic melanocortin neuropeptide system appears to act upstream of OT to facilitate and enhance central OT release [131]. There are five G-protein coupled

receptors which comprise the family of melanocortin receptors, but the melanocortin 4 receptor (MC4R) is the only one which is exclusively and widely expressed in the brain [121]. MC4Rs are expressed on magnocellular OT-producing neurons in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus [132]. Stimulation of MC4Rs with the endogenous melanocortin ligand α-melanocyte-stimulating-hormone (α-MSH) promotes somatodendritic OT release in magnocellular neurons both *in vitro* and *in vivo*. This central OT release occurs independently of action potential firing or peripheral OT release [119]. Synthetic analogues of α-MSH have been developed which have improved selectivity for MC4R, greater stability and longer half-lives, and are able to effectively penetrate the blood-brain-barrier (BBB) [133]. Furthermore, these synthetic analogues, such as Melanotan II (MTII), have already been evaluated for safety and tolerability in human clinical trials [134]. Given these much more favorable biochemical properties as compared to OT itself, it has been proposed that MC4R agonists, including MTII, could show greater efficacy in enhancing OT-dependent social behaviors, including partner preference formation in prairie voles, or social cognition in humans. Initial experiments in female prairie voles demonstrated that peripheral administration of MTII activates OT neurons in the PVN. Behaviorally, peripheral administration of MTII prior to a short cohabitation and in the absence of mating facilitates partner preference formation in female prairie voles [120]. These early results strongly suggest peripherally-administerable MC4R agonists, such as MTII, effectively modulate OT-dependent social cognition in a behaviorally- and ecologically-relevant manner. In this series of studies, we will first look at the effect of peripherally administered MTII on partner preference formation in male prairie voles. Demonstration

of equivalent efficacy in both sexes is of critical importance, especially given the large discrepancy in ASD prevalence in males compared to females. Further, we will attempt to demonstrate the social behavioral effect of MTII is centrally-mediated. Finally, we will quantify the reciprocal social interactions observed between a subject and a cohabitation partner to attempt to identify whether outward changes in the quality of social interactions themselves can be said to contribute to enhancements in partner preference formation.

Methods

Subjects

Adult (60-120 days of age) sexually naïve prairie voles from our colony were used for all experiments. Our colony is maintained at the Yerkes National Primate Research Center at Emory University and this facility is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Our colony of prairie voles all originate from wild-caught stock trapped in Illinois. Breeding is conducted within our facility, and all animals are weaned at 19-22 days into same-sex groups of 2-3 individuals. Animals are maintained under an extended light period 14/10 hour light/dark cycle at a temperature of 22 °C with *ad libitum* access to water and food (LabDiet rabbit chow). All experiments in this study were evaluated and approved by the Emory University Institutional Care and Use Committee (IACUC).

Partner Preference Testing

Partner preference testing is performed in two segments. At the start of the initial cohabitation period, experimental animals are placed in a clean cage with a novel opposite sex stimulus animal. After the cohabitation period, partner preference testing is

performed (Figure 2.1). Partner preference testing is conducted using clean, plexiglass 3-chambered arena with opaque dividers separating each chamber but still permitting free movement of the experimental subject. On one side of the arena, the cohabitation stimulus animal is tethered using a plastic collar and thus restricted to move only within that chamber. On the opposite side of the arena, a novel opposite sex stranger stimulus animal matched to the partner stimulus animal for age, sex and weight is similarly tethered. At the start of the 3-hour testing period, the experimental animal is placed in the center of the middle neutral/non-social zone. The experimental subject then has free access to all 3 chambers for the entire duration of the test. Time spent in immobile side-by-side contact (huddling) with both the partner and the stranger is scored using CleverSys Social Scan specifically programmed for this test [22]. Prairie voles which have formed pair bonds will prefer to engage in huddling behavior with their cohabitation partner over the novel stranger. A partner preference is experimentally defined as a subject spending twice as much time in social contact with their partner compared to the stranger. Distance traveled and number of cage crossings are analyzed as a control metric of general locomotor behavior.

Peripheral Administration of Melanotan II and Partner Preference Testing in Male Prairie Voles

The effect of peripheral Melanotan II (MTII) on partner preference formation was tested in adult (60-120 day old) sexually naïve male prairie voles. Prior to the start of cohabitation, males were injected intraperitoneally (IP) with either MTII (10mg/kg in 0.9% saline, n=17); (Alpha Diagnostics, San Antonio, TX), or vehicle (0.3mL 0.9% saline, n=16). Experimental subjects were then cohabitated in a clean cage with an

ovariectomized, non-receptive adult female for 6 hours, during which time no mating bouts were observed. While female prairie voles are considered induced ovulators, and only become sexually receptive following extended exposure (>48 hours) to male urine, we cannot discount the possibility that cross contamination of cages during the cage change process could unintentionally facilitate sexual receptivity in a stimulus female [135]. Therefore, all stimulus females are ovariectomized to ensure no mating bouts occur. Cohabitation periods were videotaped to further confirm the absence of mating. Immediately following cohabitation, males were tested for the formation of social attachment in the partner preference test as described above.

Central Administration of Melanotan II and Partner Preference Testing

To determine if the behavioral effects observed following peripheral (IP) MTII administration in male and female prairie voles are due central effects of MTII, we performed follow up experiments using intracerebroventricular (ICV) MTII administration. Adult, sexually naïve male and female prairie voles were implanted with 26-gauge guide cannulae (Plastics One, Roanoke, VA) targeting the lateral ventricle (A/P: + 0.4mm, M/L: +/- 1.0mm, D/V: -3.0mm) and allowed 6-7 days to recover. Prior to the start of cohabitation, subjects were lightly anesthetized using isoflurane to permit insertion of the injector needle through the guide cannula. Control vehicle artificial cerebrospinal fluid (aCSF, 2µL, Tocris Bioscience, Bristol, UK) or MTII (2µL, 1.5µg; 3nmol, dissolved in aCSF) were slowly infused over a period of 5 minutes via automatic syringe controller pump (World Precision Instruments, Sarasota, FL). The injector was left in place for an additional 2 minutes after the infusion was completed to prevent backflow and ensure sufficient diffusion away from injector needle. During the recovery

period, subjects were transported to the testing room. All subjects awoke from the light anesthesia within 5 minutes. Upon awakening, subjects were placed into a clean cage containing an opposite sex stimulus animal for cohabitation. To test the central effects of MTII in females, adult sexually naïve female prairie voles were ovariectomized and allowed to recover for 2 weeks. After recovery from ovariectomy subjects were implanted with guide cannulae targeting the lateral ventricle as described above. On test day, females were injected with either MTII (2μL, 3nmol, n=16) or aCSF (2μL, n=16) and cohabitated with an adult male prairie vole for 6 hours. After the end of the 6 hour cohabitation, subject females and male cohabitation partners were separated for a period of 24 hours before undergoing partner preference testing. The 24 hour separation period differs from the protocol used in males, and was an attempt to modify the partner preference testing paradigm further as a result of unexpected observations of partner preference formation in control subjects during early pilot tests. For a full discussion of pilot observations during PPT, see Appendix. To test the central effects of MTII in males, adult sexually naïve male prairie voles were implanted with guide cannuale targeting the lateral ventricle as described above. On test day, males were injected with either MTII (2μL, 3nmol, n=16) or aCSF (2μL, n=17) and cohabitated with an ovariectomized adult female prairie vole for 6 hours. Immediately after cohabitation, males underwent partner preference testing as previously described.

Peripheral Melanotan II and Reciprocal Social Interaction

To determine whether any behavioral effects in partner preference formation could be explained by qualitative differences in reciprocal social interactions precipitated by MTII administration, follow-up experiments were performed to permit analysis of discrete

social behaviors during a 30-minute interaction period with a novel opposite sex stimulus animal. This test was performed once with female experimental subjects and once with males. Both sexes underwent identical experimental procedures. Subjects were injected IP with either MTII (10mg/kg dissolved in 0.9% saline, males n=11, females, n=8) or saline (0.3mL, males n=11, females n=10). Immediately following IP injection experimental subjects were placed in a clean test cage allowed 30 minutes to habituate to this novel environment. At the end of the habituation period, a novel opposite sex stimulus animal (adult sexually naïve male or adult ovariectomized female) was placed in the test cage for 30 minutes. Social interactions were videotaped and later scored by a coder blinded to treatment condition using Noldus Observer software. Time spent engaged in social investigation (including anogenital investigation, nose-to-nose sniffing, and general body investigation) and allogrooming (both giving and receiving) were quantified. These reciprocal social interactions were aggregated to generate a cumulative value of time spent engaged in social interaction, and this value was used for statistical analyses.

Central Melanotan II and Reciprocal Social Interaction

Results from peripheral reciprocal social interaction tests in female prairie voles were extended and confirmed using central administration of MTII. As described above, adult female prairie voles were cannulated targeting the lateral ventricle and allowed to recover for 6-7 days. On test day, experimental subjects were lightly anesthetized to permit injection through the cannula. Either MTII (2μL, 3nmol, n=7) or aCSF (2μL, n=10) were slowly injected over a period of 5 minutes. Subjects recovered from anesthesia and subsequently were habituated to the novel environment of the clean test cage for 45

minutes after the end of the injection. After the 45-minute recovery/habituation period, a novel adult male stimulus animal was introduced into the test cage for 30 minutes of social interaction. Reciprocal social interactions (social investigation and allogrooming) were scored as described above. Cumulative social interaction time values were generated by aggregating time spent engaged in social investigation and allogrooming, and these values were used for subsequent statistical analyses.

Statistical Analysis

All analyses were performed using R 3.4.0. The effect of MTII treatment on time spent with either the partner or the stranger stimulus animal was compared using a two-way repeated measures ANOVA, with treatment as a between subjects and stimulus animal as a within subject factor. Log transformation of the outcome variable was performed to improve model fit and resulted in the residuals from the ANOVA model being approximately normally distributed. When significant interaction effects were found, post-hoc comparisons using Student's paired T-tests comparing time spent with partner and stranger were performed, with alpha-value significance threshold corrected for the number of comparisons made (2 comparisons, alpha-value significance threshold corrected to 0.025). To analyze MTII treatment effects on reciprocal social interaction, time spent engaged in social investigation or allogrooming were summed for each subject to generate a cumulative reciprocal interaction score. These aggregate social interaction times were compared using a Student's unpaired T-test with alpha-value set at 0.05.

Results

Peripheral Administration of Melanotan II and Partner Preference Testing in Male Prairie Voles

Previous studies showed peripheral MTII at a 10mg/kg dose facilitated partner preference formation in female prairie voles [120]. We attempted to replicate this effect using male prairie voles as our experimental subjects. Using suboptimal social learning conditions (6 hour cohabitation, no mating, Figure 2.1), we found that peripheral MTII facilitates partner preference formation in male prairie voles. There was a significant main effect of stimulus animal (partner/stranger; $F_{1,31}=21.5$, $p<0.001$) and a significant stimulus animal X treatment interaction effect ($F_{1,31}=5.9$, $p=0.02$) but no significant main effect of treatment ($F_{1,31}=1.2$, $p=0.28$). Post-hoc Student's paired T-test revealed that males receiving a 10mg/kg dose of MTII, but not saline, spent significantly more time engaged in social contact with the partner compared to the stranger (partner vs. stranger contact time, $p<0.001$) (Figure 2.2).

Central Administration of Melanotan II and Partner Preference Testing

Peripherally administered MTII crosses the BBB in multiple species, including prairie voles, however despite its high relative selectivity for MC4R, MTII is still able to activate other peripherally-expressed melanocortin receptors to some extent [120]. Therefore, we attempted to localize the sociocognitive effect of MTII to melanocortin agonism within the brain using a central (intracerebroventricular, ICV) administration paradigm in both female and male prairie voles. In female prairie voles, there was a significant main effect of stimulus animal ($F_{1,30}=16.7$, $p<0.001$). There was no main effect of treatment ($F_{1,30}=0.2$, $p=0.67$) and no significant stimulus animal X treatment interaction effect ($F_{1,30}=2.1$, $p=0.15$). Although no significant interaction effects were found, Student's paired T-tests showed that female subjects receiving ICV MTII (3nmol) prior to cohabitation spent significantly more time in social contact with their partner male

compared to the novel stranger male ($p<0.001$). Control subjects receiving ICV aCSF prior to cohabitation did not show a significant preference for social contact with their male partner over the novel male stranger ($p=0.12$) (Figure 2.3A). The lack of significant treatment X stimulus animal interaction effects when analyzed with a two-way repeated measures ANOVA was likely driven by a small number of control aCSF subject females that displayed markedly strong partner preferences. This contributed significant variability within the data set, and occludes our ability to accurately interpret the potential central MTII effect in this set of experiments. Multiple alternative variations of the cohabitation-partner preference testing paradigm were attempted however ultimately none of these reliably produced aCSF control groups which did not display partner preferences above the level of chance (see Appendix for detailed review of methods). As highly social animals, prairie voles that have not formed a partner preference during a cohabitation period will still engage socially with both target stimulus animals (partner and stranger) however it is generally understood that they will distribute their social engagement time equivalently to both stimulus animals, or display a preference for one or the other at the level of chance (50%). Within the dataset reported here, 12 out of the 16 female subjects receiving ICV MTII displayed a partner preference (defined as the subject spending twice as much time engaged in social contact with the partner compared to the stranger), compared to 9 out of 16 control (aCSF) females.

Similar issues with lack of reliable controls within a suboptimal social learning paradigm were present when attempting this central infusion experiment using male subjects. In male prairie voles receiving ICV MTII (3nmol) or control aCSF prior to cohabitation, we found a significant main effect of stimulus animal ($F_{1,31}=19.5$, $p<0.001$),

but no main effect of treatment ($F_{1,31}$=0.4, p=0.5) and no significant stimulus animal X treatment interaction effect ($F_{1,31}$=0.01, p=0.93) (Figure 2.3B). Despite the lack of a significant stimulus animal X treatment interaction effect, Student's paired T-tests were still performed to examine the extent of preferences formed in both groups. Males receiving ICV MTII spent significantly more time engaged in social contact with the female partner compared to the novel stranger female (p=0.006). Control males receiving ICV aCSF did not spend significantly more time with partner compared to the stranger when the alpha-value significance threshold is appropriately corrected to 0.025 for the two comparisons being made (p=0.052) however this should not necessarily be interpreted as a lack of preference in the control group. Using the definition of a 'partner preference' from above, 12 out of 16 male MTII-treated subjects showed a partner preference, compared to 11 out of 17 control male aCSF subjects. Again, the lack of an appropriate control group which fails to display partner preferences above the level of chance prevents us from drawing any significant conclusions from these central MTII partner preference experiments in both sexes.

Peripheral Melanotan II and Reciprocal Social Interaction

To investigate if peripheral MTII facilitated partner preference formation secondary to qualitative changes in reciprocal social interactions between the experimental subject and the cohabitation stimulus animal, we quantified social behaviors during a 30-minute social exposure period following drug treatment. This 30 minutes of social interaction with a novel opposite sex stimulus animal was designed to essentially replicate the conditions of the first 30 minutes of a cohabitation period. We hypothesized that peripheral MTII would not positively shift the quality of observable reciprocal social

interactions, as exogenous OT has not been shown to affect non-sexual social behaviors in male or female prairie voles. Experimental animals were injected IP with MTII (10mg/kg) or saline before being habituated to a novel clean test cage environment for 30 minutes. After habituation, a novel opposite sex stimulus animal was placed in the test cage and reciprocal social interactions were recorded for 30 minutes. Social investigation (including anogenital investigation, nose-to-nose sniffing, general body investigation) and allogrooming were scored and summed to generate a total time of cumulative reciprocal social interaction. Female prairie voles treated with peripheral MTII (10mg/kg) spent an equivalent amount of time engaged in reciprocal social interaction compared to control females receiving an injection of saline ($p>0.05$) (Figure 2.4A). Interestingly, male prairie voles treated with peripheral MTII (10mg/kg) spent significantly less time engaged in reciprocal social interaction with a novel stimulus female compared to saline injected controls ($p=0.003$) (Figure 2.4B). Therefore, we conclude that peripheral administration of MTII does not enhance partner preference formation as a result of positive shifts in reciprocal social interactive behaviors.

Central Melanotan II and Reciprocal Social Interaction

We next replicated the reciprocal social interaction test in female prairie voles using central (ICV) MTII administration. As observed with peripheral treatment, there was no difference in the amount of time spent engaged in reciprocal social interactions with a novel stimulus male. Females receiving ICV MTII (3nmol) spent an equivalent amount of time engaged in social interaction when compared with control females infused with ICV aCSF ($p>0.05$) (Figure 2.5).

Discussion

We show here peripheral administration of MTII in male prairie voles replicates the behavioral effect observed in female prairie voles within the partner preference paradigm. Peripheral MTII facilitated formation of a partner preference in male prairie voles under suboptimal social learning (cohabitation) conditions, conditions under which the saline control group did not show partner preferences. Attempts to replicate peripheral MTII findings within the partner preference paradigm using central administration of MTII were confounded by an inability to set up cohabitation conditions under which control groups consistently did not show partner preferences above the level of chance. Interestingly, peripheral or central MTII administration in female prairie voles did not affect reciprocal social interactions with a novel male stimulus animal, while male prairie voles receiving peripheral MTII injections appear to show decreases in reciprocal social interactions with a novel female stimulus animal, despite also ultimately showing increases in partner preference formation. Taken together, we interpret these results to mean that the quality or quantity of reciprocal social interactions an experimental subject experiences during a given cohabitation period does not necessarily influence subsequent partner preference formation or expression, at least in the context of melanocortinergic enhancements of social bonding. These results are in line with the existing literature on oxytocinergic manipulations and social interactive behavior in healthy rodent subjects. While disruption of OT signaling has been shown to impair social recognition and the capacity for associative learning of social rewards, enhancement of OT signaling in baseline healthy rodent subjects does not appear to alter outward social interactive behaviors.

Previous studies of the sociocognitive effect of MTII in prairie voles focusing specifically on females were predicated upon the longstanding belief within the field of prairie vole research that OT is primarily necessary for social bonding in female voles, but not males. Early studies dating back several decades reported that central administration of an OXTR antagonist blocked the formation of mating-induced partner preferences in female, but not male, prairie voles while exogenous central OT administration was shown to facilitate partner preference formation in females and males [130, 136-139]. More recent studies using sophisticated genetic manipulation techniques or more highly selective OXTR antagonists, however, have provided firm evidence for an equally potent role of OT in social bond formation in male prairie voles [140]. We therefore hypothesized MTII administration prior to cohabitation would facilitate partner preference formation in male prairie voles in a manner similar to what was previously reported in females. Additionally, there is a large sex difference in the prevalence of ASD diagnoses, with males being diagnosed at a rate 3-4 times higher than females [5]. As we have proposed MTII as a potential novel treatment for ASD, it was therefore necessary to examine the social behavioral effects of MC4R agonism in male prairie voles. Data reported here lend further support to the hypothesis that oxytocinergic manipulations can be equally effective for positively modulating social cognition in males and females.

Administration of MTII prior to the start of cohabitation period likely affects one or more of the sociocognitive processes which occur during that social learning phase, such as formation of social memory or the associative learning of social reward. In the next chapter, we will examine the cellular-level of effects of MC4R agonists in social and

non-social contexts. These experiments add to our understanding of the mechanisms by which MC4R agonists may alter an individual's perception of social stimuli in the absence of outward changes on the level of reciprocal social interactive behaviors. We will propose MTII treatment could be used in the treatment of social deficits in conjunction with behavioral therapies that can also be conceptualized as a form of social learning. While behavioral-based therapies show efficacy in the treatment of ASD, these therapies are incredibly time-intensive and can take years to produce meaningful improvements [5]. The development of pharmacological adjuvants able to enhance the speed or efficacy of these therapies would represent a critical advancement in the field of ASD treatment. As MC4R agonists, including MTII, have already been tested for safety and tolerability in humans, future studies should include clinical trials in ASD patient populations, particularly in the context of behavioral therapy.

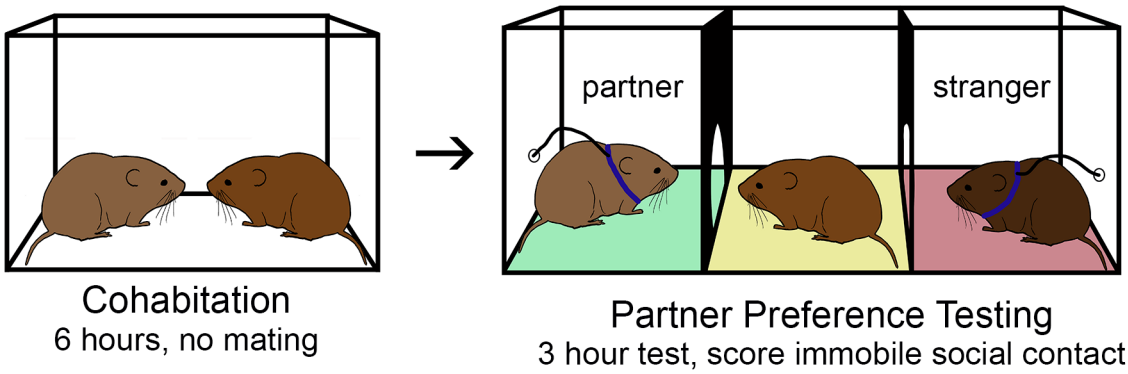

Figure 2.1. Partner Preference Testing paradigm. To test the hypothesis that Melanotan II treatment enhances the formation of pair bonds or 'partner preferences', experimental subjects are treated with Melanotan II prior to a 6 hour cohabitation with a novel opposite sex stimulus animal ("partner") in the absence of mating. Following cohabitation, Partner Preference Testing is performed. The cohabitation partner and a second novel opposite sex stimulus animal ("stranger") are tethered with a plastic collar in opposing sides of a 3-chambered arena. The tether prevents stimulus animals from leaving their respective chambers during the test period. At the start of the 3 hour test period, the experimental animal is placed in the center neutral/non-social chamber. The experimental animal has free access to all 3 arena chambers during the entire 3 hour test period. Testing is recorded with overhead video cameras and time spent in immobile social contact with both the partner and the stranger are quantified using automated software (CleverSys Social Scan) specifically programmed for this test.

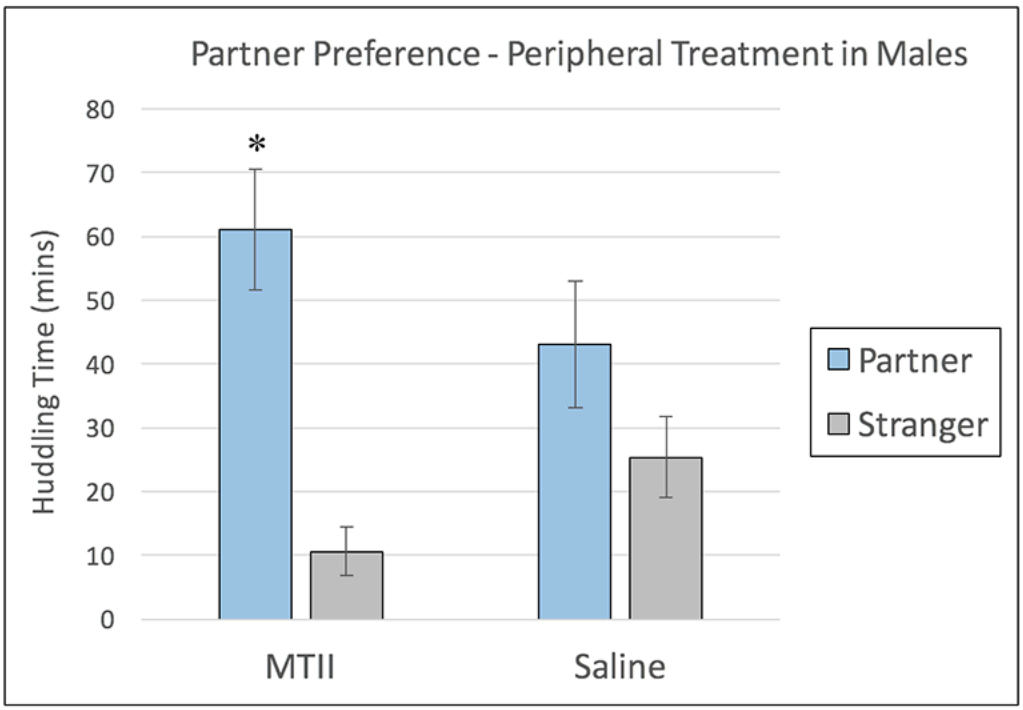

Figure 2.2. Peripheral MTII facilitates partner preference formation in male prairie voles. Male prairie voles receiving a peripheral (IP) injection of MTII (10mg/kg) spent significantly more time engaged in immobile social contact (huddling) with their cohabitation partner female compared to a novel stranger female (MTII-treated animals, partner time vs stranger time p=0.0002). Subjects receiving a control injection of saline did not show a significant preference for spending time engaged in social contact with their cohabitation partner as compared to a novel stranger female (p=0.22). Error bars are ± SEM, * denotes a statistically significant difference in post-hoc Student's paired T-test.

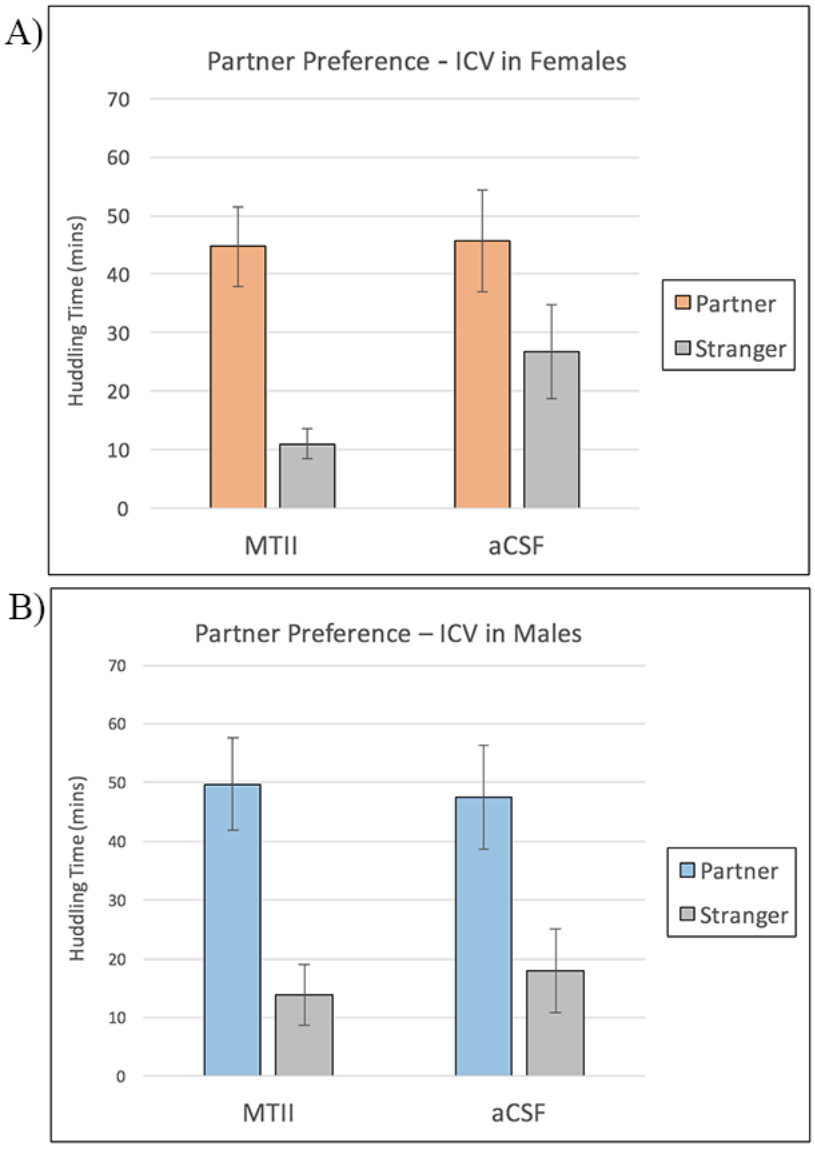

Figure 2.3. Central MTII may facilitate partner preference formation in female prairie voles. Central (ICV) administration of MTII (3nmol) may facilitate partner preference formation in female prairie voles **[A].** Due to high levels of variability within the control aCSF group, we failed to find a significant treatment X stimulus animal interaction effect in a two-way repeated measures ANOVA. Despite the lack of significant interaction effects, there still appears to be a difference in the amount of time spent engaged in huddling in MTII-treated females compared to aCSF controls. Paired Student's T-tests comparing partner time vs. stranger time in MTII-treated females are significantly different ($p=0.0008$), while aCSF-treated animals are not ($p=0.17$). Future studies should attempt to repeat this experiment to add additional subjects and determine true effects. **[B]** A similar, but more pronounced problem with the establishment of a no-preference control group was present in ICV MTII (3nmol) experiments using male subjects. Future studies should modify cohabitation parameters to determine true effects in males. Error bars are ± SEM.

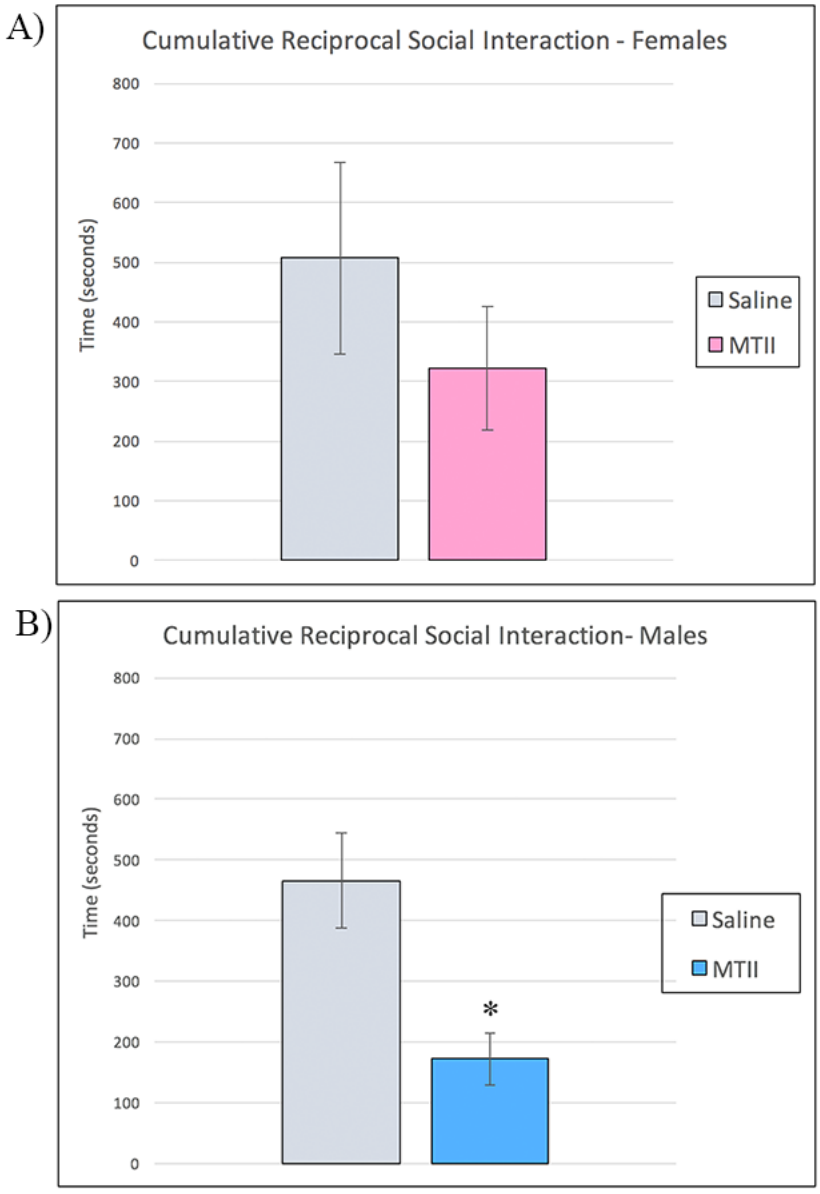

Figure 2.4. Peripheral MTII does not significantly affect Reciprocal Social Interactions in female prairie voles, but significantly reduces social interaction in males. Female prairie voles receiving peripheral (IP) injection of MTII (10mg/kg) prior to 30 minutes of social interaction with a novel stimulus male did not show significant differences in the amount of time spent engaged in reciprocal social interactions (social investigation or allogrooming) compared to control females injected with saline [A]. Male prairie voles injected with peripheral MTII (10mg/kg) spent significantly less time engaged in reciprocal social interactions compared to control males injected with saline ($p=0.003$) [B]. Error bars are ± SEM. * denotes a statistically significant difference in Student's unpaired T-test.

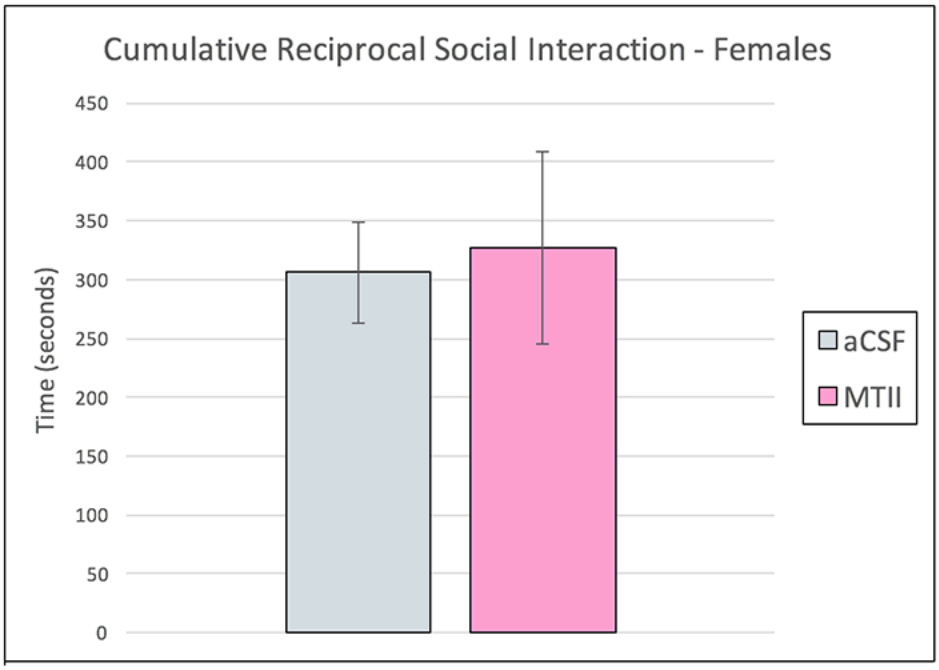

Figure 2.5. Central MTII does not significantly affect Reciprocal Social Interactions in female prairie voles. Central (ICV) administration of MTII (3nmol) does not significantly alter Reciprocal Social Interactions. Female prairie voles receiving ICV MTII prior to 30 minutes of social exposure to a novel stimulus male spend an equivalent amount of time engaged in social interaction (social investigation and allogrooming) as compared to aCSF injected control females. Error bars are ± SEM.

CHAPTER 3:

Melanocortin 4 receptor agonists and context-dependent neuronal activation

ABSTRACT

The neuropeptide oxytocin (OT) is well-known for its role in modulating and coordinating sociocognitive processing and social behavioral responding in multiple mammalian species, including humans. The importance of OT for effective social functioning has led to significant clinical interest in oxytocinergic pharmaceuticals as potential treatments of social deficits in psychiatric or neurodevelopmental disorders. Melanocortin 4 receptor (MC4R) agonists, such as Melanotan II (MTII), increase stimulus-evoked endogenous central OT release, and enhance sociocognitive functioning in multiple rodent models. To test the hypothesis that MTII enhances social cognition in the prairie vole by enhancing neuronal activity in OT-sensitive brain regions important for sociocognitive processing, we mapped neuronal activation patterns following drug treatment in homecage or social exposure contexts. Using the immediate early gene protein product Fos as our proxy marker of neuronal activation, we show peripheral MTII treatment in a social exposure context, but not in the homecage environment, increases neuronal activation in mesocorticolimbic regions important for mediating social reward and social motivation, the nucleus accumbens shell (NAcc shell) and prelimbic cortex (PLC), in both males and females. Follow-up central administration experiments in females replicate peripheral findings, and support a centrally-mediated mechanism for the context-dependent effect of MTII within the NAcc shell and PLC. Increased NAcc shell and PLC neuronal activation following MTII-treatment in a social context is blocked by co-administration of a selective OT receptor antagonist. This leads to the promising hypothesis that MC4R agonists could be used to increase context-dependent endogenous central OT release for clinical applications in the treatment of social deficits.

Introduction

The hypothalamic melanocortin system is well understood as a regulator of food intake and energy expenditure, but has also been implicated in the modulation of sexual behavior, stress responses, and natural reward [121]. Recently, several studies have described a novel role of melanocortins in social cognition, specifically through activity at the brain-exclusive melanocortin 4 receptor (MC4R) [120, 141]. Agonists of MC4R have been shown to modulate activity in several neurotransmitter or neuropeptide systems important for sociocognitive behaviors, including oxytocin (OT), dopamine, opioids, and corticotropin-releasing factor (CRF) [121]. The OT system in particular has been extensively studied as an evolutionarily conserved neuromodulator capable of coordinating neuronal responses across regions of the Social Salience Network (SSN) responsible for integrating the physiological, motivational, and associative properties of social stimuli [10, 142]. In the socially monogamous prairie vole, development of a selective social attachment to a mating partner (pair bond or 'partner preference') is an OT-dependent sociocognitive process [130, 136-138, 140, 143]. Peripherally-administered Melanotan II (MTII), a selective MC4R agonist, enhances partner preference formation in both male and female prairie voles [120]. Additionally, chronic neonatal administration of MTII in prairie voles enhances social attachment formation in adulthood [141]. It has been hypothesized that these sociobehavioral effects are a consequence of MC4R-stimulated central OT release. To further investigate the central mechanisms by which MTII enhances social cognition in the prairie vole model, we mapped the patterns of neuronal activation across regions of the SSN following drug treatment in both social and non-social contexts.

In multiple rodent models, including prairie voles, MC4R agonists induce expression of immediate early gene (IEG) products such as Fos or Egr-1 in OT-producing magnocellular neurons in the paraventricular nucleus of the hypothalamus (PVN) [119, 120, 144]. Early studies in rats using the endogenous ligand of MC4R, α-melanocyte-stimulating-hormone (α-MSH), demonstrated both *in vitro* and *in vivo* stimulation of MC4R evokes somatodendritic OT release in the hypothalamus. This somatodendritic OT release is facilitated by a rise in intracellular concentrations of Ca^{2+} [119]. Increases in intracellular Ca^{2+} also serve to promote trafficking of OT-containing large dense core vesicles from the reserve pool into the ready-to-release pool at the cell membrane, priming OT neurons for enhanced activity-dependent release in response to subsequent physiological stimulation [13]. In female prairie voles, peripheral MTII administration potentiates OT release within the nucleus accumbens shell (NAcc shell) in response to osmotic challenge, a physiological stimulus which is known to induce central and peripheral OT release [120]. The NAcc shell is a convergence point within the SSN, receiving glutamatergic inputs from the amygdala, hippocampus, and frontal cortical regions [72]. The summation of these inputs facilitates integration of the emotional, contextual, and motivational aspects of social information to generate adaptive social behavioral responses [41]. Specific oxytocinergic activity within the NAcc shell and other mesocorticolimbic reward regions such as the prelimbic cortex (PLC) is important for social reward and social motivation [76, 138, 145, 146]. Thus, sociosensory information processing and ultimate behavioral output selection, particularly as it relates to encoding of social reward or social motivation, could be enhanced through pharmacological manipulation of the endogenous OT system.

The ability to pharmacologically target and enhance endogenous OT release is of particular interest as a potential therapeutic in the treatment of social deficits in psychiatric or neurodevelopmental disorders, including autism spectrum disorder (ASD). Preliminary studies using intranasal administration of oxytocin (IN-OT) have demonstrated proof-of-principle efficacy for OT in improving sociocognitive functioning in ASD patients. Unfortunately, the potential clinical efficacy of IN-OT is limited by poor central penetrance and short-half life [110]. The use of MC4R agonists to indirectly enhance endogenous central OT release could circumvent many of the limitations of IN-OT while still serving to enhance OT-dependent sociocognitive functioning. Stable and selective brain-penetrant MC4R agonists already exist, and have been tested for general safety and tolerability in humans, including MTII [133, 134]. As many of the sociocognitive and neuromodulatory effects of OT originally delineated in rodent models can also be observed in humans, there is significant translational utility in using rodent models for the development and study of pharmacological enhancers of social cognition.

Although a great deal of work in multiple species supports the idea that OT positively modulates multiple domains of social cognition, this effect is not always directly observable as a significant change in outward behavior. Subtle changes in the perception of social stimuli can also be considered critically important for the modulation of adaptive social functioning. Using fMRI techniques, it has been reported that IN-OT in humans can augment social perception. IN-OT increases activation of reward-related mesocorticolimbic brain regions in response to positive social stimuli such as social touch, anticipating social reward, or viewing images of a partner's face [56, 61, 77, 78, 80, 81, 147, 148]. In negative social contexts, such as in the presence of social threats,

IN-OT reduces amygdala activity while increasing activation in prefrontal cortical regions important for the top-down regulation of emotional responding [49, 51, 54, 55, 61, 68, 149, 150]. In this way, it appears that OT promotes successful social adaptation by decreasing reactivity to negative socio-emotional stimuli and enhancing the encoding of positive social interactions with rewarding valences. While human neuroimaging studies show us regional activation levels as well as functional connectivity, this technique cannot provide us with the same level of cellular resolution achievable in rodent studies. To determine levels of neuronal activation within specific brain regions and across networks, many rodent studies have capitalized on the relationship between neuronal activation and the expression of IEG protein products. IEGs, such as *c-fos*, are rapid-onset markers of neuronal activity and synaptic plasticity that have been used to identify the subsets of cells which respond to a particular stimulus or experience [151]. Patterns of IEG expression within specific brain regions can tell us a great deal about neuronal network functioning, and give us important insights regarding drug effects when it may not be immediately apparent that a given drug dramatically alters external behavioral responding. In the case of MTII in prairie voles, there do not seem to be observable positive shifts in affiliative or reciprocal social interactions following drug treatment, however significant enhancements in partner preference formation can still be observed during the probe trial segment of partner preference testing (see Chapter 2). Therefore, it is possible that MC4R stimulation is somehow altering neuronal processing in regions of the SSN to affect *how* social stimuli are perceived as opposed to directly modulating social interactive behavior itself.

Previous studies have examined the effect of MC4R agonists on IEG induction in various behavioral or physiological paradigms primarily related to feeding behavior or energy expenditure. Beyond specific activation of oxytocinergic neurons in the hypothalamus, much of what we know about IEG expression in response to MC4R agonists describes activation patterns within networks which do not significantly overlap with the SSN. Therefore, in this series of studies, we will examine cellular activation patterns observed in OT receptor (OXTR) expressing regions of the prairie vole brain following MTII administration, using the IEG protein product Fos as our proxy marker for neuronal activation. We will first examine the baseline effect of MTII administration in the non-social context of an individual's homecage. Next, we investigate the potential synergistic effect between drug administration and environmental context, by adding a component of social exposure. Finally, we will probe the extent to which MTII administration affects central OXTR-dependent signaling and neuronal activation in select regions of the SSN.

Methods

Subjects

All experimental subjects or stimulus animals were adult (60-120 days old), sexually naïve male or female prairie voles from our in-house breeding colony at the Yerkes National Primate Research Center at Emory University. The breeding colony is derived from originally wild-caught stock from Illinois, USA. All animals were weaned at 21 days old into same-sex groups of 2 or 3 individuals with water and food (LabDiet rabbit chow) provided *ad libitum*. Housing rooms are temperature controlled (22°C) and on a

14/10 hour light/dark cycle. All experiments were approved by the Emory University Institutional Animal Care and Use Committee.

Pharmacological Agents

To activate central melanocortin 4 receptors, we used Melanotan II (MTII), Ac-Nle-c[Asp^{5},$Dphe^{7}$,Lys^{10}]α-MSH-NH2 (PP-1520, Alpha Diagnostics, San Antonio, TX). MTII is a stable, synthetic analogue of the endogenous MC4R ligand α-MSH with improved selectivity for MC4R, enhanced *in vivo* stability ($T^{1/2}$: 2 hrs) and greater blood-brain-barrier penetrability because of its cyclical structure [120]. To block central OXTR signaling, a highly selective oxytocin receptor antagonist, (d$(CH_2)_5^1$,Tyr(Me)^2,Thr^4,Orn^8,des-Gly-NH_2^9)-Vasotocin, was used (OTA, H-2908, Bachem, Bubendorf, Switzerland). Vehicle control for peripheral experiments was 0.9% sterile saline. For central infusion experiments, vehicle control was artificial cerebrospinal fluid (aCSF, Tocris Bioscience, Bristol, UK).

Surgical Procedure and Central Infusion Protocol

For central infusion experiments, adult, sexually naïve female prairie voles were anesthetized with isoflurane and implanted with 26-gauge guide cannulae (Plastics One, Roanoke, VA) to target the lateral ventricle (A/P: + 0.4mm, M/L: +/- 1.0mm, D/V: -3.0mm) secured to the skull with glass ionomer luting cementing (72-9169, Harvard Apparatus, Holliston, MA). Subjects recovered for 6-7 days before testing. On test day, subjects were lightly anesthetized with isoflurane while the injector needle was inserted through the guide cannula. Each 2μL infusion of control aCSF, MTII (1.5μg; 3nmol), MTII+OTA (1.5μg; 3nmol MTII, 5ng OTA), or OTA (5ng) was slowly infused over 5 minutes using an automatic syringe pump and controller (World Precision Instruments,

Sarasota, FL). Following infusion, the injector needle was left in place for 2 minutes to allow diffusion of the drug away from the needle and prevent backflow during removal.

Perfusion Protocol

Subjects were deeply anesthetized with isoflurane in the absence of added oxygen and perfused transcardially with 30mL of ice cold phosphate buffered saline (PBS; pH 7.4), followed by 30mL of ice cold 4% paraformaldehyde in PBS (pH 7.4). Immediately following perfusion subjects were decapitated and their brains removed and placed into a jar of 4% paraformaldehyde for 24 hours at 4°C. The following day, brains were transferred to 30% sucrose dissolved in PBS and stored at 4°C until sectioning.

Neuronal Activation Pattern Following Peripheral Melanotan II in the Homecage

Adult, sexually naïve male or female prairie voles were separated from their group housing cagemates into new, clean cages and left undisturbed for 48-72 hours prior to testing to allow each individual subject to establish a "homecage" with their own nest and scent. On test day, male or female subjects were injected intraperitoneally (IP) with either MTII (10mg/kg, dissolved in 0.9% saline, males: n=6, females: n=6) or vehicle control (0.3mL 0.9% saline, males: n=6, females: n=6) and returned to their homecage where they remained undisturbed for 90 minutes. At the end of 90 minutes, subjects were perfused and brains removed as described above (Figure 3.1).

Neuronal Activation Pattern Following Peripheral Melanotan II in a Social Context

Adult sexually naïve male or female prairie voles were kept in group housing until 48-72 hours prior to testing, at which point they were separated into individual cages. On test day, subjects were brought to the procedure room and injected IP with either MTII (10mg/kg, males: n=11, females: n=8) or vehicle control (0.3mL 0.9% saline, males:

n=11, females: n=10) and placed into a clean testing cage to habituate to the novel test cage environment for 30 minutes. After the habituation period, a novel opposite sex stranger stimulus animal was placed into the testing cage for 30 minutes of social interaction. For male experimental subjects, the stimulus stranger female was ovariectomized at least 2 weeks before test day. Social exposure periods were videotaped, and no mating attempts were observed in any of the test periods. Social interactions during the 30-minute exposure period were scored offline by an observer blinded to treatment group. After the 30-minute social exposure period, subjects were returned to their homecage for 60 minutes before perfusion (Figure 3.2).

Neuronal Activation Pattern Following Central Melanotan II in the Homecage

To determine the effect of restricted central MTII administration on neuronal activation patterns in the homecage environment, adult female prairie voles were implanted with guide cannulae targeting the lateral ventricle as described previously, and allowed 6-7 days to recover. As the role of OT within the PLC and NAcc shell in prairie voles has been most extensively studied in females, and similar results were observed in both males and females using peripheral MTII administration, we focused our mechanistic central infusion studies only on females. On test day, subjects were lightly anesthetized with isoflurane and injected through the guide cannula with either MTII (n=6) or control aCSF (n=6), then returned to their homecage for 90 minutes before perfusion (Figure 3.3).

Neuronal Activation Pattern Following Central Melanotan II in a Social Context

As described previously, adult female prairie voles were implanted with guide cannulae targeting the lateral ventricle and allowed 6-7 days to recover. On test day, subjects were injected with either MTII (n=8) or aCSF (n=8). Subjects were transported to the testing

room where they were allowed to recover from anesthesia and habituate to the clean novel test cage environment for 45 minutes. After recovery and habituation, a novel stimulus male was introduced to the testing cage and subjects were allowed to interact with the stimulus target for 30 minutes. Social interaction was videotaped and scored offline by an observer blinded to treatment group. After 30 minutes, subjects were returned to their homecage for 60 minutes before perfusion (Figure 3.4).

Effect of Oxytocin Receptor Blockade on Melanotan II-evoked Neuronal Activation Patterns

To determine the contribution of central oxytocin receptor signaling on MTII-evoked neuronal activation patterns in the social exposure context, we replicated our initial central MTII/Social Exposure study in female prairie voles and added two additional groups: subjects receiving central co-injections of MTII+OTA, and a control group which received only OTA. Group sizes were: MTII n=7, MTII+OTA n=8, aCSF n=7 and OTA n=8. Surgical procedure, central infusion, social exposure to a novel stimulus male, perfusion and brain extraction were performed exactly as described previously for the central MTII/ social exposure study (Figure 3.4).

Immunohistochemical Visualization of Fos Expression

To determine the effect of MTII on neuronal activation patterns across the brain in homecage or social exposure contexts, we processed collected brain tissue for immunohistochemical (IHC) visualization of the IEG protein product Fos. Collected brains were sectioned into 40μm coronal sections using a freezing microtome and stored free-floating in PBS + 0.05% sodium azide or cryoprotectant at 4°C until further IHC processing. Briefly, sections were washed thoroughly in PBS. The sections were then

blocked with 0.5% Triton-X and 5% Normal Goat Serum in PBS (PBST/NGS) for 1 hour at room temperature. Sections were then incubated in a solution of PBST/NGS plus primary antibody directed against c-Fos (ABE457, Millipore, Billerica, MA; at a concentration of 1:20,000) for 1 hour at room temperature, then transferred to a shaker at 4°C for 48 hours. After 48 hours, sections were washed in PBS, then incubated in secondary antibody solution containing 1:500 biotinylated goat anti-rabbit IgG secondary antibody (BA-1000, Vector Labs, Burlington, CA) in 2% NGS + PBS + 0.5% Triton-X for 2 hours at room temperature. After PBS wash, avidin/biotin reaction was developed using Vectastain ABC Kit (PK-4000, Vector Labs). Nickel-enhanced 3,3'-diaminobenzidine (DAB) staining was performed to visualize antibody binding using DAB Peroxidase (HRP) Substrate Kit (SK-4100, Vector Labs). After final PBS wash, sections were stored in PBS + 0.05% sodium azide until mounting. Mounted sections were dehydrated overnight before coverslipping with Krystalon Mounting Medium (64969, Millipore, Billerica, MA).

Microscopy

Microscopic images were captured using a Nikon E800 microscope with MCID Core Imaging Software (Interfocus, Ltd., Cambridge, UK). Six OXTR-expressing brain regions of the Social Salience Network (SSN) were imaged across all subjects: anterior olfactory nucleus (AON), medial amygdala (MeA), paraventricular nucleus of the hypothalamus (PVN), anterior cingulate cortex (ACC), prelimbic cortex (PLC) and nucleus accumbens shell (NAcc shell). Anatomical boundaries of each region were determined by referencing *The Mouse Brain Atlas* (Franklin and Paxinos; 3rd edition). The PVN and PLC were analyzed by taking images of 3 consecutive sections capturing

both hemispheres. The AON, MeA, ACC and NAcc shell were imaged bilaterally in 3 consecutive sections, resulting in 6 total images used for cell counting. Sections or hemispheres with damaged/absent tissue within a target region were excluded from analysis. Total Fos-positive nuclei within each image was quantified using an MCID grain count function applying a constant threshold across all subjects (see Figure 3.5 for representative example of cell counting algorithm). For each subject, Fos+ cell counts from each image of a given region were averaged to generate a single Fos+ cell count average per subject per brain region.

Statistical Analysis

All statistical analyses were performed using R 3.4.0. The effect of MTII on Fos induction in selected regions of the SSN was analyzed using a two-way repeated measures ANOVA, with brain region as a within subject factor and treatment as a between subjects factor. Planned post-hoc comparisons were performed when appropriate using unpaired Student's T-tests to compare Fos+ cell counts in drug-treated subjects versus control subjects. Alpha value significance thresholds for post-hoc comparisons for each experiment were adjusted as appropriate for multiple comparisons. All statistical analyses were performed on raw cell count values. Bar graphs of Fos expression per region in Figures 1-8 are presented showing Fos+ cell counts normalized to percent of control values to permit visualization of results from all regions in a single graph. Raw cell count values are presented in Tables 1-6. Each individual Table represents a single IHC run. As variation can be observed between individual IHC runs, care should be taken not to attempt comparisons between raw cell counts not presented within the same Table.

Results

Neuronal Activation Pattern Following Peripheral Melanotan II in the Homecage

To determine the baseline effect of MTII on Fos induction in OT-sensitive regions of the SSN, peripheral MTII (10mg/kg) was injected IP in male or female prairie voles before returning the subjects to their homecage. In male subjects, there was a significant main effect of treatment ($F_{1,10}$=13.7, $p<0.01$) and brain region ($F_{5,56}$=31.5, $p<0.001$) as well as a significant interaction effect of treatment X brain region ($F_{5,56}$=7.7, $p<0.001$). Post-hoc comparisons within region reveal peripheral MTII treatment in males significantly increased Fos induction in the PVN ($p<0.005$) and MeA ($p<0.005$) (Figure 3.6, Table 3.1). In female subjects, we observed a significant main effect of brain region ($F_{5,53}$=4.3, $p<0.005$) but no significant main effect of treatment ($F_{1,10}$=0.1, $p>0.05$) or treatment X brain region interaction effect ($F_{5,53}$=0.8, $p>0.05$) (Figure 3.7, Table 3.2). These results suggest that, in the absence of additional physiological stimuli, peripheral MTII treatment does not significantly enhance neuronal activation in OT-sensitive regions of SSN in female prairie voles known to be involved in pair bonding. The baseline effects of peripheral MTII on Fos induction in male prairie voles within the PVN is in agreement with previously published literature which shows MC4R agonists activate the PVN [152, 153]. These previous studies were also primarily conducted in male subjects, suggesting perhaps there may be a small sex difference in the organization of the hypothalamic melanocortin system, however our experiments were not designed to be able to detect sex differences. The significant MTII-evoked increase in Fos induction in the MeA of male subjects, but not females, is again perhaps suggestive of a sex difference in the organization of this region, but further study is needed to confirm this hypothesis.

Neuronal Activation Pattern Following Peripheral Melanotan II in a Social Context

It was previously reported that peripheral MTII enhances OT release in the NAcc shell of prairie voles only in response to physiological stimuli which in and of itself evokes OT release [120]. We therefore hypothesized that peripheral MTII could enhance neuronal activation patterns within OT-sensitive regions of the SSN during social interaction with a novel conspecific, a stimulus presumed to naturally stimulate OT release. Male prairie voles pretreated with peripheral MTII prior to 30 minutes of social exposure with a novel stimulus female showed robust induction of Fos expression in several regions of the SSN compared to saline treated controls. We observed a significant main effect of treatment ($F_{1,16}$=4.9, $p<0.05$), brain region ($F_{5,92}$=32.6, $p<0.001$) and a significant treatment X brain region interaction effect ($F_{5,92}$=8.2, $p<0.001$). Post-hoc comparisons revealed MTII-treated males showed significant enhancements in Fos expression in the PVN ($p<0.005$), NAcc shell ($p<0.0001$) and PLC ($p=0.01$), however when applying appropriate corrections for multiple comparisons the effect within the PLC is no longer statistically significant (Figure 3.8, Figure 3.9, Table 3.1). Future studies should attempt to replicate this experiment with increased group sizes in order to determine if the effect in the PLC is consistent and significant. Interestingly, the MTII-evoked increase in neuronal activation appears to be selective for the PVN, NAcc shell and PLC, as we observed no significant treatment effects on Fos expression within the AON, ACC, or MeA. Similarly, social exposure to a novel stimulus male markedly increased Fos expression in several regions of the SSN in female prairie voles, with significant main effects of treatment ($F_{1,14}$=22.0, $p<0.001$) and brain region ($F_{5,76}$=43.4, $p<0.001$), as well as a significant treatment X brain region interaction effect ($F_{5,76}$=12.9, $p<0.001$). Post-hoc

analysis revealed that female prairie voles treated with peripheral MTII, like males, showed significant increases in Fos expression in the PVN ($p=0.001$), PLC ($p<0.005$), and NAcc shell ($p<0.0001$) (Figure 3.10, Figure 3.11, Table 3.2). No effect of drug treatment was observed in the AON, ACC, or MeA. These results demonstrate peripheral MTII administration has context-dependent effects on neuronal activation patterns within select OT-sensitive regions of the SSN. Specifically, robust increases in neuronal activation within mesocorticolimbic reward regions of the PLC and the NAcc shell, two regions where oxytocinergic activity is critical for social reward and social motivation.

Neuronal Activation Pattern Following Central Melanotan II in the Homecage

Peripheral MTII is able to cross the blood-brain-barrier in multiple species, including prairie voles [120]. While MTII is highly selective for MC4R, it does still show activity at other melanocortin receptors, including those expressed in peripheral tissues [121]. Therefore, it is necessary to rule out peripheral off-target effects which could confound our observations of neuronal network activation following peripheral MTII administration. Using central intracerebroventricular (ICV) administration of MTII or control artificial cerebrospinal fluid (aCSF), we first replicated our baseline homecage experiment in females. In agreement with our peripheral administration findings, we observed no significant induction of Fos in the SSN in the homecage context following ICV MTII infusion. There was a significant main effect of brain region ($F_{5,48}=43.8$, $p<0.001$) but no main effect of treatment ($F_{1,8}=3.5$, $p>0.05$), and no significant treatment X region interaction effect ($F_{5,48}=0.04$, $p>0.05$) (Figure 3.12, Table 3.3).

Neuronal Activation Pattern Following Central Melanotan II in a Social Context

Central ICV administration of MTII in females prior to 30 minutes of social exposure with a novel stimulus male significantly enhances neuronal activation in OT-sensitive mesocorticolimbic reward regions of the SSN in a pattern nearly identical to the one observed following peripheral MTII administration in a social context. We observed a significant main effect of treatment ($F_{1,11}$=14.9, $p<0.005$), brain region ($F_{5,66}$=77.1, $p<0.001$), and a significant treatment X region interaction effect ($F_{5,66}$=8.9, $p<0.001$). Post-hoc comparisons show significant increases in Fos expression in the PVN ($p<0.001$), PLC ($p=0.001$) and NAcc Shell ($p<0.005$) in MTII-treated subjects compared to aCSF subjects (Figure 3.13, Figure 3.14, Table 3.4). There was no significant effect of drug treatment on Fos expression within the AON, ACC or MeA. These results support the hypothesis that context-dependent MTII neuronal activation patterns observed in peripheral social exposure experiments are likely a result of central MTII activity.

Effect of Oxytocin Receptor Blockade on Context-dependent Melanotan II Activation Patterns

It is hypothesized that enhanced endogenous central OT release underlies the observed increases in neuronal activation in select regions of the SSN following MTII treatment in a social exposure context. To determine the specific role of OXTR activation in the social exposure context, we analyzed neuronal activation patterns in a group of female subjects which received a co-injection of MTII plus a selective OXTR antagonist (MTII+OTA) compared to MTII-treated or aCSF control subjects. We observed significant main effects of treatment ($F_{2,16}$=26.2, $p<0.001$) and brain region ($F_{5,90}$=82.2, $p<0.001$), as well as a significant treatment X region interaction effect ($F_{10,90}$=4.2, $p<0.001$) (Figure 3.15, Figure 3.16, Table 3.5). Post-hoc analysis confirmed that we

successfully replicated our initial central drug treatment – social exposure findings, with MTII-treated subjects showing significant increases in Fos expression compared to aCSF controls within the NAcc shell ($p<0.0001$), PVN ($p<0.0001$) and PLC ($p<0.01$), however the effect in PLC does not remain statistically significant after applying appropriate corrections for multiple comparisons and therefore future replication attempts with increased group sizes are needed to determine if this is a genuine effect. Blockade of OXTR signaling significantly reduced Fos expression in the MTII+OTA group as compared to MTII-treated subjects in NAcc shell ($p<0.0001$) and PLC ($p=0.02$) however as was the case in statistical comparisons of MTII-treated subjects and aCSF-treated subjects, comparisons between the level of Fos induction in the PLC between MTII-treated and MTII+OTA treated subjects are not statistically significant after applying appropriate corrections for multiple comparisons. The levels of Fos expression observed in MTII+OTA subjects were not significantly different from levels observed in aCSF controls. There was no significant effect of OTA administration alone on Fos expression as compared to aCSF controls in any brain region analyzed (Figure 3.17, Table 3.6). These results confirm our hypothesis that intact OXTR signaling is a critical component of MTII-mediated increases in neuronal activation in the NAcc shell and PLC within the social exposure context.

Discussion

Peripheral administration of the brain-penetrant MC4R agonist MTII in the non-social context of an individual's homecage does not preferentially activate OT-sensitive regions of the SSN responsible for mediating social reward or social motivation in male or female prairie voles. Interestingly, it appears that peripheral MTII is able to exert context-

dependent effects on neuronal activation in specific regions of the SSN, including the PLC and NAcc shell, as well as the OT-producing region of the PVN. Pretreatment with peripheral MTII before 30 minutes of non-sexual social exposure to an opposite sex stimulus animal induces robust neuronal activation within OT-sensitive mesocorticolimbic reward regions in both males and females. This effect appears to be mediated by central activity of MTII as opposed to off-target effects secondary to activation of other melanocortin receptor subtypes found in peripheral tissue. Central ICV infusion of MTII in females within the homecage environment produced no significant increases in neuronal activation in the AON, ACC, MeA, PLC, NAcc shell or PVN, while central MTII infusion before social exposure stimulated strong Fos expression in the NAcc shell, PVN, and to a lesser extent the PLC. The similarity in Fos expression profiles within the PLC, NAcc shell and PVN obtained from social exposure experiments using either peripheral or central administration of MTII strongly support a centrally-mediated effect. Furthermore, specific blockade of OXTR signaling significantly attenuates central MTII-mediated increases in Fos expression in NAcc shell in the social exposure context, and it appears to have a similar effect in the PLC however this effect was not statistically significant after applying appropriate corrections for multiple comparisons. These results support the hypothesis that enhanced endogenous central OT release, resulting from stimulation of hypothalamic MC4Rs expressed on OT-producing neurons, positively modulates neuronal activation within select regions of the SSN responsible for mediating social motivation and social reward, the NAcc shell and the PLC. It can therefore be hypothesized that enhanced OT-mediated neuronal

processing specifically within the PLC and NAcc shell may underlie the ability of MTII to enhance partner preference formation in prairie voles.

Partner preference formation in prairie voles represents a complex sociocognitive process requiring appropriate processing of the social cues of the partner, linkage of those cues with a rewarding valence, and social motivation to remain with that partner. One of the primary aims of these Fos expression studies was to investigate how MTII administration affects neuronal processing within the SSN to gain insight to the cellular mechanisms by which MTII enhances social cognition to precipitate changes on a behavioral level. The social exposure experiments can be conceptualized as essentially replicating the first 30 minutes of a cohabitation period in a partner preference experiment, the social learning phase. Thus, by examining changes in patterns of Fos expression after 30 minutes of exposure to a novel opposite sex stimulus animal, we can then make inferences about how these cellular-level changes may affect an individual's perception of social interaction with a cohabitation partner. Increases in Fos expression within the PLC and NAcc shell following MTII administration in a social exposure context can be interpreted as enhancements in social reward processing. This would suggest interactions with a stimulus animal were perceived as having greater positive (hedonic) value for MTII-treated subjects as compared to controls.

Corticostriatal signaling is generally understood to underlie an individual's ability to effectively coordinate behavioral responses to secure or maintain access to rewards [154]. Projections from PLC to striatum innervate the rostral portion of the medial NAcc shell, where the highest density of accumbal OXTRs are found [24, 73, 155]. The PLC and NAcc shell both express OXTRs, and are innervated by oxytocinergic fibers

emanating from the PVN [15, 16, 138]. Specific activation of OXTRs in either the PLC or NAcc shell is necessary for the formation of mating-induced partner preferences in prairie voles, and electrophysiological studies reveal that mating in female prairie voles results in dynamic enhancements of PLC-NAcc shell net modulation [75, 138]. The overall strength of this PLC-NAcc shell net modulation can be causally predictive of the emergence of affiliative behavior between mates, and optogenetic stimulation of PLC projection terminals in the medial NAcc shell induces partner preference formation [75]. Beyond the specific process of partner preference formation, oxytocinergic signaling in the PLC or NAcc shell has been shown to affect several generalized domains of sociocognitive processing. OXTRs expressed on a specific class of prefrontal cortical interneurons modulate social approach behavior in mice, possibly by coordinating the activity of glutamatergic projection neurons to suppress background activity and enhance the signal-to-noise ratio [145]. Further studies in mice reveal that within the NAcc shell, activation of OXTRs located on presynaptic inputs projecting from the dorsal Raphe is necessary for social reward [76]. Additional *in vivo* electrophysiological studies in rats report central administration of OT increases mean firing rate of medium spiny neurons in NAcc shell [156]. Central OT administration is also reported to increase fMRI BOLD responses in NAcc shell in rats [157]. Thus, it is thought that oxytocinergic enhancement of neuronal network activity within the SSN, particularly the PLC and NAcc shell, facilitates appetitive and motivated social behavioral responding, particularly as it relates to positive or rewarding social experiences.

As the retention rate of OT in microdialysis experiments is only approximately 2%, it is difficult to ascertain exactly how much OT may be normally released during

non-sexual social encounters, or how significant of an increase we may evoke through the addition of MTII treatment [16]. Thus, in this series of studies, we utilized expression patterns of Fos protein as a proxy marker of neuronal activation and transcriptional activity within OT-sensitive regions of SSN. Fos is the protein product of the immediate early gene *c-fos,* which is transcribed in most neuronal subtypes in response to Ca^{2+} influx secondary to synaptic activity and/or membrane depolarization [158]. Fos protein dimerizes with other members of the c-Fos/Jun family to form one of several AP-1 transcription factor complexes which function to rapidly alter transcription of other genes, either positively or negatively, in response to cell surface signals [159]. Therefore, expression of Fos protein within the brain is thought to be reflective of cellular activation and synaptic activity, and patterns of Fos expression have been extensively used to study neuronal network responses to various physiological or environmental stimuli, however it is important to note that not all neuronal subtypes show strong Fos expression in response to a given stimulus. Therefore, although we did not observe any significant increases in neuronal activation following drug treatment and social exposure within the other regions examined (AON, ACC or MeA), we cannot definitively conclude there was no effect of MTII on neuronal processing within these regions. In fact, studies in male prairie voles show that blockade of OXTR during mating disrupts coordinated activation across all regions of the SSN, despite affecting no significant differences in mean levels of Fos activation within specific regions between aCSF-treated subjects and OTA-treated subjects [140]. Based on these results, and the known multimodal release mechanisms of OT with differential spatial and temporal resolutions within and across contexts, it is again possible differences in neuronal processing occur in response to

MTII-enhanced OT release that are unable to be captured using regional Fos analysis alone.

Activation of MC4Rs also stimulates other neurotransmitter systems know to be important for social cognition, particularly dopamine. Dopamine plays a critical role in motivation and reward processing. Oxytocinergic projections from the PVN target dopamine-producing cells in the ventral tegmental area (VTA), which then send dopaminergic projections to multiple corticostriatal regions, including PLC and NAcc, as part of the mesocorticolimbic reward pathway [160]. Stimulation of MC4Rs in the VTA evokes dopamine release within the NAcc [161]. OXTRs are expressed on both D1-like receptor expressing and D2-like receptor expressing medium spiny neurons in the NAcc shell of prairie voles (K. Inoue, unpublished data). Furthermore, synergistic activation of dopamine receptors and OXTRs facilitates pair bond formation [146]. It can therefore be hypothesized that simultaneous engagement of both OT and dopamine systems as a consequence of MC4R agonism may underlie the sociobehavioral effects of MTII, and further study on the specific role of dopamine in this context is warranted.

Pharmacological manipulation of the OT system is an attractive treatment strategy for social deficit symptoms present in psychiatric or neurodevelopmental disorders, particularly autism spectrum disorder (ASD). There are currently no FDA-approved pharmacological treatments for the social deficits characteristic of ASD, and the high rate of diagnosis represents a significant unmet clinical need. Current treatment strategies often utilize behavioral therapies which do appear to generate clinical gains however they often require up to 30 hours per week of intensive therapy over a period of many years [5]. Many of these behavioral therapies have the explicit target of nurturing and

reinforcing aspects of social engagement and social motivation, and enhancing perception of social reward [122]. Therefore, it is possible that the addition of OT-targeted therapeutics in this environment could further enhance behavioral therapy gains. Based on the results of our Fos expression studies, it appears MTII enhances OT-dependent social motivation and social reward processing in a context-dependent manner. While there are several published studies which show positive efficacy of IN-OT in the amelioration of social deficits in ASD patients, IN-OT administration is consistently tested in a manner that is context-free. It often contains more OT than the total average pituitary content of OT in humans, given in a single bolus dose 40-60 minutes before a behavioral task or imaging session [110]. Furthermore, it is currently unknown if IN-OT is able to effectively penetrate the brain to gain access to central OXTRs, particularly those expressed deep within OXTR-expressing regions of hindbrain such as the superior colliculus or the nucleus basalis of Meynert [19, 30, 110]. The ability of MTII to enhance endogenous central OT release means increased oxytocinergic signaling would occur in a context in which OT is normally released within the brain, and would be specifically targeted to brain regions which express OXTR. Pharmacological targeting of the OT system in this way could provide significantly improved therapeutic efficacy over IN-OT, particularly as an adjuvant to behavioral therapy. Furthermore, the potential synergistic role of MC4R agonist-enhanced OT and dopamine release could be an additional therapeutic benefit in the context of therapies targeting social motivation and social reward processes. In an animal model of a monogenic form of ASD, the *Cntnap2* knockout mouse, social interaction deficits are reversed by administration of either OT or an MC4R agonist [128]. MTII has already been tested in humans for safety and

tolerability, and therefore could potentially be more easily tested in a clinical trial in ASD patients. Based on the results of our studies in prairie voles, clinical trials of MC4R agonists in ASD patient populations, particularly within the context of increasing behavioral therapy gains, is a promising future direction in this field.

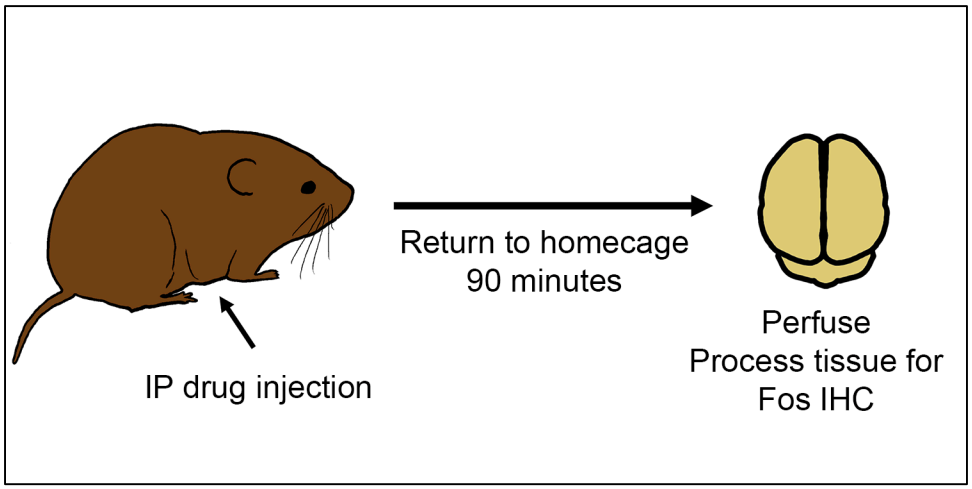

Figure 3.1. Homecage Fos expression peripheral treatment experimental design. To determine the levels of Fos expression across the brain in the homecage environment, experimental subjects were injected peripherally (IP) with either Melanotan II (10mg/kg) or Saline control, and returned to their homecage for 90 minutes. At the end of 90 minutes, animals were perfused transcardially and their brains removed and tissue processed for immunohistochemical detection of Fos protein product.

Figure 3.2. Social Exposure Fos expression peripheral treatment experimental design. To determine the effect of peripheral Melanotan II treatment on levels of Fos expression across the brain in a social context, experimental subjects were injected IP with either Melanotan II (10mg/kg) or Saline control, and placed into a clean cage to habituate to the test environment for 30 minutes. At the end of the habituation period, a novel opposite sex stimulus animal was introduced to the test cage and subjects were allowed to interact freely for 30 minutes. At the end of the 30 minute social exposure period, subjects were returned to their homecage for 60 minutes. After 60 minutes, subjects were perfused transcardially and brains removed. Brain tissue was later sectioned and processed for immunohistochemical visualization of Fos protein product expression.

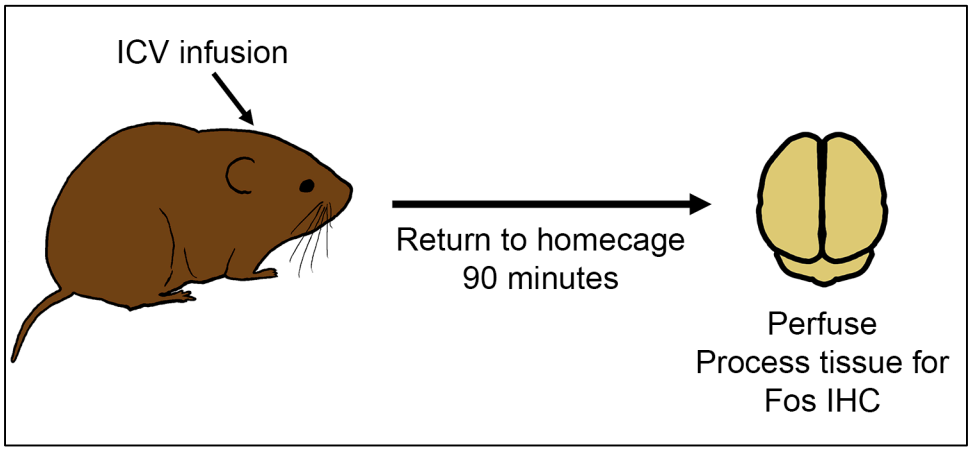

Figure 3.3. Homecage Fos expression central drug treatment experimental design. To determine the levels of Fos expression following central drug treatment in the homecage environment, subjects were lightly anesthetized with isoflurane and infused with either Melanotan II (3nmol) or artificial cerebrospinal fluid (aCSF) control through an intracerebroventricular guide cannula over a period of 5 minutes. After infusion, subjects were returned to their homecage for 90 minutes. At the end of 90 minutes, subjects were perfused and their brains collected to permit tissue processing for immunohistochemical detection and visualization of Fos protein product.

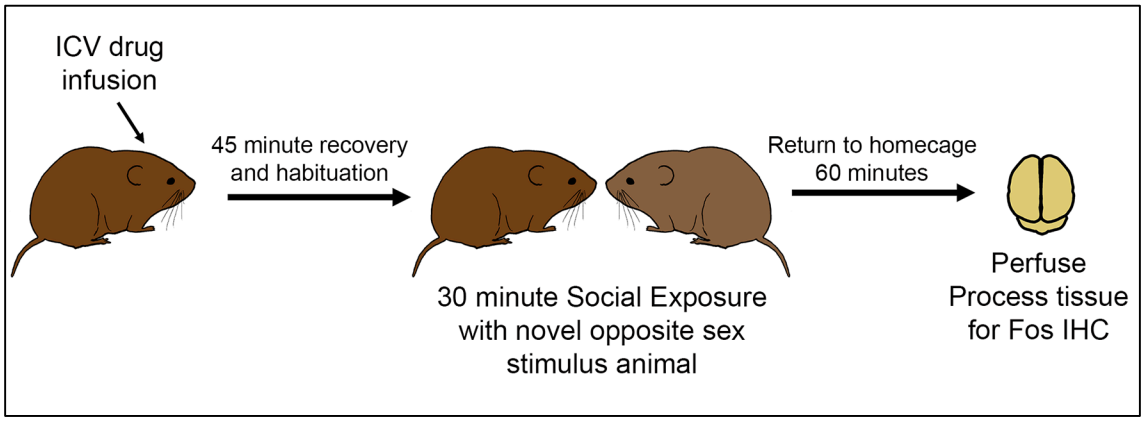

Figure 3.4. Social Exposure Fos expression central treatment experimental design. To test the hypothesis that elevated Fos expression in select brain regions in a social context is mediated by a central effect of Melanotan II, subjects were lightly anesthetized with isoflurane to permit injection through an intracerebroventricular guide cannula. Melanotan II (3nmol) or aCSF was infused over a period of 5 minutes. After infusion, subjects were placed in a clean cage for 45 minutes to recover from anesthesia and habituate to the test environment. At the end of the habituation period, a novel opposite sex stimulus animal was introduced to the test cage. Subjects were allowed to interact freely with the stimulus animal for 30 minutes before being returned to their homecage for 60 minutes. At the end of these 60 minutes, subjects were perfused transcardially and brains removed. Brain tissue was sectioned and processed for immunohistochemical detection of Fos protein product.

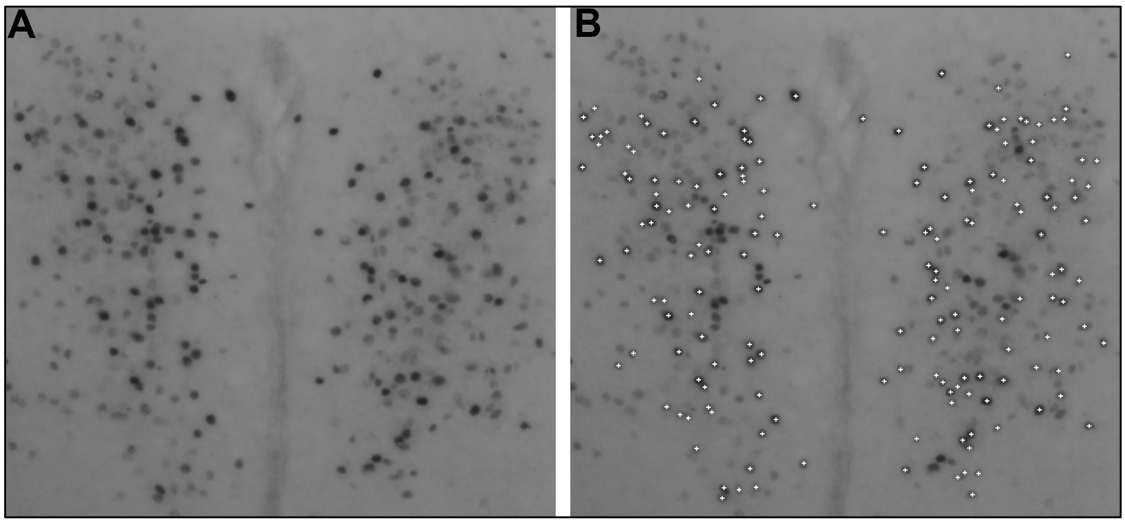

Figure 3.5. Automated cell counting algorithm example. Automated counting of Fos+ cells was performed using MCID software using a grain count function. A) shows a representative image taken of the paraventricular nucleus of the hypothalamus stained for Fos protein. B) shows which cells the MCID software counted as Fos+ (marked with a white + sign). Cells not marked with + were not counted by the software. Clusters of Fos+ cells are sometimes excluded from the software's automated counting algorithm due to an inability to distinguish them as individual cells as opposed to one solid dark mass which does not adhere to the software's shape requirements for what is recognized as a "cell". This can result in final cell counts that are under-estimates of total Fos+ cells however as the same threshold parameters are applied to all images in a given experiment the final cell count values used for statistical analyses are still considered valid and useful proxies of total actual Fos+ cell counts.

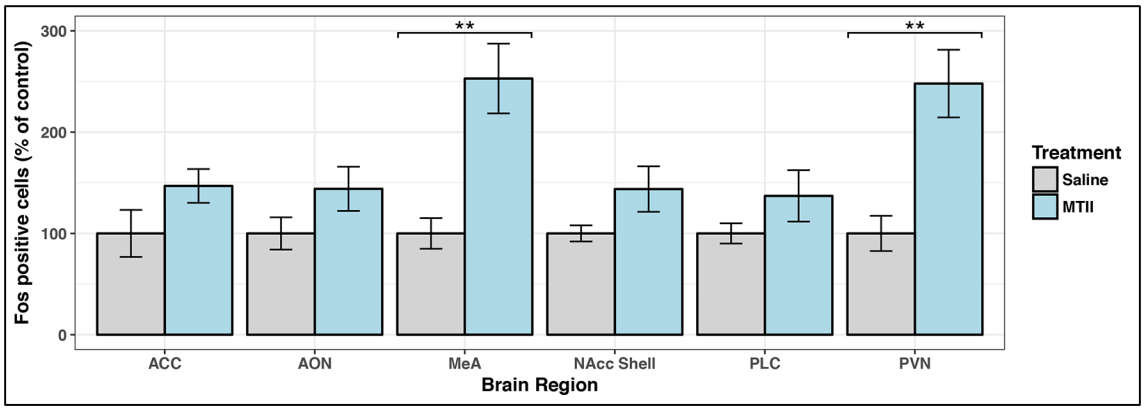

Figure 3.6. Peripheral MTII treatment in male prairie voles in the homecage environment does not significantly activate mesocorticolimbic reward regions of the Social Salience Network. Peripheral (IP) injection of MTII (10mg/kg) or saline control in the homecage environment significantly increases Fos expression in the paraventricular nucleus of the hypothalamus (PVN, p=0.002) and the medial amygdala (MeA, p=0.002) in male prairie voles. There is no significant increase in Fos expression in the anterior cingulate cortex (ACC), anterior olfactory nucleus (AON), nucleus accumbens shell (NAcc shell), or prelimbic cortex (PLC). Fos-positive cell counts are represented as percent of the averaged Fos-positive counts per region from saline treated controls. Error bars are ± SEM. ** $p<0.005$

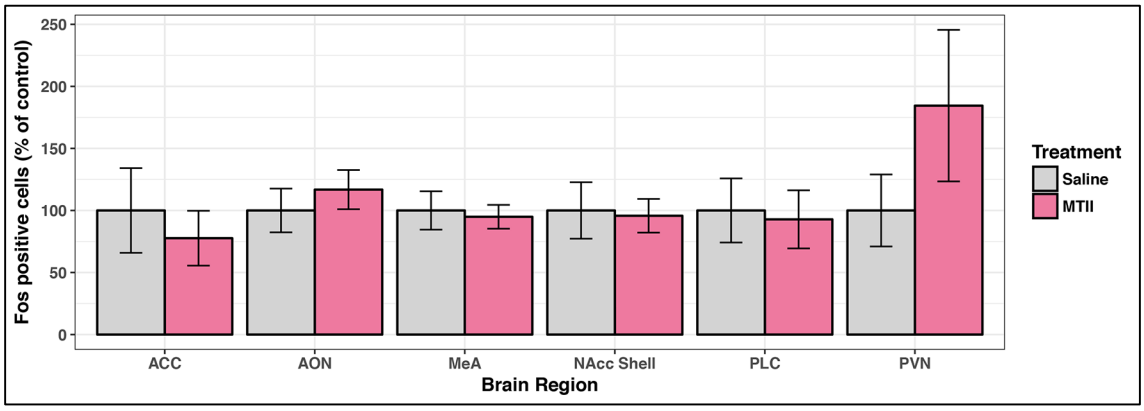

Figure 3.7. Peripheral MTII treatment in female prairie voles in the homecage environment does not significantly activate oxytocin-sensitive regions of the Social Salience Network. Peripheral (IP) injection of MTII (10mg/kg) or saline control in the homecage environment does not significantly increase Fos expression in female prairie voles in any of the regions analyzed. There were no significant differences between MTII-treated females and saline treated controls in the anterior cingulate cortex (ACC), anterior olfactory nucleus (AON), the medial amygdala (MeA), the nucleus accumbens shell (NAcc shell), the prelimbic cortex (PLC) or the paraventricular nucleus of the hypothalamus (PVN). Fos-positive cell counts are represented as percent of the averaged Fos-positive counts per region from saline treated controls. Error bars are ± SEM.

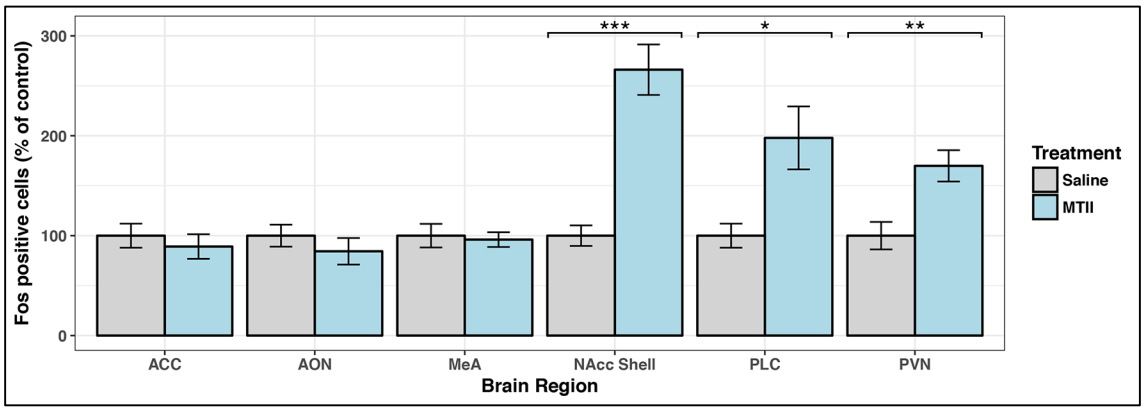

Figure 3.8. Peripheral MTII treatment in male prairie voles in a social context significantly increases Fos expression in several regions of the Social Salience network. Male prairie voles treated with peripheral (IP) MTII (10mg/kg) prior to 30 minutes of social exposure to a novel stimulus female show significant increases in Fos expression in the nucleus accumbens shell (NAcc shell, $p<0.001$) and paraventricular nucleus of the hypothalamus (PVN, $p=0.003$) compared to saline-treated controls. A significant increase in Fos expression was also observed in the prelimbic cortex (PLC, $p=0.01$) however this increase does not remain statistically significant after applying appropriate corrections for multiple comparisons. There were no significant differences in Fos expression in the anterior cingulate cortex (ACC), anterior olfactory nucleus (AON), or medial amygdala (MeA). Fos-positive cell counts are represented as percent of the averaged Fos-positive counts per region from saline treated controls. Uncorrected p-values are represented as: * $p<0.05$, ** $p<0.005$, *** $p<0.001$. Error bars are ± SEM.

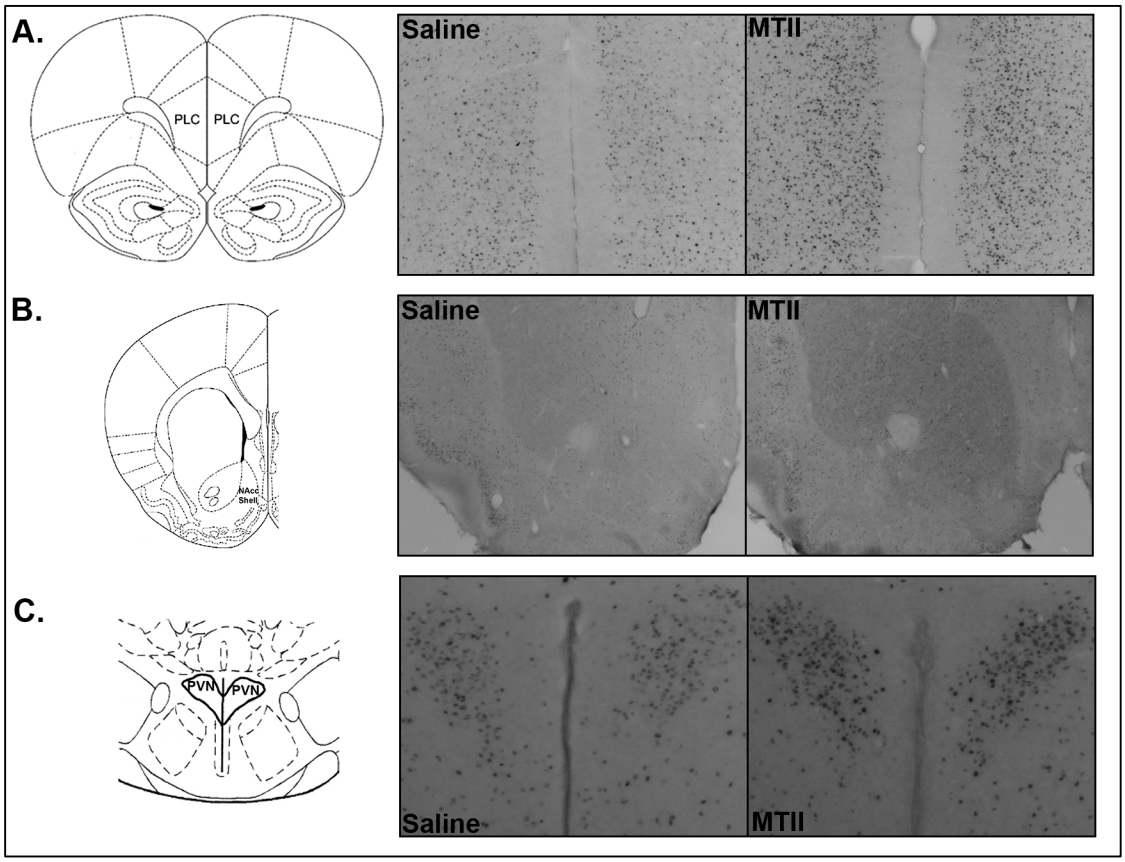

Figure 3.9. Representative micrographs of Fos+ cells following peripheral MTII treatment and social exposure in male prairie voles. Sample representative images from male prairie voles treated with peripheral MTII or Saline control from the prelimbic cortex (PLC, A), nucleus accumbens shell (NAcc Shell, B), and paraventricular nucleus of the hypothalamus (PVN, C).

Mean raw cell count values - Peripheral treatment in males

Region	*Context*	*Treatment*	*Fos+ cell count*
PVN	Homecage	Saline	58 ± 10
		MTII	145 ± 20 **
	Social Exposure	Saline	94 ± 13
		MTII	160 ± 15 **
NAcc Shell	Homecage	Saline	20 ± 2
		MTII	29 ± 4
	Social Exposure	Saline	43 ± 4
		MTII	115 ± 11 ***
PLC	Homecage	Saline	63 ± 6
		MTII	87 ± 16
	Social Exposure	Saline	204 ± 24
		MTII	403 ± 64 *
MeA	Homecage	Saline	23 ± 3
		MTII	58 ± 8 **
	Social Exposure	Saline	121 ± 14
		MTII	116 ± 9
AON	Homecage	Saline	29 ± 5
		MTII	42 ± 6
	Social Exposure	Saline	129 ± 14
		MTII	108 ± 17
ACC	Homecage	Saline	28 ± 6
		MTII	41 ± 5
	Social Exposure	Saline	141 ± 17
		MTII	126 ± 17

Table 3.1. Mean raw cell count values from peripherally-treated male prairie voles. Averaged raw counts of Fos-positive cells in male prairie voles injected peripherally (IP) with either MTII (10mg/kg) or saline control in both homecage and social exposure contexts. Number of Fos-positive nuclei ± SEM are presented for all regions analyzed: anterior cingulate cortex (ACC), anterior olfactory nucleus (AON), medial amygdala (MeA), nucleus accumbens shell (NAcc shell), prelimbic cortex (PLC) and paraventricular nucleus of the hypothalamus (PVN). * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

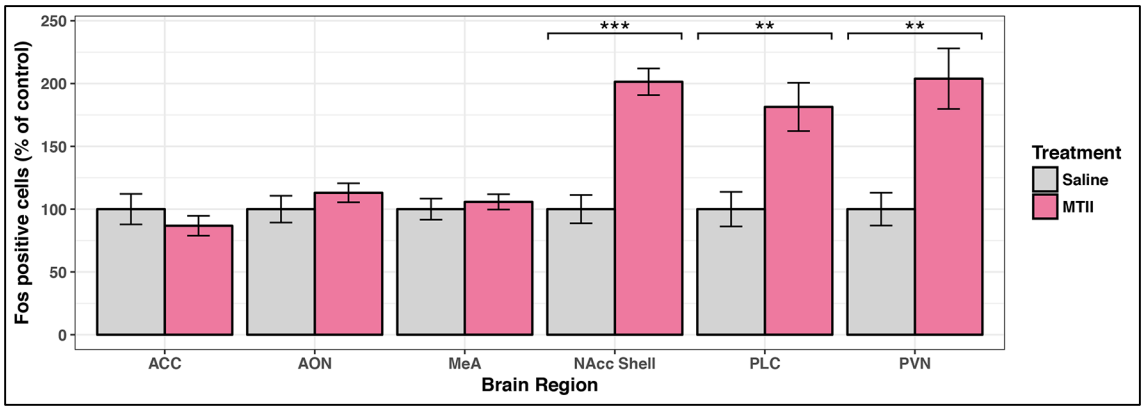

Figure 3.10. Peripheral MTII treatment in female prairie voles in a social context significantly increases Fos expression in several regions of the Social Salience network. Female prairie voles treated with peripheral (IP) MTII (10mg/kg) prior to 30 minutes of social exposure to a novel stimulus male show robust and significant increases in Fos expression in the nucleus accumbens shell (NAcc shell, $p<0.001$), the prelimbic cortex (PLC, $p=0.003$) and paraventricular nucleus of the hypothalamus (PVN, $p=0.001$) as compared to saline-treated controls. There were no significant differences in Fos expression in the anterior cingulate cortex (ACC), anterior olfactory nucleus (AON), or medial amygdala (MeA). Fos-positive cell counts are represented as percent of the averaged Fos-positive counts per region from saline treated controls. ** $p<0.005$, *** $p<0.001$. Error bars are ± SEM.

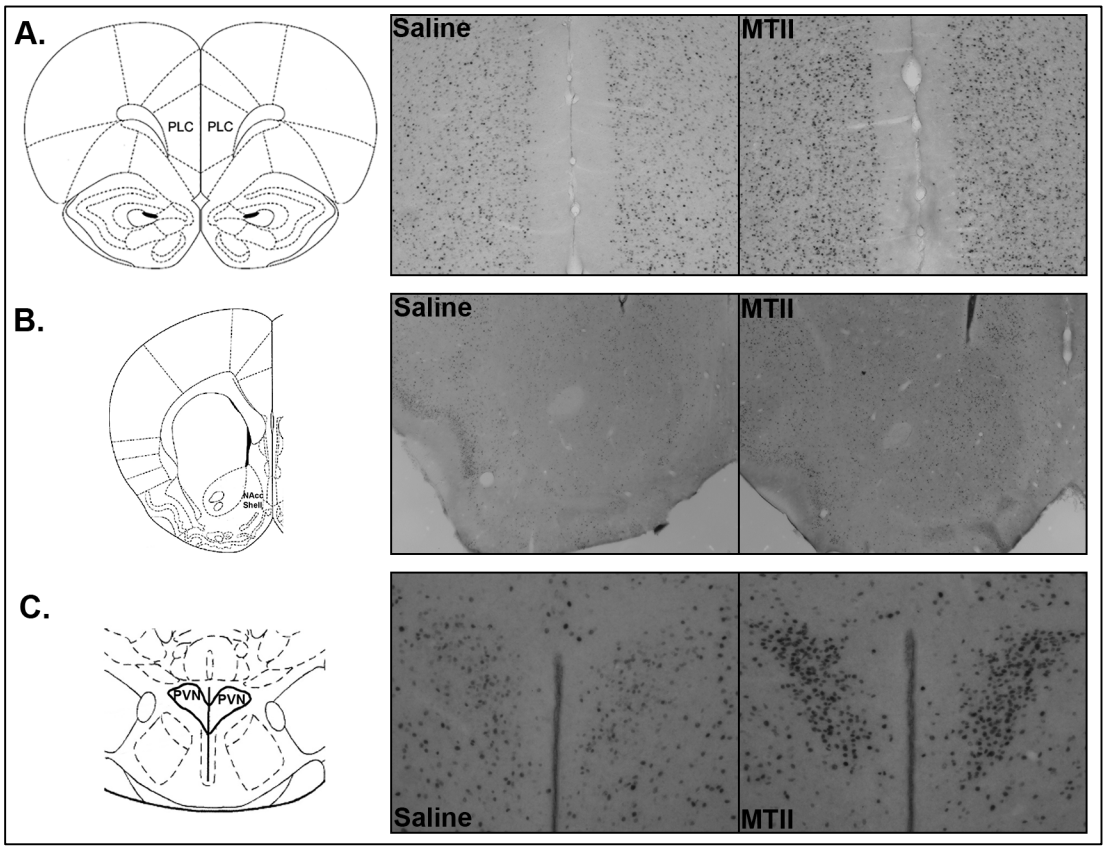

Figure 3.11. Representative micrographs of Fos+ cells following peripheral MTII treatment and social exposure in female prairie voles. Sample representative images from female prairie voles treated with peripheral MTII or Saline control from the prelimbic cortex (PLC, A), nucleus accumbens shell (NAcc Shell, B), and paraventricular nucleus of the hypothalamus (PVN, C).

Mean raw cell count values - Peripheral treatment in females

Region	*Context*	*Treatment*	*Fos+ cell count*
PVN	Homecage	Saline	35 ± 12
		MTII	67 ± 22
	Social Exposure	Saline	73 ± 10
		MTII	149 ± 18 **
NAcc Shell	Homecage	Saline	67 ± 8
		MTII	82 ± 12
	Social Exposure	Saline	152 ± 17
		MTII	305 ± 16 ***
PLC	Homecage	Saline	66 ± 21
		MTII	67 ± 17
	Social Exposure	Saline	216 ± 30
		MTII	392 ± 42 **
MeA	Homecage	Saline	57 ± 9
		MTII	54 ± 5
	Social Exposure	Saline	156 ± 13
		MTII	165 ± 10
AON	Homecage	Saline	47 ± 8
		MTII	55 ± 7
	Social Exposure	Saline	107 ± 11
		MTII	121 ± 8
ACC	Homecage	Saline	34 ± 12
		MTII	27 ± 7
	Social Exposure	Saline	143 ± 17
		MTII	124 ± 11

Table 3.2. Mean raw cell count values from peripherally-treated female prairie voles. Averaged raw counts of Fos-positive cells in female prairie voles injected peripherally (IP) with either MTII (10mg/kg) or saline control in both homecage and social exposure contexts. Number of Fos-positive nuclei ± SEM are presented for all regions analyzed: anterior cingulate cortex (ACC), anterior olfactory nucleus (AON), medial amygdala (MeA), nucleus accumbens shell (NAcc shell), prelimbic cortex (PLC) and paraventricular nucleus of the hypothalamus (PVN). ** $p<0.005$, *** $p<0.001$.

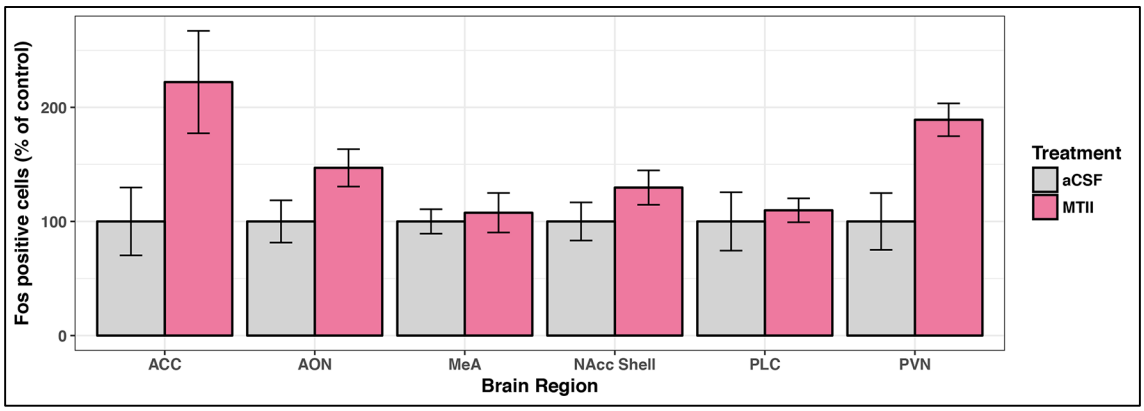

Figure 3.12. Central MTII treatment in female prairie voles in the homecage environment does not significantly activate oxytocin-sensitive regions of the Social Salience Network. Central (intracerebroventricular) injection of MTII (3nmol) or saline control in the homecage environment does not significantly increase Fos expression in female prairie voles in any of the regions analyzed. There were no significant differences between MTII-treated females and aCSF-treated controls in the anterior cingulate cortex (ACC), anterior olfactory nucleus (AON), the medial amygdala (MeA), the nucleus accumbens shell (NAcc shell), the prelimbic cortex (PLC) or the paraventricular nucleus of the hypothalamus (PVN). Fos-positive cell counts are represented as percent of the averaged Fos-positive counts per region from saline treated controls. Error bars are ± SEM.

Mean raw cell count values - Central treatment in females

Region	*Context*	*Treatment*	*Fos+ cell count*
PVN	Homecage	aCSF	95 ± 24
		MTII	180 ± 14
NAcc Shell	Homecage	aCSF	94 ± 16
		MTII	122 ± 14
PLC	Homecage	aCSF	347 ± 89
		MTII	381 ± 36
MeA	Homecage	aCSF	25 ± 3
		MTII	27 ± 4
AON	Homecage	aCSF	22 ± 4
		MTII	33 ± 4
ACC	Homecage	aCSF	10 ± 3
		MTII	23 ± 5

Table 3.3. Mean raw cell count values from centrally-treated female prairie voles in the homecage environment. Averaged raw counts of Fos-positive cells in female prairie voles injected centrally (intracerebroventricularly) with either MTII (3nmol) or aCSF control in the homecage context. Number of Fos-positive nuclei ± SEM are presented for all regions analyzed: anterior cingulate cortex (ACC), anterior olfactory nucleus (AON), medial amygdala (MeA), nucleus accumbens shell (NAcc shell), prelimbic cortex (PLC) and paraventricular nucleus of the hypothalamus (PVN).

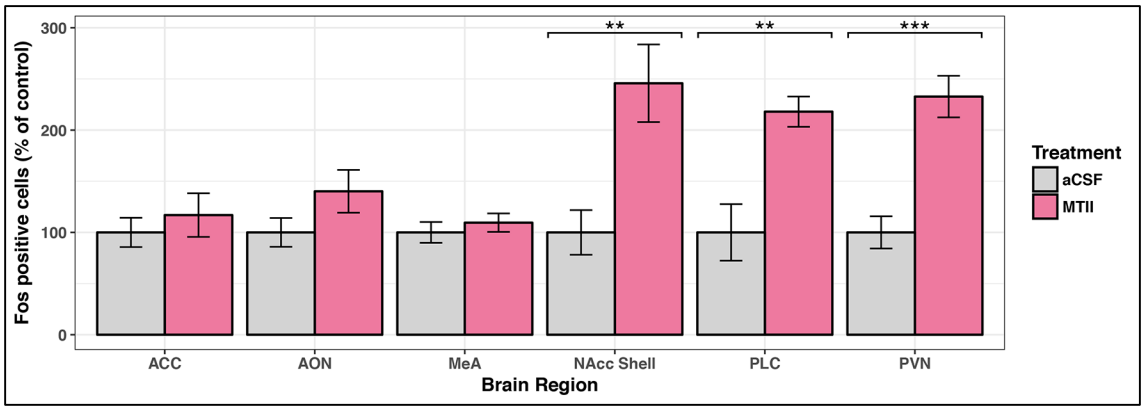

Figure 3.13. Central MTII treatment in female prairie voles in a social context significantly increases Fos expression in several regions of the Social Salience network. Female prairie voles treated with central (intracerebroventricular) MTII (3nmol) prior to 30 minutes of social exposure to a novel stimulus male show robust and significant increases in Fos expression in the nucleus accumbens shell (NAcc shell, $p=0.004$), the prelimbic cortex (PLC, $p=0.001$) and paraventricular nucleus of the hypothalamus (PVN, $p=0.0001$) as compared to aCSF-treated controls. There were no significant differences in Fos expression in the anterior cingulate cortex (ACC), anterior olfactory nucleus (AON), or medial amygdala (MeA). Fos-positive cell counts are represented as percent of the averaged Fos-positive counts per region from saline treated controls. ** $p<0.005$, *** $p<0.001$. Error bars are ± SEM.

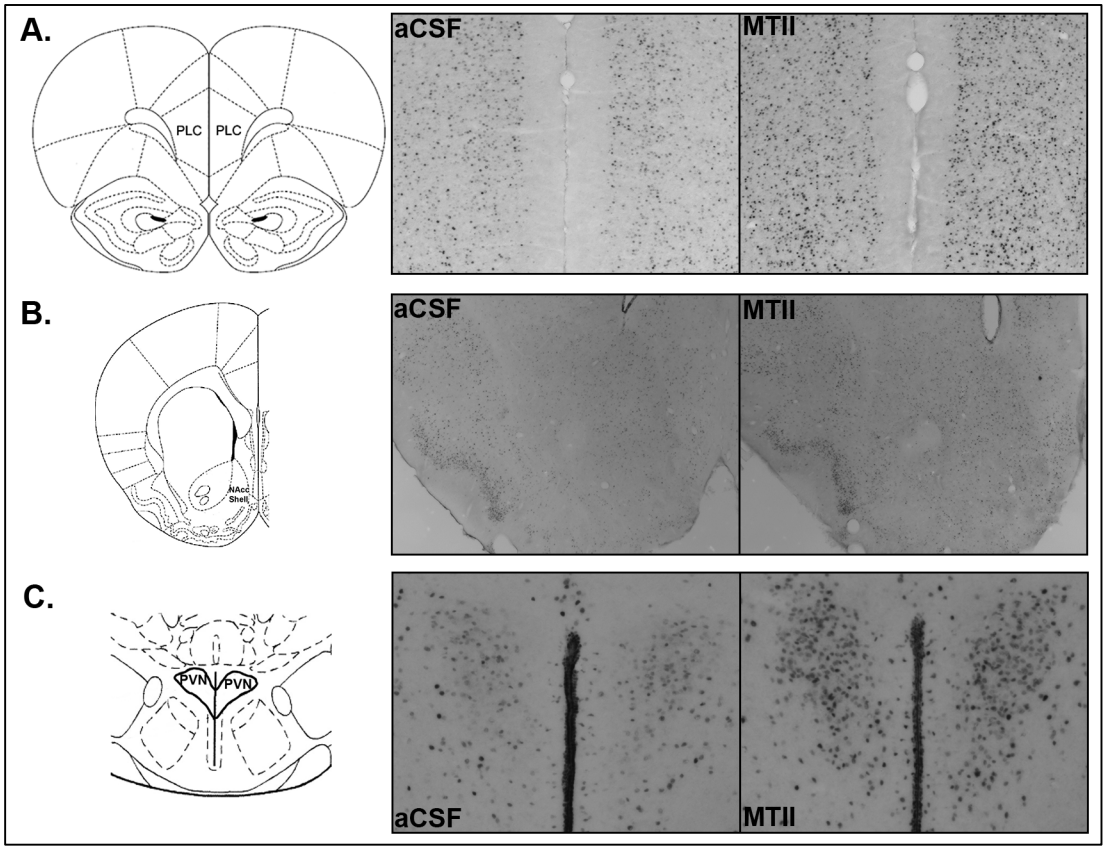

Figure 3.14. Representative micrographs of Fos+ cells following central MTII treatment and social exposure in female prairie voles. Sample representative images from female prairie voles treated with central (ICV) MTII or aCSF control from the prelimbic cortex (PLC, A), nucleus accumbens shell (NAcc Shell, B), and paraventricular nucleus of the hypothalamus (PVN, C).

Mean raw cell count values - Central treatment in females

Region	*Context*	*Treatment*	*Fos+ cell count*
PVN	Social Exposure	aCSF	42 ± 7
		MTII	98 ± 9 ***
NAcc Shell	Social Exposure	aCSF	33 ± 7
		MTII	82 ± 13 **
PLC	Social Exposure	aCSF	178 ± 49
		MTII	388 ± 26 **
MeA	Social Exposure	aCSF	240 ± 24
		MTII	263 ± 22
AON	Social Exposure	aCSF	59 ± 8
		MTII	83 ± 12
ACC	Social Exposure	aCSF	50 ± 7
		MTII	58 ± 11

Table 3.4. Mean raw cell count values from centrally-treated female prairie voles in the social exposure context. Averaged raw counts of Fos-positive cells in female prairie voles injected with central (intracerebroventricular) MTII (3nmol) or control aCSF prior to 30 minutes of social exposure with a novel stimulus male. Number of Fos-positive nuclei ± SEM are presented for all regions analyzed: anterior cingulate cortex (ACC), anterior olfactory nucleus (AON), medial amygdala (MeA), nucleus accumbens shell (NAcc shell), prelimbic cortex (PLC) and paraventricular nucleus of the hypothalamus (PVN). ** $p<0.005$, *** $p<0.001$

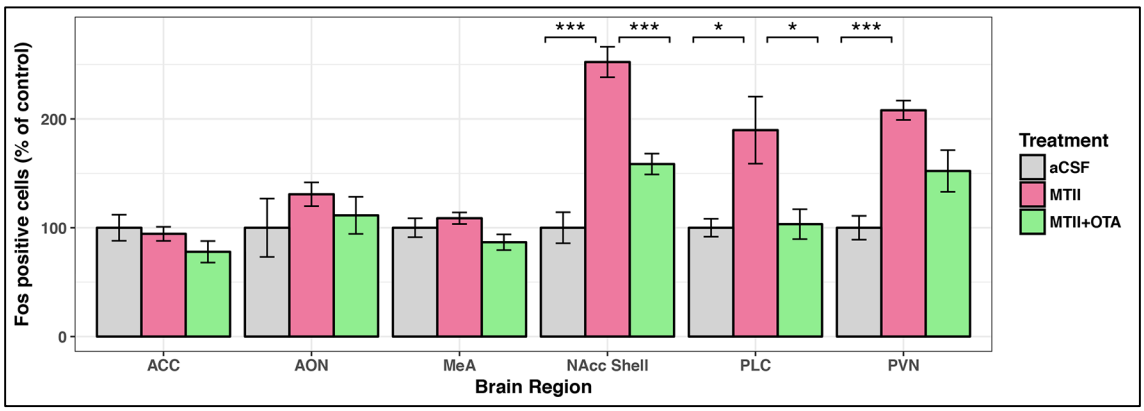

Figure 3.15. Selective blockade of oxytocin receptor signaling significantly attenuates MTII-mediated increases in Fos expression in mesocorticolimbic reward regions of the Social Salience Network in a social context. Central (intracerebroventricular) injection of MTII (3nmol) in female prairie voles prior to 30 minutes of social exposure to a novel stimulus male significantly increases Fos expression compared to control females treated with aCSF in the nucleus accumbens (NAcc shell, $p<0.001$) and paraventricular nucleus of the hypothalamus (PVN, $p<0.001$). Significant increases in Fos expression in the prelimbic cortex (PLC, $p=0.01$) were observed in MTII-treated females compared to aCSF-treated controls however this increase does not remain statistically significant after applying appropriate corrections for multiple comparisons. Co-injection of MTII with a selective oxytocin receptor antagonist (MTII+OTA; MTII 3nmol + OTA 5ng) significantly attenuates Fos expression compared to MTII-treated females in NAcc shell ($p<0.001$). Fos expression is also reduced in MTII+OTA-treated females compared to MTII-treated females in PLC ($p=0.02$) however this reduction does not remain statistically significant after applying appropriate corrections for multiple comparisons. There were no significant differences in Fos expression between any groups in the anterior cingulate cortex (ACC), anterior olfactory nucleus (AON) or medial amygdala (MeA). Fos-positive cell counts are represented as percent of the averaged Fos-positive counts per region from saline treated controls. Uncorrected p-values are represented as: * $p<0.05$, ** $p<0.005$, *** $p<0.001$. Error bars are ± SEM.

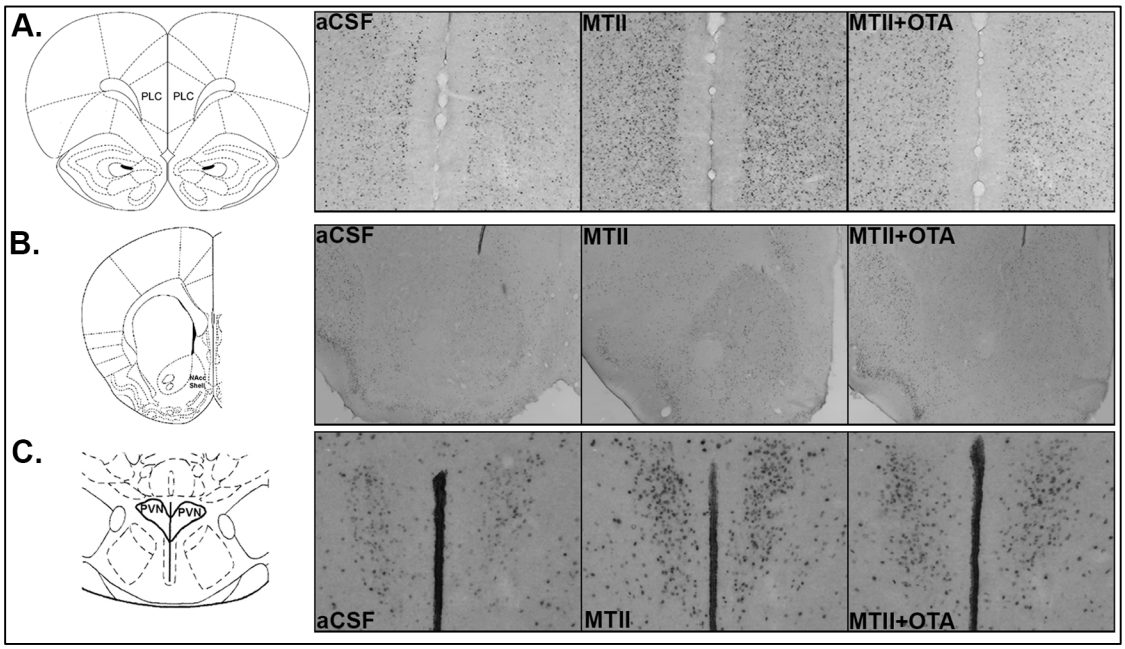

Figure 3.16. Representative micrographs of Fos+ cells following central drug treatment and social exposure in female prairie voles. Sample representative images from female prairie voles treated with central (ICV) MTII, MTII+OTA, or aCSF control from the prelimbic cortex (PLC, A), nucleus accumbens shell (NAcc Shell, B), and paraventricular nucleus of the hypothalamus (PVN, C).

Mean raw cell count values - Central treatment in females

Region	*Context*	*Treatment*	*Fos+ cell count*
PVN	Social Exposure	aCSF	83 ± 9
		MTII	126 ± 16 ***
		MTII + OTA	173 ± 7
NAcc Shell	Social Exposure	aCSF	37 ± 5
		MTII	93 ± 5 ***
		MTII + OTA	59 ± 4 △△△
PLC	Social Exposure	aCSF	215 ± 18
		MTII	407 ± 66 *
		MTII + OTA	222 ± 29 △
MeA	Social Exposure	aCSF	268 ± 23
		MTII	282 ± 15
		MTII + OTA	232 ± 19
AON	Social Exposure	aCSF	51 ± 14
		MTII	66 ± 6
		MTII + OTA	57 ± 9
ACC	Social Exposure	aCSF	98 ± 12
		MTII	93 ± 6
		MTII + OTA	76 ± 10

Table 3.5. Mean raw cell count values from centrally-treated female prairie voles in the social exposure context. Averaged raw counts of Fos-positive cells in female prairie voles injected with central (intracerebroventricular) MTII (3nmol), MTII plus a selective oxytocin receptor antagonist (MTII+OTA; 3nmol MTII + 5ng OTA) or control aCSF prior to 30 minutes of social exposure with a novel stimulus male. Number of Fos-positive nuclei ± SEM are presented for all regions analyzed: anterior cingulate cortex (ACC), anterior olfactory nucleus (AON), medial amygdala (MeA), nucleus accumbens shell (NAcc shell), prelimbic cortex (PLC) and paraventricular nucleus of the hypothalamus (PVN). Statistical comparisons between MTII-treated females and aCSF-treated females are represented by: * $p<0.05$, ** $p<0.005$, *** $p<0.001$. Comparisons between MTII-treated females and MTII+OTA treated females are represented by: △ $p<0.05$, △△△ $p<0.001$.

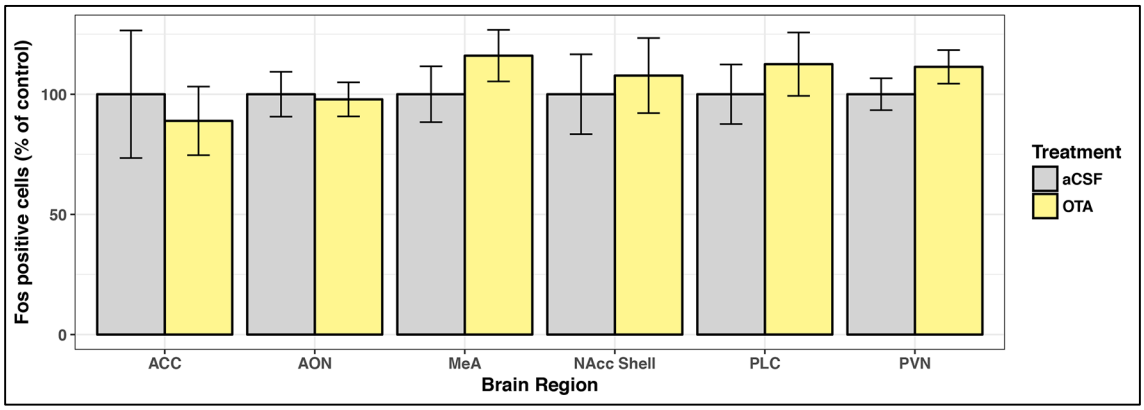

Figure 3.17. Oxytocin receptor blockade in a social exposure context does not significantly alter Fos expression in the Social Salience Network. Female subjects injected with central (intracerebroventricular) oxytocin receptor antagonist (OTA, 5ng) or control aCSF prior to 30 minutes of social exposure to a novel stimulus male show no significant differences in Fos expression in all regions analyzed: anterior cingulate cortex (ACC), anterior olfactory nucleus (AON), medial amygdala (MeA), nucleus accumbens shell (NAcc shell), prelimbic cortex (PLC), paraventricular nucleus of the hypothalamus (PVN). Fos-positive cell counts are represented as percent of the averaged Fos-positive counts per region from saline treated controls. Error bars are ± SEM.

Mean raw cell count values - Central treatment in females

Region	*Context*	*Treatment*	*Fos+ cell count*
PVN	Social Exposure	aCSF	137 ± 9
		OTA	153 ± 10
NAcc Shell	Social Exposure	aCSF	32 ± 5
		OTA	35 ± 5
PLC	Social Exposure	aCSF	215 ± 27
		OTA	242 ± 28
MeA	Social Exposure	aCSF	89 ± 10
		OTA	103 ± 9
AON	Social Exposure	aCSF	107 ± 10
		OTA	105 ± 8
ACC	Social Exposure	aCSF	75 ± 20
		OTA	67 ± 11

Table 3.6. Mean raw cell count values from centrally-treated female prairie vole control subjects in the social exposure context. Averaged raw counts of Fos-positive cells in female prairie voles injected with central (intracerebroventricular) OTA (5ng) or aCSF prior to 30 minutes of social exposure with a novel stimulus male. Number of Fos-positive nuclei ± SEM are presented for all regions analyzed: anterior cingulate cortex (ACC), anterior olfactory nucleus (AON), medial amygdala (MeA), nucleus accumbens shell (NAcc shell), prelimbic cortex (PLC) and paraventricular nucleus of the hypothalamus (PVN).

CHAPTER 4:

General Conclusions and Future Directions

Conclusions

The evolutionarily conserved central OT system mediates social information processing and social reward and motivation to generate adaptive social functioning. The Social Salience Network (SSN) is comprised of several OT-sensitive brain regions responsible for integrating the physiological, emotional, contextual and motivational properties of social stimuli [143]. Alterations in SSN function, regionally or across the entire network, can affect multiple domains of social cognition. Targeted genetic or pharmacological disruptions of central OT signaling in animal models generates deficits in sociobehavioral functioning, while augmenting central OT signaling enhances social cognition. Augmented central OT signaling improves sociocognitive functioning in several animal models of healthy social interactive behaviors, animal models of diseases characterized by social deficits, healthy human populations, and human patient populations with social deficit symptoms. Furthermore, pharmacological manipulation of central OT signaling does not appear to disrupt or alter other essential physiological functions and appears to have few, if any, off-target effects [162]. Thus, pharmacological targeting of the central OT system is an attractive therapeutic strategy for the amelioration of social dysfunction in psychiatric or neurodevelopment disorders, particularly autism spectrum disorder (ASD).

Direct pharmacological manipulation of the central OT system is hindered by the biochemical properties of OT itself. Studies in humans have attempted to circumvent some of these issues through intranasal OT administration (IN-OT), which is proposed to increase central penetrance of neuropeptides [112]. While behavioral studies in healthy and ASD patient populations have reported improvements in several domains of

sociocognitive functioning following IN-OT administration, recent studies cast doubt on the idea of a "privileged nose-to-brain" route of neuropeptide entry [116]. Therefore, while IN-OT studies have provided excellent proof-of-principle efficacy for the manipulation of central OT signaling as a pharmacological enhancer of social cognition, clinical benefits could be significantly increased through a more precise targeting of the central OT system.

One attractive potential method of achieving this goal is through indirect manipulation of the central OT system. These methods could include positive allosteric modulators of OXTRs, synthetic small molecule OT agonists, or compounds targeted to receptors expressed on OT-producing neurons which then promote increases in downstream OT release, such as MC4R agonists. Approaching the goal of manipulating central neurochemical functioning in an indirect manner is a common theme in psychiatry. For example, serotonin is implicated in the underlying etiology of depression, however exogenously administered serotonin is not able to effectively cross the BBB. Instead, the most common serotonergic treatments of depression in use today are selective serotonin reuptake inhibitors. By slowing reuptake of serotonin at the synapse, these compounds function to effectively increase serotonergic signaling indirectly [163]. In this same vein, MC4R agonists which increase physiologically-evoked endogenous central OT release are proposed as a viable treatment method for the amelioration of social deficits. Some MC4R agonists, such as Melanotan II (MTII), have already been tested for safety and tolerability in humans, making the transition from preclinical animal studies to human clinical trials significantly easier.

While *in vitro* and *in vivo* microdialysis studies confirm application of MC4R agonists evokes endogenous somatodendritic OT release and primes OT neurons for enhanced subsequent release, it is important to demonstrate this physiological effect is behaviorally relevant. Preclinical studies in animal models of OT-dependent sociocognitive functioning are a critically important step in the identification and development of novel pharmacotherapies. Models which specifically recapitulate disease states, such as monogenic forms of ASD, or models of healthy social cognition are both informative and often complimentary in their utility. Formation of selective social attachments (pair bonds or 'partner preferences') in the socially monogamous prairie vole represent a complex sociocognitive process that requires intact central OT signaling. Furthermore, studies of social bonding in this model capture multiple domains of social cognition relevant to human social behavioral functioning, including social information processing, social reward and reinforcement, and social learning and memory. These sociocognitive domains are not exclusively involved in social bond formation and hence this model has wide translational applicability to human sociality in general. There is a high degree of evolutionary conservation of function in the neurochemical systems which modulate social functioning, particularly the OT system. In general, as social behavioral complexity increased across evolutionary time, it is often observed that additional layers of functionality seem to be endowed to existing "older" circuitry. For example, it is hypothesized that the capacity for social bond formation between adult mating partners in socially monogamous species likely arose from an evolutionary "repurposing" of the circuitry responsible for mother-infant bonds, which is present in all mammalian species [14]. Thus, while the complexity and diversity of human sociocognitive functions is

unparalleled in other mammalian species, mechanistic studies in rodent models can still provide key insights to basic neuronal network functioning as it relates to social cognition. These mechanistic studies can provide the preclinical justification needed to advance pharmacological treatment trials into human populations.

Project 1: Behavioral effects of a melanocortin 4 receptor agonist in the prairie vole

We report here peripheral MTII in male prairie voles facilitates partner preference formation in a manner identical to that previously reported in females. Peripherally administered MTII crosses the BBB in prairie voles, however it is not entirely selective for MC4R and is thus capable of simultaneously activating peripherally-expressed melanocortin receptor subtypes [120]. Therefore, we next attempted to demonstrate a restricted central effect of MTII on partner preference formation using intracerebroventricular (ICV) administration in both males and females. These experiments were not able to be accurately interpreted due to difficulties with establishing a reliable control group in which partner preferences were not observed to form (for further discussion of this issue, see Appendix). This problem was less pronounced in female subjects compared to males, and there appears to be a trend towards facilitation of partner preference formation in females following ICV MTII administration, however again these results cannot be conclusively interpreted. There are several potential mechanisms by which MTII could act to enhance partner preference formation. In our experiments, and previous experiments using MTII in prairie voles, drug treatment was given prior to the start of the social learning phase of cohabitation. Having MTII on board during this social learning phase, and presumably enhancing OT release during this time, could affect several OT-dependent sociocognitive processes;

such as social recognition and memory or social reward and reinforcement. It was unclear, however, if modifying OT release, and by proxy any of these physiological processes underlying social cognition, could also affect observable social interactive behaviors. Therefore, we conducted a separate series of experiments to quantify reciprocal social interactions between opposite sex pairs of prairie voles. In these experiments, females administered either peripheral or central MTII showed no significant change in the amount of time spent engaged in reciprocal social interactions with a novel male stimulus animal. Interestingly, male prairie voles receiving peripheral MTII showed a significant decrease in the amount of time spent engaged in reciprocal social interactions with a novel female compared to saline injected controls. It is unclear from the experiments presented here why males show a differential acute behavioral response to MTII as compared to females. Furthermore, despite the observations of an apparent negative shift in reciprocal social interactive behavior following acute MTII treatment, male MTII-treated subjects still ultimately show enhanced social cognition as measured by partner preference formation. These seemingly incongruent results suggest there may be several parallel processes activated by MTII treatment, a hypothesis which should be further investigated in future studies.

Project 2: Context-dependent neuronal network activation in response to MC4R agonism

Building upon previously published results, as well as the results from Project 1, we attempted to further understand the cellular mechanisms by which MTII modulates social cognition in the prairie vole. Strong evidence exists to support the hypothesis that MC4R agonists administered prior to the social learning phase of cohabitation significantly enhance the acquisition or encoding of socially relevant information to promote social

bond formation. Interestingly, this effect cannot be associated with positive shifts in observable reciprocal social interactions between cohabitating opposite sex prairie voles. We therefore hypothesized that MTII administration affects an individual's *perception* of social interactions by modifying cellular mechanisms responsible for processing sociosensory information or encoding the emotional and hedonic valences of social stimuli through an OT-mediated mechanism. Using immunohistochemical visualization of the immediate early gene (IEG) protein product Fos as a proxy marker of neuronal activation, we mapped the patterns of regional SSN engagement in response to MTII administration. In a non-social homecage environment, peripheral administration of MTII in males and females does not significantly increase neuronal activation in OT-sensitive regions of the SSN responsible for processing the rewarding or motivational aspects of social stimuli. Previous studies in female prairie voles reported peripheral MTII evokes increases in detectable OT release in the NAcc shell in response to hypertonic saline, a physiological stimulus known to stimulate OT release on its own. There was no observable increase in accumbal OT release in response to MTII in the absence of additional physiological stimulation [120]. Therefore, we hypothesized that MTII would increase neuronal activation in the context of social exposure, a stimulus which naturally engages the central OT system. In the context of social exposure, we observed peripheral MTII in both males and females robustly increases neuronal activation within the NAcc shell and PLC, as well as the OT-producing region of the PVN. This effect appears to be centrally mediated, as ICV MTII administration in the social exposure context, but not in the homecage environment, induces the same potent effect on neuronal activation within the PLC and NAcc shell. Finally, this context-

dependent effect of MTII appears to be mediated at least in part by oxytocinergic signaling, as a central co-injection of a selective OXTR antagonist with MTII (MTII+OTA) significantly attenuates MTII-induced increases in Fos expression in the NAcc shell and PLC. Based on these results, we conclude that MTII administration increases central OT release in a context-dependent manner. This context-dependent evoked central OT release then modifies neuronal processing within specific mesocorticolimbic reward regions, and this is likely to underlie MTII's facilitatory effect on social bond formation the prairie vole model.

General Summary

The SSN contains several OT-sensitive brain regions implicated in sociosensory information processing, socioemotional salience and valence encoding, and social motivation. We observed a significant context-dependent effect of MTII on neuronal engagement in two specific SSN regions, the NAcc shell and the PLC. Oxytocinergic activity in the PLC or NAcc shell is necessary for social bond formation in the prairie vole model, and has also been shown to modulate social motivation and social reward in multiple model systems. The formation of social bonds can be conceptualized as a type of learned association, where the specific sociosensory cues of a partner stimulus animal are linked to a rewarding or appetitive valence. This positive association can then be observed in the partner preference test, where bonded animals will preferentially spend time engaged in social contact with their cohabitation partner over a novel stranger. It is hypothesized that learned associations are encoded within sparse ensembles of neurons distributed across functional networks. Engagement of specific neuronal ensembles relevant to distinct contexts or behavioral outputs can be visualized using whole-brain

expression mapping of IEG protein products, such as Fos. Fos expression patterns can be interpreted as a read-out of the summation of afferent inputs to a given brain region. These afferent inputs convey information about interoceptive or exteroceptive stimuli, as well as previous experiences, and once summated will aid in generating appropriate behavioral responses within a given context. Neurons in a given brain region which receive the strongest integrated stimulatory input will likely show the strongest level of Fos expression, and can be considered as part of the activated ensemble underlying a specific behavior [151]. Social exposure itself, in the absence of MTII treatment, increases Fos expression across all OT-sensitive regions of the SSN we examined: AON, ACC, MeA, NAcc shell, PLC and PVN. Interestingly, MTII treatment in the social exposure context does not generate global increases in neuronal network engagement across the SSN. Instead, increases in Fos expression are observed only in the PLC, the NAcc shell, and the PVN, suggesting that some regions of the SSN underlying specific domains of social cognition may be more responsive to pharmacological OT interventions than others. The PLC and NAcc are integrative hubs in the SSN, receiving convergent inputs from multiple SSN nuclei, including the hippocampus and amygdala [72]. It is therefore possible that MTII, and by proxy elevated central OT, increases the strength of incoming synaptic inputs within the PLC and NAcc shell, thereby increasing neuronal activation and elevating Fos expression. Convergence of strong excitatory inputs to these regions could be further modified by fine-tuning within local circuits.

Neuromodulators such as OT have been shown to facilitate the engagement of synaptic plasticity mechanisms in multiple regions of the SSN, and aid in the synaptic balancing of glutamatergic drive and GABAergic tone. Precise neuronal firing,

contingent upon appropriate balancing of these excitatory and inhibitory drives, is critical to the neuronal processing which contextualizes sociosensory information and recruits attentional or salience encoding mechanisms. In the past several years a common theme has begun to emerge wherein activation of OXTRs expressed on inhibitory interneurons is observed to increase the signal-to-noise ratio in excitatory glutamatergic projections, enhancing the fidelity of social information transfer. Within the hippocampus, activation of OXTRs located on fast-spiking interneurons increases spontaneous GABA release to suppress background firing of excitatory CA1 pyramidal neurons. Elevation of this inhibitory tone serves to reduce random circuit noise. Furthermore, elevations in spontaneous GABA release from fast-spiking interneurons would serve to partially deplete GABA stores available for stimulated release, further boosting the efficacy of incoming signals to CA1 pyramidal neurons and strengthening subsequent excitatory output [84]. As both the PLC and NAcc shell receive excitatory inputs from the hippocampus, stronger incoming glutamatergic drive, as a consequence of elevated central OT, could be an important contributor to our observed increases in neuronal activation within these regions. A similar effect of oxytocinergic synaptic balancing is observed within the olfactory system, where OT acting within the AON augments top-down recruitment of inhibitory interneurons within the main olfactory bulb. As is the case in the hippocampus, elevations of inhibitory tone serve to enhance social odorant coding by improving the signal-to-noise ratio [28]. Within the PLC itself, OXTRs are expressed almost exclusively in GABAergic interneurons, and activation of OXTRs in prefrontal cortical interneurons is necessary for normal expression of specific social approach behaviors in rodents [145]. Finally, within the NAcc shell, activation of

presynaptic OXTRs located on serotonergic projections from the dorsal Raphe engage synaptic plasticity mechanisms by modulating local 5-HT release and subsequent activation of 5-HT1B receptors expressed on incoming glutamatergic terminals. Blockade of OXTR or 5-HT1BR signaling in the NAcc shell prevents the formation of learned associations of social reward [76]. Taken together, mounting evidence suggests that across multiple regions of the SSN oxytocinergic neuromodulation balances the temporal profile of inhibition and excitation. Heighted levels of central OT in a social context therefore fine-tune neuronal processing across the SSN, strengthening excitatory synaptic inputs projecting to the PLC and NAcc shell, as well as further refining local processing within these regions. Additionally, although discrete, region-specific increases in Fos expression were not observed in the AON, MeA or ACC, it is still possible that neuronal processing within these regions was affected by increased OT release but that this effect is not observable through the specific measurement of Fos expression.

Translationally, disruption of the balance of glutamatergic drive and GABAergic tone has been proposed to play a critical role in the neurobiological etiology of ASD [164]. Anatomical, physiological, genetic, and behavioral findings provide strong evidence for this hypothesis [165-167]. Postmortem brain tissue from ASD patients show decreases in the number of parvalbumin-positive interneurons in the medial prefrontal cortex, as well as reductions in GABA mRNA or the ratio of GABA to glutamate [168-170]. Frequent comorbidity with epilepsy further suggests excitatory:inhibitory (E:I) imbalance may be a common underlying etiology of ASD [171]. Optogenetic manipulation of the E:I balance in the PLC of mice can disrupt social

exploration behavior. Selective elevation of activity within excitatory prefrontal cortical neurons reduces social exploration of a conspecific and abolishes preferences for social interaction. Concomitant elevation of inhibitory interneuron activity within this paradigm partially restores social exploratory behavior and social preferences, suggesting that interventions able to modulate inhibitory tone could be efficacious in the treatment of social deficits. Social dysfunction as a consequence of elevated excitatory tone seems to be selective to activity within the mPFC, as control manipulations of E:I balance in the primary visual cortex do not affect social behavior [172]. Thus, the extensively interconnected mPFC might be particularly sensitive to disruptions in E:I balance, at least as it relates to social interactive behavior. EEG studies in ASD patients show heighted resting state activity in the frontal cortex, particularly in the high-frequency gamma range [173-177]. Interestingly, electrophysiological studies in female prairie voles document low-frequency coherence between PLC and NAcc shell, and the strength of this PLC-NAcc shell net modulation was casually predictive of the emergence of affiliative behaviors across the duration of cohabitation. Recapitulation of this low-frequency drive using optogenetic stimulation of PLC projection terminals in the NAcc shell facilitates pair bond formation, providing causal evidence that this specific emergence of network coherence can influence social bonding [75]. Taken together, it can be hypothesized that elevations in high-frequency neuronal network oscillations in the frontal cortices of ASD patients, as a result of disruptions in E:I balance, may be partially causative of social deficit symptoms.

As we have already discussed, there is little evidence to suggest oxytocinergic signaling mechanisms are generally disrupted in ASD. This leads to the exciting

possibility that pharmacological enhancement of oxytocinergic neuromodulation could partially restore the integrity of E:I balance or other synaptic plasticity mechanisms to promote long-term changes in social behavioral functioning for ASD patients. While altered cortical E:I balance in ASD pathology is strongly supported by existing data, pharmacological targeting of the GABA system itself has not been shown to improve clinical social deficit symptoms [178]. This may be due to the fact that pharmacological manipulation of GABAergic function broadly targets both excitatory and inhibitory neuron subtypes, as both express GABA receptors. Alternatively, more precise alterations in cortical processing could be achieved via pharmacological targeting of neuromodulators such as OT, which instead affect overall E:I balance (i.e. the relative number of activated excitatory or inhibitory neurons). Studies in the *Cntnap2* knockout, a mouse model of a monogenic form of autism, strongly support this idea. *Cntnap2* KO mice show deficits both in total social interaction time during behavioral testing, as well as decreases in duration of social exploration per-bout, suggesting *Cntnap2* KO mice have lower overall motivation to interact socially, and potentially find social interactions they do engage in not rewarding enough to sustain engagement. Optogenetic restoration of mPFC E:I balance in *Cntnap2* KO mice rescues social interactive behavior [179]. Amazingly, treatment with MC4R agonists also completely restores normal social functioning in the *Cntnap2* KO mouse, providing compelling evidence that MC4R agonists may have efficacy in ASD patients [128].

Future Directions

A significant and obvious future direction of this work is clinical trials which attempt to apply MC4R agonists as novel treatments for social deficits in ASD or other psychiatric

or neurodevelopmental disorders. Based on the robust context-dependent effects observed in the experiments described here, a critical component of these clinical trials should be the combination of MC4R agonist treatment with behavioral-based therapy. The specific choice of a behavioral-based therapy within these trials should consider explicit targeting of social reward or social motivational based on the brain regions we have observed to be most responsive to MC4R agonist treatment. One clinical trial is currently wrapping up using another selective MC4R agonist compound, PT-141, in ASD patients. Unfortunately, this clinical trial does not pair MC4R agonist treatment with any type of behavioral therapy. The results of this trial are still being analyzed however if no significant effects are found it would be worth additional study using a design where MC4R agonists are administered as an adjuvant rather than a stand-alone treatment. In parallel with clinical trials, additional rodent studies can be performed to further clarify the cellular and systems-level mechanisms underlying the behavioral effects of MC4R agonists.

Social Memory and Ventral Hippocampal CA1 Region

One important brain region within the SSN which was not included in our Fos analysis is the ventral hippocampus, and future studies should investigate hippocampal involvement in the social behavioral effect of MTII. The hippocampus projects to the PLC and NAcc shell to provide environmental and contextual information critical for social decision making [41, 72, 85]. A recent elegant study by Okuyama et al. reports that specific projections from the ventral hippocampus to the NAcc shell are necessary for the formation of social memories of individual conspecifics. Inactivation of ventral CA1 projection neurons, or their terminals in the NAcc shell, significantly impairs social

discrimination performance in mice. Using a system which labels cells by driving effector molecules under the *c-fos* promoter, it was further shown that ventral CA1 neurons activated during initial exposure to a specific individual are preferentially reactivated by re-exposure to this same mouse as compared to exposure to a second novel mouse, representing an engram of this specific social memory. Using this same *c-fos* promoter to drive the excitatory optogenetic receptor Channelrhodopsin 2 in ventral CA1 projection neurons initially activated by exposure to a novel mouse, and later optogenetically stimulating these previously activated neuron's projection terminals within the NAcc shell permits retrieval of a social memory long after it has become inaccessible to natural recall [85]. The findings reported by Okuyama et al. were published in late September 2016, at which time most of the tissue analyzed for our Fos studies had already been collected. As essential the role of ventral CA1 for social memory was previously undescribed, tissue capturing this very posterior brain region was not collected and we were therefore unable to modify our analyses to include it. In light of these findings showing that specific ventral CA1 projections to the NAcc shell are both necessary and sufficient for social memory recall, future studies investigating pharmacological enhancement of social cognition should include assessments of cellular activation effects in the ventral CA1 region of the hippocampus.

Contribution of Direct Extrahypothalamic MC4R Stimulation

In addition to its hypothalamic interactions with the OT system, it is important to consider our results in the context of other potential consequences of MC4R activation. MC4R is widely and exclusively expressed within the central nervous system [121]. *In situ* hybridization studies in prairie voles document MC4R expression within multiple

hypothalamic nuclei, PLC, NAcc core and shell, MeA, CA1-3 hippocampal fields, and ventral tegmental area, as well as multiple other cortical, mesencephalic, rhombencephalic, thalamic and olfactory nuclei [120]. MC4R is a G-protein coupled receptor, and activation of MC4Rs can trigger coupling to any of the 3 downstream G-protein signaling molecules (Gs, Gi/o, or Gq) in a manner that appears to be specific for cell type and ligand, however there also appears to be some measure of species-specific variation in G-protein coupling [121]. As multiple neuromodulators can often be observed to act in concert to contextualize, induce and ultimately reinforce behaviors, direct activation of MC4Rs within the PLC or NAcc shell could contribute to the cellar activation patterns we observe in our Fos expression studies. Although the direct effects of MTII on MC4R activation and downstream signaling should not be context-dependent, the ultimate consequence of MC4R agonism within the specific "neural context" of all the various neurochemical systems engaged during social interaction is likely distinct from its effects in the homecage environment. Site-specific manipulations of MC4R signaling, such as microinjection of MC4R antagonists directly into PLC or NAcc shell prior to MTII administration and social exposure could reveal direct region-specific effects of MC4R agonism on neuronal activation patterns.

Interactions with Mesocorticolimbic Dopamine Pathway

In the same vein, melanocortinergic stimulation of other neuromodulatory systems, particularly dopamine, could also serve to influence neuronal activation patterns across the SSN and modify sociocognitive processing, particularly in the domains of social motivation and social reward. Central administration of the endogenous MC4R ligand α-MSH, either ICV or directly into the ventral tegmental area, increases dopamine release

in the NAcc, and central administration of MC4R antagonists attenuate the reinforcing properties of psychostimulant drugs of abuse such as cocaine [161, 180-182]. Dopamine-producing cells of the ventral tegmental area send projections to the NAcc and mPFC (including PLC) as part of the mesocorticolimbic reward pathway [41, 183, 184]. The mesocorticolimbic dopamine system has been extensively implicated in reward processing, and psychostimulant drugs of abuse known to elevate central dopamine levels increase Fos expression across the mesocorticolimbic pathway [185]. Dopaminergic signaling has also been linked to social reward and social motivation specifically in the prairie vole model. Administration of dopamine receptor agonists into the NAcc shell facilitates partner preference formation in the absence of mating, and blockade of dopamine receptors prevents the formation mating-induced partner preferences. Importantly, the facilitatory action of NAcc shell infusions of dopamine receptor agonists on partner preference formation requires intact OXTR signaling. Simultaneous infusion of dopamine receptor agonists and selective OXTR antagonists into the NAcc shell do not facilitate partner preference formation, and dopamine receptor antagonists similarly block OT-induced partner preference formation [146]. Thus, it appears that partner preference formation relies on synergistic activity in both the OT and dopamine pathways specifically within the NAcc shell. Interestingly, this synergy of dopaminergic and oxytocinergic signaling within the PLC does not appear to be necessary for partner preference formation. Lack of evidence for *necessity* of simultaneous dopamine and OT activity in the PLC, however, does not inherently imply these two neurotransmitter systems do not functionally interaction within the PLC to underlie pair bond formation, and further study is needed. Based on these past findings, one can hypothesize that

MTII-mediated central elevations in both OT and dopamine concentrations, specifically acting in the mesocorticolimbic reward pathway, could contribute to our observations of enhanced sociocognitive functioning at the behavioral level and increased neuronal engagement on the cellular level. Future studies should investigate the consequences of simultaneous central co-administration of MTII and selective dopamine receptor antagonists on Fos expression within the SSN in the social exposure paradigm to attempt to elucidate the relative contribution of dopaminergic signaling in our model.

Possible Caveats to Clinical Applicability of MC4R Agonists

The potential clinical utility of MC4R agonists as indirect modulators of the central OT system is not without caveats or possible negative side effects. Some of the potential side effects could be particularly problematic in the context of specific symptoms common in ASD patients. The central melanocortin system, and specifically MC4R, regulates feeding and energy homeostasis. Stimulation of MC4R produces anorectic effects, with MC4R agonists potently reducing food intake for up to 24 hours after central or peripheral administration [152]. Children with ASD often have feeding problems in general and typically will prefer to consume a more narrow range of food choices compared to typically developing children [186]. As we have proposed the use of MC4R agonists specifically in the context of augmenting behavioral therapy, this potential negative side effect could be attenuated by a restrictive dosing schedule as opposed to daily use.

Another common category of behavioral problems present in ASD patients includes stereotyped or repetitive behaviors. MC4R agonists can induce stereotypies in rodent models, including excessive scratching and self-grooming [187-189]. These

stereotypies are typically interpreted to be indicative of an anxiety-like emotional state. There is currently no available clinical evidence to suggest that MC4R agonists in humans similarly evoke stereotypic behaviors however we are also unaware of any clinical studies that have directly examined this potential effect. Therefore, future clinical trials should take care to include measurements of stereotypic or repetitive behaviors following administration of MC4R agonists. In addition to the anxiety-like behaviors of repetitive self-grooming and scratching, MC4R agonists also show potent interaction effects with the hypothalamic-pituitary-adrenal (HPA) stress axis. Central administration of MTII stimulates *de novo* gene transcription of corticotropin-releasing factor in the PVN, and dose-dependently increases levels of plasma corticosterone. This elevation in plasma corticosterone can be attenuated by pretreatment with a selective MC4R antagonist [190]. Behaviorally, central administration of α-MSH or MTII evokes anxiety-like responding in the Elevated Plus Maze, decreasing the percentage of open arm entries and total time spent in the open arms [191]. These findings appear to be mediated at least in part by neuronal activity within the MeA, as restraint stress induces robust expression of *c-fos* mRNA in the MeA, and a majority of these *c-fos* positive neurons also express MC4R. Microinjection of an MC4R agonist into the MeA recapitulates findings observed with ICV administration of MC4R agonists, elevating plasma corticosterone levels and evoking anxiety-like behavioral responding in the Elevated Plus Maze [192]. Care should be taken in clinical trials to monitor the potential consequences of activation of the HPA axis, either behaviorally or physiologically.

The hypothalamic melanocortin system is also a potent regulator of sexual responding in both males and females, stimulating penile erections and female sexual

receptivity in multiple species [193-199]. In early clinical trials, 17 out of 20 men who received a peripheral injection of MTII developed erections in the absence of physiological or psychological stimulation [200]. Ongoing clinical trials have also noted a potent stimulatory effect on female sexual responding in post-menopausal women [194]. ASD patients have been reported to show higher incidences of inappropriate sexual behaviors displayed in public [9]. These behavioral problems could therefore potentially be increased during periods of MC4R agonist treatment. Although as we have discussed above some of the negative behavioral or physiological consequences of MC4R agonist treatment could be attenuated by the use of restrictive dosing schedules, it has been argued by the original developer of MTII that the reported 1.5-2 hour half-life is too conservative, and it may actually have biological activity for up to 24 hours after administration (Victor Hruby, personal communication). Additional strategies for addressing the concerns of unwanted negative side effects of MC4R agonists have been proposed to include alternative routes of administration (including intranasal administration) or carefully selected low doses based on controlled dose-response studies in healthy subjects.

Final Conclusions

The central OT system has tremendous therapeutic potential for addressing social deficit symptoms in ASD or other psychiatric or neurodevelopmental disorders. Strong evidence from animal models and human clinical trials support the idea that manipulation of oxytocinergic signaling can positively modulate multiple subdomains of social cognition. The use of peripherally-administerable MC4R agonists is a novel strategy to indirectly target the central OT system and circumvent the physiological limitations of

exogenous OT administration. Given the high degree of evolutionary conservation of function for OT in modulating social cognition, we believe studies in rodent models have strong translational applicability to humans. The results presented here demonstrate that MC4R agonists show behavioral efficacy for enhancing social cognition in both sexes using the prairie vole model of social bonding. Furthermore, the results from our analyses of neuronal activation in the SSN demonstrate context-dependent effects of MC4R agonism on cellular-level processing in brain regions critical for the integration of the sensory, contextual and motivational aspects of social stimuli. Increased neuronal activation in mesocorticolimbic reward regions following MTII administration in a social context is dependent on intact OXTR signaling, and likely reflects a strengthening of multimodal signal integration across the SSN. Refinement of signal-to-noise ratio and enhanced fidelity of social information transfer can invigorate the process of social learning, intensifying the perception and encoding of social reward and motivation. Future clinical trials using appropriate contextualized study designs and careful controls should be initiated based upon preclinical results from our rodent work, and other studies. Pharmacological interventions able to target social reward and social motivation represent a significant advancement in the identification and development of novel ASD treatment methods. The successful translation of preclinical results to human patient populations will address a current critically unmet clinical need in the field of psychiatry, and could significantly improve the quality of life for persons currently affected by deficits in social functioning.

APPENDIX

Observations on Conducting Partner Preference Testing in Suboptimal Conditions

The utility of the prairie vole model for the identification of compounds able to enhance social cognition is inherently linked to the stability of the model's "companion" behavioral testing paradigm, the Partner Preference Test (PPT). As we have described previously, PPT is a two-component assay. The first segment, cohabitation, is easily amenable to modifications which support the testing of various underlying hypotheses regarding pair bonding behavior, while the choice assay itself is more rigidly structured and does not generally vary from experiment to experiment or in different laboratories. Since its inception in the 1990s, PPT has been extensively used to document the relative contributions of multiple neurobiological systems in the development of pair bonds or partner preferences, including oxytocin, vasopressin, dopamine, corticotropin-releasing factor, opioids, and glutamate [3]. Laboratory colonies of prairie voles established across the world are derived from wild-caught stock originating from field studies in southern Illinois. As interest and usage of the prairie vole model has expanded over the years, multiple independent laboratory colonies have been established. In some research groups, indoor laboratory colonies are regularly outbred with fresh wild-caught stock to maintain genetic diversity and prevent in-breeding however this is not the case for most prairie vole laboratories, and the frequency of outbreeding with wild caught stock is likely highly variable across different research groups in the United States. Anecdotal conversations with other prairie vole researchers suggest each independent colony shows subtle behavioral variation in the PPT paradigm. This necessitates the establishment of

independent PPT parameters within a given laboratory and hinders one's ability to simply consult the Methods section of another lab's publications for guidance. Even within a given laboratory, multiple variables may affect pair bonding behavior such that each individual experimenter would be well advised to run preliminary pilot tests strictly evaluating control parameters before beginning experimental manipulations. Some of the potential influencing factors for PPT have been suggested to include: colony inbreeding or genetic drift, the sex of the experimenter, time of year, environmental housing conditions, and other specifics related to pre-experimental manipulations of subjects or the testing parameters themselves.

The only truly successful experiment I have performed which relied on the generation of a no-preference control group was the first experiment I ran in 2013, using peripheral MTII to facilitate partner preferences in male prairie voles. All subsequent experiments were negatively affected to some degree by an inability to successfully and consistently generate a no-preference control group, with these problems being significantly more pronounced when using female subjects. Table I describes several factors that were found to anecdotally affect the propensity with which control groups would show partner preferences. A vast majority of attempts to use PPT in general used female subjects, and thus Table I describes anecdotal observations from female-focused experiments. Several rounds of male-focused PPT experiments were attempted after numerous failures with female subjects however these too were unsuccessful. All male PPT experiments were performed using a 6 hour cohabitation in the absence of mating with PPT performed immediately after cohabitation.

It is important to first consider how the lack of sufficient colony outbreeding or small colony size in the Young lab can lead to complications. The Young lab prairie vole colony was previously outbred in 2008 however laboratory records and conversations with previous lab members differ as to whether or not this outcross was to laboratory bred voles or wild-caught voles. Other well-known vole labs are outbred much more frequently than the Young Lab (see Table II for a list of known recent colony outbreeds in other prairie vole laboratories across the United States). Aside from the consequences of generalized inbreeding, more subtle variations or complications may arise from genetic drift, where the frequency of an existing allele within a given population changes over time. Genetic drift is heavily influenced by the smallest size a population experiences, which creates a bottleneck effect [201]. As the needs and size of our laboratory can vary significantly over time, periods of low animal usage are often accompanied by a culling of the breeding colony and a reduction in the number of breeding pairs. This creates significant opportunities for the unintended selection of alleles which may influence partner preference formation. Genetic drift is typically considered in the context of evolutionary selection pressures that favor beneficial alleles, although 'hitch-hiking' on these positively selected beneficial mutations can facilitate the simultaneous spread of deleterious mutations [201]. As lab-bred prairie vole colonies are not subject to evolutionary selection pressures, and assignment to breeding pairs is largely random aside from considerations of recent hereditary lineage, it is impossible to know in advance if pairs will generate offspring homozygous for alleles that impact partner preference formation, positively or negatively. To date, only one genetic variant, a single nucleotide polymorphism in the *Oxtr* gene, has been linked to the expression of partner

preference formation in the laboratory [24]. It is highly unlikely that this is the only genetic variation that manifests as variation in laboratory partner preference behavior, and anecdotal observations from my own thesis research as well as common practices used in other vole labs across the US strongly suggest the prairie vole colony currently housed at the Yerkes National Primate Research center is in desperate need of outbreeding if future experimenters wish to rely on effective and stable use of the PPT paradigm. In addition to the potential behavioral consequences of genetic drift or inbreeding, the colony has also suffered from a dramatic increase in incidence of "hydrocephaly" (enlarged ventricles) over the past several years. Recent estimates from my own work suggest ~25%-35% of the general colony population is afflicted by hydrocephaly, however certain cohorts of experimental subjects from my own work have shown prevalence rates as high as 45%. Based on discussions with other prairie vole labs which participate in frequent outbreeding, this problem seems to be relatively unique to the Young lab, with some labs reporting to have never observed this anomaly and others reporting it is extremely rare. Although it is not possible for me to say with complete certainty that this problem will be completely ameliorated with outbreeding, I believe it is significant that labs which incorporate frequent outbreeding as part of their regular colony maintenance do not appear to encounter this issue with nearly the same frequency or consistency as the Young lab.

Aside from possible complications arising from inbreeding or genetic drift, conversations with other Young lab members have relayed a great deal of PPT "folklore" passed down through the years that may or may not influence test results. It has been argued that the sex of the experimenter themselves may somehow affect PPT results. A

2014 paper by Sorge et al. reported that rodents exposed to male experimenters demonstrated pain inhibition, and this effect was not observed when the experimenters were female. These results were replicated when rodents were exposed to T-shirts worn by men [202]. Some of my own experiments were performed with the assistance of different male colleagues, including several of my ICV MTII experiments in female and male prairie voles, as well as several rounds of pilot testing attempting to determine the potential efficacy of MDMA in facilitating partner preference formation in female voles. Although it is possible to identify from memory a few distinct rounds of testing in which I am confident I had the assistance of a male colleague, inadequate notation particularly from ICV MTII experiments prevents thorough analysis of any potential male/female experimenter effect. Early experiments with peripheral MTII in male subjects were performed solely by me, as well as pilot studies investigating the use of a positive allosteric modulator of the oxytocin receptor (OXTR-PAM) in females. Both of these experiments generated relatively decent control groups although the overall results from OXTR-PAM experiments in female subjects trended towards an "over-bonding" effect in controls. The first round of pilot testing attempting to use MDMA in female subjects was performed solely by me, as my male collaborator was sick on test day. This pilot test worked beautifully and we had excellent controls with no preferences above the level of chance. The second two follow-up rounds were performed with the help of my male collaborator, and in these two rounds the problem of lack of no-preference controls reappeared. Based on these anecdotal analyses of my results, care should be taken by future vole experimenters to consider the sex of themselves, as well as any colleagues assisting them in their behavioral work.

It has further been suggested that seasonal breeding patterns could carry over into laboratory bred vole colonies even in the absence of the traditional cues used by wild voles, such as variations in photoperiod length. At the start of my thesis research several then-senior Young lab graduate students relayed to me the common practice of only attempting suboptimal PPT experiments in females during summer months and only testing on male subjects in the winter months. From my own anecdotal experience, I have not found this rule to be particularly useful, however it is possible that other factors such as sex of experimenter or assistants could have had such strong effects so as to occlude the observation of these potentially more subtle seasonal effects. Therefore, should future Young lab investigators encounter difficulties which cannot be explained by the other factors I have described, the time of the year in which one is performing experiments could be taken into consideration.

Some other prairie vole labs have suggested that the environmental conditions of housing may strongly influence behavioral test results, particularly temperature and humidity. Some vole labs have found that when they have had to move their housing facilities into ones with slightly different temperature/humidity conditions they have had to modify their PPT parameters. In writing earlier sections of this thesis while using another previous student's thesis as a guide for certain Methods sections, I discovered a discrepancy where in one chapter this student had indicated our colony was housed at 20°C and in another chapter they indicated it was 22°C. I contacted the Yerkes Department of Animal Resources for clarification of the temperature of our colony housing and was told by Sunday Buge, the Operations Manager for the Yerkes National Primate Research Center in an email, "the temperature range is 68-79 degrees F. This is

consistent with the center's SOP and also the Guide for the Care and Use of Laboratory Animals. (NIH) 8th edition page 44." (Sunday Buge, personal communication). This is an incredibly large temperature range and is actually not consistent with what is written in the NIH Guide. The Guide for the Care and Use of Laboratory Animals provided by the NIH (8th edition, p. 44) does state that the dry-bulb range for rodent colony housing should be within the range of 68°-79°F, however it also further clarifies, "The dry-bulb range temperatures listed in Table 3.1 are broad and generally reflect tolerable limits for common adult laboratory animal species, provided they are housed with adequate resources for behavioral thermoregulation; temperatures should normally be selected and maintained with minimal fluctuations near the middle of these ranges." Based on my reading of this section of the guide, it is impossible for me to be satisfied with the Department of Animal Resources' assertion that simply maintaining colony housing within the broad range of tolerable limits for rodent housing is adequate or sufficient to support repeatable and generalizable rodent studies, particularly in species that may be acutely sensitive to small fluctuations in temperature or humidity. I attempted to follow up with the Operations Manager with my concerns and offered as support for my opinions the above quoted section of the guide but I received no response. Future rodent researchers should either attempt to clarify if the initial response I received to my temperature question is indeed reflective of the Yerkes Department of Animal Resources Standard Operating Procedures and, if it is, attempt to solicit modifications to more accurately reflect common laboratory practices and NIH guidelines.

Based on my experiences and results obtained from my own PPT studies in both male and female prairie voles, I reiterate several important suggestions for future Young

lab students. First, the colony should be outbred, ideally with wild-caught voles or to laboratory bred voles that are frequently outbred to wild-caught stock. Table II provides a list of some of the known recent outbreeding schemas from other prairie vole laboratories in the United States as a guide. Second, each individual experimenter that may attempt experiments requiring a 'no-preference' control group should begin their work by establishing their own individual parameters for testing which generates stable, replicable results. These parameters may take into consideration: length of cohabitation, separation of partners before PPT, sex of the experimenter, time of year, the housing conditions and temperature/humidity stability available at Yerkes, age of the subjects and stimulus animals, or other pre-experimental manipulations such as ovariectomy. Table I provides some general observations from experiments in female subjects however these observations are provided only as a guide and may not reflect every experimenter's outcomes when using PPT.

Table I Partner Preference Testing Parameters

Variable	Effect on Partner Preference Formation in controls
Overall age	↑ age ↑ partner preference formation
Age at ovariectomy	↑ age ↑ partner preference formation
6hr cohab, PPT immediately	Controls will show partner preference
6hr cohab, 24hr separation	Controls may still show partner preference
3hr cohab, PPT immediately	↑ overall variability in partner preferences

Table I. Observations from Partner Preference Testing in female prairie vole subjects. Anecdotal observations from PPT experiments suggest that certain manipulations may influence the results with respect to the ability to generate 'no-preference' control groups. Based on these observations, female subjects should be ovariectomized as close to 60 days of age as possible, and in general tested as young as possible. Furthermore, some period of separation from the partner in between cohabitation and PPT may attenuate to some extent the problem of 'over-bonding' in controls.

Table II Recent Outbreeding Schedules in US Prairie Vole Labs

Principal Investigator	*Notes on Outbreeding*
Steve Phelps, UT Austin	Outbred to wild-caught stock every 3 generations
Alex Ophir, Cornell University	Outbred to wild-caught stock every 3 generations
Sue Carter, Indiana University	Last outbred to voles from Alex Ophir (2014)
Karen Bales, UC Davis	Last outbred to voles from Lisa McGraw (2014). Planned outbreed to voles from Zuoxin Wang (2017)
Andrey Ryabinin, OHSU	Recent outbreeds to voles from Lisa McGraw (2014), Karen Bales (2008), and Philip Smith (2007)
Tom Curtis, OSU	Last outbreed to F1 wild-caught voles from Alex Ophir (2014)
Lisa McGraw, NCSU	Colony derived from Young lab, est. 2011
Zuoxin Wang, FSU	Last outbred to voles from Bruce Cushing (2011)
Zoe Donaldson, CU Boulder	Colony est. 2010 from Young lab voles, outbred to voles from Karen Bales (2015), planned outbreed to voles from Miranda Lim (2018)

Table II. Recent outbreeding schedules in laboratory housed prairie vole colonies in the United States. Based on my own preliminary survey of outbreeding schedules across different prairie vole labs, the Young lab has fallen behind in terms of regularity of outbreeding as compared to other research groups. Given the growing issue of the prevalence of hydrocephaly and the importance of maintaining genetic diversity, the Young lab should strongly consider outbreeding the Yerkes colony as soon as possible.

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