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Identifying Novel Therapeutic Strategies for Enhancing Social Cognition Using Functional Animal Models

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Identifying Novel Therapeutic Strategies for Enhancing Social Cognition Using Functional Animal Models

By

Meera Modi

B.S./B.A., Binghamton University, 2005

Advisor: Larry Young, PhD

An abstract of

A dissertation submitted to the Faculty of the

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Abstract

Identifying Novel Therapeutic Strategies for Enhancing Social Cognition Using Functional Animal Models

By Meera Modi

Currently, there are no FDA approved drugs for the treatment of the social impairments characteristic of many psychiatric disorders, including autism and schizophrenia. One potential approach to treating social impairments is to target the neural systems that underlie functional social motivation and information processing in normative populations. Oxytocin (OT) has emerged as a central modulator of social behavior, and the OT system is being actively explored as a pharmacological target for the enhancement of social cognition clinically. However, the therapeutic potential of OT is limited by the biophysical properties of the peptide, include its poor penetration of the blood-brain barrier and its metabolic instability. Consequently, novel methods of enhancing the OT system and the social brain circuit are required to realize the pharmacological treatment of social impairments. Through my doctoral work, I have evaluated and developed novel methods of pharmacologically enhancing social cognition and characterized a functional animal model with predictive validity for prosocial therapeutics.

Alternate methods of enhancing the central OT system were tested. The relative efficacy of peripheral routes of OT administration on increasing central peptide levels to evoke behavioral effects was measured in a rhesus monkey model. In prairie voles, a novel method of increasing central OT through the pharmacological stimulation of oxytocinergic neurons was tested. Peripherally administered melanocortin receptor agonists, Melanotan II and Pf-446687, are able to recapitulate the behavioral effects of central OT in this model, through an OT-dependent pathway. These findings suggest indirect stimulation of the OT system can result in a functional enhancement of social cognition. Social cognition can also be enhanced in the prairie vole model through the glutamatergic activation of the brain areas involved in the regulation of OT-dependent social behavior. Administration of the NMDA receptor partial agonist, D-cycloserine, prior to a social learning experience also facilitates social cognition through brain areas that regulate the expression of social behavior, the nucleus accumbens and the amygdala. Importantly, preliminary studies indicate that D-cycloserine reduces some of the social impairments in individuals with autism, suggesting that partner preference in the prairie vole may have predictive validity for identifying drugs that enhance the acquisition of social information.

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The oxytocin system in drug discovery for autism: animal models and novel therapeutic strategies.

Abstract

Animal models and behavioral paradigms are critical for elucidating the neural mechanism involved in complex behaviors, including social cognition. Both genotype and phenotype based models have implicated the neuropeptide oxytocin (OT) in the regulation of social behavior. Based on the findings in animal models, alteration of the OT system has been hypothesized to play a role in the social deficits associated with autism and other neuropsychiatric disorders. While the evidence linking the peptide to the etiology of the disorder is not yet conclusive, evidence from multiple animal models suggest modulation of the OT system may be a viable strategy for the pharmacological treatment of social deficits. In this review, we will discuss how animal models have been utilized to understand the role of OT in social cognition and how those findings can be applied to the conceptualization and treatment of the social impairments in ASD. Animal models with genetic alterations of the OT system, like the OT, OT receptor and CD38 knock-out mice, and those with phenotypic variation in social behavior, like BTBR inbred mice and prairie voles, coupled with behavioral paradigms with face and construct validity may prove to have predictive validity for identifying the most efficacious methods of stimulating the OT system to enhance social cognition in humans. The widespread use of strong animal models of social cognition has the potential yield pharmacological, interventions for the treatment social impairments psychiatric disorders.

Keywords: social cognition, oxytocin, autism, therapeutics, buspirone, serotonin, prairie voles, animal models

Introduction

Autism spectrum disorders (ASD) are a class of neurodevelopmental disorders characterized by communication deficits, repetitive interests or stereotyped behavior and most saliently, impairments in social cognition(Bartz et al., 2008). The diagnostic prevalence of ASD in the United States has increased dramatically over the last 20 indicating the disorder is growing and significant public health concern(Yeargin-Allsopp et al., 2003). Presently, 1 in 88 eight year-old children have received a diagnosis of an ASD(Baio, 2012). It is unclear, though, whether this increase in prevalence is due to an increase in clinical awareness and diagnosis or due to an actual increase in occurrence. Irrespective of the cause of increased rates, the growing ASD population places a substantial burden on the nations social, educational and medical systems. Unfortunately, the pace of diagnosis has not been matched by the development of pharmacological treatments for the disorders(Posey et al., 2008). To date there are no FDA approved drugs for the treatment of any of the core features of the disorder(Bartz et al., 2008). In particular there is a notable lack of treatments for the social impairments, which are a hallmark of ASD, but are also present in a number of other psychiatric disorders including schizophrenia and depression. Most existing pharmacotherapies available to the ASD population are re-labeled drugs used to treat other psychiatric disorders that primarily target co-morbid conditions, like anxiety and irritability(Posey and McDougle, 2002).

The lack of progress in the development of pharmacological treatment strategies is likely due, at least in part, to the poor understanding of the diseases etiology. Animal models and behavioral paradigms with face, construct, and predictive validity are essential for the development of novel, biologically-based drugs to enhance social function. Consequently, increased attention has been given to the development of animal models that enable the study of social cognition and social dysfunction. Many of the most promising animal models do not model ASD *per se*, but were developed to study the basic neurobiology of social cognition. The neural systems identified in

these models that regulate social cognition provide an entry point to look at systems that may be disrupted in ASD and other psychiatric disorders with social impairments. The oxytocin (OT) system has been identified in several models to be a potent regulator of prosocial behavior. As a result of these findings, investigation in ASD subjects has revealed some associated alterations in the OT system (for a thorough exploration of the role of OT in ASD see Appendix 1). More importantly, preliminary findings suggest that the OT system may be a viable pharmacotherapeutic target for the treatment of the social impairments of the disorder regardless of specific etiology. In this review, we will discuss how animal models have been utilized to understand the role of OT in social cognition and how those findings can be applied to the conceptualization and treatment of the social impairments in ASD.

Using Animal Models to Understand ASD

Animal models are used to characterize the biological mechanisms of disease or to understand the normative processes disrupted in disease. The ultimate goal of using animal models is to identify pharmacological targets for interventions. As ASD, like most psychiatric diseases, is a genetically and phenotypically complex disorder, no one animal model can capture all of the core features of the disorder, see Table 1. Animal models relevant to the *social impairments* of ASD can be divided into three categories: 1) genotype based, in which genes that regulate social cognition are altered in transgenic rodents, 2) phenotype based, in which normative or dysfunctional social behaviors are modeled, and 3) environmental based, in which environmental insults linked with social impairments are recapitulated. Animal models are used in conjunction with behavioral paradigms that enable the quantification of specific behaviors reflecting the animal's cognitive or emotional state. Strong models have a combination of three features: 1) face validity, the model shares behavioral phenotypes with the disorder, 2) construct validity, the model shares a common biological mechanism with the disorder, and 3) predictive validity, drugs that have a specific effect in the model have a parallel effect in humans. Behavioral paradigms

relevant to the social impairment in ASD should enable the quantification of variability in social cognition (face validity) based on evolutionarily common neurobiological mechanisms (construct validity). Ideally these behavioral paradigms will also prove to have predictive ability for identifying drugs capable of enhancing social cognition in human subjects in ASD and other psychiatric conditions with impairments in the social domain. Identifying the strengths and limitations of the individual animal models and behavioral paradigms may be useful in identifying the most clinically efficacious pharmacological methods of enhancing social functioning through the modulation of the OT system, see Table 2.

ASD Phenotypes in Genotype Based Models

Pharmacological manipulation of the OT system in functional model systems has indicated that this neurohypophyseal peptide not only coordinates the physiology of reproduction, but also promotes several elements of prosocial behavior, including parental behavior and complex social interactions between adults. Studies in mice with a genetically altered OT system have confirmed the role for OT in the regulation of social behavior. The establishment and verification of behavioral paradigms that capture variation in social cognition enables the attribution of specific phenotypes to the neurochemical system. Three lines of mice with genetically altered OT systems have been evaluated in a battery of social behavioral paradigms that capture a number of features relevant to the ASD phenotype. Each mouse line models the perturbation of a different level of OT signaling, release of the peptide in CD38 knock-out mice (CD38KO; the CD38 enzyme regulates the Ca^{2+} dependent secretion of OT, see Higashida in this issue for a full review), the peptide itself in OT knock-out mice (OTKO) and the peptide receptor in OT receptor knock-out (OTRKO) mice, to produce a detailed picture of the contributions of the OT system to discrete components of functional social cognition.

Social Information Processing and Recognition

Impairments in social cognition have been identified in all three models of altered OT signaling in multiple behavioral paradigms. The most robust social deficits can be seen in the social recognition paradigm. Social information in rodents is primarily conveyed through olfactory cues. Rodents can differentiate between individuals based on their olfactory signature and maintain that memory for up to two hours. Social recognition of a conspecific can be detected by a decrease in olfactory investigation of the familiar animal (habituation) compared to a novel animal (Winslow and Camacho, 1995). Injection of OT into the lateral septum (Popik et al., 1992), medial preoptic areas (Popik and van Ree, 1991) and olfactory bulb (Dluzen et al., 1998) of rats enhances social recognition. Global elimination of OT, the OT receptor or CD38 in genetically engineered knock out mice prevents the recognition of familiar conspecifics (Ferguson et al., 2000b, Takayanagi et al., 2005, Jin et al., 2007, Higashida et al., 2011a). Male knock-out mice showed complete social amnesia, with no decrease in olfactory investigation after repeated exposures to a single animal, see Figure 1 (Ferguson et al., 2000b, Takayanagi et al., 2005, Jin et al., 2007). Despite a profound social memory deficit, the knock-out mice have normal sensory and nonsocial learning and memory capabilities. These mice habituate normally to non-social odors, like lemon scent. Mice with altered OT systems appear to have a specific deficit such that they are unable to use perceived social cues to recognize familiar individuals. The selective social impairments of the knock-out mice provide strong face validity for the models within the social recognition paradigm for the disruption of social cognition in ASD. The contribution of OT to functional social recognition may even be more subtle than social vs. non-social discrimination. Forebrain-specific OT receptor knock-down indicates that OT-dependent social recognition deficits are specific to intra but not inter mouse strain comparisons. This suggests the OT receptor is primarily involved in “fine” social discriminations, like those between specific mice, as opposed to “gross” between strain judgments (Macbeth et al., 2009b). The “fine tuning” function of OT corresponds to the often intact pedantic emotion recognition amongst

individuals with social deficits despite impairments in the context of a complex social interaction (Bartz et al., 2011).

The deficits in the social recognition paradigm can be rescued in all three knock-out lines by a single intracerebroventricular injection of OT given prior to, but not after the initial social exposure (Ferguson et al., 2001b, Jin et al., 2007). This suggests that OT facilitates the encoding and not the consolidation or recall of social information. We hypothesize that this enhancement of encoding may be due to OT's effect of enhancing the saliency of social stimuli. While this effect is consistent with the genetic alterations in CD38KO and OTKO, which have decreased levels of circulating OT, the efficacy of the OT in restoring social recognition with the OTRKO line is particularly intriguing. Central injections of either OT or vasopressin rescue the impaired social phenotypes in this model questioning the role of strict OT-OT receptor interactions in social deficits of this model. Co-administration of a vasopressin receptor 1a (V1aR) antagonist along with OT in this model prevents the rescue effects, implicating an alternative pathway (Sala et al., 2011). The overlap of the OT and vasopressin systems highlighted by the findings in the OTRKO mouse emphasizes the complicated pharmacology involved in developing OT-related pharmacotherapies for ASD and the potential alteration of both neuropeptide systems in the disease state (Ring, 2011). Evidence from the knock-out models suggests that while OT may be a viable therapeutic strategy for the treatment of social impairments, further exploration into the cross talk between that OT and vasopressin system that may occur in ASD is necessary for the determination of the ideal pharmacological intervention.

Social Comfort

OT also plays a role in social phenotypes, like separation-induced vocalization and locomotion, which are measures of isolation-induced anxiety. Separation-induced vocalization is used as a measure of perceived distress and desire for reunification in response to social isolation. Mouse pups emit these types of ultrasonic calls when separated from their dam and littermates. In this

behavioral paradigm, the subsequent vocalizations are quantified, with frequency of calls reflecting the distress associated with isolation. OTKO, OTRKO and CD38KO mouse pups emit fewer of these distress calls when separated from their mother than wild–type littermates, with the most profound reduction seen in the OTKO pups (Winslow et al., 2000, Takayanagi et al., 2005, Liu et al., 2008, Higashida et al., 2011b). In response to the same separation paradigm, OTKO pups make fewer attempts to reunite with their dam by crossing under a barrier (Ross and Young, 2009). The reduced reunification driven locomotion, is interestingly accompanied by an increase in general locomotor and exploratory behavior in OTRKO and CD38KO pups compared to wild-type mouse pups. Increased locomotion is thought to reflect a decrease in isolation-induced anxiety. Thus mouse pups with genetically altered OT systems appear to be less anxious than wild-type mice in response to social isolation. Abnormal responses to social stress are also seen in individuals with autism. Children with high-functioning autism have altered an neuroendocrine response to the Trier Social Stress Test, showing decreases in cortisol whereas the controls show increases in cortisol in response to the stressor, suggesting a diminished social stress response (Levine et al., 2012).

Anxiety

In contrast to decreased isolation induced anxiety, though, mice with genetically altered OT systems have increased general anxiety levels. OTKO mice spend less time in the open arms of the elevated plus maze (Mantella et al., 2003), a behavioral paradigm assessing anxiety, and show heightened corticosterone reponses to environmental stressors compared to wild-type mice (Amico et al., 2008). The anxiety related behavior could be decreased with central injections of OT (Mantella et al., 2003). The anxiolytic effects of OT can be seen in both genetically modified and wild-type mice. Central and peripheral OT injections decrease anxiety behavior the elevated maze paradigm and in the four-plate paradigm in wild type mice, the effects of which can be blocked with a centrally acting OT receptor antagonist (Ring et al., 2006). Based on the anti-

anxiety effects of OT, it has been speculated that the pro-social effects of OT may be mediated through a reduction in socially induced anxiety. Further work is needed to determine the extent to which OT promotes social behavior independently from its anxiolytic effects.

Other ASD Relevant Phenotypes

Interestingly, alteration of the OT system has recently been found to affect other clinical features associated with ASD. The OTRKO mouse displays non-social phenotypes that are consistent with ASD. OTRKO mice exhibit impaired cognitive flexibility. In an appetite-motivated T-maze test, the OTRKO animals are significantly slower to learn reversals of the baited arm than wildtype littermates, despite similar initial acquisition rates (Sala et al., 2011). This phenotype is consistent with the limited behavioral patterns inflexible to environmental demands frequently seen in individuals with ASD. Epileptic seizures are comorbid with ASD in 10-30% of cases (Gabis et al., 2005). OTRKO mice also have an increased vulnerability to pentylenetetrazole-induced seizures. Injection of the pro-epileptic drug induces spike train activity associated with clonic-tonic seizures in the knockout animals as opposed to the milder myoclonic seizure activity seen in wildtype littermates (Sala et al., 2011). These co-morbid findings in the OTRKO mouse suggest there may be overlap in the neurobiological systems that contribute to the complex phenotypes associated with ASD.

Evidence for Construct Validity

Based the profound social impairments associated with genetic modifications of the OT system in animal models, OT has been proffered as a candidate neurochemical system in the etiology ASD. Variation in the molecular, genetic and epigenetic regulation of the OT system has been associated with ASD. Single nucleotide polymorphisms in the OT receptor gene and haplotypes of these polymorphisms have been found within ethnically divergent populations to be associated with ASD, though no single haplotype has been consistently identified (Wu et al., 2005, Jacob et al., 2007a, Lerer et al., 2008b, Liu et al., 2010b, Wermter et al., 2010). Several genome-wide

linkage studies have been used to analyze autistic populations, the largest of which found a linkage peak over the 3p25 chromosomal, the location of the OTR gene (Barrett et al., 1999, Phillippe et al., 1999, McCauley et al., 2005, Lauritsen et al., 2006, Ylisaukko-oja et al., 2006, Campbell et al., 2011). Interestingly, deletions or hypermethylation of the p25 region of chromosome 3 has also been found in a few autism cases (Sebat et al., 2007, Gregory et al., 2009b). Hypermethylation of the *OXTR* promoter is associated with decreased *OXTR* mRNA in the temporal cortex. Peripheral levels of the peptide have also been reported to be altered in the ASD population compared, however the direction of this alteration is unclear (Modahl et al., 1998, Jansen et al., 2006). Despite the evidence linking alterations of the OT system with ASD, OT has neither emerged as a biomarker for the disorder nor a major genetic contributor. The critical role of OT in the regulation of social behavior in genetic mouse models, though, reinforces its contribution to the production of functional social behavior. The absence of dramatic alteration of the OT system in the ASD population, though, suggests the feasibility of enhancing the OT system to promote functional social cognition, irrespective of the cause of the social impairments in the disorder. Novel tools for investigating OT receptor density in the brains of patients, such as OTR PET ligands, would be potentially useful for identifying those who would likely be unresponsive to OT based therapies.

Oxytocin and Monogenic Forms of ASD

Of all rodent models of ASD, those based on monogenic forms of autism have the highest level of construct validity. Whereas alterations of the OT receptor gene are *associated* with ASD, alterations of genes including FMR1, UBE3A, DHCR7, and MeCP2 are considered *causative* of specific monogenic forms of autism (Kotulska and Jozwiak, 2011). Interestingly, mouse models recapitulating the genetic variation associated with monogenic autism disorders often show deficits in behavioral paradigms of social cognition (Ey et al., 2011). Despite some phenotypic similarities with OT deficient mouse models, most monogenic forms of autism are not associated

with dysregulation of the OT system. Only Smith-Lemli-Opitz Syndrome (due to a mutation of the DHCR7 gene) may contain OT dysfunction in its etiology due to cholesterol's modulatory role in the function of the OT receptor (Bukelis et al., 2007). Preliminary evidence, though, suggests that pharmacological manipulation of the OT system could enhance social function in monogenic forms of autism irrespective of the peptide's etiological contribution. Intranasal OT increases eye gaze to social interactors and decreases cortisol response to social interactions in individuals with Fragile X Syndrome (Hall et al., 2011). The efficacy of OT in a population not characterized by OT dysregulation suggests the peptide may be beneficial in a number of disorders with social impairments and that mouse models of monogenic forms of autism may be useful for assessing the efficacy of OT based drug therapies (including those discussed in the Therapeutic Strategies section) in behavioral paradigms of social cognition.

ASD Related Phenotype Based Models

As ASD is a disorder currently diagnosed by distinct behavioral phenotypes rather than genetic or physiological biomarkers, an alternative approach in modeling the disorder is to study animal models with relevant phenotypes, in this case variation in social behavior. Social cognition in humans, like most behavioral traits, exists on a continuum, such that some individuals are extremely adept at navigating social encounters, while others have limited abilities. People with ASD fall at the low end of this continuum and are diagnosed, in part, by how they deviate from the mean in social functioning. The same variation in social behavior can be seen in animal models that contain behavioral diversity. Animal models that encompass a continuum of social behavior, with both highly social and asocial constituents, allow for the comparative investigation of the mechanisms that contribute to variation in social cognition. OT has emerged as a distinguishing feature between constituents that fall at the high and low end of social function. Two animal models, inbred mice and microtine rodents, have been used to phenotypically

characterize variation in social behavior and comparatively identify the neurobiological structures that underlie this variation.

Inbred Mouse Models of Social Variability

Laboratory mice have been selectively bred to create hundreds of genetically distinct inbred lines. The genetic and phenotypic variation between these lines has been mined to identify inbred strains of mice with phenotypes relevant to autism based on high throughput behavioral screening assays. Several strains of inbred mice have been identified to have lower levels of sociability and the species typical preference for social novelty compared to other strains of mice, using tests of social recognition, reciprocal social interactions, social approach and social preference tasks (Brodkin, 2007, Silverman et al., 2010b). Thus these inbred strains have face validity in the sense that their behavioral phenotype is relevant to the endophenotype in ASD, but it is unclear if they have construct or predictive validity for drug discovery. Of all the strains identified to have phenotypical similarities with ASDs, only the BTBR T+tf/J (BTBR) inbred strain, also shows alterations in the OT system, therefore we will limit our discussion to this strain.

BTBR mice have low levels of social play initiation as juveniles (Bolivar et al., 2007) and preference for social interaction, as measured in the social preference test, as adults (Moy et al., 2007). The language impairments and repetitive and stereotyped behaviors of ASD are also recapitulated in BTBR mice, which have atypical ultrasonic vocalizations during social interactions and high levels of repetitive grooming. Unlike the vocalization alterations seen in the OT related deficit models, BTBR pups emit louder and more frequent calls when isolated than other more social inbred stains of mice (Scattoni et al., 2008). As adults, though, the BTBR mice vocalize less and scent mark, a species-typical form of olfactory communication, less than more highly social strains in response to a social stimuli (Wohr et al., 2011). Interestingly, these behavioral similarities to ASD are accompanied by an increase in OT peptide levels in the

paraventricular nucleus of the hypothalamus (Silverman et al., 2010b). The contribution of the altered peptide levels in the low social BTBR mice has yet to be examined.

When using comparative variation models, it is important to keep in mind that the variation in the model arose through an independent selection history that does not necessarily match the evolution of the disorder. The altered systems in the model may not be altered in the disease to which it is phenotypically similar. In particular, inbred mice were all developed through human guided artificial selection and not through natural selection, which shapes non-domesticated species. Consequently, the selective pressures that led to a divergence of brain structure between high social and low social inbred mice are not the same as those that could have led to the same outcome in the natural environment. This may limit the construct and perhaps the predictive validity of inbred mouse strains for the development of pharmacological approaches to enhance social function.

Social Variation in Microtine Rodents

In contrast to the comparative exploration of inbred mice strains, microtine rodents (voles) allow for the investigation of natural variation in social behavior. The Microtus genus exhibits a large amount of natural diversity in social behavior within its constituent species, from the highly affiliative, socially monogamous prairie voles (*Microtus ochrogaster)* to the asocial, non-monogamous meadow (*M. pennsylvanicus)* and montane voles (*M. montanus)*. Additionally, as these are outbred species, there is also a large amount of intra-species social behavioral diversity. Both inter- and intra-species variation in social behavior have been linked to variation in the OTR system (Ross and Young, 2009). Highly social prairie voles have much higher levels of OT receptors in the nucleus accumbens, a reward center of the brain, than the asocial meadow voles, see Figure 2 (Insel and Shapiro, 1992b). In contrast, the distribution of the OT peptide is highly conserved among rodents (Ross et al., 2009a). Central administration of OT in prairie voles promotes the formation of social bonds between conspecifics (Williams et al., 1994b).

Correspondingly, administration of an OT receptor antagonist into the nucleus accumbens or the prefrontal cortex inhibits the formation of these bonds (Young et al., 2001b). It is hypothesized that the presence of OT receptors in the nucleus accumbens allows for the pairing of neural encoding of social information, with reinforcement learning, mediated by dopamine signaling, thereby promoting a conditioned partner preference (Young and Wang, 2004b). Variation in the density of OT receptors in the nucleus accumbens within prairie voles is also associated with intra-species variation in social motivation. Female prairie voles with high density of receptors in the nucleus accumbens are more likely to display alloparental behavior, in spontaneous parental care tests, than those with low receptor densities (Olazabal and Young, 2006b, Olazabal and Young, 2006a). Females with experimentally increased OTR expression in the nucleus accumbens form pair bonds more quickly, but interestingly do not show elevated alloparental behavior if the OTR manipulation is performed in adults (Ross et al., 2009c). However, if OTR levels are increased as juveniles, both partner preference and alloparental behavior is enhanced, suggesting a cumulative or "organizational" role of OTR in this regions for shaping some aspects of social behavior (Ross et al., 2009c, Keebaugh and Young, 2011).

While behavioral paradigms assessing prosocial behavior, like spontaneous parental care tests and the partner preference test, were originally developed to explore basic neurobiology in the prairie vole, they may have important face, construct and predictive validity for screening drugs that may enhance social motivation and social cognition more generally. For example, pair bonding is a type of social learning in which the social cues of the partner become associated with the reinforcing aspects of their interaction. This process requires that the social cues of the partner be highly salient and the encoding of those cues be linked to the reward system. Therefore, systems involved in social motivation, social information processing and learning and memory are all involved in the formation of a pair bond. The partner preference test utilizes the natural pair bonding behavior of the prairie vole to give a discrete experimental readout of social learning and

expression. The conditions under which social bonds can be learned and expressed within the behavioral test are well established. These conditions can be augmented such that the learning phase of social bonding occurs under suboptimal conditions (e.g. decreased time and value of social stimuli). Experimental manipulations (genetic, behavioral or pharmacological) consequently can be made to try to promote the formation of social bonds under the suboptimal conditions, allowing for the identification of prosocial manipulations. This suboptimal pairing paradigm was used to first identify the critical role of OT in partner preference formation, as a central injection of OT was able to induce a partner preference under a brief cohabitation condition in which it would not otherwise form (Williams et al., 1994b). More recently, this paradigm has been used specifically to screen for drugs that enhance social learning, which could be used in combination with social skills behavioral therapy to treat the social impairments of ASD (Modi and Young, 2011a). We propose that drugs given prior to the social learning phase of the partner preference paradigm (e.g. prior to cohabitation) that accelerate bond formation, perhaps by modulating social motivation, social information processing, or learning and memory, may also work in accelerating the beneficial effects of behavioral therapies in ASD based on social reinforcement. Thus the microtine rodent natural variation phenotype model functions both comparatively for the identification of neural substrates underlying different levels of sociability and may also have predictive validity for the identification of novel prosocial therapeutics.

Therapeutic Strategies for Targeting the OT system.

Based on the work in animal models, we propose that OT plays a critical role in 1) increasing the saliency of social stimuli and 2) linking the encoding of those stimuli to social reward and reinforcement. Thus OT can rescue social recognition in the deficit OTKO mouse, and also accelerate partner preference formation in a prairie vole with a functional OT system. This is important because targeting the OT system to increase the saliency of social stimuli and potentially enhance the interaction of the social processing and reward systems should be

effective irrespective of the role of the OT system in the pathophysiology of ASD. Consequently, targeting the central OT system has considerable therapeutic potential for ameliorating the social deficits of not only ASD, but a number of psychiatric diseases characterized by social impairments (Ring, 2011).

The most direct approach to enhancing the OT system is peripheral administration of the peptide or its analogues. Due to its peripheral use in accelerating labor, biologically stable analogues, like pitocin, have been developed and approved for clinical use (Page, 1954). The initial studies of the therapeutic use of OT in the treatment of ASD made use of this compound. Intravenous administration of high doses of pitocin was found to both improve the retention of social information and reduce repetitive and stereotypic behaviors in individuals with high functioning autism and Asperger's (Hollander et al., 2003, Hollander et al., 2007). However, while these studies provided proof of concept that the OT system can be targeted to treat ASD, there are still several limitations to its widespread clinical use based on the physiochemical properties of the peptide. OT, due to the actions of aminopeptiases, is metabolically unstable and has short half-life both in central (20 minutes) and peripheral circulation (about 5 minutes) (Mens et al., 1983b). In addition, as a large, charged peptide, OT has poor penetration of the blood brain barrier (Ermisch et al., 1985, Landgraf and Neumann, 2004). Both properties of OT limit the potential for peripheral administration of the peptide to promote central neurotherapuetic effects (Ring, 2009). Thus, alternative strategies are needed to increase the central activation of the OT system for the treatment of the social impairments associated with ASD.

Intranasal OT

To circumvent the limitations posed by OT's poor penetration of the blood brain barrier, several groups have administered the peptide intranasally. It is hypothesized that intranasal administration of peptides allows for passage through clefts in the nasal epithelium into the cerebral spinal fluid (Illum, 2000). Intranasal administration of other peptides, including

vasopressin, results in a sustained increase in central levels for over an hour (Born et al., 2002), but there is not yet direct molecular evidence that OT accumulates in the brain in the same fashion. Recently, a large number of studies have looked at the behavioral effects of intranasal OT in humans and found that the peptide enhances a broad range of social and perceptual abilities. Intranasal OT increases the perception of socially important information, like emotion recognition (Domes et al., 2010), empathic perception (Bartz et al., 2010), facial identity (Rimmele et al., 2009a) and the response to biological motion (Keri and Benedek, 2009), see (Bos et al., 2011) for a detailed review. It also promotes interpersonal relationships though the enhancement of socially reinforced learning (Hurlemann et al., 2010b), trust (Kosfeld et al., 2005b) and the experience of attachment (Buchheim et al., 2009). These effects may be, in part, mediated by increased reward associated with social encounters, due the interaction of the striatal OT and dopamine systems after intranasal administration (Rilling et al., 2011). Three studies have also looked at the prosocial effects of OT in ASD subjects. Guastella and colleagues found that OT improves the recognition of emotions from faces in autistic populations (Guastella et al., 2010). Similarly, Hollander et al., found that the peptide increases the retention of social content in speech (Hollander et al., 2007). Most recently, Andari et al., found that OT increases social interaction, trust and attention to socially informative stimuli (Andari et al., 2010a). However, whether the effects of intranasal OT are mediated by activation of central OT receptors, or OT receptors in peripheral tissues, which then impact brain activity, remains to be determined. This distinction is currently one of the most important clinical questions in guiding therapeutic approaches for manipulating the system. Non-human primate models will likely be useful in making this critical determination. A more thorough understanding of the mechanism of action will be essential to inform the further development of drug strategies targeting the OT system.

Non-peptide Agonists

Alternatives to synthetic OT are non-peptide, small molecule agonists, partial agonists or positive allosteric modulators, which could target and activate the OT receptor without the physical limitations of peptide agonists. The benefit of non-peptide agonists lies in the potential for increased stability, central penetration and oral bioavailability, making the agonist more amenable to classical routes of pharmacotherapeutic administration (Pitt et al., 2004, Ashworth et al., 2006). In addition, the increased epitope specificity possible with small, non-peptide agonists potentially allows for increased selectivity for the OTR over the structurally similar vasopressin receptors (Hawtin et al., 2001). Two groups have identified non-peptide molecules with high affinity for the OTR and have potent agonistic properties. WAY-267464 is a first generation agonist developed by Wyeth Pharmaceuticals with 87% of the intrinsic efficacy of OT and is 100 times more selective for the OTR than any other members of the OT/vasopressin receptor family (Ring et al., 2010). The compound produces anxiolytic effects in rodent behavioral paradigms similar to those seen with OT, which can be blocked by application of the OTR antagonist (Ring et al., 2010). However, the effects of WAY-267464 have not yet been reported in paradigms assessing social cognition. Ferring Pharmaceuticals also developed a potent nonpeptide agonist, which maximally stimulates the OTR to the same degree as OT and is 25 times for selective for the OTR than any other receptors in the family (Pitt et al., 2004). Behavioral efficacy of the Ferring agonist, though, has yet to be demonstrated. Behavioral effects of positive allosteric modulators of the OTR have not been reported, but these would have the advantage that they would only stimulate the OTR in the context of endogenous OTR activation, making that activation more potent.

Enhancing Endogenous OT Release

As an alternative to exogenous administration of OT or OT agonists, the endogenous OT system can be manipulated to increase central levels. One method of increasing central OT levels is by promoting the release of the peptide from OT producing neurons. Stimulation of receptors that

regulate oxytocinergic neurons, through pharmacological manipulation, has been used to promote both central and peripheral OT release (Bagdy and Kalogeras, 1993, Sabatier et al., 2003). Receptors identified as being expressed on oxytocinergic neurons or on cells that interact with oxytocinergic neurons can be assayed to determine their ability to stimulate OT release and their subsequent therapeutic potential. Receptor systems are targeted based on their interactions with the OT system and not based on their other endogenous functions, though those functions should be considered as potential side effects at the clinical level. For example, stimulation of melanocortin receptors on the oxytocinergic neurons of the supraoptic neurons (SON) induces central, but not peripheral release of OT in rats, and a melanocortin 4 receptor (MC4R) antagonist blocks this effect (Sabatier, 2006). Thus MC4R agonists would presumably increase central OT release, circumventing the limitations of peripheral peptide administration. Furthermore, if this mechanism promotes both somatodendritic release and release from axon collateral dense core vesicles, OT would be elevated in brain regions most responsive to the peptide. Preliminary studies suggest that administration of a melanocortin receptor agonist promotes partner preference development in prairie vole (Modi and Young, unpublished data). However, the MC4R also has been targeted in drug development to reduce appetite and to increase sexual arousal, which may be confounding side effects in the treatment of disorders such as autism. These other activities of MC4R agonists must be taken into consideration in a drug discovery context.

The serotonin system is also involved in the regulation of OT secretion. Both serotoninergic fibers and receptors are located in the oxytocinergic SON and paraventricular nuclei of the hypothamaus, where they regulate the release of neurohypophyseal hormones (Jorgensen et al., 2003). Specifically, agonists of the 5-HT1a receptor, including 8-OH-DPAT, DOI and buspirone, cause substantial increases in plasma OT levels, see Figure 3a (Bagdy and Kalogeras, 1993, Uvnas-Moberg et al., 1996). 5-HT1a receptor-induced release of OT is even thought to underlie

the prosocial effects of the hyperserotonergic drug of abuse, MDMA (Thompson et al., 2007). Buspirone, a clinically available partial 5-HT1a agonist, in addition to increasing peripheral levels of OT, promotes OT-dependent prosocial behaviors. As a proof of principle for the idea that stimulating OT release may be a viable means of enhancing social cognition, we performed a study to determine whether buspirone would accelerate partner preference formation in a suboptimal paradigm in female prairie voles.

Twenty-nine female prairie voles were ovariectomized and given two days to recover. Females were then injected intraperitoneally with either vehicle (saline), 8mg/kg or 30 mg/kg buspirone hydrochloride (Sigma-Aldrich, St. Louis, MO) and immediately cohabitated with an adult sexually experienced male prairie vole. After six hours of cohabitation, the experimental females were tested for partner preference, as previously described using an automated behavioral analysis system, see Figure 3b (Ahern et al., 2009b, Modi and Young, 2011a). Animals receiving saline or the high dose of buspirone failed to display a partner preference. However, those animals receiving 8mg/kg displayed a significant partner preference, see figure 3c. Thus buspirone, which acts on the 5-HT1a receptors to stimulate OT release, has the same behavioral effect on social bonding in female prairie voles as does a central injection of OT. We hypothesize that the accelerating effects of buspirone on partner preference are mediated through the OT system, but confirmation of this through co-infusion with OT antagonist is needed. The serotonin system is also intimately involved in the regulation of social interactions and anxiety thus it is possible that this effect is mediated by an OT independent mechanism. It is possible based on buspirone current clinical use as an anxiolytic that the effect on partner preference could be due to a reduction in anxiety rather than the promotion of social cognition. However, buspirone is just one example of indirectly stimulating the OT system to achieve the potential social behavioral benefits of the peptide. There are potentially numerous drugable receptor targets on oxytocinergic neurons that could be used to enhance OT release. This potential highlights the

need for a systematic characterization of the receptors on OT neurons, so that the pathway with the greatest efficacy and least off target effects can be identified for pharmacotherapeutic use.

The OT system can also be pharmacologically enhanced by activating intracellular mechanisms, like the CD38 enzyme. Retinoids are a class of compounds related to vitamin A (retinol) including all-*trans*-retinoic-acid (ATRA), which has been proposed as a novel therapeutic strategy targeting the OT in the treatment of ASD. ATRA, a high-affinity ligand for retinoic acid receptors, is a potent inducer of CD38 and thus presumably OT release (Kishimoto et al., 1998). Cell lines derived from the lymphoblastoid cells of individuals with ASD have lower expression of CD38 mRNA than those derived from a control population (Lerer et al., 2010). Treatment of the ASD derived cells lines with ATRA results in increased CD38 mRNA expression, the same occurs in the control derived lines to a lesser extent (Ebstein et al., 2011). This suggests that ATRA could potentially be used to increase CD38 levels in individuals with autism, and in turn increase OT release through the endogenous inducer function of the protein.

Oxytocinase inhibitors

In addition to regulation of extracellular concentrations of OT through regulation of release, OT levels are also regulated through enzymatic degradation. Extracellular OT is limited by the enzymatic actions of aminopeptidases, which degrade the peptide to limit its functional activity. Placental leucine aminopeptidase (P-LAP, also known as insulin regulated amino peptidase) preferentially degrades OT by cleaving the peptide bond between the N-terminal cysteine and the adjacent tyrosine residue to inactivate the hormone (Tsujimoto and Hattori, 2005). Peripherally, P-LAP is released from the placenta and increases in maternal serum during pregnancy to maintain appropriate OT levels throughout pregnancy. P-LAP is also expressed in the brain, selectively in neurons (Matsumoto et al., 2001). High levels of P-LAP are expressed in selected olfactory regions, throughout the hippocampus and co-localized with OT and vasopressin neurons in the hypothalamus (Fernando et al., 2005). The overlapping distribution of the enzyme with

OTR rich brain regions suggests that the enzyme plays a role in maintaining brain OT levels. Recently, competitive peptide inhibitors of the enzyme have been identified, including amastatin, angiotensin IV and LVV-hemorphin 7, which bind specifically with high affinity to the catalytic site to prevent aminopeptidase activity (Mizutani et al., 1992, Lew et al., 2003). The inhibitors have been used to facilitate memory in a number of paradigms and thus may be a viable strategy for promoting behavioral effects by inhibiting brain peptide degradation (Chai et al., 2008, Albiston et al., 2011). To further investigate therapeutic potential, the effect of P-LAP inhibitors should be compared to those of central OT in behavioral paradigms of social cognition, like social recognition in mice or partner preference in the prairie vole. However, it must be considered that aminopeptidases are not specific for OT and therefore, like many potential pharmacological approaches, the effects on other systems, and the consequential side effects, must be scrutinized.

Oxytocin Manipulations as Adjuncts to Behavioral Therapies

OT based therapies of any class may be most beneficial for the treatment of social impairments as a pharmacological adjunct to behavioral therapies. It is hypothesized that OT acts to enhance the saliency of social information and to assign social stimuli a positive valence. In that capacity, many of the initial studies of OT on functional human social cognition have demonstrated short-term prosocial effects evident in discrete experimental measures, including socially reinforced learning(Hurlemann et al., 2010b). However, it is unclear if OT treatment alone can induce long-term improvement in measures of holistic social cognition. We propose that the saliency and valence effects of OT could enhance the acquisition of social skills taught as a part of structured behavior therapy program, like applied behavioral analysis. Many of the sub-skills already taught as a part of social skills training paradigms, like maintaining eye contact and understanding facial expressions, are modulated by OT (Guastella et al., 2008c, Van Ijzendoorn and Bakermans-Kranenburg, 2011). Administration of a drug that enhances the function of the OT system may enable the patient to better attend to and form positive associations with the relevant social

information presented. This treatment model is reflected in the design of one of the behavioral paradigms discussed, the partner preference test, in that potential prosocial drugs are administered prior to the social learning phase, so that the animals are receiving both pharmacological and behavioral stimulation simultaneously. The partner preference test could be used predictively to identify OT based therapies that would be most efficacious in a combined pharmacological/ behavioral treatment strategy (Modi and Young, 2011a).

Conclusion

Irrespective of the contribution of the OT system to the pathophysiology of the disorder, the OT system has a strong therapeutic potential for the treatment of social impairments in ASD and a number of other psychiatric disorders, based on its critical role in the modulation of social cognition in animal models. The potential benefits of OT, though, are limited by the biophysical properties of the peptide. This necessitates the development and validation of animal models and behavioral paradigms of social cognition to identify the most efficacious methods of upregulating the OT system. The potential use of genetic based and phenotype based animal models in the context of the various therapeutic approaches for targeting the OT system, can be seen in Table 2. Thus far, the potential benefits of pharmacologically enhancing the OT system have been limited by the scale of the available methods to manipulate the system. The development of more potent methods stimulating the OT system could have profound effects on social cognitive processes, which could be harnessed clinically. Thus, while OT may play a negligible role in the induction of social impairments of ASD it may still have a meaningful effect on the manifestation of the disorder. It is imperative that the field work to fill in the knowledge gaps in the relationship between the OT system and human social cognition, including characterization of the human functional and disorder OT system, the mechanism of action of intranasal OT, and the long-term efficacy of OT administration to realize the clinical potential of OT in the treatment of social impairments.

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Terminology	Type	Definition	Example
Animal model *Laboratory animals used to characterize a disease or the normative processes disrupted in a disease.*	Genotype based	Mice (in most cases) genetically engineered to contain genetic alterations either associated with ASD or causative of monogenic forms of ASD	•FMR1 KO mouse (*causative*) •DHCR7 KO mouse (*causative*) •OTR KO mouse (*associated*) •OT KO mouse (*associated*) •CD38 KO mouse (*associated*)
	Environment based	Animals (typically rodents or primates) that have undergone an environmental manipulation linked with ASD	•Valproic acid treatment •Borna disease virus infection *Not discussed in this review*
	Phenotype based	Animals social behavior parallels either functional or disordered human phenotypes	•BTBRT+ tf/J mice (*disordered*) •BALB/c mice (*disordered*) •Microtine rodents (*functional*)
Validity *Relationship of the animal model or behavioral paradigm to the disease or behavior it is intended to represent.*	Construct validity	Model replicates etiological or neurobiological bases of the human condition being represented	•FMR1 KO mouse (*high*) •DHCR7 KO mouse (*high*) •OTR KO mouse (*moderate*) •CD38 KO mouse (*moderate*) •OT KO mouse (*low*)
	Predictive validity	The outcome of a manipulation in an animal model is indicative of the effect on the human condition	Partner preference in prairie voles (*TBD*) Social preference in mice (*TBD*)
	Face validity	The measured phenootypes in the model are phenomenologically similar to the human condition	Social recognition in OTRKO mice (*disordered*) Partner preference in prairie voles (*functional*)
Behavioral paradigm *An experimental test used to quantify a specific behavior in the laboratory, usually representative of an animal's cognitive or emotional state.*	Isolation induced vocalization	Frequency and intensity of ultrasonic vocalizations emitted by pups, when separated from their dams, is indicative of social anxiety	•Wild-type mice ✓ •OT KO mice ✖ •OTR KO mice ✖ •CD38 KO mice ✖
	Social recognition test	Ability of a rodent to recall a familiar individual, as measured by decreased time spent in olfactory investigation of a familiar as opposed to novel conspecifics, as a measure of social memory	•Wild-type mice ✓ •Rats ✓ •OT KO mice ✖ •OTR KO mice ✖ •CD38 KO mice ✖
	Social preference test	Degree of preference expressed, as measure by time in proximity, to social *vs.* nonsocial and novel *vs.* familiar stimuli a proxy for social motivation	•Wild-type mice ✓ •BTBR mice ✖ •BALB/c ✖
	Partner preference test	Formation of a social bond, as measured by twice as much time spent huddling with familiar as opposed to novel animal.	•Prairie voles ✓ •Meadow voles ✖

Table 1: Using of Animal Models. Basic terminology used to describe how animal models and behavioral paradigms are classified, evaluated and used to assess social cognition. ✔ indicates species typical performance in the behavioral task. ✖ indicates abnormal performance associated with social impairment.

Treatment strategy	Description	Potential predictive model					
		Genotype based models				Phenotype based models	
		OT-KO	OTR-KO	CD38-KO	Monogenic ASD models	Inbred mice	Microtine rodents
Intranasal OT	Peripheral route of administration with hypothesized preferential access across the BBB.	+	−*	+	+*	+	+
Non-peptide agonists	Small molecule agonists for the OT receptor that cross the BBB.	+	−*	+	+*	+	+
OT releasers	Agonists for receptors on OT neurons or intracellular signaling molecules that promote OT release.	−	−*	+	+*	+	+
P-LAP inhibitors	Inhibits the enzymatic activity of placental leucine aminopeptidase, to prevent the degradation of OT	−	−*	−	+*	+	+

Table 2: Relevance of Animal Models for Oxytocin-based Drug Discovery. The potential efficacy of OT based therapeutics can be best evaluated using different animal models of social cognition (+ indicates validity of testing that form of OT-based therapy in that specific model, - indicates lack of validity of testing that form of OT-based therapy in that specific model). OTKO and CD38KO mice are best utilized to test the effects of OT and OT receptor agonists, as the effect of OT in the social recognition test in these models is well characterized. Phenotype base models can be used to test all classes of OT-based therapies, though the best characterized behavioral endpoint uses the partner preference in female prairie voles. The use of OTR-KO mice has the most construct validity for the small subpopulation of ASD patients altered OTR expression, though the mechanism of action of OT-based drugs would be less clear in this model (-* indicates the model couldn't be used to identifying therapeutics that act at OT receptors, but the model could be useful in understanding signaling through alternative pathways).

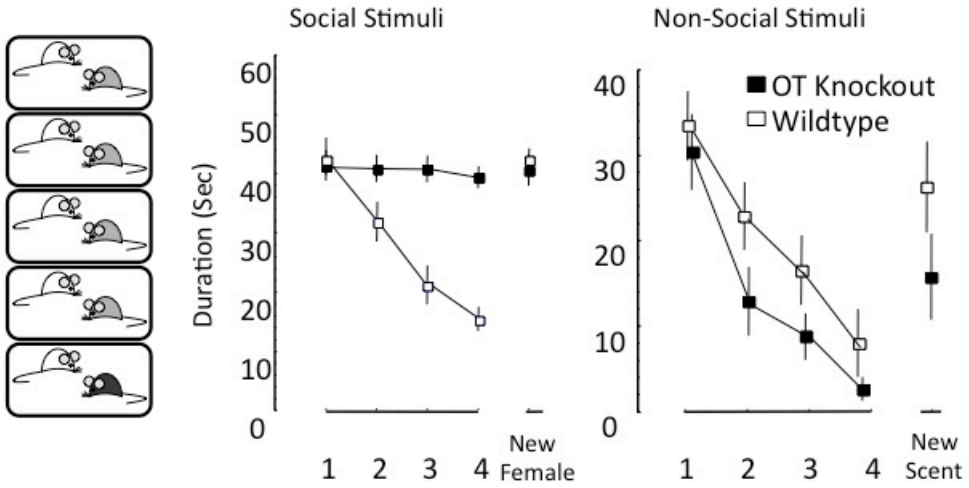

Figure 1. Social Recognition Behavioral Paradigm. Recognition of a familiar odor (either social or non-social) is indicated by a decrease in duration of time in olfactory investigation of the scent. In the paradigm, the test animal is presented with either a single mouse (left graph) or cotton swab scented with a non-social odor (right graph) four times sequentially. The fifth presentation is a novel mouse or novel non-social odor. Recognition of the repeated stimulus should elicit a decrease in investigation duration. Presentation of the novel stimulus should result in a return to baseline levels of investigation. The OTKO mouse fails to show a reduction in the duration of olfactory investigation of social stimuli, but does show a habituation to the non-social stimuli. Similar patterns of olfactory investigation for social and non-social stimuli can be observed in comparisons of OTRKO and CD38KO mice to wild-type littermates.

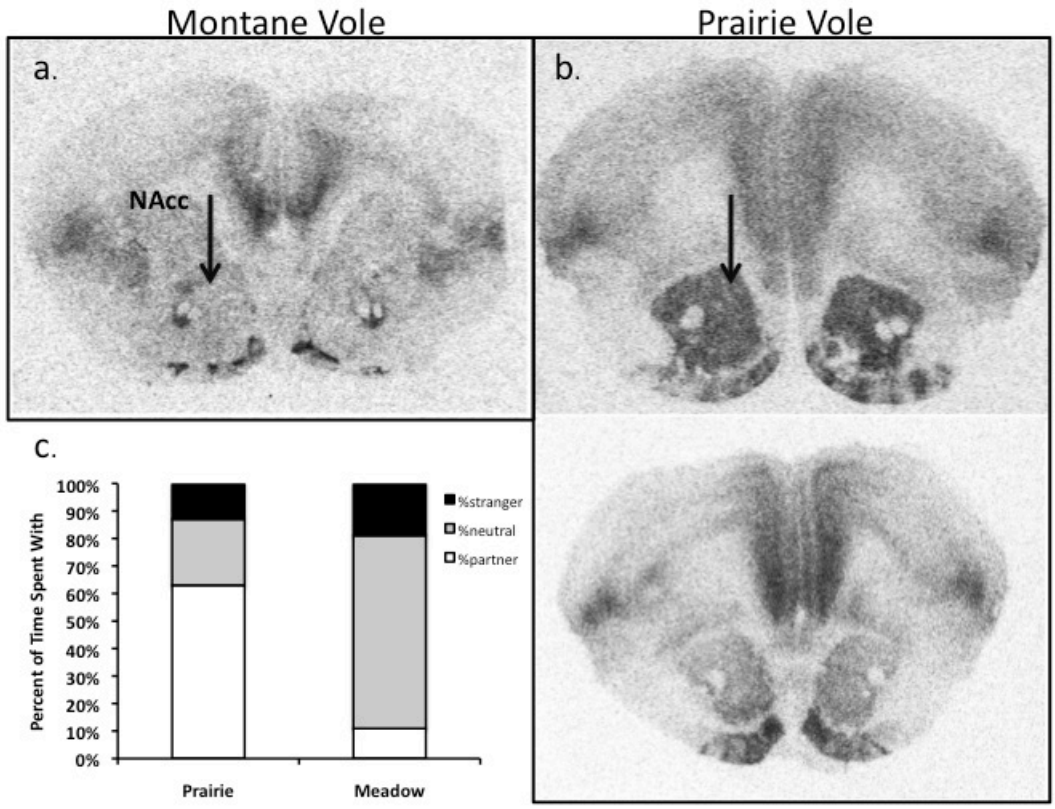

Figure 2. Variation in Oxytocin Receptor Levels and Partner Preference. Asocial meadow voles (a) have lower levels of OT receptors in the nucleus accumbens (NAcc-indicated by black arrow) than highly social prairie voles (b), as seen in autoradiographic images of oxytocin receptor density. There is also individual variation within prairie voles in the levels of OT receptor in the NAcc (b, high d, low). The variation in receptor density, both across species and within prairie voles, corresponds to variation in partner preference behavior and alloparental behavior. Prairie voles spend more time in proximity of their familiar partner than either alone or with a novel stranger, while meadow voles spend more of their time alone than with either conspecifc while in the partner preference test (c).

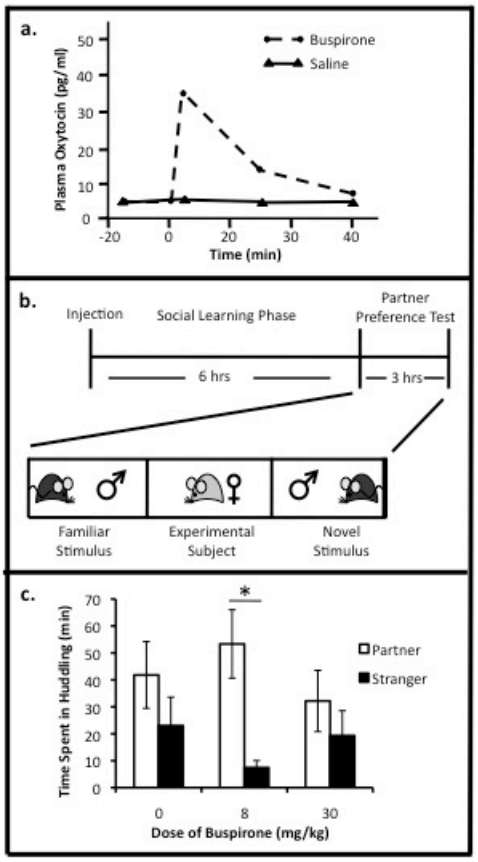

Figure 3. The effects of buspirone on OT release and partner preference formation in prairie voles. (a) Peripheral administration of buspirone (0.7mg/kg intravenous), a 5-HT1a partial agonist, induces an increase in plasma OT levels (graph modified from (Bagdy and Kalogeras, 1993). (b) The partner preference paradigm was used to assess the effect of buspirone on the formation of OT-dependent pair bonds. Buspirone (0,8, 30mg/kg) was administered to non-sexually receptive, adult female prairie voles via intraperitoneal injection immediately prior to a six-hour cohabitation with an adult, sexually experienced male prairie vole. After the social learning phase, the formation of a partner preference by the experimental subject was tested in the partner preference test. During the test, the male partner was tethered in one arena of a three-chambered linear test box and a novel male was tethered in the opposite chamber. The experimental female was allowed to freely wander the test box for three hours and the amount of time spent huddling with either the partner or stranger was recorded using TopScan behavioral analysis software (CleverSys Inc. Reston, VA). (c) Females receiving an 8 mg/kg dose of buspirone but not a control injection or 30 mg/kg dose formed a robust pair bond under suboptimal social learning conditions, as indicated by spending greater than twice the amount of time with the partner than the stranger (* indicates a significant partner preferences). A comparison using a 2-way ANOVA revealed a significant main effect of stimulus animal ($F(1,27)=9.262$, $p=0.004$) no other significant main effects or interactions were found. To determine which treatments resulted in significantly more time spent with the partner than the stranger, post-hoc Student's t-tests were performed with Bonferroni corrections of the p-value. Peripheral administration of 8mg/kg of buspirone ($p<0.003$; Student's t-test, Bonferroni level set at $p<0.01$).

CHAPTER 2:

Intranasal oxytocin increases plasma but not lumbar cerebrospinal fluid oxytocin concentrations in rhesus monkeys.

Abstract

Oxytocin (OT) modulates complex social behaviors in rodents as well has humans. However, until recently, investigation into the effect of central OT using non-invasive means has been limited by OT's poor permeability of the blood-brain-barrier (BBB). It has been hypothesized that intranasally (IN) administered OT can reach the central nervous system via a compromised BBB in the nasal epithelium. IN OT in humans enhances trust, emotional perception, social memory and empathetic behavior and IN oxytocin has been proposed as a potential pharmacotherapy to enhance social functioning in ASD. However, there is no molecular evidence that IN delivered OT actually increases central OT concentrations. To address this limitation, we have used rhesus macaques to determine the extent to which IN and intravenous (IV) OT administration effects plasma and cerebrospinal fluid (CSF) OT concentrations. Anesthetized rhesus monkeys were given the synthetic oxytocin analogue, Syntocinon (24 or 48 IU, Novartis), or its placebo, IV or IN. Serial CSF samples and plasma samples were collected over a two hour post-administration window. CSF and plasma samples were assayed for OT and vasopressin (AVP) levels by radioimmunoassay. Peripheral cortisol was measured via mass spectronomy. Neither IN nor IV OT had a significant effect on the level of OT in the lumbar CSF of rhesus monkeys as compared to placebo administration or pretreatment levels. Both OT treatments, however, did dramatically increase plasma levels of OT, which were maintained throughout sampling, compared to placebo treatments and over the pre-administration time points. IV OT also increased plasma cortisol levels, counter to the existing dogma. Neither treatment nor route of administration had a significant effect on either CSF or plasma levels of AVP. There are two possible explanations for this finding in light of the behavioral effects of IN OT. Either IN OT does penetrate the BBB, perhaps via the CVO, and accesses CNS receptors, but bypasses CSF circulation or IN OT does not reach the CNS in appreciable amounts and has behavioral effects via peripheral mechanisms. Further studies are needed to distinguish these two possibilities.

Introduction

The neuropeptide oxytocin (OT) modulates complex social behavior in a number of animal models. The effect on social behaviors, including parental care, social discrimination and bonding, is thought to be primarily mediated through central mechanisms. In primates, though, the contribution of the peptide to social cognition has, until recently, been unclear. The central effects of OT in primates have been difficult to determine due to the inability for peripherally administered OT to cross the blood-brain-barrier (BBB), preventing experimental administration through the typical routes (Landgraf and Neumann, 2004). Intranasal (IN) administration of OT has been used in a number of recent studies to circumvent this limitation, based on the hypothesized property of increased brain penetration resultant from this route of administration. While IN administration has been used to increase both CSF and brain levels of several large molecules and peptides, the efficacy of this method has not been systematically tested for OT. The goal of this study is therefore, to measure contribution of different routes of OT administration to central and peripheral OT levels.

The BBB inhibits the penetration of endogenous neuropeptides in physiologically relevant quantities, such that OT exists at significantly different concentrations in the brain and blood. This impermeability allows for independent regulation of central and peripheral levels of OT resulting in discrete behavioral and physiological functions (Landgraf and Neumann, 2004). Peripheral administration of OT, in rodents, results in the entry of less than 1% of the peptide into brain areas protected by the BBB (Ermisch et al., 1985). This rate of penetration, while insufficient to alter the endogenous gradient of the peptide, could potentially penetrate at minute but functionally significant levels after administration of pharmacological relevant doses (Landgraf and Neumann, 2004).

It has been proposed that intranasal administration of peptides allows for privileged access into the brain compartment through the nasal epithelium. The mechanism through which molecules

administered in this fashion are able to gain access to the CNS, though, is unclear. It is hypothesized that the rapid turn over of nasal epithelium cells leads to the loosening of tight junctions facilitating the transport of large molecules into the lamina propria (Altner and Altner-Kolnberger, 1974). Once in the lamina propria the molecules could either be absorbed into blood vessels and enter peripheral circulation or could diffuse into perineural or perivascular spaces and gain access to the central nervous system (Lochhead and Thorne, 2011).

Several peptides including insulin, α-melanocyte stimulating hormone, and vasopressin (a peptide structurally similar to OT) have been reported to achieve access to the brain or cerebrospinal fluid after human intranasal administration (Born et al., 2002). A recent study demonstrated an increase in CSF levels of OT after aerosolized administration of the peptide in a small number of monkeys (Chang et al., 2012). Strikingly, despite a limited number of studies showing physiological penetration of IN OT into the CNS, a large number of studies have shown behavioral effects after IN administration consistent with central activity. IN OT in humans is reported to enhance trust, emotional perception, social memory and empathetic behavior (Kosfeld et al., 2005b, Rimmele et al., 2009b, Bartz et al., 2010b, Domes et al., 2010). Based on these findings, IN oxytocin has been proposed as a potential pharmacotherapy to enhance social functioning in autism spectrum disorders (ASD) and other psychiatric disorders characterized by social impairments. Four studies have reported improvements in measures of social function in individuals with ASD, including the monogenic syndrome Fragile X, after IN OT administration (Bartz and Hollander, 2008a, Andari et al., 2010c, Guastella et al., 2010b, Hall et al., 2011). In light of these findings, it is important to determine the mechanism of action through which intranasal OT administration impacts social behavior, particularly for its advancement as a putative therapeutic.

The aim of this study is to characterize the effect IN and intravenous (IV) OT administration on blood and CSF levels of OT in rhesus monkeys (*Macaca mulatta*). The effect of OT on the

closely related peptide, vasopressin, and the steroid hormone, cortisol were also measured as potential intermediaries to OT behavioral effects. The rhesus monkey shares a number of anatomical similarities in nasal passage morphology with humans that are not seen in rodent models, including a relatively low surface to volume ratio, the distribution of epithelial cell populations and facultative nasal breathing (Harkema, 1990). Thus the findings from this model should provide insight not only into the distribution of OT after IN administration in monkeys but also in humans, providing mechanistic evidence supporting a mechanism for the function effect of OT on behavior.

Materials and Methods

In accordance with Emory IACUC policies, adult, male rhesus monkeys (*Macaca mulatta)* were anesthetized via ketamine induction and propofol maintenance under veterinary supervision. After a sufficient depth of anesthesia was achieved, baseline blood and cerebrospinal fluid samples were collected. Animals were dosed with the synthetic oxytocin analogue, Syntocinon (Novartis), or its placebo (Novartis) using intravenous or intranasal administration routes. Serial CSF samples were taken from the lumbar region 60 and 120 minutes after administration. Plasma samples were taken at the same time points for the IN administration and at 5, 15, 60 and 120 minutes for IV administration. Time points were chosen based on the time course of vasopressin central penetration after IN administraton (Born et al., 2002). Each blood sample was 3ml and each CSF samples was 300µl in volume. Blood was collected into tubes containing 10mg of EDTA and stored on ice until the conclusion of the session. CSF was place immediately on dry ice after collection. At the conclusion of each session, the blood samples were spun at 3000 rpm at 4°C for 15 minutes after which the plasma fraction was collected. All samples were stored at -80°C until analysis. The same subjects received all IN and IV Syntocinon and placebo conditions during separate sampling sessions allowing for a repeated measures within-subjects design.

CSF and plasma samples were assayed for OT and vasopressin (AVP) levels by a highly specific and highly sensitive radioimmunoassay in the laboratory of Dr. Rainer Landgraf (Max Plank Institute of Psychiatry, Munich, Germany). All samples directly compared were analyzed within the same run. The limit of detection of the assay is 0.1pg per sample and the cross reactivity is <0.7%.

Plasma samples were also analyzed for cortisol levels using liquid chromatography mass spectronomy by the Yerkes National Primate Research Center Biomarkers Core. Samples were treated with the internal standard d4-Cortisol provided by CDN Isotopes (Pointe-Claire Quebec, Canada) for quantitation.

Results

Oxytocin and Vasopressin

Neither IN nor IV OT had a significant effect on the level of OT in the lumbar CSF of rhesus monkeys as compared to placebo administration or pretreatment levels (Figure 1a and 1b). Both OT treatments, however, did dramatically increase plasma levels of OT compared to placebo treatments [$F(3,5)=23.515$, $p<0.0001$] and over the pre-administration time points [$F(2,5)=15.685$, $p=0.001$] (Figure 1b and 1d). Corrected post-hoc Student's T-tests indicate a significant increase in both treatment conditions (IN and IV) in plasma levels of oxytocin at all time points after administration (IN-60 $p<0.001$; IN-120 $p<0.01$; IV-5 $p<0.0001$; IV-15 $p<0.0001$; IV-60 $p<0.001$; IV-120 $p<0.0001$) minutes. Plasma levels of OT increased 100 fold after IN administration and 1000 fold after IV administration. Neither treatment nor route of administration had a significant effect on either CSF or plasma levels of AVP (Figure 2a-d).

Cortisol

Administration of IV OT resulted in an increase in plasma cortisol levels compared to administration of the IV placebo condition (Figure 3). A Student's T-test comparing the area under the curve of the OT and the placebo conditions over time indicates a trend towards an

increase in cortisol levels with IV OT administration with a p=0.08. At the 60-minute time point 6 out of 6 animals show higher levels of cortisol with drug treatment compared to the placebo. This trend is much less pronounced in the IN conditions.

Discussion

Both IN and IV administrations of OT resulted in robust and long lasting increases in blood OT concentrations. Both routes of administration showed a prolonged elevation of OT plasma levels (>120 minutes), despite the short half-life of the peptide in the blood (~2 minutes) (Mens et al., 1983a). However, neither route of administration significantly increased the concentration of OT in the lumbar CSF of rhesus monkeys. While these findings appear to be incongruent with the behavioral effects seen after IN OT administration, there are two possible explanations. Either IN OT does penetrate the BBB and accesses CNS receptors, but bypasses lumbar CSF circulation or IN OT does not reach the CNS in appreciable amounts and has behavioral effects via peripheral mechanisms.

Undetected Central Penetration

Despite the lack of measurable change in CSF OT levels after IN or IV administration, there could be penetration of the peptide into the CNS. Penetration of many molecules, including peptides, into the CNS after IN administration has been well documented (Lochhead and Thorne, 2011). The failure to detect OT in our paradigm could be the result of the limitations of our animal model or limitations in the method of sampling.

In this study CSF was used as a proxy for central penetration, however it is possible the peptide entered into the brain parenchyma and extracellular space, bypassing CSF circulation and subsequent detection. One proposed mechanism for the entry of intranasally administered large molecules into the CNS is via the perinerual space that surrounds the cranial nerves innervating the nasal epithelium. Labeled compounds applied to the nasal epithelium can be detected within the sheath surrounding the olfactory nerve bundle in the olfactory bulb minutes after

administration (Jansson and Bjork, 2002, Thorne et al., 2008). Evidence of radiotracers administered in a similar fashion cannot, though, be detected in cisternal CSF, despite the access of this region to the CSF (Thorne et al., 2008). The ability to detect substances administered intranasally in CSF appears to be highly variable between molecules and does not always correlate with its levels in the brain tissue (Lochead, 2011). It is possible that while vasopressin is detectable in CSF after IN administration (Born et al., 2002), OT is unable to penetrate into CSF circulation and remains sequestered in brain tissue. An analysis of OT levels after IN administration within the tissue of specific regions, either using in vivo microdialysis or post-mortem tissue quantification would be required to address this hypothesis. Evidence of OT penetration to olfactory bulb via the olfactory nerve or the brain stem via the trigeminal nerve after IN application still begs the question of the method of transport from these sites to areas of behavioral relevance.

In addition to the inability to detect brain levels of OT, there are other methodological limitations to the model that may not allow for the direct translation of these results to humans. The proposed efficacy of IN administration is based on deposition of the molecule across a specific region of the nasal epithelium. Optimal penetration to the CNS via neuronal pathways requires targeting of the drug to the upper-third of nasal cavity ((Dhuria et al., 2009, Lochhead and Thorne, 2011). In this study the animals were unconscious and therefore couldn't be made to "sniff" forcefully to drive the nasal spray further into the nasal passage, as was done in the human IN studies. The lack of "sniffing" could result in suboptimal deposition patterns and subsequent central penetration. Altering the deposition pattern through head position or delivery method could result in increased CSF OT accumulation. A recent study found that aerosolized OT administration significantly increased CSF OT levels in two monkeys (Chang et al., 2012). Aerosolized OT could result in a more widespread and penetrative deposition pattern allowing for increased access to areas of the nasal passage more permissive of central penetration. Further

systematic exploration of methods of IN delivery including head position, dosage volume, drug vehicle and delivery method should be explored further with specific respect to OT to determine which method, if any, result in consistent CNS increases in OT.

Potential Peripheral Mechanism

Irrespective of the CSF accumulation, the increase in peripheral OT after administration provides a potential mechanism for the behavioral effects of IN OT. The accumulation of the peptide in the plasma is not surprising, as the IN delivery method was originally developed to facilitate the penetration of orally unstable compounds into peripheral circulation utilizing the highly vascular nature of the nasal epithelium. The nasal vasculature is so dense, it can act as a sink for some IN administered compounds, decreasing the probability of them reaching the CNS (Chang et al., 2012). Upon entry into systemic circulation, OT can act in the periphery through receptors located in male and female reproductive tissue, the kidney, the heart and vascular tissue (Gimpl et al., 2008). The endogenous peptide is additionally found in adrenals, the vagus nerve, thymus and pancreas, suggesting multifarious peripheral functions (Gimpl et al., 2008, Llewellyn-Smith et al., 2012). Through these peripheral sites of actions there are three potential mechanisms through which OT could exert behavioral effects: the autonomic nervous system, the hypothalamic-pituitary-adrenal (HPA) axis and the peripheral sensory nervous system. OT affects the activity of both the sympathetic and parasympathetic arms of the autonomic nervous system, through the modulation of heart rate and blood pressure (Pardini et al., 1989, Higa et al., 2002). It has been proposed that activation of autonomic nervous system, in particular the vagally mediated reduction of heart rate, can promote prosocial behavior (Porges, 2001). Through this mechanism, the heart rate modulation by peripheral OT could affect behavior consistent with the manner seen in IN OT studies. In combination with the autonomic nervous system, the hypothalamic-pituitary-adrenal axis can also modulate behavior through peripheral OT activity. OT can act directly at all three levels of the HPA axis, including the peripheral adrenal site, to modify cortisol release and

the expression of stress associated behaviors (Legros et al., 1988). Peripheral administration of OT at a dose 10 times lower than those used for most IN studies results in a measurable reduction in plasma ACTH and cortisol levels (Legros et al., 1984). It has been hypothesized that many of the "social" effects of IN OT could in fact be mediated through OT's anxiolytic effects on the HPA axis, by disinhibiting social engagement (Churchland and Winkielman, 2011). Lastly, OT can act in tissues innervated by the peripheral nervous system to mediate behavioral responses. For example, OT receptor activation of the cervix and uterus enhances the production of female sexual behavior. This peripheral effect of OT is mediated through the pelvic nerve and resection of the nerve prevents the OT induced enhancement. This suggests that peripheral activation of OT receptors feeds back to the CNS via the pelvic nerve to initiate sexual behavior (Moody and Adler, 1995). Similar mechanisms of peripheral receptor activation feeding back into central regulation of behavior could be involved in the production of other social behaviors after peripheral OT administration. It is unknown whether any of these mechanisms actually contribute to the behavioral effects seen after IN OT administration, but all represent a viable mechanism should exogenous OT not accumulate in the CNS.

Two lines of evidence support the viability of peripheral OT administration as a behaviorally efficacious route. First, in the clinical setting, peripheral OT has been used to evoke behavioral changes. An acute IV dose of OT reduced repetitive behaviors in individuals with autism spectrum disorders (Hollander et al., 2003). A similar protocol also resulted in increased retention of social information in the same population (Hollander et al., 2007). Second, several studies using IN OT have reported peripheral effects in addition to behavioral effects after administration. IN OT increased autonomic cardiac control and reduced the cortisol response to a variety of paradigmatic stressors (Heinrichs et al., 2003, Norman et al., 2011). The behavioral effect of peripheral OT and the autonomic effects of IN OT suggest a systemic mechanism of action, but they could also be mediated through central mechanisms. Due to the relatively large

doses administered, IV OT could result in undetectably small yet significant increases in central OT levels, which contributed to the behavioral effects. Similarly the autonomic effects could be regulated centrally through the brain stem nuclei, particularly through IN transport along the trigeminal nerve. Regardless of functional mechanism, the increases in plasma levels of OT resulting from both routes of administration in this study are likely evoke behaviorally relevant effects.

Effect of OT Administration on Vasopressin and Cortisol

In addition to its direct effects, OT interacts with a number of neurochemicals that affect behavior. There is a high degree of homology in the OT and vasopressin systems. The two peptides are both structurally similar and to an extent functionally redundant. Central OT can act on vasopressin receptors in the brain to modulate vasopressin release. A decrease in peripheral vasopressin levels after exogenous OT administration could be indicative of a central activation of the endogenous neurohypophyseal hormone system (Neumann et al., 2006). This would suggest that exogenous OT administration could promote the release of endogenous OT. However, neither route of OT administration had significantly altered central or peripheral vasopressin levels. This suggests, though does not conclude, that the increases in peripheral OT are not due to an enhancement of the endogenous OT system.

Interactions with the vasopressin, though, may underlie the paradoxical effect of OT on cortisol levels in this study. IV administration of OT resulted in a trend towards increased plasma cortisol levels not seen with placebo treatment. Typically OT functions as an anxiolytic, reducing the activity of the HPA axis and subsequent corticotropin release (Lightman and Young, 1989). However, OT acting through vasopressin 1b receptors in the neurohypophysis can stimulate the release of the HPA regulating hormone, adrenocorticotropin (ACTH; (Schlosser et al., 1994). ACTH acts on the adrenal to increase the release of cortisol (Stokes and Sikes, 1991). It is possible that OT, under the conditions of this study, induced an increase in peripheral cortisol

through the upregulation of ACTH. The increase in cortisol primarily after IV administration, though, suggests that this unusual response could be due to unknown effects of extremely high levels of circulating OT at the adrenals themselves. The OT receptor, as a g-protein coupled receptor, undergoes desensitization and internalization after prolonged stimulation (Robinson et al., 2003). The high plasma OT concentrations over the course of this study could have altered the activity of the OT receptors regulating cortisol release, resulting in increased plasma cortisol levels. Increased peripheral levels of cortisol after OT administration, regardless of cause, could have a meaningful impact on the expression of social behavior.

Therapeutic Use of OT

IN administration has been touted as the means though which the therapeutic potential of OT can be harnessed. However, in this study IV and IN routes of administration had parallel effects on CSF and plasma levels of OT. In addition, both IN and IV administrations of OT have been used to experimentally enhance social cognition in individuals with ASD (Hollander et al., 2007, Andari et al., 2010b, Guastella et al., 2010). This suggests, that despite the evidence from animal models, both peripheral routes of administration may be viable methods for the pharmacological treatment of social impairments with OT. To determine the relative efficacy of these routes, studies should be conducted directly comparing the effect of IV and IN OT on social behavioral measures in both typical and affected populations. A similar comparison of the social behavioral effects resulting from exogenous OT or clinical anxiolytic administration could also be made to determine if the prosocial effects of OT are mediated through the HPA axis.

To facilitate the use OT clinically additional parameters associated with IN administration should also be addressed. The effective dose size (i.e. the amount of OT that is taken up into the blood or the CNS after dosing) and the consistency of that dose size between subjects after IN administration should be determined. If IN OT administration results in a consistent increase in OT levels, either in the periphery or the CNS, it may be the preferred way to clinically administer

OT due to its ease of use. IN OT administration in this study also resulted in an extended release of OT into the blood stream. Whereas IV OT levels at 120 minutes were a third of their levels at 60 minutes, the 120 OT levels after IN OT were only 10% less than at 60 minutes. IN application may allow for a slower rate of diffusion of the peptide from the nasal epithelium into systemic circulation. This could result in a more consistent elevation of OT blood levels, despite the rapid degradation of the enzymes mediating the typically short half-life of the peptide in the blood.

Conclusion

Both IN and IV routes of OT administration resulted in substantial increases in peripheral OT levels without a measurable change in lumbar CSF OT levels. Counter to what was initially expected based on the behavioral efficacy of IN OT, these findings open the door to a potential peripheral mechanism for OT's effects on social behavior. Further work is needed elucidate the mechanisms through which OT could gain access to the CNS in primates. However, attention should also be given to evaluating the role of peripheral OT in human social behavior.

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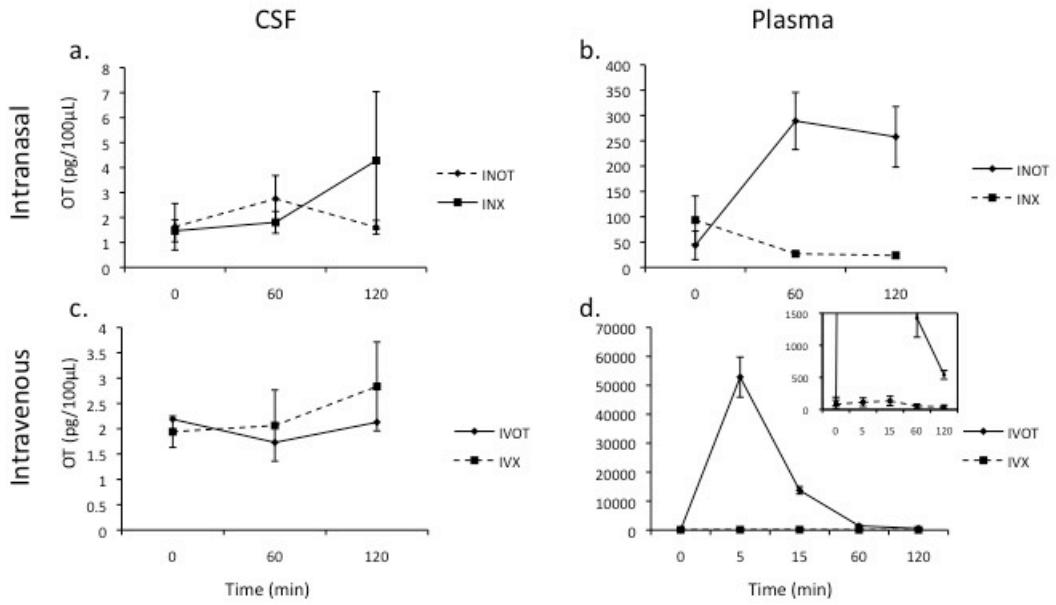

Figure 1. Effect of IN and IV OT on Central and Peripheral Oxytocin (OT) Levels. Administration of neither IN nor IV OT resulted in significant measurable increases in central OT levels compared to either baseline or placebo, as measured in lumbar CSF samples (a,c). However, both routes of administration did dramatically increase plasma levels of OT compared to placebo treatments [$F(3,5)=23.515$, $p<0.0001$] and over the pre-administration time points [$F(2,5)=15.685$, $p=0.001$] (b,d). Plasma levels of OT increased 100 fold after IN administration and 1000 fold after IV administration. * Indicates a significant difference in OT levels in post-hoc comparisons with a probability of error <0.005.

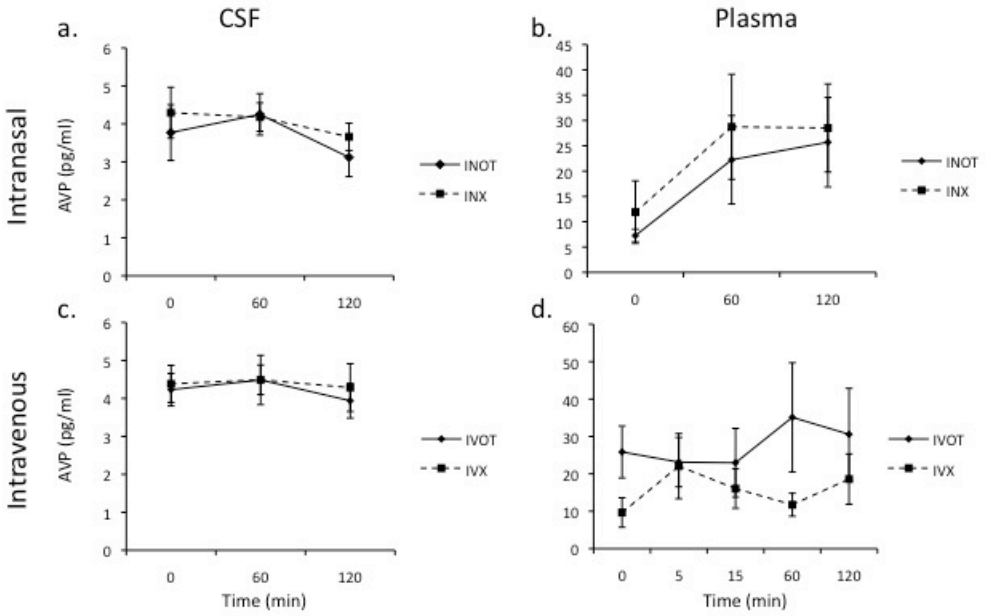

Figure 2. Effect of IN and IV OT on Central and Peripheral Vasopressin (AVP) Levels. Due to the cross affinity of the OT and AVP peptides for both OT and AVP receptors in the brain and periphery, the effect of OT on AVP levels was measured to assess for potential feed forward activation of the vasopressin system. Administration of neither IN nor IV OT resulted in significant measurable increases in central (a,c) or peripheral (b,d) AVP levels compared to either baseline or placebo, as measured in lumbar CSF samples.

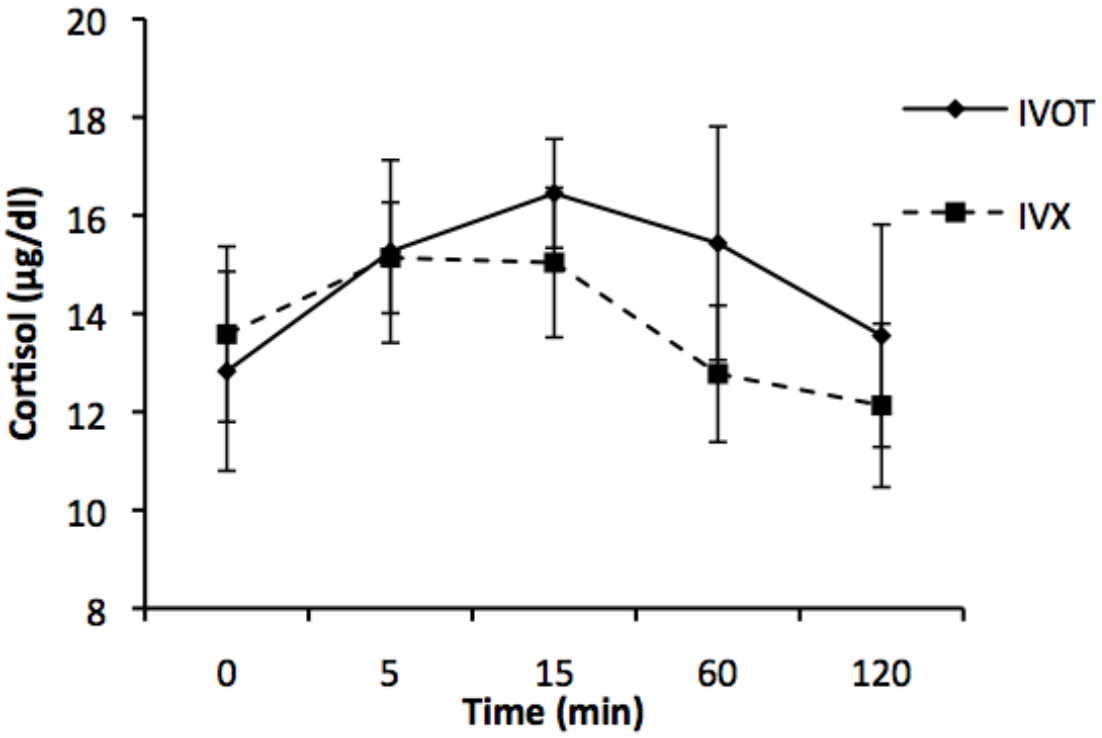

Figure 3. Effect of IV OT on Plasma Cortisol Levels. OT contributes to the regulation of cortisol. To look for the effect of OT on cortisol in this paradigm, plasma cortisol levels were compared between the treatment and route of administration conditions. A Student's T-test comparing the area under the curve of the OT and the placebo conditions over time indicates a trend towards an increase in cortisol levels with IV OT administration with a p=0.18. This trend is much less pronounced in the IN conditions.

CHAPTER 3

Enhancing oxytocin dependent behavior through the stimulation of melanocortin receptors.

Abstract

Oxytocin (OT) enhances prosocial behavior in animal models and in both normative and autistic human populations. The therapeutic potential, though, of oxytocin is limited by its poor penetration of the blood-brain-barrier. An alternative approach to enhance social cognition via the central OT system, is to induce OT release by the pharmacological stimulation of neurochemical receptors on oxytocinergic neurons. Endogenously, α-melanocyte-stimulating- hormone (α-MSH) acts via melanocortin 4 receptors (MC4R) on hypothalamic neurons to selectively induce central but not peripheral OT release. To test the hypothesis that indirect stimulation of OT neurons would facilitate OT-mediated social behaviors, we examined the effects of melanocortin agonists on OT-dependent partner preference formation in prairie voles.

First we used *in situ* hybridization (ISH) to confirm the expression of MC4R in regions that contain OT neurons in the prairie vole. Administration of a melanocortin receptor agonist, Melanotan II (MTII), increased neuronal activation in the same OT containing brain nuclei, as indicated by expression of the immediate early gene, Egr-1. Behaviorally, MTII facilitated the expression of a partner preference in female prairie voles, but not male prairie or female asocial meadow voles, in a pattern consistent with the direct effects of OT. MTII also enhanced the long-term acquisition of social information under both acute and developmental conditions. The facilitatory effect of MTII is likely mediated through the OT system, as co-administration with an OT receptor antagonist blocks its effects. Administration of Pf-446687, a highly-specific MC4R agonist with increased brain penetrance relative to MTII, also analogously enhanced partner preference formation and is a strong candidate for translational applications. This novel paradigmatic approach of indirectly inducing OT release to facilitate prosocial behavior represents a new strategy for the development of drugs to treat social impairments.

Introduction

The prosocial effects of oxytocin (OT) in animal models and in sociobehavioral tasks in humans suggests that the oxytocin system may be a viable pharmacological target for the treatment disorders characterized by social deficits, including autism spectrum disorders (ASD) and schizophrenia (Modi and Young, 2011b). In healthy subjects, intranasal (IN) OT has been shown to increase trust (Kosfeld et al., 2005b), generosity (Zak et al., 2007), empathy (Hurlemann et al., 2010b) and attention to and comprehension of emotional expression (Domes et al., 2010, Guastella and Macleod, 2012). The prosocial effect of OT is particularly pronounced in clinical and subclinical populations with social deficits (Bartz et al., 2010). IN OT increases feelings of trust, reciprocal social play and the recognition of emotional expression in individuals with ASD (Andari et al., 2010b, Guastella et al., 2010). In schizophrenia, IN OT reduces symptom severity for positive and negative symptom including those related to social cognition not typically affected by antipsychotics (Feifel et al., 2010, Pedersen et al., 2011). The efficacy of OT in enhancing aspects of social behavior makes the neuropeptide system a promising pharmacological target for the treatment of social impairments. Importantly, OT based therapies appear to have prosocial effects in populations with social deficits irrespective of the presence of OT dysregulation in the etiology of the disorder. This is particularly relevant for genetically heterogenous disorders like ASD, as the treatment strategy is likely to be effective in a larger proportion of the population since it is not based on correcting a specific biological deficit.

Despite its broad potential application, the maximal therapeutic potential of OT is hindered by its poor penetration of the blood-brain-barrier (BBB) (Landgraf and Neumann, 2004). As OT is a large polar molecule, only a small percentage of peripheral OT is able to cross BBB to elicit central effects (Churchland and Winkielman, 2011). The impermeability of the BBB, though, allows OT to occupy functionally distinct roles in the brain and in the periphery (Landgraf and Neumann, 2004). Most of the social behavioral effects of OT in animal models are mediated

through the central OT system (Williams et al., 1994b, Ferguson et al., 2001c, Ring et al., 2006). A central mechanism of action is a barrier to clinical use of OT, as most traditional routes of drug administration are ineffective at increasing brain levels of the peptide. Neuropharmacological studies have recently suggested that peptides gains better access to the brain through IN administration (Born et al., 2002). Though many behavioral studies have been conducted using this technique, the mechanism through which this route of administration elicits behavioral effects is unclear (Churchland and Winkielman, 2011). An alternative approach to increase the central OT to evoke behavioral effects, is to pharmacologically stimulate endogenous OT release (Modi and Young, 2011b).

Central levels of endogenous OT can be increased through the selective pharmacological targeting of the mechanisms that regulate OT release. In addition to separation of central and peripheral OT circulation by the blood-brain barrier, release of OT into the two systems is thought to be regulated semi-independently. For peripheral release, OT synthesized in magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) is transported via axonal projections to the posterior pituitary where it is secreted into blood circulation. Independently, OT is also released from the dendrites of the magnocellular neurons where the peptide diffuses to have "neurohormonal-like" effects at distal OT receptor sites in the brain (Ludwig and Leng, 2006, Ross and Young, 2009). Stimulation of discrete receptor populations on oxytocinergic neurons can differentially activate either of the independent release mechanisms. The neuropeptide α-melanocyte stimulating hormone (α-MSH) promotes the release of central but not peripheral OT release through the melanocortin 4 receptor (MC4R) (Sabatier et al., 2003). Administration of MC4R agonists, therefore, can be used to increase central OT in a physiologically relevant manner.

To determine if MC4R agonists can recapitulate the behavioral effects of central OT administration, we look at partner preference formation in the prairie vole. Prairie voles are

highly affiliative socially monogamous rodents that form enduring pair bonds, which can be measured and experimentally manipulated in the laboratory. In contrast, meadow voles are a closely related asocial species that typically does not form pair bonds. Pair bonds, an experimental proxy for complex social cognition, are assessed using the partner preference test. Pair bonded animals prefer to spend time in close contact with their partner compared to a novel stimulus animal.

Central OT receptor activation is necessary for the formation of pair bonds in prairie voles. Intracerebroventricular (ICV) infusion of OT in female prairie voles facilitates partner preference formation under conditions in which bonds wouldn't otherwise form (Williams et al., 1994b). Conversely, ICV infusion of an OT antagonist (OTA) prevents the formation of a partner preference under optimal conditions (Insel et al., 1998). Peripheral injection of OT, however, fails to induce a partner preference, likely due to the poor penetration of the blood brain barrier by the peptide (Williams et al., 1994b). Because it is an OT-dependent behavior, the partner preference test can to be used to assess the ability of peripherally administered MC4R agonists to recapitulate the behavioral effects of central OT administration.

Compounds that enhance OT-dependent behavior in prairie voles are likely to have similar effects in humans due to the evolutionary conservation of the OT system (Donaldson and Young, 2008b, Meyer-Lindenberg et al., 2011). The partner preference test, therefore, can be used as a predictive assay for the identification of drugs that may have prosocial effects in humans (Modi and Young, 2011a). Drugs identified in this study to enhance partner preference formation have strong translational potential and are a viable alternative to IN OT for the clinical treatment of social deficits. Furthermore, evidence that indirect stimulation of the endogenous OT system has behavioral consequences validates a new paradigmatic approach for the therapeutic utilization of neuropeptide systems.

In this study, we look at the effect of MC receptor agonists, currently approved for use in humans, on the formation of partner preference in the highly social prairie vole and the asocial meadow vole. The long-term effect of MC agonists (Figure 1) on the expression of social behavior is assessed in both adult and developing animals. Finally, the oxytocinergic mechanisms through which MC agonists are hypothesized to exert prososcial effects are validated.

Methods

Subjects

Subjects were adult (60-120 days of age) sexually naïve prairie voles from our colony maintained at the Yerkes National Primate Research Center at Emory University. This facility is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Prairie voles in our colony are derived from Illinois field caught stock. All animals were weaned at 19-21 days, maintained in same-sex groups of 2-3 under a 14/10 light/dark cycle with a stable environmental temperature of 22°C with access to food (LabDiet rabbit) and water ad libitum. All procedures used in this study were approved by the Institutional Care and Use Committee of Emory University.

Partner Preference Test

To test the formation of a pair bond, experimental subjects are tested in the partner preference test. After a co-habitation period, the experimental animal is allow to freely roam a three-chambered partner preference arena with two opposite sex stimulus animals, the partner with whom she was cohabitated and an age/experience matched stranger, tethered on opposite sides of the arena. The female is free to move throughout the environment, interact with either male or stay isolated in the central neutral cage. The time the subject spent in immobile side-by-side contact (huddling) and in the social and non-social zones was scored using the TopScan Behavioral Analysis System (CleverSys, Inc. Reston, VA)(Ahern et al., 2009b). As highly social

animals, prairie voles that have not formed bonds will huddle with either the partner or the stranger at chance levels (50% of the population will choose the partner, 50% will choose the stranger). In populations that have formed pair bonds, more animals will prefer to huddle with the familiar partner. A partner preference within a population is defined statistically as significantly more time spent with the partner than the stranger, and is used as a measure of prosocial behavior in the prairie vole. Total distance moved by the female during the test is also recorded as a measure of the effect of the drugs tested on general activity.

Localization of the Melanocortin 4 Receptor (MC4R) in the Prairie Vole

MC4R were localized in the prairie vole brain using in situ hybridization (ISH) techniques. Probes for the MC4R were generated from pCRII-TOPO Vector plasmids (Invitrogen, Carlsbad, CA) containing a 920 bp sequence prairie vole MC4R cDNA. The antisense and sense probes were linearized with *BamHI* and *NotI* restriction enzymes and transcribed in vitro using T7 and SP6 polymerases the presence of S^{35}UTP, respectively. ISH was carried out on fresh frozen brain sections from female prairie voles. Brain sections were fixed in 4% paraformaldehyde, rinsed in Proteinase K, deaminated in acetic anhydride, dehydrated in ascending concentrations of ethanol and delipidated with chloroform. Sections were then hybridized overnight at 57°C with the S^{35}-labeled MC4R cRNA antisense or sense probes. The next day the sections were incubated in 5X SSC+dithiotheitol, 2XSSC+formamide, RNAse A, washed in 2x SSC and 0.1X SSC, and then dehydrated in ascending concentrations of ethanol. MC4R mRNA signal was visualized using the BAS-5000 Phosphoimager (FujiFilm, Valhalla, NY).

Activation of OT-Positive Neurons after MTII Administration

To look at neuronal activation after MTII administration, female prairie voles were injected with 10mg/kg of MTII or a vehicle control (n=6, MTII; n=5, Sal) dissolved in a volume of 0.1ml 0.1% sterile saline. After administration the animals were returned to their home cage. 90 minutes after the initial injection the females were deeply anesthetized with a combination of ketamine

(120mg/kg) and dormitory (10mg/kg) and perfused transcardially. The perfusion consisted of 50ml of phosphate buffered saline (PBS; pH 7.4), followed by 50ml of 4% paraformaldehyde in 0.1M phosphate buffer. Immediately following the perfusion the brains were removed and placed in a bath of 4% paraformaldehyde in 0.1M phosphate buffer for six hours after which they were stored at 4°C in 30% sucrose until sectioned. The brains were cute into 35μm coronal sections with a freezing microtome and stored free-floating in a cryoprotectant solution at 4°C until immunohistochemical processing.

To determine if MTII activated OT-positive neurons in the PVN of the hypothalamus, the brain sectioned underwent immunohistochemical processing to detect the presence of Egr-1 (Early Growth Response protein 1) and OT. Briefly, sections were removed from the cryoprotectant solution, rinsed extensively in PBS. The sections were then blocked with 0.3% Triton-X, 5% Normal Goat Serum (PBS/NGS) for 30 minutes. Sections were then incubated in a primary antibody solution (PBS/NGS) directed against Egr-1 (sc-189, Santa Cruz Biotechnology, Santa Cruz, CA; at a concentration of 1:8,000) and OT (mAb5296, Millipore, Billerica, MA; at a concentration of 1:10,000) overnight at 4°C. The next day the sections were washed in PBS and then incubated for 3 hours in a secondary solution (PBS/NGS) containing alexaflour conjugated antibodies directed against the mouse and rabbit primaries (Alexa Flour 568 Goat Anti-Rabbit binding the Egr-1 Ab, fluorescing red and Alexa Flour 488 Goat Anti-Mouse bindings the OT Ab, fluorescing green, Life Technologies, Grand Island, NY) at a concentration of 1:1,000). The sections were then washed sequentially in PBS and PBST (PBS+1%Tween-20) then transferred to 75% PBS until mounted. Mounted sections were covered VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA), coverslipped and visualized under a confocal microscope and captured using ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland). Four bilateral sections from each subject containing the PVN were

quantified. The ratio of Egr-1+OT to total OT positive cells was quantified in each section and then averaged to yield a total ratio for each animal.

Peripheral Administration of Melanotan I and Melanotan II in Prairie Voles

The effect of Melanotan I (MTI) and Melanotan II (MTII) on partner preference formation was tested in ovariectomized female (N=10/group) and male (N=12/group) prairie voles. Male and female prairie voles were injected intraperitoneally with either Melanotan I (1, 10mg/kg; Sigma Aldrich, St. Louis, MO), Melanotan II (1,10 mg/kg; Sigma Aldrich, St. Louis, MO), or a saline control dissolved in a volume of 0.1ml 0.1% sterile saline. Initial doses were based the doses given to elicit behavioral responses in rodent models (Rossler et al., 2006, Klenerova et al., 2008). Females were cohabitated with sexually experienced males for six hours. Males were cohabitated with ovariectomized female prairie voles for 24 hours. Both experimental and stimulus females were ovariectomized to prevent mating from occurring, as ovariectomized females do not come into estrus and are consequently not sexually receptive. The difference in cohabitation time is due to sex differences in the time typically required for the formation of a pair bond. Immediately following cohabitation, the formation of a bond was measured in the partner preference test.

Peripheral Administration of MTII in Meadow Voles

The effect of MTII in an asocial species was tested in female meadow voles that were injected with MTII (n=9-11/group; 10mg/kg) or saline control dissolved in a volume of 0.1ml 0.1% sterile saline. Females were cohabitated with sexually inexperienced males for 24 hours and then tested for the formation of a partner preference. Following the test, the animals were re-paired for an additional 48 hours and tested again for a preference. The long cohabitation periods reflected the reduced sociality of this species and were chosen to increase the likelihood of pair bond formation.

Long-term Effect of MTII Administration in Female Prairie Voles

The effect of MTII on partner preference after the clearance of the drug was tested in adult ovariectomized female prairie voles that were injected intraperitoneally with MTII (n=9-11/group; 10mg/kg) or saline control dissolved in a volume of 0.1ml 0.1% sterile saline. The experimental animals were then paired with stimulus males for six hours. After the cohabitation period the animals were separated and singly housed for seven days and then reunited for partner preference testing. The animals were then separated for an addition seven days and then retested for partner preference 14 days after the initial drug administration. The repeat partner preference test was performed to determine how long the facilitatory effect of MTII was present.

Effect of Neonatal MTII on Adult Social Behavior

To determine the developmental effects of early life MTII administration neonatal, prairie vole pups were injected with MTII (N=20) or a saline (N=16) control every day for the first seven days of life. Neonates received 28 μg MTII in volume of 35 μl 0.1% sterile saline for the first 2 days of life, 40 μg MTII in volume of 50μl for days 3-5 and 52μg MTII in volume of 65μl for days 6-7 or an equivalent volume of saline. These doses are roughly equivalent to the 10mg/kg doses given to adults. The pups were weighed each day prior to drug injection. Weights were taken again at weaning and then prior to sacrificing. Pups of both sexes were dosed and weighed due to the difficulty distinguishing the sex of this species early in life. Only female animals were used for subsequent behavioral testing. After the first week of life, the pups were allowed to mature to weaning without intervention. As adults, the female prairie voles were cohabitated with opposite sex stimulus animals from the general colony for 6 hours and tested in the partner preference test. The animals were than repaired for another 18 hours and tested again for partner preference.

Co-administration of Melanotan II and Oxytocin Receptor Antagonist

To test the hypothesis that MTII facilitated partner preference through the central OT system, the ability of an OT receptor antagonist (OTA) to block the effects of MTII on partner preference was tested. Adult, ovariectomized female prairie voles were bilaterally cannulated into the nucleus accumbens (NA) using stereotaxic methods. Subjects were anesthetized using isoflurane and 26 gauge bilateral guide cannulas (Plastics One, Roanoke, VA) aimed at the NAcc (anterior 1.7mm, bilateral ±1mm, ventral -3.5mm to bregma), were implanted. Location of the cannulas was verified post-experimentally in frozen brain sections. After 3 days of recovery, subjects received microinjections with a 33-gauge internal cannula (Plastics One, Roanoke, VA) that extended 1mm below the guide cannula into the target area. The needle was connected to a Hamilton syringe (Hamilton, Reno, NV) via polyethylene-20 tubing (Plastics One, Roanoke, VA), through which the central treatment was injected. The internal needle was left in place for 2 minutes after the injection to prevent backflow.

Animals received either a bilateral control injection under isoflurane anesthesia of Ringer's Solution or 10μg per side of the OTA (d(CH2)51,Tyr(Me)2,Thr4,Orn8,des-Gly-NH29)-Vasotocin (H-2908 Bachem, Torrance, CA) dissolved in 500nl of Ringer's Solution (Fisher Scientific, Pittsburg, PA) per side into the NA (N=11-12/treatment). Simultaneously the females received injections of either Melanotan II (10mg/kg) or saline control dissolved in a volume of 0.1ml 0.1% sterile saline. Immediately after the injections, the females were cohabitated with sexually experienced males for six hours and then tested in the partner preference test.

Central Penetration of MC Agonists

Female prairie voles (n=3/group) were given Melanotan II (1mg/kg or 10mg/kg) or Pf-446687 (1mg/kg or 10mg/kg) intraperitoneally and then sacrificed after 60 minutes. Immediately after sacrifice, approximately 300μl of trunk blood was collected in heparinized capillary tubes and transferred to chilled microcentrafuge tubes. Brains were rapidly dissected out and flash frozen

on dry ice. Within 90 minutes of collection, the blood was spun at 3000 rpm for 15 minutes at 4°C after which the plasma fraction was collected for analysis.

Plasma and brain were frozen at -20°C until bioanalysis. Prior to acetonitrile (ACN)-mediated matrix precipitation, thawed brain tissues were weighed, diluted 4-fold (w/v) in 40% H_2O in isopropanol and homogenized in a bead beater. Standard curves were prepared in respective matrix (plasma and brain homogenate) via serial dilution at a concentration of 0.5-1000 ng/mL (PF-00446687 plasma), 0.5-1000 ng/g (PF-00446687 brain homogenate), 3.9 -1000 ng/mL (Melanotan II plasma) and 7.8-1000 ng/g (Melanotan II brain homogenate). For plasma samples, an aliquot (50 µL) was precipitated with ACN (300 µL) containing an internal standard. Samples were vortexed (1 min) and centrifuged (1811 rcf for 10 min) to afford supernatant (250 µL), which was transferred to a 96-well plate, evaporated under N_2 at 37°C and reconstituted in 25% ACN in H_2O (100 µL). For brain samples, a mixed-matrix approach was employed. To generate the brain homogenate standard curve, control brain homogenate (50 µL) was added to an aliquot (50 µL) of each plasma standard curve sample. Likewise, control plasma (50 µL) was added to each brain homogenate sample (50 µL). These brain samples were then processed as described above for plasma.

Samples were analyzed by an LC-MS/MS consisting of an AB Sciex API 4000 or API 5500 tandem quadrupole mass spectrometer with a TurboIon Spray probe, tertiary Shimadzu LC20AD pumps and a CTC PAL autosampler. Instrument settings and potentials were adjusted to provide optimal data. The ion pairs monitored were 471.2/415.2 for PF-00446687 and 512.8/110.2 for Melanotan II. Chromatographic separation was achieved using a Kinetex C18, 3x30mm, 2.6µ column for PF-0446687 and a Synergi Max RP, 2x30mm, 4µ, 80A column for Melanotan II. Both compounds were run under gradient conditions using ACN and 0.1% formic acid in 10mM

ammonium formate as the mobile phase. All raw data was processed using Analyst Software version 1.5.2.

Peripheral Administration of PF-446687 in Female Prairie Voles

To determine the specific receptor through which melanocortin agonists affect social behavior, the highly specific melanocortin 4 receptors agonist, PF-66887 (Pfizer, Inc, New York, NY), was tested. Adult, ovariectomized female prairie voles (n=10/group) were injected intraperitoneally with PF-446687 (1mg/kg or 10mg/kg) dissolved in a volume of 0.1ml of 10% β-cyclodextrin in 0.1% sterile saline or a vehicle control. The females were then cohabitated with sexually experienced males for six hours and then tested in the partner preference test.

Statistical Analysis

The effect of drug treatment on time spent with either the partner or the stranger was compared in a two-way ANOVA, with treatment and stimulus animal as the two factors. Significant effects were followed up with post-hoc tests comparing total time spent with the partner vs. the stranger using a Student's T-test with an alpha value corrected for the number of comparisons made. A partner preference was defined as significantly more time spent by the treatment group in immobile social contact with the partner compared to the stranger.

Results

Localization of the Melanocortin 4 Receptor (MC4R) in the Prairie Vole

MC4R mRNA was visualized in the PVN and the SON of the hypothalamus, the primary hypothalamic sites of central OT production (Figure 2a). Both the PVN and the SON typically contain high levels of both OT mRNA and peptide (Figure 2c; Ross, 2009). The specificity of the ISH probe for MC4R mRNA was verified by the lack of signal in sections hybridized with the sense probe (Figure 2b).

Activation of OT-Positive Neurons after MTII Administration

MTII administration resulted in a significant increase in the expression of the immediate early gene EGR1 in cells also expressing OT in the paraventricular nucleus of the hypothalamus (Figure 3), but no differences in the total number of OT neurons. Statistical comparison of the number of cells containing both OT and EGR1 after saline or MTII administration revealed a significant difference (Student's T-test p=0.0049). Only 0.8% of the cells containing OT in brain sections from animals that received saline also expressed EGR1, while 13.8% of cells contain EGR1 in the MTII treated animals.

Peripheral Administration of Melanotan I and Melanotan II in Prairie Voles

MTII administration resulted in a significant difference between stimulus groups when compared in a two-way ANOVA (Figure 4a; drug X stimulus; $F(1,53)=11.041$, $p=0.002$). Female prairie voles receiving a high dose (10mg/kg) of MTII (partner vs. stranger p=0.003) spent significantly more time with the partner male vs. the stranger male, thus they are considered to have formed a "partner preference". Animals given a control saline injection or a low dose of MTII (partner vs. stranger p=0.428) did not show a preference. MTI administration did not result in any significant differences between groups when compared in a two-way ANOVA (Figure 4c; drug X stimulus; $F(1, 69)=0.15$, $p=0.700$), neither a low (partner vs. stranger p= 0.85) or high dose of MTI (partner vs. stranger p=0.923) induced a partner preference. No difference from control treatment was seen in the locomotor patterns under any of the drug conditions (data not shown).

Neither MTI (Figure 4d; partner vs. stranger p=0.93) nor MTII (Figure 4b; partner vs. stranger p=0.77) had resulted in a significant partner preference in male prairie voles.

Peripheral Administration of MTII in Meadow Voles

After 24 hours, a significant difference between time spent in the partner zone, the stranger zone and the non-social zone one determined in a one-way ANOVA (partner vs. stranger vs. nonsocial

$p<0.001$) after MTII administration in meadow voles (Figure 5a). The experimental animal spent over five times as much time in the non-social chamber than with either stimulus animal. There was no difference between time spent with in either the partner zone or the stranger zone ($p= 0.77$). The same trend was seen after the placebo treatment, the experimental animal spent significantly more time in the nonsocial zone and did not show a preference for either stimulus animal.

After 72 hours there was still no difference between the time allocations each zone between the two drug treatments (Figure 5b). In both treatments, the experimental animal spent more time in the nonsocial zone than with either stimulus animal (MTII-partner vs. stranger vs. nonsocial $p<0.01$; Saline partner vs. stranger vs. nonsocial $p <0.001$). Unlike the 24 hour time point, after 72 hours the experimental animals spent more time with the stranger than with the familiar partner.

Long-term Effect of MTII Administration in Female Prairie Voles

A single dose of MTII (10mg/kg) followed by a 6 hour cohabitation period is sufficient to induce a partner preference in the experimental animal after a 7 day period of separation (Figure 6). A two-way ANOVA indicated a trend towards an interaction between stimulus animal and drug treatment ($F(1,45)=3.172$, $p=0.082$).Comparison of time spent with the partner vs. the stranger after MTII ($p<0.05$) but not saline ($p=0.85$) results in a significant difference between groups, with an average of thrice more time spent with the partner than the stranger.

Effect of Neonatal Melanotan II on Adult Social Behavior

Early life administration of MTII to female prairie voles accelerated the formation of a partner preference as adults (Figure 7a). A comparison of time spent with the partner vs. the stranger by animals receiving either MTII or saline after a six-hour cohabitation revealed a significant main effect of stimulus animal as determined by a two-way ANOVA ($F(1,67)=5.104$, $p=0.027$). A

significant interaction was also seen between drug and stimulus animal ($F(1,67)=7.597$, $p=0.008$). A post-hoc Student's T-test indicates a significant partner preference in animals that received MTII as pups ($p=0.0005$), but no preference in animals that received saline ($p=0.75$). After a 24-hours cohabitation, there was no longer an effect of drug on partner preference but there was still a strong significant main effect of stimulus animal ($F(1,67)=71.001$, $p<0.0001$). Post-hoc Student's T-tests indicate that after a 24-hour cohabitation both groups of animal formed a significant partner preference (MTII-$p<0.0001$; Saline-$p<0.0001$). In addition, pups treated with MTII displayed side effects associated with chronic neonatal administration not seen in the saline treated animals. MTII treated animals were significantly lower in weight on the last day of administration saline treated animals (Figure 7b; $F(1,31)=7.447$, $p<0.0001$). However by 21 days there were no longer significant differences in weight ($p=0.20$). Pups that received MTII also had darker pigmentation in the fur at weaning (Figure 7c)

Co-administration of Melanotan II and Oxytocin Receptor Antagonist

The facilitatory effect of MTII administration on partner preference was inhibited by the administration of the OT receptor antagonist, (d(CH2)51,Tyr(Me)2,Thr4,Orn8,des-Gly-NH29)Vasotocin into the NA (Figure 8a). A significant main effect of stimulus animal group was determined by a three-way ANOVA ($F(1,29)= 4.342$, $P=0.04$). Female prairie voles receiving vehicle injections both peripherally and centrally did not show a group preference in a post-hoc Student's T-test ($p=0.22$), with 5 of the 10 animals showing a partner preference (Figure 8b). Females receiving MTII peripherally and vehicle centrally did show a group preference in a post-hoc Student's T-test ($p=0.008$) with 9 of the 12 animals showing a partner preference. Females that received both MTII peripherally and the OT receptor antagonist centrally did not show a partner preference in a post-hoc Student's T-test ($p=0.18$) with 4 of the 10 animals showing a partner preference.

Central Penetration of MC Agonists

MTII accumulates in the plasma at an average of 2400 ng/ml (2343.34 nM) after a dose of 1mg/kg and 17550 ng/ml (17135.66 nM) at a dose of 10mg/kg as determined by PK analysis. In the brain, a 1mg/kg dose results in an accumulation of 47.45 ng/g (46.33nM) and a 10mg/kg dose results in 379.5 ng/g (370.54nM) of peptide. This results in a brain to plasma ratio of 0.02, which is not indicative of a brain penetrant compound (Table 1).

Pf-446687 accumulates in the plasma at an average of 21.13 ng/ml (44.91 nM) after a dose of 1mg/kg and 371.33 ng/ml (789.06 nM) at a dose of 10mg/kg. In the brain, a 1mg/kg dose results in an accumulation of 10.94 ng/g (23.25nM) and a 10mg/kg dose results in 324.77 ng/g (690.11 nM) of peptide. The brain to blood ratio is 0.68 for total Pf-446687 indicating the compound is able to cross the blood brain barrier (Table 1).

Peripheral Administration of PF-446687 in Female Prairie Voles

Administration of PF-446687 resulted in a significant difference between stimulus groups as determine by a two-way ANOVA (Figure 9; $F(1,29)= 11.789$, $P=0.001$). Female prairie voles receiving a low dose (1mg/kg) of MTII (partner vs. stranger $p=0.007$) spent significantly more time with the partner male vs. the stranger male. 9 out of 10 animals receiving the low dose formed a preference as can be indicated by greater than twice as much time spent with the partner than the stranger. Animals receiving the high dose of 10mg/kg trended towards a partner preference, 7 out of 10 animals formed a preference under this condition with a nearly significantly difference between time spent with the partner and stranger ($p=0.08$). Only 5 out of 10 animals formed a preference with the vehicle injection (partner vs. stranger $p=0.15$).

Discussion

Effect of Melanocortin Agonists on Partner Preference Formation

Peripheral administration of MC agonists recapitulates the behavioral effects of central OT administration in the partner preference paradigm. Both MTII and the MC4R specific agonist

PF-446687, induced the formation of a partner preference under conditions in which they would not otherwise form in female prairie voles. The drugs likely act through the MC4R, as the receptors are expressed in the major oxytocinergic nuclei in the prairie vole, and induce activation in neurons in these areas. This facilitatory effect can be blocked by the central administration of an OT receptor antagonist, suggesting the effects of MC agonists work through the enhancement of the central OT system. MTII did not promote pair bond formation in male prairie voles or female meadow voles, which is consistent with the relatively minor role OT plays in the regulation of bonding behavior in these animals. The administration of melanocortin agonists has both long lasting developmental effects and acquisitional effects. Activation of the melanocortin system during the neonatal period promotes social bonding in adult female prairie voles. MTII facilitates the acquisition of social information, and not the expression of social behavior based on the efficacy in promoting partner preference a week after administration and social exposure. Taken collectively, MC4R agonists are a mechanism for enhancing the OT system that can be utilized clinically either alone or in combination with behavioral therapy to treat social deficits.

Mechanism of Action

Stimulation of hypothalamic MC4R induces central, but not peripheral release of OT in rats (Sabatier, 2006) and likely has a similar effect in prairie voles based of similar patterns of MC4R mRNA expression (Figure 2). Activation of MC receptors is thought to differentially effect the regulation of these independent mechanisms of OT release. An intracerebroventricular injection of α-MSH, the endogenous ligand of the melanocortin receptors, induces the expression of c-fos, a neuronal marker of excitation, in OT containing neurons of SON (Sabatier et al., 2003). However, in contrast with this marker of activity, electrophysiological recordings of OT neuron cell bodies show an inhibition of electrical activity in response to α-MSH or a specific MC4R agonist. Functionally, MC4R dependent electrical inhibition results in a significant decrease in peripheral levels of circulating OT. Stimulation with an MC4R agonist, though, induces an

increase somato-dendritic OT from brain sections including OT neurons. It is hypothesized that stimulation of MC4Rs upregulates dendritic release of OT, by increasing intracellular concentrations of Ca^{+2}, but downregulates axonal release of OT by inhibiting electrical activity. Accompanying the changes in intracellular of Ca^{+2} in OT containing neurons, is the expression of immediate early genes, Fos (Sabatier et al., 2003) and Egr-1 (Figure 3). While expression of this class of proteins is typically associated with electrical activation, it is also has associated with the central secretion of OT in the absence of depolarization (Sabatier, 2006). This proposed mechanisms allows for the administration of MC4R agonists to specifically increase brain levels of OT but not peripheral levels. This allows for the selective modulation of brain mediated behavioral changes but not peripheral functions. The selective enhancement of the OT system through the use of MC4 agonists could maximize therapeutic potential of therapeutic OT while minimizing side-effects (MacDonald et al., 2011).

Differential Effects of MTI, MTII and PF-446687

Significant research has gone into the development of synthetic analogs of α-MSH due to the wide array of behavioral and physiological effects mediated by the endogenous peptide. This foundational work has led to the development of a number of MC agonists with different pharmacokinetic profiles, several of which are currently in clinical use. Based on the proposed mechanism of OT release via centrally located MC4R receptors, agonists selective for and potent at the MC4R and able to cross the blood brain barrier should be most efficacious in enhancing OT-dependent behavior. The three MC receptor agonists were used in this study, MTI, MTII and Pf-446687 vary in pharmacokinetic properties and the variability is reflected in their ability to promote partner preference formation (Figure 1). MTI, [Nle^4,$Dphe^7$] α-MSH, is considered the most potent analog of α-MSH with activity at the MC1, MC3, MC4 and MC5 receptors and good in vivo stability and biodistribution (Hruby, 2011). MTII, Ac-Nle-c[Asp^5,$DPhe^7$,Lys^{10}] α-MSH -NH2, is of similar potency and promiscuity as MTI but with 10 fold greater affinity at the MC4R

and enhanced in vivo stability due to its cyclic structure (Sawyer et al., 1980, Al-Obeidi et al., 1989, Oosterom et al., 1999). MTII has been reported to able to cross the blood brain barrier, whereas MTI cannot (Hruby et al., 2011). Due to this difference in BBB permeability, MTI has been primarily used clinically for the peripheral function of therapeutic tanning, while MTII has been utilized for its central regulation of sexual function (Hadley and Dorr, 2006). The differential usage of MTI and MTII in their commercial development supports the activation of independent receptor systems. The peripheral activation of the MC1 receptors is associated with the production of melanin and skin darkening (Garcia-Borron et al., 2005). Whereas the MC3 and 4 receptors are more closely tied to the hypothalamic functions of α-MSH, including the regulation of food intake and sexual response (Voisey et al., 2003, Hruby et al., 2007).

The duality in the mechanisms of MTI and MTII supports their incongruent effects on the formation of a partner preference, despite the pharmacological similarities of the analogs. The lack of behavioral effect of MTI in this paradigm supports a central mechanism of action, as peripheral administration of MTI is unlikely to have resulted in penetration of the BBB and activation MC receptors in the brain(Hruby et al., 2011). The behavioral effects of MTII in the prairie voles suggest the compound is able to evoke central effects, however the pharmacological data is indicative of poor BBB permeability. The conflicting evidence for the ability of MTII to cross the blood brain barrier is also present in literature, suggesting MTII may be able to evoke behavioral effects at low concentrations (Trivedi et al., 2003, Hruby et al., 2011). While the ratio of MTII in the plasma compared to the brain in this study is low, the absolute levels of MTII accumulated in the brain should be sufficient to activate hypothalamic receptors as the EC50 of MTII at the MC4R is only 2.87nM while the measured concentration in the brain is 370nM (Grieco et al., 2007). MTII has an increased affinity for the MC3 and MC4 receptor compared to MTI, but it also has activity at the MC1 and MC5 receptors (Hruby et al., 2011). The lack of selectivity of MTII is reflected in the unintended side effects seen after chronic neonatal

administration. The pups receiving MTII were smaller and darker in coloring than the saline injected animals, likely due the pigmentation effects through the MC1 receptor and the anorexigenic effects of the MC4 receptor (Figure 6 b,c).

In contrast to some of the limitations of MTI and MTII, Pf-446687 is a highly selective, brain penetrant, non-peptide MC4 agonist (Lansdell et al., 2010). Combined those properties should enable the compound to effectively stimulate MC4R on the oxytocinergic neurons of the PVN and SON to induce central OT release. Indirect evidence of a pro-OT effect is seen in the facilitation of partner preference with Pf-446687 in female prairie voles. Interestingly, despite greater accumulation of the drug in the brain with the higher 10mg/kg dose, Pf-446687 was more behaviorally effective at the lower dose of 1mg/kg, suggesting there may be alternate intracellular pathways activated at higher doses. MC receptors are able to couple to alternate g-proteins and activate different intracellular signaling pathways depending on the ligand (Cai et al., 2004). Peptide and non-peptide agonists have been shown to activate alternate signaling pathways at the MC4 receptor, which could account for the difference in dose dependent effects between the compounds (Cai et al., 2004). Despite the potential difference in intracellular mechanisms, both MTII and Pf-446687 are able to promote the expression of OT-dependent and both likely act through the MC4 receptor.

Lack of Effect in Male Prairie and Female Meadow Voles

Partner preference in this study is used as a proxy for the activation of the central OT system. However, the role of OT in partner preference formation varies across model systems. Therefore, we propose that the partner preference test, specifically in female prairie voles, is the best predictive model for the identification of OT related prosocial therapeutics (Modi and Young, 2011a). In this model, there is a strong correlation between central OT activity during specific critical periods and well-defined behavioral outputs. The formation of partner preference in male prairie voles or female meadow voles, however, is not as tightly linked with central OT function,

and consequently is not a reliable marker for the activation of the OT system. Meadow voles lack OT receptors in the NA, a brain region critical for bond formation, and subsequently do not have the necessary neural structures to show an enhancement in pair bonding after OT treatment (Insel and Shapiro, 1992b). Male prairie voles, on the other hand, have accumbal OT receptors but partner preference in this model is primarily mediated through the vasopressin receptor system (Winslow et al., 1993). As a result, both models are less likely to show social behavioral effects after manipulation of the OT system. Consequently, the lack of effect of MTII on the formation of partner preference in either of these models is consistent with diminished role of OT their prosocial behavior.

Further, we hypothesize that OT's role in human social cognition is most evolutionarily similar to the role of OT in pair bonding in female prairie voles. OT functions as a mediator of affiliative and attachment processes in a wide array of taxonomically diverse species (Donaldson and Young, 2008b). The role of OT in higher order social cognition in humans is potentially an evolutionary adaptation of the attachment circuits that allows them to function in a more general prosocial capacity. As partner preference in female prairie voles is a measure of affiliation and attachment, it therefore should have good predictive validity for compounds that enhance human social cognition.

Facilitation of the Acquisition of Social Information

In addition to promoting acute prosocial effects, OT facilitates the long-term acquisition of social information. In female prairie voles, a single administration of either central OT or peripheral MC4R agonists results in the display of a partner preference immediately after an abbreviated cohabitation period (Williams et al., 1992). However, it is unclear if this effect is due to the pharmacological enhancement of the acquisition of social information or expression of social behavior. In this study, MTII administered prior to a social exposure was able to enhance the formation of a partner preference to the extent that it was still detectable after a one-week

separation. This suggests, as the half-life of the drug is approximately 1.5 hours, that MTII need only be present during the acquisition of bond for a long-lasting and robust partner preference to develop. This is consistent with the conceptualization that central OT increases the saliency of social information. OT knock-out mice have specific deficits in fine social discrimination but not gross discrimination (Macbeth et al., 2009b). Correspondingly, people who have taken intranasal OT are better able to recognize and discriminate different faces and emotions (Domes et al., 2007b, Savaskan et al., 2008). These properties may enhance the efficacy of current social training paradigms, like computerized social games or even applied behavior analysis, if the MC4R agonists are given concurrently with therapy sessions by making the social stimuli and reinforcement given more profound. The social skills acquired during the drug facilitated therapy sessions are likely to be better retained and potentially applied in a real world setting. Additionally the administration of the agonists could be limited to only within the setting of the therapy session decreasing likelihood of side-effects without impairing the efficacy.

MC4R Agonists as a Developmental Pharmacotherapy

The long term effects of MC4R agonist administration could be further utilized therapeutically if administered during critical periods in the development of social behavior. Evidence from animal models suggests that activation of the OT system during early in life, either through endogenous mechanisms or pharmacological interventions, can have a life-long impact on social behavior and neuroendocrine function (Stribley and Carter, 1999). Administration of a single peripheral dose of OT to female prairie voles on the first day of life has effects on a number of social behavioral measures including maternal care and partner preference (Bales et al., 2007b). Early OT treatment facilitates the establishment of a partner preference in a dose dependent fashion as adults. These findings parallel the effect of neonatal MTII administration on adult pair bond formation and support an OT based mechanism of action. Molecularly, neonatal OT treatment is associated with reduced expression of vasopressin 1a receptors but not with alterations in OT

receptors expression in adult prairie voles (Bales et al., 2007a). Conversely, neonatal OT exposure increases OT peptide levels of juveniles, while decreasing vasopressin peptide levels in the hypothalamus of adult prairie voles (Yamamoto et al., 2004, Bales et al., 2007a). The presence of behavioral effects, resulting from neonatal MTII administration, as adults suggests a developmental alteration of the social brain circuit. It is hypothesized that many of the same systems altered by early life OT would be similarly changed by MTII administration. Elucidation of the actual cellular and molecular resulting from this early life manipulation is critical. This is particularly true as MTII or other MC4R agonists are being developed as a treatment childhood developmental disorders like ASD.

The developmental effects of neonatal MTII administration in the prairie vole supports clinical use of OT-based therapeutics, like MTII or Pf-446687, as an early childhood intervention for young children diagnosed with social disorders. ASD can be characterized as a disorder resulting from a disruption of the normative process of social development (Jones and Klin, 2009). For example, failure to attend to the eyes of interactors during certain critical periods can lead to a deviation from normal social growth that multiplies into a myriad of social impairments later in life (Volkmar et al., 2004). A therapeutic intervention administered during this critical period could reset the developmental trajectory of the child preventing the acquisition of second order social impairments. As OT increases attention to social stimuli in adults, it likely has similar effects in children, which could facilitate the normative development of complex social cognition. MC4R agonists, based on their social developmental effects in prairie voles and the acute effects of OT in adult humans, could be used in children or infants showing early signs of ASD to correct abnormal social development resulting in more favorable long-term outcomes.

Melanocortin 4 Receptor Agonists as an Alternative to Intranasal Oxytocin

OT enhances many facets of social cognition in both normative and clinical populations, making the peptide system one of the most promising pharmacological targets for disorders characterized

by social impairments, like ASD and schizophrenia. The native peptide OT, though, has several biophysical properties that limit its potential for clinical use, including poor brain penetration and metabolic instability. Enhancing social cognition through administration of exogenous OT either intravenously or intranasally requires the peptide cross the blood brain barrier and to diffuse considerable distances through the brain to reach the biologically relevant receptor targets. OT is degraded by aminopeptidases and has a half-life of only 2-5 minutes in the blood and about 20 minutes in CSF, requiring large amounts of peptide administration to achieve behavioral effects (Leng and Ludwig, 2008). The current dose of intranasal OT given in the human behavioral studies is 24-40 IU in a single dose, which can be 10-fold higher than the doses given for effects related to parturition (Balki et al., 2006, Macdonald and Macdonald, 2010). To circumvent some of these limitations, central OT can be increased indirectly through the stimulation of receptors on oxytocinergic neurons. Indirect stimulation enables the peptide to be released directly into the brain, where the molecule has a longer half-life, in proximity to its receptor targets and at biologically relevant concentration. This may allow for profound behavioral effects to be achieved at a low effective dose, diminishing the potential for peripheral side effects (Heinrichs et al., 2009).

The predictive validity of the partner preference test in prairie voles suggests that indirect activation of the OT system by stimulation of MC4R, either through MTII or the Pfizer compound, may have similar prosocial effects in people (Modi and Young, 2011a, b). Based on the types of prosocial effects seen after IN OT administration in ASD populations, MC4R agonists should increase attention to social stimuli and the perception of socially relevant information (Andari et al., 2010b, Guastella et al., 2010). In addition, the MC4R agonists should have a broader window of efficacy based on the increased stability of the compounds compared to OT itself and increased effect size based on the discrete release of OT in physiologically appropriate brains areas. However, a direct comparison of the behavioral effects resulting from

IN OT and peripherally administered MC4R agonists is necessary to determine the most efficacious route for clinical development.

Like IN OT, there are also limitations to the therapeutic use of MC4R for the treatment of social deficits. α-MSH and the MC4R also play a major role in the regulation of feeding and sexual behavior. The MC4R is a negative regulator of food intake, such that disruption of the receptor leads to obesity in animal models and humans (Pritchard et al., 2002). Conversely, stimulation of the receptor suppresses eating and induces weight loss, representing a significant side effect of MC4R agonism. MTII, in animal models, can cause a conditioned taste aversion and in humans nausea is dose limiting (Wessells et al., 2000, Benoit et al., 2003). Nausea, however, is reported in substantially lower frequencies after Pf-446687 administration (Lansdell et al., 2010). Unwanted sexual effects are likely to be the more limiting factor for the clinical use of MC4R agonists. Both MTII and Pf-446687 were originally developed clinically for the enhancement of sexual function in both men and women and are associated with the induction of erection, ejaculation and vaginal arousal (Diamond et al., 2004, Diamond et al., 2006, Lansdell et al., 2010). Enhanced expression of a sexual response resulting from MC4R agonist administration may be particularly limiting in adolescents with ASD, as this population often has problems with inappropriate sexual behavior (Stokes and Kaur, 2005). However, the undesired sexual side effects could be mitigated through use of the drugs primarily in prepubescent populations and through limited administration primarily in the context of behavioral therapy. Interestingly, though, OT itself has very similar effects on both feeding and sexual behavior, suggesting these may be limitations to any OT-based therapy (Sabatier, 2006).

Alternative Paradigmatic Approach

Clinical efficacy of MC4R agonists aside, the strategy of indirectly inducing endogenous OT release represents a new paradigm for looking at ways to enhance social behavior through the oxytocin system that does not rely on penetrance of the blood brain barrier. The concept of

indirectly stimulating central OT release by activation of receptors on OT neurons is applicable outside the melanocortin system. For example, we have also found that administration of the 5-HT1a partial agonist, buspirone, a drug that has been shown in increase peripheral OT levels, also accelerates the formation of partner preference in prairie voles (Modi and Young, 2011b). Identification of the full complement of receptors on oxytocinergic neurons enables a screen for the most potent regulators of OT release. The identified receptor systems could be subsequently screened for prosocial effects using the partner preference test in prairie voles, with those that facilitated the formation of a pair bond becoming translational candidates. Through this system the pharmacological target for enhancing central OT with the maximal social behavioral effects but minimal side effects could be identified for clinical use.

Conclusion

There is considerable therapeutic potential for the use of central OT in the treatment of social impairments, the main remaining challenge, though, is how best to pharmacologically enhance that system. The upregulation of central OT levels through the stimulation of receptors on oxytocinergic neurons is a viable pharmacological strategy. Activation of the MC4R in the brain of female prairie voles results in the facilitation of social behavior, in a manner that is parallel to central OT administration. MC4R agonists have acute, long-term and developmental effects on the acquisition and expression of socially important information. Furthermore, the translation of these findings into the clinical setting is aided by the preclinical development of both MTII and Pf-446687. The relative efficacy of MC4R agonists in enhancing human social cognition can and should therefore be evaluated in both normative and clinical populations. Irrespective of the therapeutic efficacy of MC4R agonists, OT is likely to be the key to the treatment of social disorders it is our responsibility to find innovative ways of harnessing it.

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	Dose (mg/kg)	Time (h)	Cp (ng/mL)	Cb (ng/g)	Cp (nM)	Cb (nM)	Cb/Cp
Melanotan II	1.00	1.00	2400.00	47.45	2343.34	46.33	0.02
	10.00	1.00	17550.00	379.50	17135.66	370.54	0.02
	Overall	---	---	---	---	---	0.02
Pf-446687	1.00	1.00	21.13	10.94	44.91	23.25	0.50
	10.00	1.00	371.33	324.77	789.06	690.11	0.86
	Overall	---	---	---	---	---	0.68

Table 1. PK for MTII and Pf-446687 in the brain and blood.

Cp=concentration in plasma; Cb=concentration in brain; u=unbound; Cb/Cp=ratio of brain and blood concentrations

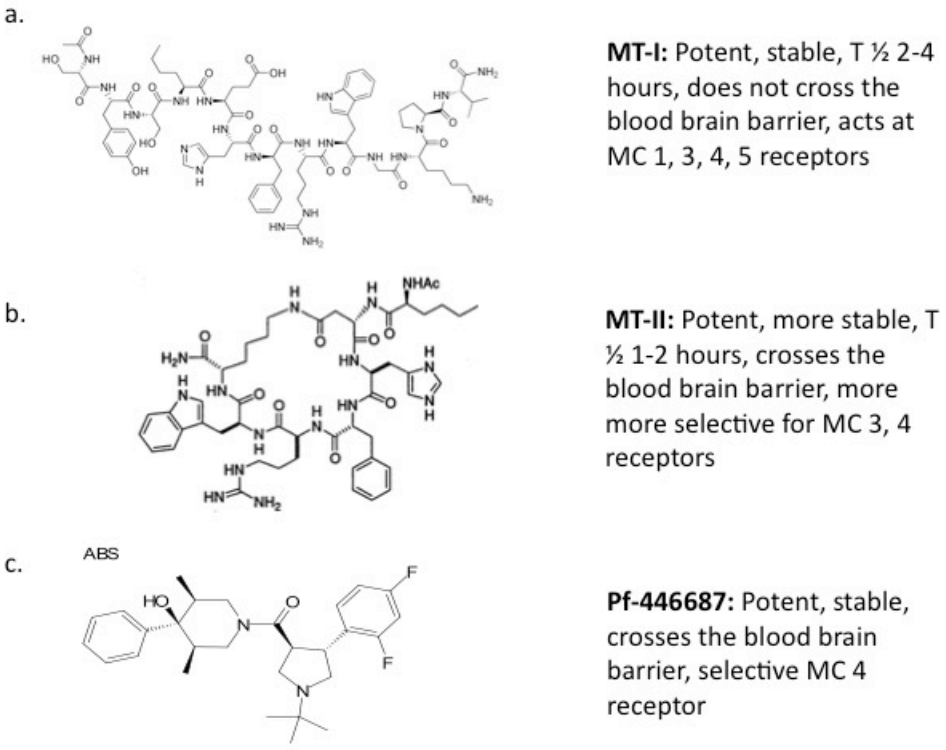

Figure 1. Melanocortin receptor agonists. The three melanocortin receptor agonists whose effects were tested in partner preference in this study. All three have been used for clinical indications unrelated to social behavior and are viable therapeutic options if they prove efficacious in the partner preference test. Melanotan I (MTI; [Nle^4,$Dphe^7$] α-MSH) is a highly potent, non-specific, non-brain penetrant linear peptide analogue of α-MSH (a). Melanotan II (MTII; Ac-Nle-c[Asp^5,$DPhe^7$,Lys^{10}] α-MSH -NH2) is a highly potent, stable, poorly brain penetrant, cyclic peptide analogue with increased specificity for the MC3R and MC4R (b). Pf-446687 is a potent, stable, brain penetrant non-peptide agonist highly specific for the MC4R (c)(Lansdell et al., 2010, Hruby et al., 2011).

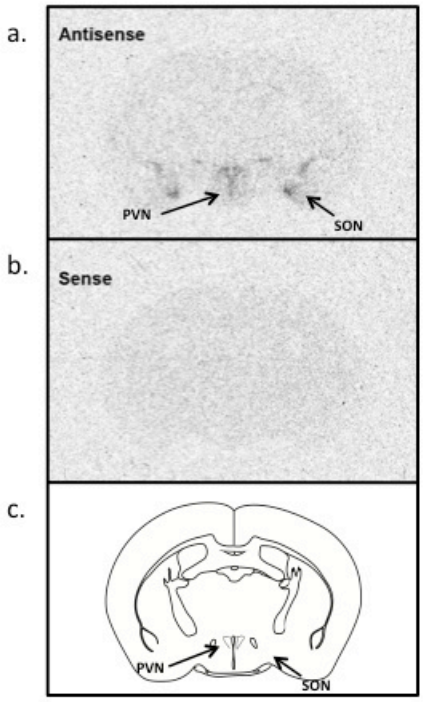

Figure 2. MC4R mRNA is present in the PVN and SON of the prairie vole brain. In situ hybridization revealed the presence of MC4R mRNA in the paraventricular (PVN) and supraoptic (SON) nuclei, the two primary hypothalamic sites of OT synthesis of the prairie vole brains (a,c). The specificity of the probe for MC4R mRNA is indicated by the lack of binding by the sense probe (b).

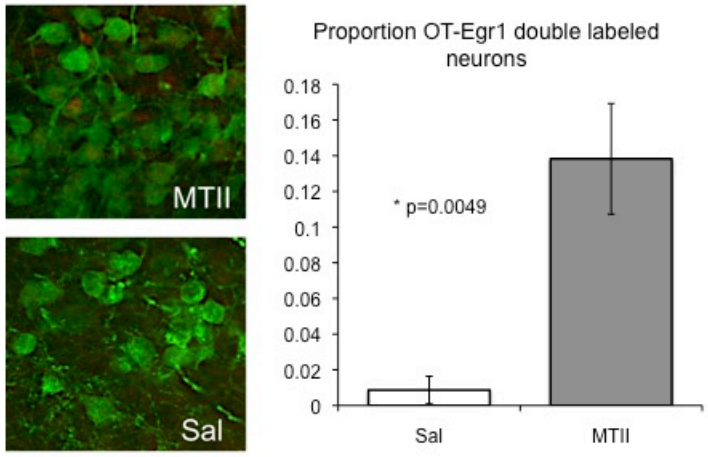

Figure 3. MTII activates OT-positive neurons in the paraventricular nucleus. In paraventricular hypothalamic cells containing OT (a; green fluorescence), MTII administration increases the expression of the immediate early gene Egr-1 (red fluorescence) relative to saline (Sal) treated animals (b; Student's T-test p=0.0049).

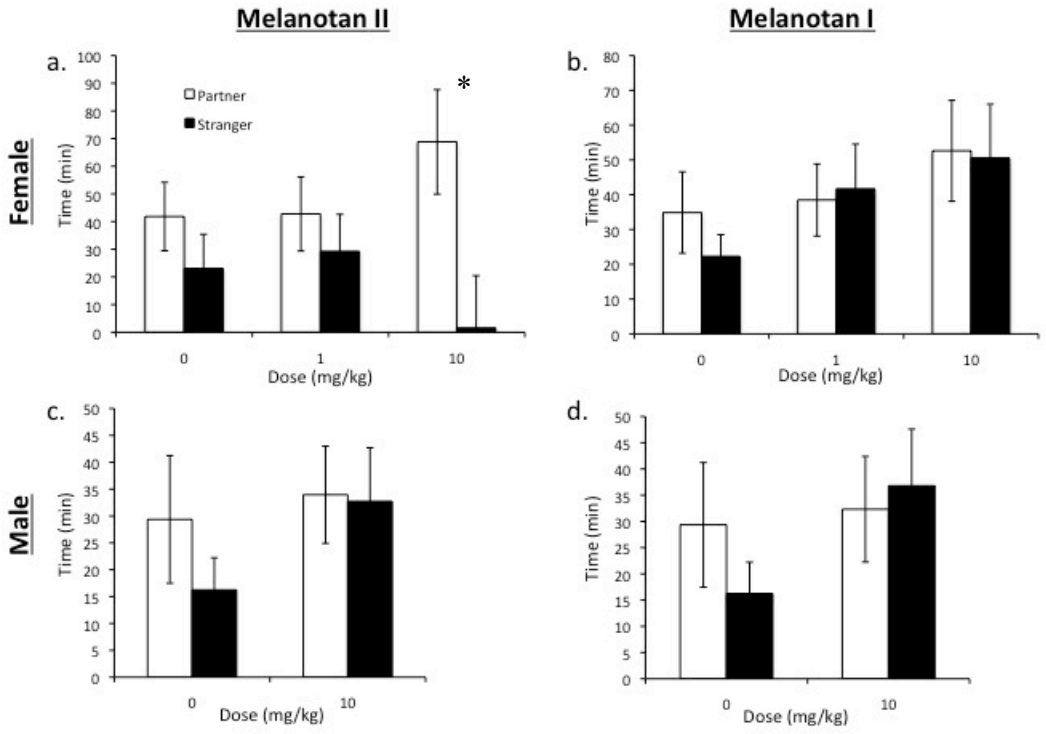

Figure 4. MTII facilitates partner preference in female prairie voles. Female prairie voles receiving a high dose (10mg/kg) of MTII (a; partner vs. stranger p=0.003) spent significantly more time with the partner male vs. the stranger male, thus are considered to have formed a "partner preference". Animals given a control saline injection, a low dose of MTII (a; partner vs. stranger p=0.428) or a low (b; partner vs. stranger p= 0.85) or high dose of MTI (b; partner vs. stranger p=0.923) failed to spend significantly more time with either stimulus animal. Neither MTI (d; partner vs. stranger p=0.93) nor MTII (c; partner vs. stranger p=0.77) resulted in a significant partner preference in male prairie voles. * indicates a partner preference in post hoc comparisons.

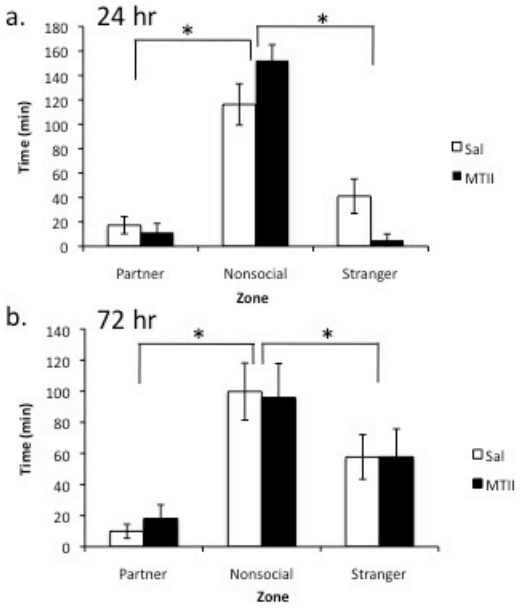

Figure 5. MTII does not facilitate partner preference in meadow voles. MTII was unable to facilitate the formation of a partner preference in female meadow voles. The drug treatment did not alter the species typical behavior in the partner preference test. After both a 24 (a; partner vs. stranger vs. nonsocial $p<0.001$) and a 72 (b; partner vs. stranger vs. nonsocial $p<0.01$) hour cohabitation the experimental animals spent significantly more time in the non-social zone than with either stimulus animal. * indicates a significant difference between time allocations in zones.

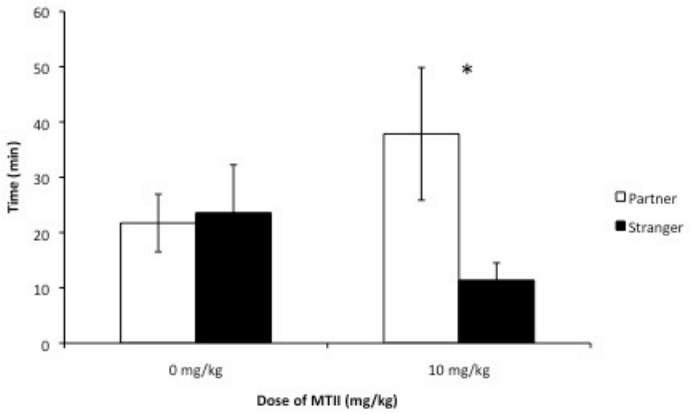

Figure 6. MTII facilitates long-term expression of partner preference. Administration of TII (10mg/kg) followed by a 6 hour cohabitation period is sufficient to induce a partner preference in the experimental animal after a 7 day period of separation. Comparison of time spent with the partner vs. the stranger after MTII ($p<0.05$) but not saline ($p=0.85$) results in a significant difference between groups, with an average of thrice more time spent with the partner than the stranger. * indicates a partner preference in post hoc comparisons.

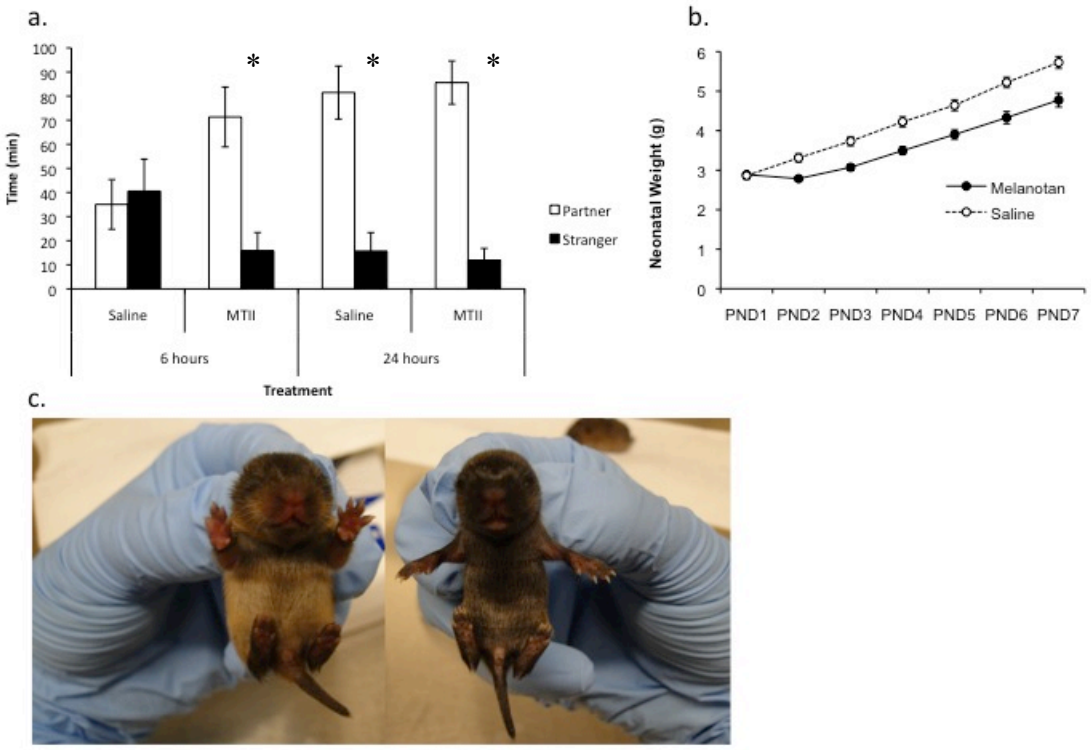

Figure 7. Neonatal administration of MTII facilitates adult partner preference. Early life administration of MTII accelerates partner preference formation as an adult indicated by a significant interaction between drug and stimulus animal in a 2-way ANOVA ($F(1,67)=7.597$, $p=0.008$). Post hoc comparisons revealed that animals receiving MTII, but not saline, as pups formed a partner preference after 6 hours (a; $p=0.0005$). After a 24-hours cohabitation, both treatment groups formed a significant partner preference (a; MTII-$p<0.0001$; Saline-$p<0.0001$). In addition, pups treated with MTII displayed side effects associated with chronic neonatal administration not seen in the saline treated animals. MTII treated animals were significantly lower in weight on the last day of administration saline treated animals (b; $F(1,31)=7.447$, $p<0.0001$). Visual inspection reveals that pups that received MTII (c-right) had darker pigmentation in the fur and were visibly thinner than animals receiving saline (c-right). * indicates a partner preference in post hoc comparisons.

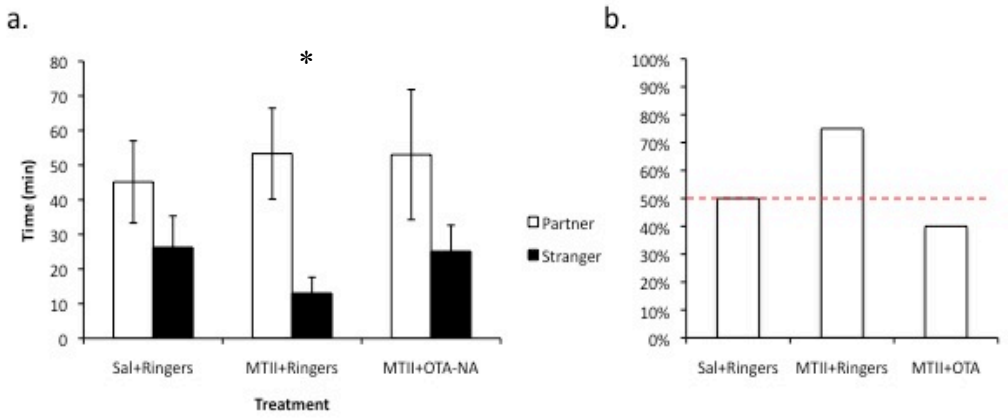

Figure 8. OT receptor antagonist blocks MTII induced partner preference. The facilitatory effect of MTII administration on partner preference was inhibited by the administration of the OT receptor antagonist, (d(CH2)51,Tyr(Me)2,Thr4,Orn8,des-Gly-NH29)Vasotocin into the NA (OTA-NA). Females receiving MTII peripherally and a vehicle injection centrally formed a preference, as expected (a; p=0.008), however those that received MTII peripherally but the OTA-NA centrally showed a preference closer to the levels of controls (a; sal+ringers-p=0.22, MTII+OTA-NA-p=0.18). In a population of prairie voles that have not formed pair bonds, roughly 50% of animal will show a partner preference and 50% will show a stranger preference. Of the three treatment conditions, only those that received MTII and a central vehicle injection showed a percentage of animals exhibiting a partner preference over statistical chance (b). Red line indicates chance. * indicates a partner preference in post hoc comparisons.

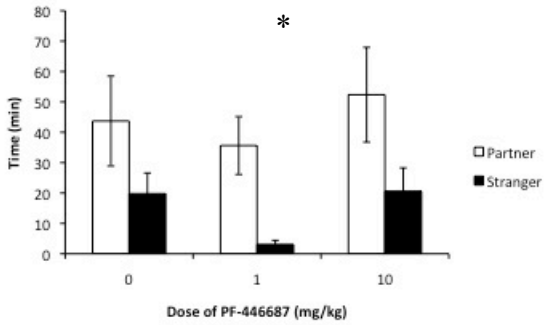

Figure 9. Pf-446687 facilitates partner preference in prairie voles. Administration of PF-446687 resulted in significant partner preference ($F(1,29)= 11.789$, $P=0.001$). Female prairie voles receiving a low dose (1mg/kg) of MTII (partner vs. stranger $p=0.007$) spent significantly more time with the partner male vs. the stranger male. The 10mg/kg doses trended towards the formation of a preference but did not reach significance ($p=0.08$). * indicates a partner preference in post hoc comparisons.

CHAPTER 4

D-cycloserine facilitates socially-reinforced learning in an animal model relevant to autism spectrum disorders: Implications for serving as an adjunct to behavioral therapies.

Abstract

Background: There are no drugs that specifically target the social deficits of autism spectrum disorders (ASD). This may be in part due to a lack of behavioral paradigms in animal models relevant to ASD. Partner preference formation in the prairie vole represents a social cognitive process involving socially reinforced learning. D-cycloserine (DCS) is a cognitive enhancer that acts at the N-methyl-D-aspartate receptor to promote learning and memory. If DCS enhances socially reinforced learning in the partner preference paradigm, it may be useful in combination with behavioral therapies for enhancing social functioning in ASD.

Method: Female prairie voles were given DCS either peripherally or directly into one of three brain regions: nucleus accumbens, amygdala, or caudate putamen. Subjects were then cohabitated with a male under conditions that do not typically yield a partner preference. The development of a preference for that stimulus male over a novel male was then assessed using a partner preference test. The effects of peripheral administration of DCS on partner preference formation in asocial meadow voles was also examined.

Results: A low dose of DCS administered peripherally enhanced preference formation in prairie voles under conditions in which it would not otherwise occur. These effects were replicated by microinfusions of DCS into the nucleus accumbens, which is involved in reinforcement learning, and the amygdala, which is involved in social information processing, but not into the caudate putamen. DCS administration did not induce partner preference in meadow voles.

Conclusions: Partner preference in the prairie vole may provide a behavioral paradigm with face, construct and predictive validity for identifying prosocial pharmacotherapeutics. DCS may be a viable treatment strategy for social deficits of ASD when paired with social behavioral therapy.

Introduction

Despite the growing public health concern over autism spectrum disorders (ASD), there have been few advances in the development of pharmacotherapuetic treatment options for these neurodevelopmental disorders. Most existing pharmacotherapies for children and adults with ASD are simply re-labeled drugs commonly used for the treatment of other neuropsychiatric disorders, which in ASD, target only peripheral co-morbid symptoms rather than key features like social impairment (Bartz and Hollander, 2008). Consequently, there is a significant need for the use of animal models and behavioral paradigms relevant to ASD to gain understanding of the fundamental neurobiology of the core endophenotypes of ASD so that informed pharmacotherapies based on biology can be developed. Given the heterogeneous nature of ASD, targeting individual endophenotypes may be a more viable approach for drug development than targeting the global etiology. For this reason, we have focused on behavioral paradigms that may be useful in screening drugs that enhance social cognitive function in animal models, with the presumption that similar pharmacotherapeutic approaches may enhance social cognition in patients with ASD, and therefore may be useful adjuncts to behavioral based therapies.

Cognitive enhancers such as d-cycloserine (DCS) have gained considerable attention in recent years for their potential in facilitating selective cognitive processes in the treatment of psychiatric disorders such as phobias, social anxiety, obsessive-compulsive disorder and post-traumatic stress disorder (Heresco-Levy et al., 2002, Guastella et al., 2008b, Wihlem et al., 2008, Jorstad-Stein and Heimberg, 2009). D-cycloserine (DCS) is a partial agonist of the N-methyl-D-aspartate (NMDA) glutamate receptor that binds to the glycine site of the NMDA receptor, enhancing receptor activation only in the presence of glutamate (Watson et al., 1990). The NMDA-R plays a pivotal role in long-term plasticity, the neuronal correlate of memory (Kauer et al., 1988). DCS enhances many different forms of learning and memory, including spatial memory (Win-Shwe et al., 2010), extinction of fear conditioning (Davis et al., 2006b), drug associated memory (Lee et

al., 2009), declarative memory (Onur et al., 2010) and episodic-like memory (Zlomuzica et al., 2007). The versatile application of DCS in memory enhancement suggests the drug may also be effective in improving social memory and cognition.

Currently there are two rodent behavioral paradigms that are particularly well suited to the investigation of the neurobiological mechanisms underlying social cognition and for screening compounds that may enhance social cognition: social recognition in the mouse (*Mus musculus)* and partner preference formation in the socially monogamous prairie vole (*Microtus ochrogaster)* (Hammock and Young, 2006, Silverman et al., 2010a).

Social recognition paradigms in mice have revealed an important role for the amygdala (Amyg) in social information processing. Mice discriminate novel from familiar mice using olfactory cues and habituate to a familiar mouse following repeated social exposures (Macbeth et al., 2009a). Mice genetically deficient in oxytocin (OT) fail to habituate to a conspecific after repeated exposure and therefore fail to discriminate familiar from novel conspecifics (Ferguson et al., 2000a). This deficit is specific to social learning since the same mice perform normally on other non-social cognitive tests, including olfactory based habituation tasks (Ferguson et al., 2000a). Oxytocin acts in the medial amygdala to facilitate social recognition. Silencing OT receptor expression or infusion of OT receptor antagonist into the Amyg disrupts social recognition in wildtype mice, while microinjections of OT directly into the Amyg rescues social recognition (Ferguson et al., 2001a, Choleris et al., 2007). D-serine, a compound related to DCS, increases social recognition in rats at high doses(Shimazaki et al., 2010). Correspondingly, antagonists of the NMDA receptor including phencyclidine, dizocilpine and CPP prevent the expression of social memory(Hlinak and Krejci, 1994).

Social bonding in monogamous prairie vole, which is assessed in the laboratory using a partner preference paradigm, is a higher-order and multi-dimensional social cognitive process, that involves functional circuits for social recognition, social reward and reinforcement, and associative social learning (Lim and Young, 2006). The partner preference paradigm may

therefore more closely model complex social cognitive processes of human interactions, including socially reinforced learning. In female prairie voles, social bonding is facilitated by mating, although partner preferences can develop without mating with longer periods of cohabitation. In this paradigm, the social learning phase (e.g. the initial cohabitation) can be manipulated pharmacologically to either accelerate or inhibit the formation of the social bond, consequently enabling the identification of compounds and neural circuits that affect social learning. In female prairie voles, OT and dopamine interact in the nucleus accumbens (NAcc) to promote partner preference formation (Young et al., 2001a, Liu and Wang, 2003a, Ross et al., 2009b). Direct infusion of OT into the brain during the social learning phase accelerates partner preference formation in the absence of mating (Williams et al., 1994a). Infusion of an OT antagonist, or a D2 dopamine antagonist directly into the NAcc prevents mating-induced partner preference formation (Liu and Wang, 2003a). It has been postulated that OT enhances the salience of social stimuli (e.g. olfactory cues of the partner) while dopamine modulates the reinforcing properties of the social interaction, thereby enhancing socially reinforced learning by incentivizing the social stimuli (Young and Wang, 2004a).

Although a role for glutamate in partner preference formation has not yet been demonstrated, we hypothesize that DCS, acting in the Amyg and NAcc, will facilitate social learning during the initial cohabitation, thereby accelerating partner preference formation. G-protein coupled receptors, like dopamine and OT receptors, can potentiate the action of NMDA receptors in the encoding of long term behavioral changes(Wolf, 2003). The effect of DCS on the enhancement of social learning should therefore be most profound in brain areas that mediate OT dependent social functions, like the Amyg and NAcc.

An alternative animal model relevant to ASD is the meadow vole (*Microtus pennsylvanicus*), which despite being closely related to the prairie vole, is relatively asocial and does not typically display partner preferences following mating. The lack of OT receptors in the NAcc and the low levels of social motivation in meadow voles likely underlie, in part, their inability to form partner

preferences in our laboratory paradigm(Young and Wang, 2004a). It is likely that DCS will not facilitate partner preferences in meadow voles since they lack some of the neural substrates essential for socially reinforced learning of olfactory cues. We propose that DCS will accelerate bonding in female prairie voles, by enhancing socially reinforced learning through modulation of NMDA receptors in socially relevant brain regions. If our assertion is correct, and DCS accelerates partner preference formation during the learning phase of the paradigm, then DCS may be a useful adjunct to behavioral based therapies currently utilized to enhance social function in ASD based on the conserved neural mechanisms underlying social cognition and the face and predictive validity of the partner preference paradigm in prairie voles.

Materials and Methods

Subjects

Experimental subjects were adult (60-120 days of age), sexually naïve female prairie voles or meadow voles, and stimulus males were adult (90-180 days of age), sexually experienced prairie voles or sexually naive meadow voles. All prairie voles were generated from an in-house breeding colony originally derived from a wild caught population in Illinois, USA. All meadow voles were generated from our outbred colony of meadow voles originating from northwest Pennsylvania, USA. After weaning at 21 days of age, subjects were housed in same sex sibling pairs or trios with water and Purina rabbit chow provided ad libitum. All cages were maintained on a 14:10 light:dark cycle with the temperature at 20°C. All experiments were done in accordance to the Institutional Animal Care and Use Committee at Emory University.

Peripheral Effects of DCS on Partner Preference in Prairie Voles

Adult, gonadally intact female prairie voles were injected intraperitoneally with either physiological saline or d-cycloserine dissolved in saline (DCS; Signma-Aldrich C6880; 0mg/kg N=6; 10mg/kg N=7; or 20mg/kg N=7). The doses used were based on the functional doses of DCS for appetitive learning in other rodent models (Pussinen and Sirvio, 1999). Immediately

after the injection the females were placed into the cage of a novel, sexually experienced stimulus male for a 6-hour co-habitation period. As female prairie voles are induced into estrus after 24 hours of exposure to male pheromones, the females were non-receptive and should not have mated. Following the co-habitation period the subjects were tested for partner preference. Partner preference was tested using a 3-cage apparatus with each cage linearly linked by passage tubes. The familiar "partner" male and the novel "stranger" male were tethered one in each of the two end cages. The female was then placed in the center "non-social" cage and allowed to freely move through the three cages. The amount of time the female spent in social proximity with each male was recorded using the VoleTracker beam-break infrared monitoring system (Aragona and Wang, 2004). The infrared beams were placed just beyond the reach of each tethered animal, such that a beam break indicated that the female entered the "social proximity zone" in which social contact was possible. The amount of time the female spent in the social proximity zone for each stimulus animal was counted and used as a measure of time spent with the "partner" and the "stranger" for the determination of a partner preference. All other time was counted as "non-social" time.

Central Effects of DCS on Partner Preference in Prairie Voles

The effort to further specify the area of action of DCS we made some refinements in the partner preference paradigm. All experimental females were ovariectomized to ensure non-receptivity throughout the testing. The pairs were video recorded during the social learning period to verify that they did not mate. The method of quantifying social interaction between the female and males was also refined through a move to automated computerized scoring. To accomplish effective automated scoring the testing arenas were redesigned to a single testing arena divided into three chambers by opaque doorways. This allowed for overhead video recording, which could be analyzed by the automated computerize scoring system SocialScan 2.0 (CleverSys, Inc., Reston, VA), which can track the location of each of the three animals in the

apparatus. The use of two different animal monitoring systems is representative of an enhancement in behavioral scoring technique over the course of the study. The validity of the automated computerized scoring was previously assessed. SocialScan 2.0 correlated highly with manual scoring of partner preference (R=0.904)(Ahern et al., 2009a).

Adult, ovariectomized female prairie voles were bilaterally cannulated into either the nucleus accumbens (NAcc), the amygdala (Amyg), or the caudate-putamen (CP) using stereotaxic methods. The CP cannulated females served as anatomical controls. Ovariectomy was performed at approximately 60 days and animals were allowed to recover for 14 days prior to beginning the study. Subjects were anesthetized using isoflurane and 26 gauge bilateral guide cannulas (Plastics One, Roanoke, VA) aimed at the NAcc (anterior 1.7mm, bilateral ±1mm, ventral -3.5mm to bregma), Amyg (anterior -1.3mm, bilateral ± 2.7mm, ventral -6.1mm) or CP (anterior 1.7mm, bilateral ±1mm, ventral -2.5mm to bregma) were implanted. Location of the cannulae was verified post-experimentally in nissl-stained brain sections (Figure 3g, h, i). The coordinates used for the Amyg group targeted specifically the medial amygdala, however any cannulae that hit within the amygdala were included in the analysis. After 2-3 days of recovery, subjects received microinjections with a 33-gauge internal cannula (Plastics One, Roanoke, VA) that extended 1mm below the guide cannula into the target area. The needle was connected to a Hamilton syringe (Hamilton, Reno, NV) via polyethylene-20 tubing (Plastics One, Roanoke, VA), through which the solution was injected slowly over the course of 1 minute. The internal needle was left in place for 2 minutes after the injection to prevent backflow.

The effect of DCS on partner preference was tested in each brain location independently. Animals received either a bilateral control injection of Ringer's Solution or 10µg of DCS dissolved in 500nl of Ringer's Solution (Fisher Scientific, Pittsburg, PA) per side into the NAcc (N=11-12/treatment), Amyg (N=11/treatment) or CP (N=12/treatment). The 10µg dose is based on the effective dose needed for intra-amygdalar infusion in studies examining fear learning in

rats(Walker et al., 2002). Immediately after the injection the females were placed into the cage of a novel sexually experienced male for a 6-hour co-habitation period. Subjects were video recorded during the cohabitation to ensure no mating occurred. Mating was not observed in any of these animals. Following the co-habitation period the subjects were tested for partner preference (Figure 1).

Partner preference testing was conducted in a three-chamber arena in which a novel male "stranger" was tethered at one end and the familiar "partner male" was tethered at the other. The test female was then placed in the arena and allowed to freely wander. The amount of time the female spent in side-by-side immobile social contact, "huddling", with either male was measured using SocialScan 2.0. Huddling time is more reflective of partner preference than time in social proximity as it precludes non-social or agonistic behavior in the social proximity zone. Distance traveled by the stimulus animal was also recorded as a measure of general levels of activity to control for possible locomotor effects of the drug.

Peripheral Effects of DCS on Partner Preference in Meadow Voles

Adult, gonadally intact female meadow voles were injected intraperitoneally with either physiological saline or d-cycloserine dissolved in saline (DCS; Signma-Aldrich C6880; 0mg/kg N=11; 10mg/kg N=11). For two days prior to drug injection, the females were hormonally primed with estradiol benzoate (Sigma-Aldrich E8515; 2μg/day) dissolved in 0.1ml sesame oil via subcutaneous injection. On the day of the experiment, the females were injected with an additional dose of estradiol benzoate at the same time as the drug injection. Immediately after the injection the females were placed into the cage of a novel, stimulus male for a 24-hour co-habitation period. Mating during the co-habitation period was promoted by hormonal priming to induce sexual receptivity. Receptivity was desired in the meadow vole experiment, as opposed to the prairie vole experiments, to increase the likelihood of detecting an enhancement of partner preference in this asocial species. Following the co-habitation period the subjects were tested for

partner preference as described for the prairie vole. Previous studies demonstrate that meadow voles spend more time in the central non-social arena than in social proximity to the stimulus animals. To assess potential effects of DCS on sociability, we divided the testing arena into three zones (partner zone, stranger zone, non-social zone), and the amount of time the female spent in each zone was measured using SocialScan 2.0. The partner or stranger zone was defined as the area in which the tethered stimulus could engage in social contact with the experimental animal.

Data Analysis of Partner Preference

Time spent in social proximity (peripheral injection experiments) or immobile social contact (central injection experiment) with the partner male was compared with that spent with the stranger male for each experiment using a 2-way ANOVA in which stimulus (partner, stranger or non-social) and treatment (control or DCS) were factors. In addition, Student's T-tests were used to compare time spent with the partner and stranger within each treatment condition. Bonferroni corrections for the level of significance of the Student's T-tests were made for each experiment in order to correct for multiple comparisons. Significantly more time spent with the partner than the stranger constituted a partner preference (Aragona and Wang, 2004). For the meadow vole experiment, a Bonferroni post hoc comparison was used to compare time spent in each zone.

Results

Peripheral DCS Administration in Prairie Voles

We predicted that DCS would accelerate social bonding in the prairie vole, by inducing partner preference under conditions in which it would typically not form. A comparison using a 2-way ANOVA revealed a significant main effect of stimulus animal ($F(1,19)=11.359$, $p=0.002$) no other significant main effects or interactions were found. To determine which treatments resulted in significantly more time spent with the partner than the stranger, Student's t-tests were

performed with Bonferroni corrections of the p-value. Peripheral administration of DCS at a low dose of 10mg/kg ($p<0.001$; Student's t-test, Bonferroni level set at $p<0.01$; Figure 2), but not a high dose of 20mg/kg ($p=0.359$; Student's t-test, Bonferroni level set at $p<0.01$), resulted in the females spending significantly more time with the familiar partner than the novel stranger on a group level. Animals receiving saline did not spend more time with the partner than the stranger ($p=0.419$; Student's t-test, Bonferroni level set at $p<0.01$).

Central DCS Administration in Prairie Voles

DCS was injected site-specifically into two brain regions known to be involved in social learning and reinforcement learning, the NAcc and the Amyg. The CP served as an anatomical control site to control for drug diffusion. The effect of DCS microinjection on time spent with partner and stranger was compared using a 2-way ANOVA that revealed a significant main effect of stimulus animal in both brain areas (NAcc-$F(1,22)=15.923$, $p<0.0001$; Amyg-$F(1,21)=8.959$, $p=0.005$) no other significant main effects or interactions were found for this comparison. To determine the cause of the large effect of stimulus animal, time spent in immobile social contact with the partner and the stranger was directly compared in each of the brain regions. Injection of 10μg of DCS into the NAcc and Amyg resulted in significantly more time spent with the partner than the stranger (NAcc $p=0.002$; Amyg $p=0.003$; Student's t-test, Bonferroni level set at $p<0.01$; See Figures 3a,b). Animals injected with saline into these areas did not display a partner preference, though there was a trend towards more time spent with the partner when saline was injected into the NAcc ($p=0.04$, failed to meet the corrected Bonferroni level of $p<0.01$). Injection of the same dose of DCS into the CP, however, failed to induce partner preference under these conditions, no significant interactions were seen in a 2-way ANOVA comparing stimulus animal and treatment. Direct comparison of time spent with partner and the stranger also failed to show a significant difference (CP $p=0.287$; Student's t-test, Bonferroni level set at $p<0.01$; Figures 3c). There were

no significant differences in locomotion between control and drug injections in any of the three anatomical areas (Figure 3d,e,f; NAcc p=0.04; Amyg p=0.544, CP p=0.460).

Peripheral DCS Administration in Meadow Voles

Peripheral DCS had no effect on partner preference or time spent in close social proximity to either stimulus animal in female meadow voles. A comparison using a 2-way ANOVA showed a significant main effect of zone (partner, stranger, or non-social zone; $F(2,33)=1.100$, $p=0.301$; see Figure 4). A post-hoc Bonferroni test for multiple comparisons revealed significantly more time spent in the non-social zone than time spent in social proximity with either the partner ($p<0.0001$) or the stranger ($p<0.0001$), but no difference in time spent in social proximity of either the partner or the stranger ($p=0.982$). There were no significant main effects of drug ($F(1,22)=0.312$, $p=0.579$). Meadow voles spent on average 70% of the test in the non-social zone, while prairie voles, in comparison, only spent on average 23% of the test, which highlights the profound species differences in social behavior.

Discussion

D-cycloserine Facilitates Partner Preference Formation in Prairie Voles

We demonstrated for the first time, that when administered immediately prior to a social cohabition, DCS enhances the social cognitive processes involved in the development of a partner preference in female prairie voles, leading to a partner preference after just six hours of cohabitation without mating. The effects were dose dependent, as the low dose of DCS (10mg/kg) enhanced partner preference formation while the higher dose (20mg/kg) did not. This dose effect may be due to the mixed agonist/antagonist properties of the drug. DCS binds to the strychnine insensitive glycine site of the NMDA receptor. At low doses the drug increases occupancy of this binding site, thereby increasing glutamate neurotransmission. However, at

high doses DCS out-competes the endogenous ligand at the site but only yields 40-50% of the maximal receptor activation achieved with glycine saturation (Watson et al., 1990).

To better understand the neural processes through which peripheral DCS may be modulating to facilitate partner preference formation, we infused DCS into two candidate brain sites likely to be involved in social bond formation, the Amyg and the NAcc. These sites were chosen because of their known role in OT-mediated social learning in mice and prairie voles and our hypothesis that the OT and glutamate systems interact to facilitate social learning processes at the level of the synapse.

Partner preference formation in prairie voles likely involves at least two distinct processes: i) processing the olfactory signature of the partner to form a social memory (e.g. social recognition)(Curtis et al., 2001) and ii) linking that recognition memory to the reinforcing nature of the social interaction(Young et al., 2001a). This results in socially reinforced learning of olfactory cues. In mice, the first process involves OT acting in the Amyg (Ferguson et al., 2001a), and in prairie voles the second process involves OT acting in the NAcc (Liu and Wang, 2003a). We hypothesized that infusion of DCS into either of these regions, but not into the CP, which contains OT receptors but where OT does not promote partner preferences, would promote the bond formation despite an abbreviated social learning period. Our hypotheses were supported by our findings in this study, DCS infused into the Amyg or NAcc, but not the CP accelerated partner preference formation.

These findings suggest the Glu system, along with the OT and DA systems, converge in the NAcc, and other social relevant brain regions, to facilitate the cellular and behavioral changes associated with bonding in female prairie voles, adding to our basic understanding of the underlying neurobiological events mediating social bonding.

Partner Preference as a Drug Discovery Paradigm for Social Cognition Enhancers

The partner preference paradigm in prairie voles has face, construct, and predictive validity as a potential drug screen for compounds that accentuate the beneficial effects of behavioral therapies aimed at reducing social deficits. Regarding face validity, one may consider the social learning phase, in which the prairie vole pair cohabitates and interacts, to be analogous to the acquisition of social information either in a spontaneous social interaction or in the context of a social behavioral therapy session in humans. In both situations, socially relevant information must be identified and encoded, so that it can be retrieved and utilized appropriately in future social encounters. Regarding the construct validity, converging lines of evidence suggest there is a considerable amount of evolutionary conservation in the systems that regulate social behavior and social cognition from rodent to man (Donaldson and Young, 2008a). Extensive work on the neurobiology of social bonding in the prairie vole has led to a theoretical model in which complex social cognition involves three core domains mediated by somewhat independent neurochemical systems: social information processing (OT and vasopressin), social reward (DA and opioids) and social learning/synaptic plasticity (Glu and neurotrophins), converge to encode the positive valence of species-specific social behavior, and to enhance socially reinforced learning. Conceptualizing the cognitive processes involved in social learning paradigms, such as partner preference formation, in this way may be constructive in identifying the neural systems underlying human social behavior and those that may be compromised in disorders of social behavior. Administration of OT and DA and, as we have demonstrated here, NMDA receptor agonists to prairie voles accelerates partner preference formation (Williams et al., 1994a, Liu and Wang, 2003a). Correspondingly, genetic and epigenetic modifications of the oxytocin, glutamate and dopamine systems have all been linked with ASD (Gunter and Warren, 1998, Jacob et al., 2007b, Hettinger et al., 2008, Lerer et al., 2008a, de Krom et al., 2009, Gregory et al., 2009a, Blundell et al., 2010, Liu et al., 2010a). OT has also been implicated in several recent studies in a number of human social cognitive processes, including emotion recognition(Domes et al., 2007a),

face memory(Rimmele et al., 2009b), trust(Kosfeld et al., 2005a), socially reinforced learning(Hurlemann et al., 2010a) and empathy(Hurlemann et al., 2010a).

Finally, there is preliminary evidence for the predictive validity of the partner preference test. Three independent studies have now reported some improvement in social cognition in ASD subjects following intranasal OT administration (Andari et al., 2010b, Bartz et al., 2010a, Guastella et al., 2010a). In addition a preliminary study has shown that DCS decreases social withdrawal in individuals with ASD as measured by the Aberrant Behavior Checklist (Posey et al., 2004). Promisingly for the use of DCS in the treatment of ASD, the drug has also been shown to reduce repetitive behaviors in a genetically modified mouse model of autism (Blundell et al., 2010). Therefore, we propose that the partner preference paradigm in prairie voles may have widespread potential in identifying multiple classes of drugs for the treatment of social impairments based on its face, construct and predictive validity.

Selection of Animal Model

ASD is four times more prevalent in males than females. Therefore, one might argue that partner preference formation in male prairie voles might be more relevant to identifying drugs to treat ASD. Furthermore, ASD is characterized by decreased social motivation and social reciprocity. Thus the asocial meadow vole may be argued to be a better model of ASD than the prairie vole. However, it should be noted the goal of the present study was to use a behavioral paradigm with the highest degree of construct and predictive validity as a screen for drugs that may be useful for the enhancement social cognition in humans. Female prairie voles were used in this paradigm because partner preference in females is well established to be an OT-dependent process (Williams et al., 1994a, Liu and Wang, 2003a) and since OT enhances social cognitive processes in humans (most studies have used male human subjects), we felt that partner preference formation in females could be considered to have more established predictive validity for

screening drugs to enhance social cognition than partner preference formation in males, which has not been shown to be dependent on OT.

Based on our results, prairie voles appear to be better suited than meadow voles for use in the partner preference paradigm to identify drugs that enhance social cognition. This is likely because meadow voles do not express OT receptors in the NAcc and fail to show a partner preference after long cohabitation periods(Insel and Shapiro, 1992a). However, their brain is not dysfunctional, but rather evolutionarily adapted for an asocial, sexually promiscuous mating strategy. The meadow vole brain may simply lack some of the neural substrates essential for socially reinforced learning of olfactory cues that are involved in partner preference formation. Thus while meadow voles may be useful in identifying therapeutic approaches to enhance social motivation, they are less appropriate as a screen to identify social cognitive enhancers. DCS had no effect on sociability in our meadow vole study. This is in contrast to a recent report that DCS increase sociability in the BALB/c mouse, a mouse strain with low sociability(Deutch et al., 2011). It should be noted that in our study DCS was administered 24 hours prior to the partner preference test. Thus we were specifically detecting the effect on partner preference formation, not social motivation.

The brain differences between typical and ASD individuals, though, are likely far more subtle than the species differences between meadow and prairie voles. While meadow voles are evolutionarily adapted to not form partner preferences, a high percentage of ASD subjects benefit from behavioral therapies such as applied behavioral analysis, thus demonstrating the presence of some the neural mechanisms necessary for the acquisition of social information and their viability as a target for potential pharmacological therapies.

Pharmacological Adjuncts to Behavioral Therapies

The limited effects of compounds identified as pro-social therapeutics by the partner preference paradigm may be enhanced by combination with social behavioral therapies. DCS has been

successfully used in the psychiatric setting for the treatment of several disorders including phobias (Grillon, 2009), social anxiety disorder (Guastella et al., 2008b), obsessive-compulsive disorder (Wihlem et al., 2008, Storch et al., 2009), drug cue extinction (Santa Ana et al., 2009) and schizophrenia (Tsai and Lin, 2010). The crux of the successful use of DCS in the treatment of these disorders is the combination of pharmacotherapy with behavioral therapies, including cognitive behavioral therapy (Davis et al., 2006a), cue exposure therapy(Santa Ana et al., 2009) and exposure and response prevention(Storch et al., 2009). This treatment model is reflected in the design of the partner preference paradigm, in that potential prosocial or cognitive enhancing drugs are administered prior to the social learning phase, so that the animals are receiving both pharmacological and behavioral stimulation simultaneously. Based on successful use of the drug in the psychiatric setting, and the effect of the drug on social bonding in this study, we propose that DCS could be used in combination with applied behavioral analysis (ABA) for the treatment of the social impairments associated with ASD. DCS may to have a positive effect on many of the skills taught in ABA sessions, including extinction of repetitive behaviors and initiation of communication, but it may have a particularly profound effect on social skills training due to its potential interaction with the other neurochemical systems mediating functional social behavior. Many of the sub-skills taught in social skills training, like maintaining eye contact and understanding facial expressions, are modulated by OT(Weiss and Harris, 2001, Domes et al., 2007a, Guastella et al., 2008a). DCS could interact with the endogenous OT system activated during these processes in subjects to produce accelerated acquisition of these skills. Alternatively, DCS could be co-administered with intranasal OT, to increase both the saliency of social stimuli and memory for the socially reinforced learning facilitated by ABA. Drugs that target any of the three systems underlying complex social cognition (OT, dopamine and glutamate) could potentially be targeted either alone or combination to enhance the effects of ABA in reducing the social impairments of ASD.

Conclusions

Through the findings in this study we have identified a role for glutamate neurotransmission in the formation of social bonds in prairie voles. Enhancement of the glutamate system, through the use of the NMDA receptor agonist DCS, accelerates the acquisition of social information in the prairie voles. DCS may therefore be a promising candidate for the treatment of the social impairments associated with disorders of social behavior particularly if combined with social behavioral therapy. The partner preference paradigm has face, construct and predictive validity for the identification of drugs that enhance social cognition and should be utilized in the generation of novel pharmacotherapies for the treatment of ASD and psychiatric diseases characterized by social impairment.

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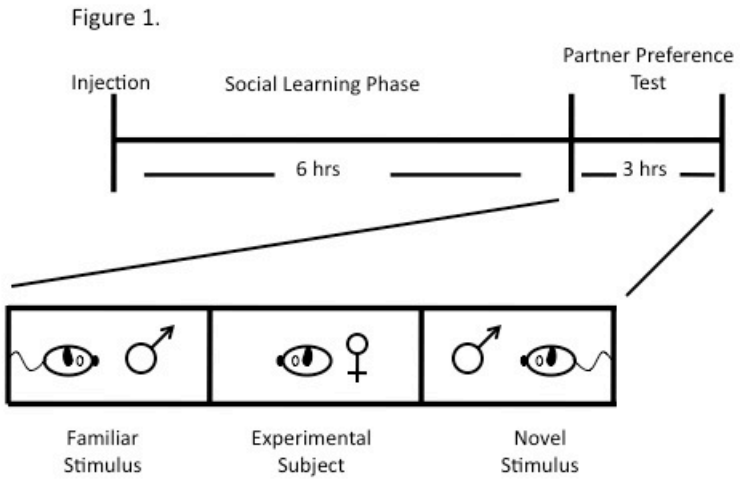

Figure 1. The Partner Preference Paradigm. Drug manipulation occurs immediately prior to animal pairing. This is followed by the social learning phase in which the experimental female is allowed to freely interact with a stimulus male for six hours during which the animals do not mate. This level of social interaction is typically insufficient to induce a social bond (bonding usually requires 24 hours of cohabitation with mating). This sub-threshold paradigm allows for the testing of drugs that accelerate social bonding. The formation of a social bond can be assayed in the laboratory using the partner preference test. In the test phase of the paradigm, the male with which the female was cohabitated is tethered to one end of a three-chambered arena and a novel male of equal stimulus value is tethered to the opposite end of the arena. The female is placed in the center of the arena and allowed to freely wander for three hours. The amount of time the female spends in social proximity or huddling with either male is recorded.

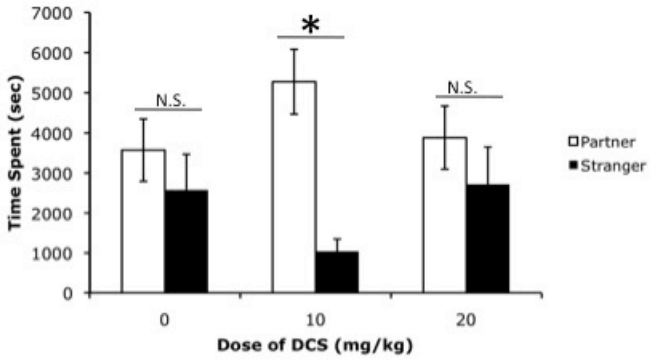

Figure 2. Effect of Peripheral D-Cycloserine (DCS) Administration on Partner Preference Formation in Prairie Voles. 10 mg/kg of DCS facilitated partner preference formation, as measured by time in social proximity, in female prairie voles after an abbreviated cohabitation with a male in the absence of mating ($p<0.001$). Both the higher dose of 20mg/kg and the control injection failed to induce a partner preference (20mg/kg-$p=0.359$; saline-$p=0.419$). Time spent with stimulus animals was compared using a Student's t-test with a Bonferroni correction for multiple comparisons. Asterisk indicates $p<0.01$.

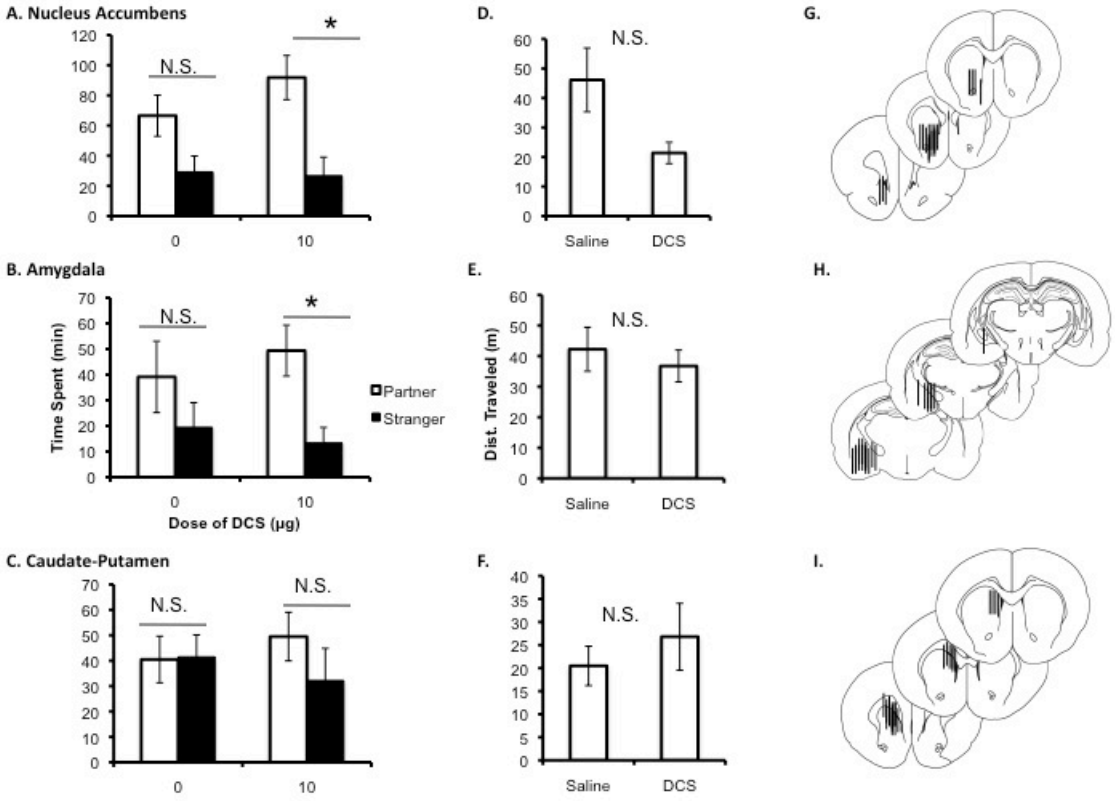

Figure 3. Effect of Central D-Cycloserine (DCS) Administration of Partner Preference. Injection of 10µg of DCS bilaterally into both the nucleus accumbens (3a) and amygdala (3b) accelerated partner preference formation as indicated by significantly more time spent huddling with partner than the stranger (NAcc $p=0.002$; Amyg $p=0.003$; Student's t-test, Bonferroni level set at $p<0.01$). The same injection into the caudate-putamen (3c) failed to induce a preference ($p=0.287$). There were no differences in total locomotion induced by DCS administration in any site tested (3d-NAcc $p=0.04$; 3e-Amyg $p=0.544$, 3f-CP $p=0.460$; Student's t-test, Bonferroni level set at $p<0.01$). Location of the cannulae placement for each site verified post-experimentally are shown (3g; 3h; 3i). Asterisk indicates $p<0.01$.

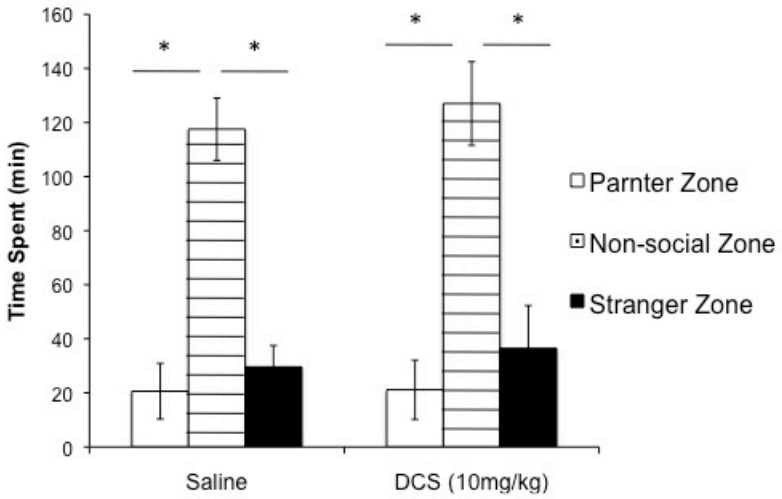

Figure 4. Effect of Peripheral D-Cycloserine (DCS) Administration on Partner Preference Formation in Meadow Voles. DCS had no effect on partner preference formation in the female meadow vole. Meadow voles, regardless of treatment, spent significantly more time in the non-social zone than in social proximity of either the partner (non-social vs. partner; $p<0.0001$) or stranger (non-social vs. stranger; $p<0.0001$). Time spent in social proximity of either stimulus animal or the non-social zone was compared using a 2-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. Asterisk indicates $p<0.0001$.

CHAPTER 5

General conclusions and future directions.

Conclusions

In the development of therapeutics for a disorder with an unknown or heterogeneous pathophysiology, a viable strategy is to target the systems regulating the affected process. The etiology of social impairment in ASD is as of yet undefined, but animal models are still useful for defining neurochemical contributors to complex prosocial behavior. The study of a complex behavior, like social cognition, may be facilitated by conceptually dividing it into discrete components that can be defined both phenotypically and mechanistically. For example, intensive study of social bonding in the monogamous prairie vole has led us to conceptually split the process into three core domains mediated by independent neurochemical systems: social information processing (OT and vasopressin), social reward and reinforcement (dopamine and opioids) and social learning/synaptic plasticity (glutamate and neurotrophins) (Figure 1) (Modi and Young, 2009). Dysregulation of any of these processes may contribute to the deficits in social cognition found in ASD and other psychiatric disorders characterized by social impairments. While the precise etiology of these disorders may be different for each affected individual, pharmacotherapies that enhance social cognition through stimulation of any of these systems either independently or perhaps in combination, may improve functional social cognition.

Of the three domains regulating social behavior, the OT system is the most promising candidate for the pharmacological enhancement of social cognition. In animal models, OT can be experimentally manipulated to alter social behavior without disrupting other essential biological functions (Young et al., 1997, MacDonald et al., 2011). Critical to the translation of any basic science finding to clinical application is the determination of the most efficacious method of targeting that system. The pharmacological enhancement of the OT system is inhibited, though, by the biophysical properties of the peptide and the functional redundancy of the OT and vasopressin (AVP) system. As a result, direct peripheral administration of OT is unlikely to have

profound behavioral effects. Consequently, novel ways of modulating the social brain, including the neurochemicals that regulate social behavior and the brain circuits that mediate the transmission of social information, must be developed in animal models with translational validity. Through my dissertation work, I have evaluated and developed novel methods of pharmacologically enhancing social cognition in functional animal models using compounds that have undergone preclinical testing in humans and are therefore viable candidates as drugs. Therapeutic strategies that are found to be efficacious in enhancing prosocial behavior in animal models are strong candidates for translational consideration in clinical human populations for the treatment of social impairments.

Project 1: Efficacy of Direct Routes of Oxytocin Administration The most straightforward method of stimulating the OT system is through the administration of OT analogues to act directly on OT receptors to evoke behavioral effects. In prairie voles, a single central injection but not a peripheral injection of OT facilitates the development of social bonds (Williams et al., 1994b). Consequently, as it is hypothesized that OT exerts behavioral effects through centrally located receptors, it is critical that a potential prosocial drug be able to access the brain compartment. It has been proposed that intranasal (IN) administration of peptides allows for preferential access to the brain compared to other peripheral routes of administration. Based on the central penetrance of other closely related peptides, like AVP, after IN administration, IN OT has been used to enhance aspects of human social cognition in a number of behavioral tasks (Born et al., 2002, Macdonald and Macdonald, 2010). Despite the striking social behavioral effects of IN OT, there is currently no physiological evidence that it increases central levels of the peptide. To fill this gap in the literature, the first aim of my dissertation work directly compared the efficacy of IN OT administration and intravenous (IV) administration in increasing central and peripheral levels of OT and other related molecules. In rhesus monkeys, neither IN nor IV OT was to found to increase OT levels in lumbar CSF samples. Both routes of administration resulted in profound

increases in plasma OT levels that were maintained for two hours after administration. While these findings appear to be incongruent with the behavioral effects after IN OT administration, there are two possible explanations. Either IN OT does penetrate the blood brain barrier and accesses central receptors, but bypasses lumbar CSF circulation or IN OT does not reach central nervous system in appreciable amounts and has behavioral effects via peripheral mechanisms. A better understanding of the mechanisms through which IN OT is able to affect human social behaviors is necessary before being scaled up to widespread clinical use.

Project 2: Behavioral Efficacy of Indirect Methods of Increasing Central OT

In light of the uncertainty regarding the mechanism through which IN OT is able to evoke social behavioral effects, alternative methods of increasing central OT levels should be considered for therapeutic indications. One potential method for manipulating the central OT system is to stimulate endogenous OT release. Activation of the melanocortin 4 receptors on the oxytocinergic neurons of the hypothalamus is able to specifically induce central while inhibiting peripheral OT release in *in vitro* models (Sabatier et al., 2003). To test the viability of this alternative method of evoking OT-dependent behavioral effects, the second portion of my dissertation work addressed whether indirect OT stimulation could recapitulate the behavioral effects of central OT. In female prairie voles, the melanocortin agonists Melanotan II (MTII) and Pf-446687, are able to facilitate the formation of partner preferences in a manner similar to intracerebroventricular injections of OT. The facilitatory effect of MTII is due to its actions during the social learning phase of the partner preference paradigm, in the acquisition of social information. MTII administered prior to an abbreviated social learning phase enhances the formation of bonds when tested one week later after the clearance of the drug. The acquisitional effect of MTII suggests MC4R agonists could be used in combination with behavioral therapy to facilitate the learning of social skills. The long-term effects of MTII can be amplified by administration during a developmentally relevant critical period. Administration of MTII during

the first week of life accelerated the formation of partner preference in adult female prairie voles. The long-term effects suggest early life manipulation of this system can alter the development of functional social behavior. Interestingly, though, the chronic early life administration of MTII revealed potential unintended side-effects of the non-specific MC agonists. The pups that received MTII were darker in pigmentation and weighed less than their control littermates, indicating some potential limitations to the therapeutic use of MC agonists.

Mechanistically, the prosocial effects of MC agonists in this model are likely driven by indirect activation of the OT system. MC4R mRNA is present in both the paraventricular and supraoptic nuclei of the hypothalamus in the prairie vole, enabling MC agonists to stimulate oxytocinergic neurons. In addition, MTII administration increases activity in OT containing hypothalamic neurons, as indicated by Egr-1 expression. Co-administration of peripheral MTII with an OT receptor antagonist prevents the enhancement of partner preference, further implicating OT receptors in the MTII's mechanism of action. Taken together with the previously established role of the MC4R in the regulation of OT release, these findings suggest MC agonists can be used to evoke OT-dependent behaviors. The prosocial effects of indirect OT stimulation are not limited to the melanocortin system, as any receptor system that alters the activity of oxytocinergic neurons is a potential therapeutic target. Stimulation of the 5-HT1a receptor, which is expressed in the paraventricular nucleus, by the agonist buspirone also facilitates partner preference (Modi and Young, 2011b). The ability of peripherally administered agonists for receptors on the oxytocinergic neurons of the brain to enhance social behavior represents a novel paradigmatic approach for therapeutically targeting the OT system.

Project 3: Behavioral Efficacy of Enhancing the Circuits Associated with Oxytocin Dependent Prosocial Behavior

Based on the critical role of OT in the regulation of prosocial behavior, the first two aims of my research have focused on direct manipulation of the OT system for the development of novel

pharmacological strategies to treat social disorders. An alternative, though, to manipulating the OT system, is to enhance the neural circuits that regulate social learning. Cognitive enhancers, typically glutamate receptor agonists, have been used in animal models and clinically to enhance many different forms of learning and memory, which suggest the drug may also be effective in improving social memory and cognition. The third portion of my dissertation work tests the hypothesis that the acquisition of social information, like other forms of learning, can be enhanced by through the administration of glutamate receptor agonists specifically in areas of the brain involved in processing of social stimuli. Administration of the NMDA receptor agonist, D-cycloserine (DCS) prior to the social learning phase of the partner preference test accelerated the formation of social bonds. To test if DCS had a specific effect within the social brain circuit, as opposed to a general cognitively enhancing affect, the drug was given site-specifically into brain regions regulating social cognition. DCS facilitated the formation of pair bonds when injected in both the nucleus accumbens and medial amygdala, areas involved in the acquisition of social information, but not in the caudate putamen, a structurally similar but functionally distinct control region. Interestingly, both areas in which DCS had a prosocial effect are also sites that mediate OT-dependent social behaviors, suggestion an interaction between the two systems. The overlap in areas of efficacy of OT and DCS in enhancing partner preference also supports the conceptual model of convergent neurochemical regulating functional social cognition and the potential for therapeutic enhancement of each domain. The acquisitional effects of DCS suggest that like OT-based therapies, glutamate-based therapies may be most beneficial in the context of behavioral therapy and social skills training if used as a treatment for social impairments and is a viable therapeutic alternative for the treatment of social impairments.

General Findings

Elucidation of the brain circuits and the neurochemical systems that regulate social behavior in animal models, like the prairie vole, has opened the door for the development of

pharmacotherapies to treat social impairments. My dissertation work has built upon this mechanistic understanding of social behavior to highlight three independent lines of potential prosocial therapeutic interventions and has characterized partner preference in the prairie vole as a predictive model for the treatment of disorders characterized by social impairments (Figure 2). Drugs, including centrally administered OT, MTII, Pf-446687 and DCS, that facilitated the formation of a partner preference within the suboptimal pairing paradigm, are likely to also enhance aspects of social cognition in humans. The predictive validity of the partner preferences test is supported by the use of IN OT, IV OT and DCS in preliminary studies of social cognition in the autistic population with cautious success. The therapeutic potential of the drugs in the preliminary clinical studies, though, was likely limited, as they were given outside of a social learning context. Their facilitatory role in the social learning phase of the partner preference test suggests both OT and glutamate based therapies may be more efficacious if used in combination with social behavioral therapies to enhance the acquisition of explicitly taught social skills. As each of the drugs explored in my dissertation work have been developed pre-clinically for use in humans, findings from the prairie vole model can be directly translated into human functional studies. Hypotheses regarding the relative efficacy of the different therapeutic lines in enhancing social cognition and the potential for increased efficacy in combination with behavioral therapy can be directly tested in both normative and affected human populations. Concurrently, the partner preference test can be used to generate hypotheses for alternative mechanism for enhancing social cognition based on our increasing understanding of the neurobiological underpinnings of prosocial behavior. Thus we have developed a pipeline for developing novel prosocial therapeutics, from basic science in the prairie vole, to predictive validation in the partner preference test, to assessing the translational potential in non-human primate models, culminating in tests of clinical efficacy in human behavioral assays.

A Case for Functional Animal Models

A functional animal model displays a behavior that can be experimentally manipulated and reliably measured in the laboratory setting. The face and construct validity of the model is evaluated relative to the functional behavior in humans. Behavioral measures within the functional model can also have predictive validity, as is seen in the Forced Swim Test for the development of anti-depressants (Castagne et al., 2011). Based on the findings of my dissertation work, I propose that prairie voles have face, construct and predictive validity for identifying drugs that enhance social cognition. The highly social nature of prairie voles, exemplified by communal living, high parental investment and the formation of socially monogamous bonds, demonstrates higher face validity for human social behavior than most traditional laboratory species. Pair bonding, as measured by the expression of a partner preference, requires several different components of social cognition, including social motivation, social information processing and social attachment. These processes are also essential for human social interaction and are often disrupted in disorders like ASD. Preliminary evidence suggests that environmental, genetic and pharmacological manipulations that modify these behaviors in prairie voles have analogous effects in humans.

However, many of these parallel effects are specific to female prairie voles, as opposed to males. The central administration of OT to female prairie voles has more robust effects on the formation of pair bonds than it does in males (Insel et al., 1995). Additionally, in the studies presented above, both DCS and MTII facilitated partner preference formation in female but not male prairie voles (Figure 3). The sex specific effect in prairie voles is not seen in human studies. The majority of the IN OT studies and a DCS study showing prosocial effects were conducted in men (Posey et al., 2008, Striepens et al., 2011). These contradictory findings suggest that only partner preference in female prairie voles has predictive validity for identifying prosocial therapeutics. The sex-specific effect in prairie voles underlies the differences in the primary mechanism mediating male and female pair bonding behavior. OT preferentially facilitates female partner

preference formation, while AVP primarily mediates male preference. The lack of dichotomy in the role of OT in human social behavior suggests that only partner preference formation in female prairie voles has construct validity with human social cognition. Human prosocial behavior is likely an evolutionary extension of the affiliative drive of OT as opposed to the agonistic drive of AVP, consequently is only reflected in the molecular mechanism of female prairie vole partner preference. Therefore drugs that enhance social behavior in the female prairie vole are likely to have a similar in humans, irrespective of effect in male prairie voles.

Despite the face and construct validity female prairie voles have for functional human social cognition, they lack both of those features in respect to disorder social cognition. The behavior of prairie voles is not similar to the behavior exhibited by individuals with ASD. Therefore, partner preference in the prairie is not a "model of autism" per se, but rather a model with relevance to autism. But you could create models by manipulations that compromise social behavior, like early life separation or siRNA knockdown of receptors. Based on the behavioral effects of both OT and glutamate receptor manipulations, the functional prairie vole model has better predictive validity than the disordered meadow vole model (Ross et al., 2009c, Modi and Young, 2011a). Neither the enhancement of OT receptors in the nucleus accumbens of meadow voles nor the indirect release of central OT using MC agonist or the administration of an NMDA receptor agonist induced meadow voles to show social preference (Figure 3)(Ross et al., 2009c, Modi and Young, 2011a). Despite the surface level behavioral similarities between the asocial meadow vole and individuals with ASD, the meadow vole is unlikely to be a good predictive model for the development of prosocial therapeutics. This unsuitability of effect is likely due to dramatic differences in the organization of the social brain circuit that developed after the evolutionary split from the ancestral vole species. The neural differences resulting from speciation are far more profound than those resulting from a genetic disorder. Thus, just as individuals with ASD have innumerable similarities to the normative population they also likely have conservation of some

of the mechanisms that mediate prosocial behavior in the prairie vole. Partner preference in the female prairie vole not only predictive validity with respect to the typical population but also for those afflicted with social impairments, as demonstrated by the efficacy of IN OT and DCS in ameliorating some of social deficits associated with ASD. The validity of this model encourages the future use of the female prairie vole in both the elucidation of the mechanisms underlying social behavior and the identification of drugs that can enhance it.

Future Directions

Based on my contributions to the field of OT and the treatment of disorders of social cognition, a number of new questions have arisen whose answers are necessary for the development of the most efficacious OT-based therapeutic strategies. In relation to the studies of IN OT, further work is needed to understand if and how intranasal administration facilitates the passage of OT into the brain compartment. In vivo microdialysis in primates from brain areas containing OT receptors could be used to directly measure if there are changes in central OT levels after intranasal administration undetectable in the CSF. However, this method is still hampered by the limitations of monkey models of inhalation. Ultimately, a systematic study in humans, which parallels the Born (2004) study, must be undertaken to determine if IN OT increases CSF levels of OT similarly to the increase in CSF AVP levels after IN administration (Born et al., 2002). Further the behavioral efficacy of IN administration should be directly compared to IV administration in well-characterized human behavioral endpoints to determine if IN administration is even necessary to achieve prosocial behavioral effects.

To further the development of therapeutics that induce the indirect release of OT to facilitate social cognition, the behavioral efficacy of this method should also be compared to IN OT administration. The ultimate goal of these therapeutic strategies is to treat social impairments, so it is imperative to know which method leads to the greatest behavioral benefit with the fewest undesired side effects. There is even the potential for undiscovered neurochemical systems to

more potently stimulate OT release and facilitate OT-dependent behavior. A characterization of all the neurochemical receptors expressed on hypothalamic oxytocinergic neurons and a systematic testing of the effect of their stimulation of partner preference could identify novel therapeutic targets.

The evidence necessary to transition these treatment strategies into human testing could be facilitated by the development of disordered female prairie vole model. Using early life manipulations, genetic selection or siRNA manipulation the OT receptor system can be downregulated in female prairie voles, such that the system is impaired but not absent (as it is in meadow voles). A disordered version of a model that already shows predictive validity has the potential to have more translatable implications for populations in which there is known OT dysregulation, such as the subset of ASD patients with genetic alterations of the OT receptor gene. Drugs that facilitate partner preference in both functional and disordered prairie vole models would be extremely strong candidates for testing in human clinical populations.

In addition to testing the efficacy of these treatment strategies in single-endpoint behavioral paradigms, their use should also be evaluated on scales of clinical impairment and in the context of social behavioral therapy. Clinical scales can be used to evaluate treatment regimens, to determine if the drugs need to be given chronically or only in combination with other therapies. Evidence from the prairie vole model suggests both DCS and OT-based therapies facilitate the acquisition of social information potentially through increasing the salience of social stimuli. As many of these drugs have been approved for use in humans, small-scale tests of their efficacy in combination with behavioral therapy could be undertaken relatively easily.

Remaining Questions for the Field

The future development of prosocial therapeutics is limited by a number of key gaps in our understanding of functional social cognition. To best inform future clinical pursuits, a better understanding of two main characteristics of the OT-dependent social cognition is required: 1) the

strengths and limitations of the biophysical properties of the OT system 2) the conceptual and mechanistic basis of how OT enhances social behavior. Elucidation of these facets of social behavior will enable better treatment through both pharmacological and behavioral interventions for the treatment of disorders characterized by social impairments.

Oxytocin: Why is it so complicated?

Many of the complicating factors in pharmacologically targeting the OT system arise from evolutionary history. OT related peptides have existed for at least 700 million years and play a role in the regulation of social and reproductive behaviors in diverse taxa ranging from hydra to insects, birds and mammals (Donaldson and Young, 2008b). Prior to the divergence of vertebrate animals, there was a gene-duplication event of the ancestral OT gene that gave rise to the closely related peptide, AVP. Genetic and molecular redundancy of these two nonapeptides accounts for many of the complexities that limit their use therapeutically. OT and AVP differ from each other at only two amino acid positions and both have activity at the other's receptors. OT has one identified receptor (OTR), while AVP has two central receptors (V1a and V1b) and one peripheral receptor (V2). There is a high degree of cross reactivity between the two systems making it difficult to design synthetic ligands with specificity for just one receptor subtype(Donaldson and Young, 2008b). One of the original OT agonists, d(CH2)5[Tyr(Me)2]OVT has since been shown to be five times more potent as a V1a antagonist than as an OT antagonist (Manning et al., 2008). Ligand promiscuity between OT and central AVP receptors can be counterproductive in achieving behavioral effects, as the V1a receptor is thought to mediate opposing social behaviors from the OT receptor. Whereas the central OT receptor activation underlies affiliative behaviors, the V1a receptor is associated with agonistic social behaviors like mate-guarding, maternal aggression and scent marking. The two peptides are also inverse regulators of anxiety behaviors, with OT being anxiolytic and AVP being anxiogenic (Churchland and Winkielman, 2011).

The selectivity of synthetic agonists is further compromised by structural differences between the primate and rodent neuropeptide receptors, such that ligands with high specificity for the rodent OT receptors may not retain that property when used translationally. Several ligands with high specificity in rodents, including those used in early characterization of the primate OT receptor system, have since been shown to have reduced selectivity in primate tissue (Tence et al., 1990, Young et al., 1999). This has resulted in a still ambiguous characterization of the human OT receptor distribution. The receptors differences have also hindered the development of clinically useful agonists and antagonists as rodents cannot be used to pharmacological and behavioral validation of candidate compounds.

In addition to specificity considerations, pharmacological manipulation of the OT system is also limited by the evolution of the system to serve dichotomous biological roles. OT mediates discrete functions in the brain and in the periphery. Independent concentrations of OT are maintained in the blood and in the extracellular fluid of the brain that respond differentially to oxytocinergic stimuli (McEwen, 2004). The functional duality is maintained by the physical separation between the two systems by the blood-brain barrier and by the independent mechanism governing central and peripheral release. The same mechanisms that enable independent regulation, prevent the pharmacological manipulation of the central OT system through classical methodologies. OT is a large hydrophilic molecule and consequently has poor permeability of the blood-brain barrier, enabling less than 1% of peripherally administered peptide to enter central circulation (Ermisch et al., 1985). Consequently, pharmacological manipulation of the OT must be targeted through agonist selection and route of administration to either central or peripheral circulation.

Thus as a result of the structural redundancy between the OT and AVP systems, the structural variability between the primate and rodent OT receptor and the independent regulation of central and peripheral OT, the full therapeutic potential of this neuropeptide system has not yet been

realized. Considerable effort, at both the academic and pharmaceutical levels, has been put into developing novel synthetic ligands that are able to overcome the biophysical limitations of neuropeptides to stimulate the OT receptor without any major success. There are currently no reported synthetic OT receptor agonists in clinical development that are able to be administered peripherally to activate central OT receptors to elicit social behavioral effects. It is, therefore, likely that the successful pharmacological enhancement of the OT system will be reliant on non-traditional methods similar to those outlined above.

How Does OT Facilitate Social Cognition?

OT affects a number of distinct aspects of human social cognition, including the perception of social stimuli, trust and generosity, empathy and social anxiety (Striepens et al., 2011). In animal models, OT affects more basal analogues of these behaviors including, social discrimination, attachment and the socially induced anxiety (Modi and Young, 2011b). It is still unknown, though, how OT functions to alter behavioral responses in social interactions in either humans or animal models. On a high level, the current literature suggests that OT enhances the saliency of social information, such that socially derived sensory information carries greater weight than non-social stimuli. Several alternate hypotheses, though, have been proposed to account for OT's function on social cognition (Churchland and Winkielman, 2011). OT may facilitate social behavior through a reduction in anxiety that inhibits social interaction or it may generally enhance affiliative drive. A more thorough understanding of how OT affects the processing of social stimuli generally will guide its therapeutic usage. For example, if OT enhances the salience of social cues, it may be most efficacious if given along with social skills training. However, evidence that it increases general affiliation or reduces social anxiety suggests a chronic administration may be more beneficial. A better characterization of the type of prosocial effect OT exerts maybe attainable through an appreciation of how OT functions at a cellular and molecular level to alter behavior.

At the mechanistic level, there are several brain nuclei in which OT could facilitate the processing of social stimuli. In rodents, the perception of social odors (the most ethologically relevant sensory stimuli) is relayed from the olfactory bulb to the medial amygdala (and other amygdaloid nuclei) then to either the extended amygdala (including the bed nucleus of the stria terminalis and the nucleus accumbens) or the hypothalamus (Sanchez-Andrade and Kendrick, 2009). Not surprisingly, all of the brain areas that respond to social stimuli also contain OT or AVP receptors (Sanchez-Andrade and Kendrick, 2009). The activation of OT or AVP receptors concurrently with the processing of specific sensory stimuli may enable socially relevant stimuli to be processed differently form other forms of sensory input. At the primary sensory level, both neuropeptides reduce the output from the olfactory bulb to higher order processing centers, which could act as a socially selective sensory filter. For example, olfactory neuropeptide receptor activation could selectively filter the neural response to familiar as opposed to novel conspecifics, consequently reducing the social behavioral response in the social recognition paradigm (Wacker and Ludwig, 2011). OT also acts at the level of the amygdala in the categorization of olfactory stimuli. OT in the medial amygdala, a nucleus categorically responsive to biologically relevant odors, is necessary and sufficient for social discrimination (Samuelsen and Meredith, 2009, Gabor et al., 2012). In the central amygdala, OT and AVP function differentially to modulate the emotional valence of sensory information. Through the activation of distinct neuronal populations in the central amygdala, OT inhibits and AVP enhances the activity of the nuclei in response to fearful stimuli (Viviani and Stoop, 2008). OT-dependent inhibition of this area is consistent with the reduction of amygdala activation in response to fearful visual stimuli after IN OT administration in humans (Kirsch et al., 2005). Modified sensory information from the amygdala then activates circuits in the ventral striatum and hypothalamus involved in social behavior. The basolateral amygdala has projections to the nucleus accumbens, an area that is differentially enriched with OT receptors in high social species (Shapiro and Insel, 1992). In the accumbens, both OT and DA are necessary for the formation of pair bonds in prairie voles (Liu

and Wang, 2003b). It is hypothesized that the co-activation of the two systems mediates as association between the social stimuli and a rewarding, or reinforcing, valence (Young and Wang, 2004c). The hypothalamic nuclei that mediate the production and release of OT have reciprocal connections with the limbic processing areas, such that OT tone could be modulated based on environmental and physiological factors to affected the subsequent behavior. Central administration of exogenous OT or the pharmacological increase of endogenous OT could mimic the regulatory role of the hypothalamic nuclei to facilitate behavioral change.

Overall, bits and pieces of how OT contributes to the cellular processing of social information are known, but it is unclear which, if any, of these mechanisms are responsible for the changes in behavioral phenotypes seen after OT administration. It is possible that the different functions of OT at different levels of sensory information processing lead independently to the multifarious behavioral effects associated with central administration. OT could promote social behavior through sensory gating in the olfactory bulb but also through anxiolytic effects mediated in the central amygdala and reinforcing effects in the nucleus accumbens. The role of these cellular systems, though, is even more unclear in relation to the effect of OT in promoting human social behavior due to the uncertainties of the distribution of OT receptors in the primate brain. OT receptor distributions underlie profound social behavioral differences in rodents and so it is critical to know if the human distribution looks more like prairie voles or meadow voles. While the development of pharmacotherapeutics that target the OT system can proceed without knowledge of the direct cellular and molecular systems involved, will enable the development of more focused treatment strategies and realistic behavioral expectations.

Final Conclusions

The OT system has a strong therapeutic potential for the treatment of social impairments in ASD and a number of other psychiatric disorders, based on its critical role in the production of functional social behavior in animal models. The potential benefits of OT, though, are limited by

the biophysical properties of the peptide. This necessitates the use of animal models, like the prairie vole, and behavioral paradigms of social cognition, like the partner preference test, to indentify the most efficacious methods of upregulating the OT system. Direct administration of OT, both intranasally and intravenously, have been effective in enhancing many aspects of human social behavior. It is still unclear, though, whether these behavioral effects are the result centrally mediated actions. Alternatively, central OT can be increased through the stimulation of MC receptors driving OT release to evoke OT-dependent effects. The relative efficacy of these two methods of enhancing the OT system to promote social cognition should be evaluated with respect to clinical use in the treatment of social impairments. Social cognition can also be enhanced, bypassing the OT system, through the enhancement of excitatory signaling in areas of the brain mediating social behavior. Each of the three lines of prosocial therapeutics, developed and validated in an animal model with face, construct and predictive validity, are the first step towards a biologically based treatment for a whole class of yet untargeted impairments pervasive across psychiatric disorders.

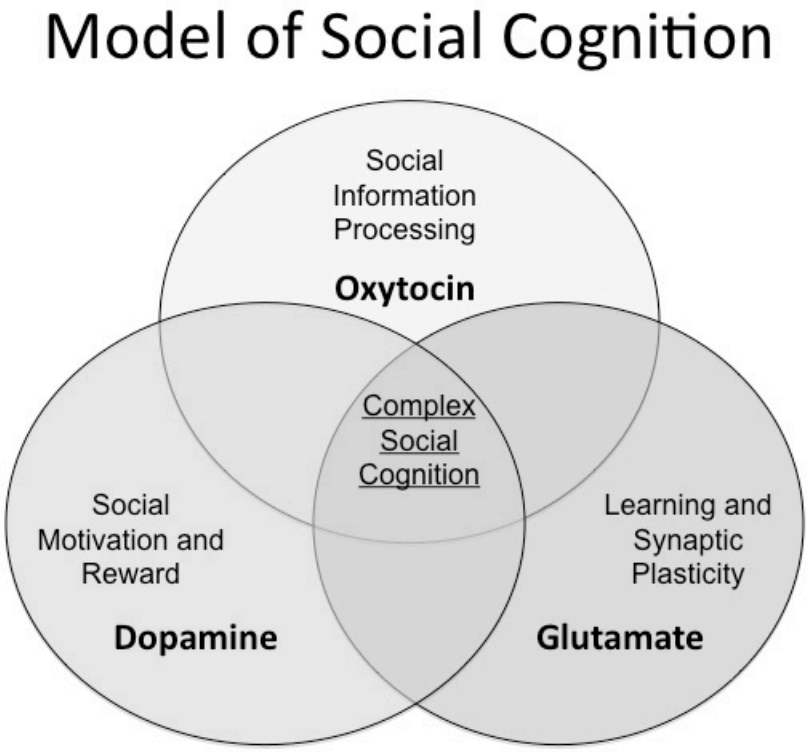

Figure 1. Model of social cognition. A conceptual model of complex social behavior. Social behavior is comprised of three subdomains: social motivation and reward (mediates by dopamine and the opioids), social information processing (mediates by oxytocin and vasopressin) and social learning/plasticity (mediates by glutamate and the neurotrophins). Each of these domains contributes to the complexity of social behavior through separate but converging systems. The study of each of these components independently it utilized to simplify the study of the biological mechanisms underlying social behavior. Alterations of any of these three systems can contribute to the expression of social impairments. Correspondingly, pharmacological stimulation any system can also enhance the expression of functional social cognition.

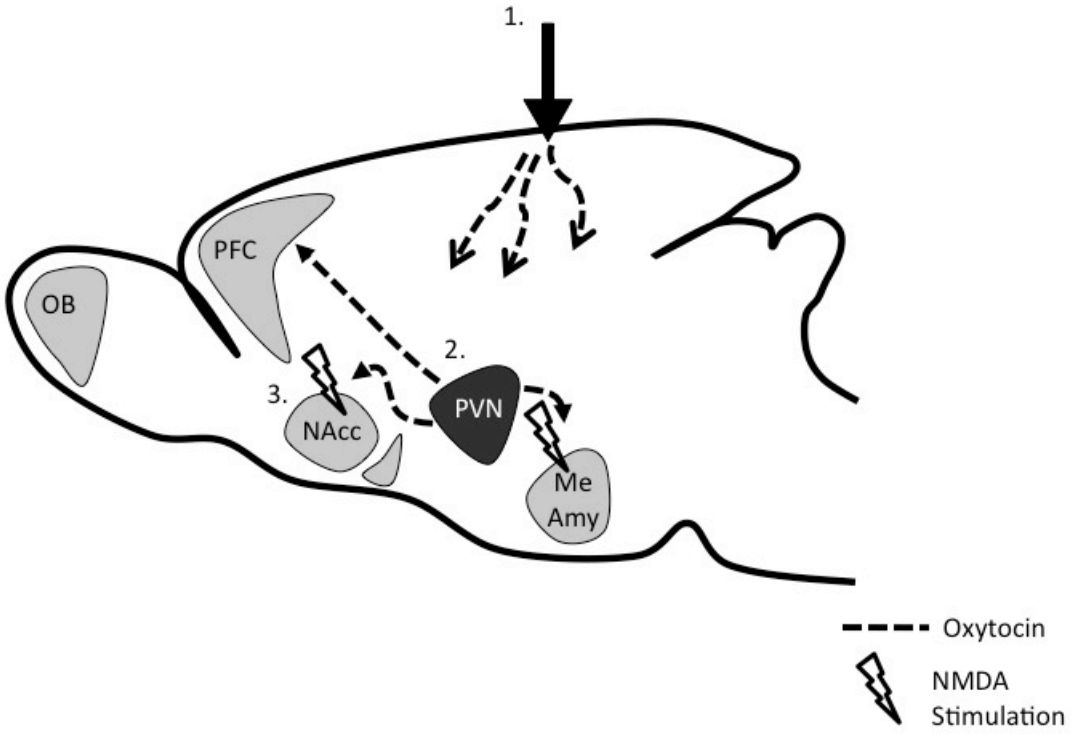

Figure 2. Alternate routes of enhancing prosocial cognition. 1. Central OT administration either through direct injection in rodent models or through IN administration in humans increases circulating brain levels of exogenous OT which must diffuse to distal receptor sites in the prefrontal cortex (PFC), nucleus accumbens (NAcc) and the medial amygdala (Me Amy) to faciliate social cognition. 2. Indirect stimulation of OT producing neurons in the paraventricular nucleus (PVN) through drugs that target either the melanocortin 4 or serotonin 1a receptors induces endogenous OT release specifically into physiologically relevant brain areas (NAcc, Me Amy, PFC) to facilitate social cognition. 3. Alternatively the activity of brains areas that regulate OT-dependent social cognition can be enhanced through administration of an NMDA receptor agonist to facilitate social cofnition. All three routes have been utilized to accelerate partner preference formation and are likely to have similar effects in social learning paradigms in humans based on the predictive validity of the model.

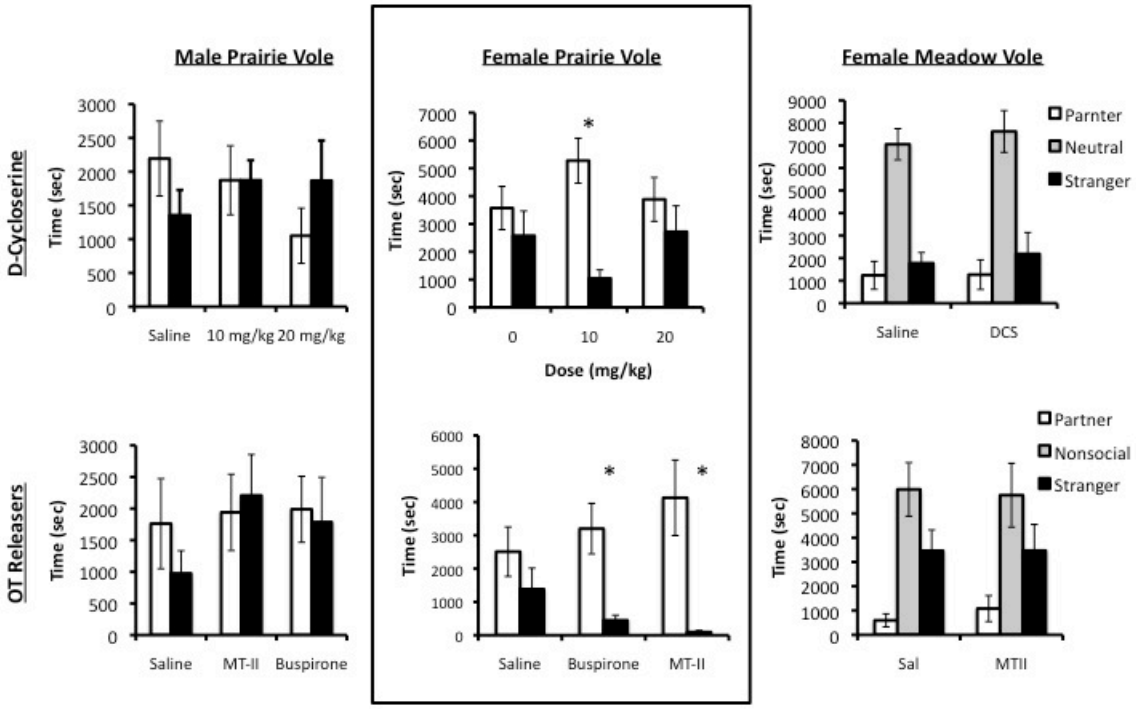

Figure 3. The female prairie voles as a model of functional social cognition. Only the female prairie vole has predictive validity for the development of oxytocin based therapeutics to treat social impairments. Neither male prairie voles nor female meadow voles show an increase in partner preference behavior after treatment with drugs that enhance social behavior through either the OT system (Melanotan II-MTII; Buspirone) or the social brain network (D-cycloserine, DCS). This is likely due to the relatively small role oxytocin plays in the regulation of partner preference formation in either of these two models.

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APPENDIX 1

Oxytocin, vasopressin and social behavior: implications of autism spectrum disorders.

Abstract:

The neuropeptides oxytocin and vasopressin have a long evolutionary history of regulating social and reproductive behaviors in vertebrates. This chapter will cover the involvement of oxytocin and vasopressin in mediating the key components of affiliative behavior: social motivation, social information processing, and social attachment as demonstrated in animal models and supported by human research. Several lines of evidence suggest that disregulation of these neuropeptides systems may contribute to the etiology of the social impairments seen in autism and related disorders. Thus, these neuropeptides may represent viable targets for the treatment of social impairments.

Key Words:

Oxytocin
Vasopressin
Affiliation
Social Behavior
Social Bonding
Social Motivation
Social Recognition
Animal Models
Prairie Voles

Introduction

Elucidation of the basic neurobiological mechanisms underlying social cognition and social behavior is pivotal for understanding the biology mediating normal human social behavior and may also provide valuable insights into the pathophysiology and potential treatment of disorders of social behavior. An extensive literature has implicated the neuropeptides oxytocin and vasopressin as being key modulators of species-typical social behavior. This chapter will review the evidence from animal and human research demonstrating that these neuropeptides modulate several aspects of social cognition and behavior, including social motivation, social information processing, and social attachment.

Oxytocin (OT) and arginine vasopressin (AVP) are evolutionarily conserved molecular modulators of complex social behavior (Donaldson & Young, 2008). These nine-amino acid peptides have been implicated in the regulation of many different aspects of affiliative behavior including social recognition and social memory in rodents, communication, parental care and social bonding (Insel & Young, 2001). Variation in the OT and AVP systems have been linked with both interspecies and individual variation of sociality in animal models which has inspired researchers to look for parallel relationships in human populations. Despite the complexity of human social behavior, OT and AVP have also been linked to trust, altruism, emotional expression and perception and social bonding.

Due to their involvement in social behavior, it has been suggested that disregulation of these systems may contribute to the disruptions in the social domain in autism spectrum disorder (ASD) and other psychiatric disorders characterized by social impairment (Hammock & Young, 2006). Even if alterations in the OT and AVP systems are not involved in the etiology of ASD, pharmacological manipulation of these systems may be a viable strategy for enhancing social cognition and interpersonal relationships in this disorder (Bartz & Hollander, 2006). Social impairments are the most defining characteristic of ASD. In clinical populations social

impairments are seen as gaze aversion, limited affective expression, improper coordination of gestures with speech and impairment in interpreting these non-verbal cues in others (Bartz & Hollander, 2006) Animal models have shown that inhibition of the OT and AVP systems, either through genetic or pharmacological manipulation, results in impairments of social investigation, recognition and motivation that are analogous to the deficits seen in individuals with autism. To understand the nature of the complex relationship between these neuropeptides and social behaviors numerous studies have looked at their effect on three facets of affiliative behavior: social motivation, social information processing, and social attachment.

This chapter will look at the role OT, AVP and their related systems play in mediating the core components of social behavior. Their involvement in both normal social behavior and how their disregulation leads to disturbances in behavior will be examined. Parallels will be drawn between the neurobiological findings in animal models and the genetic and imaging studies done in both typical and clinical human populations. Finally the direct evidence implicating a role for these neuropeptide systems in autism spectrum disorders will be presented with a discussion of how these findings may shape the development of novel pharmacological therapies for treating social impairment.

Oxytocin and Vasopressin: The Social Peptides

Oxytocin

OT is a nine amino acid peptide (nonapeptide) that is synthesized in the magnocellular neurons of the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus and transported to the posterior pituitary where it is stored and secreted into systemic circulation (Gimpl & Fahrenholz, 2001). Peripherally, OT acts as a reproductive hormone to facilitate uterine contractions during parturition and milk letdown during lactation. OT is released during orgasm and ejaculation in humans (Carmichael et al., 1987). OT functions centrally as a neuromodulator, acting at discrete receptor sites throughout the brain on a relatively slow time scale, mediating

socio-sexual behaviors (Landgraf & Neumann, 2004). In the brain, oxytocin is released both axonally and dendritically from the hypothalamic oxytocinergic neurons targeting a number of distant regions, including the septal region, nucleus accumbens, hippocampus, amygdala, medio-basal hypothalamus, and even the brains stem and spinal cord in rodents (Burbach, Young, & Russell, 2006). While the innervation of oxytocin fibers is conserved among species, OT receptor (OTR) distribution is highly species-specific, with significant inter- and intraspecies variation that may contribute to the variation in social behavior (Burbach et al., 2006).

Vasopressin

Arginine vasopressin (AVP) is an evolutionarily homologous molecule that differs from the structure of OT at only two amino acids positions (See Figure 1). Like OT, AVP is also a neurohypophyseal peptide that it is synthesized in the magnocellular neurons of the PVN and SON and is released from the posterior pituitary into peripheral circulation. Vasopressin is also released within the brain from neuronal projections arising from the parvocellular neurons of the PVN, the superchiasmatic nucleus, the bed nucleus of the stria terminalis (BNST) and the medial amygdala (MeA) (De Vries & Miller, 1998). The vasopressinergic neurons in the BNST and MeA are regulated by androgens, making the AVP system one of the most sexually dimorphic neurotransmitter systems (De Vries & Miller, 1998). Within specific brain regions, like the BNST and MeA, males have significantly more AVP than do females (Van Leeuwn, Caffe, & De Vries, 1985). Peripherally, AVP plays an important role in maintaining fluid homeostasis and blood pressure. AVP acts at three different receptor subtypes: vasopressin receptor 1a (V1aR), vasopressin receptor 1b (V1bR) and vasopressin receptor 2 (V2R). While all three receptors types are expressed in varying densities in both the brain and the periphery, V1aR is the most widely distributed AVP receptor in the brain and is thought to be the most behaviorally relevant. The V1b receptor is expressed primarily in the pituitary and some restricted brain regions and the V2 receptor is expressed primarily in the kidney where it regulates water balance (Caldwell,

Wersinger, & Young, 2008). The similarity in structure of the AVP and OT molecules enables each to bind and activate the other's receptor, creating a degree of redundancy in these systems, which complicates the interpretation of experiments using these peptides.

Evolutionary Conservation of Neuropeptide Structure and Social Function

The OT and AVP family of neuropeptides have been inextricably linked with social behaviors throughout evolutionary history. Homologues of these peptides have been identified in animals as diverse as hydra, worms, insects, fish, amphibians, birds and mammals and have been shown to regulate social and reproductive behaviors (Donaldson & Young, 2008). The genes encoding these two molecules are thought to be originally derived from a single common ancestral gene through a gene duplication event early in vertebrate evolution. Arginine vasotocin (AVT), an evolutionary antecedent of AVP, is present in all non-mammalian vertebrates examined to date, and may have been modulating social and reproductive behaviors as early as the Precambrian era almost 600 million years ago (Acher, 1995). It is thought that the gene encoding AVT underwent duplication in the evolution of early fish approximately 450 million years ago, resulting in a second oxytocin-like peptide, isotocin that is still present in bony fish. In male goldfish (*Carassius auratus*), both isotocin and vasotocin modulate social approach and levels of these peptides have been associated with levels of sociality (Thompson & Walton, 2004). Isotocin was replaced by mesotocin during the water to land transition and is currently present in amphibians, reptiles and birds. Mesotocin has been shown to regulate aggression differentially in songbirds, based on the social organization of the species (Goodson, 1998). OT is present in all utherian and protherian mammals and despite the evolutionary divergence still only differs from AVT by one amino acid. AVP also arose from a single peptide substitution in AVT in placental mammals. The conservation of the structure and function of these neuropeptides throughout evolution is quite remarkable and the conservation of regulation of socio-sexual behaviors in

animals from goldfish to rodents suggests that OT and AVP may also play a role in regulating human social behavior.

Neuropeptides and their Role in Mammalian Social Behavior

Social behavior is a complex term encompassing several different aspects of interaction between conspecifics. To better understand the complexities of social behavior, the social domain can be parsed into discrete, identifiable and most importantly, analytical components. To be useful for scientific research, each of these components must consist of a specific behavioral phenotype that can be measured in a controlled laboratory setting, and must provide a reflection of the organism's total social repertoire. In both animals and humans, social motivation, social information processing and social attachment have been indentified as subdomains contributing to the complexity social behavior (Figure 2). The study of each of these components independently simplifies the examination of the biological underpinning of complex social behavior. Here we will examine each of these subdomains separately.

Social Motivation

The motivation to interact with conspecifics is the first step needed to engage in a successful social relationship. Inherent in every social encounter is the possibility of a negative outcome, such as indifference or aggression. Thus, there are strong pressures limiting engagement in social interactions. Social motivation is a measure of desire to overcome these negative factors to actively engage in a social interaction. OT is thought to play a role in enhancing social motivation. One potential mechanism for this is related to its anxiolytic actions. OT has been shown to reduce the behavioral and neuroendocrine responses to social stress enabling an animal to approach and interact with conspecifics. Alternatively, OT may alter the valence of social interactions, making them more rewarding and ultimately promoting social interaction.

In Rodents

In animal models, social motivation can be assessed by measuring behaviors that promote social contact, the latency of the subject to approach another individual and the amount of time spent in social contact with the individual. Social motivation can be measured in adult conspecific, offspring-parent and parent-offspring interactions. For example, rodent pups emit ultrasonic vocalizations when separated from their nest or mother to protest social isolation. The role of OT in social motivation was examined using this offspring-parent paradigm through the comparison of vocalizations produced by wild type mice and OT knock-out (OTKO) mice after separation. OTKO mice emit significantly fewer distress vocalizations than their wild type litter mates (Winslow et al., 2000). In a similar reunion task, where pups are separated from their mothers by a divider with small holes that allow the pups cross though but not the mothers, wild type mice quickly learn to cross through holes to reunite with their mothers while the OTKO mice do not. As both the mutant strain and the wild type strain are able to emit normal vocalizations and show similar locomotor activity, the data suggest that the knock out pups may not find social isolation as distressing or make lack the social motivation to engage in these behaviors.

Initiation of parental behavior, though, represents one of the most dramatic shifts in social motivation, as in many rodent species the female shifts from an aversion to, to an attraction to infants. Thus maternal nurturing behavior may be a useful assay for understanding the neurobiology of social motivation. Maternal behavior, which for rodents includes nest building, licking and grooming of pups, and crouching over pups, is induced at the time of labor and parturition (See figure 3). Virgin female rats find pups highly aversive and will avoid or attack them if encountered. Only after the birth of a female's first litter will she be motivated to interact with pups. Though after that moment, for the rest of her life, regardless of reproductive status, she will find all pups highly rewarding and can even be trained to lever press for access to the pups in the same way rats can be trained to work for drugs of abuse (Fleming & Anderson, 1987; Lee, Clancy, & Fleming, 1999). The switch from indifferent to nuturing behavior towards pups is

thought to be mediated in part by OT. Lesions of the hypothalamic PVN prior to parturition, which effectively eliminates the brain oxytocin system, prevents the initiation of maternal behavior (Insel & Harbaugh, 1989). Correspondingly, infusion of OTR antagonists into the ventricles of psuedopregnant female rats, attenuates the expression of maternal behavior (Fahrbach, Morrell, & Pfaff, 1985). Specific infusions of the antagonist directly into the medial preoptic area (MPOA) and ventral tegmental area (VTA) also inhibit maternal behavior indicating that OT in these regions is necessary for the normal expression of maternal behavior (Pedersen, Caldwell, Walkder, Ayers, & Mason, 1994). It was also shown that not only is OT necessary to promote maternal behavior in virgin rodents, but it is sufficient for inducing it. Ventricular injections of OT stimulates maternal behavior in females rats and mice independent of their sexual experience or hormonal state (McCarthy, 1990; Pedersen, Ascher, Monroe, & Prange, 1982).

The hormones of pregnancy are thought to prime the brain's ability to respond to the OT surge experienced at parturition, thus enabling OT to initiate the onset of maternal behavior. OTR expression is increased by changes in estrogen, such that during pregnancy when estrogen levels increase, OTR expression increases in the hypothalamus and preoptic area (Meddle, Bishop, Gkoumassi, Van Leeuwen, & Douglas, 2007). Individual variation in levels of OTR expression in brain regions such as the bed nucleus of the stria terminalis, the amygdala, and the MPOA have been correlated with variation in maternal care (Champagne, Diorio, Sharma, & Meaney, 2001; Francis, Champagne, & Meaney, 2000; Francis, Young, Meaney, & Insel, 2002).

While the switch to maternal care is based on the hormonal changes associated with pregnancy in mice and rats, spontaneous parental behavior exists in other species like the prairie vole (*Microtus orchogaster*). Prairie voles are highly social, monogamous rodents that display both biparental and alloparental behavior. Female prairie voles display spontaneous juvenile parental behavior in which non-reproductively active adolescent voles provide care for successive sibling litters

(Solomon, 1991). This parental behavior recedes in approximately 50% of females once they reach sexual maturity. Spontaneous alloparental behavior is also thought to be OT dependent, as injections of OTR antagonist directly into the nucleus accumbens, an area of the brain that mediates reward and reinforcement, blocks the expression in adult nulliparious females (Olazabal & Young, 2006a). The expression of spontaneous alloparental behavior in both juvenile and adult prairie voles is positively correlated with the density of OTR binding in the nucleus accumbens (See Figure 4) (Olazabal & Young, 2006a, 2006b). Juvenile females with high levels of OTR binding show more time spent crouching over pups than those with low levels (Olazabal & Young, 2006b). Viral vector upregulation of OTR in the nucleus accumbens of adult female prairie voles, though, is not sufficient to increase the expression of spontaneous care however it is sufficient to accelerate social bond formation (Ross et al., 2009). This suggests that the developmental influence of OTR in the nucleus accumbens, as opposed to simply receptor number in adulthood, may regulate this behavior.

As a biparental species, male prairie voles are also highly motivated to care for pups, unlike mice, rats and non-monogamous meadow voles. In males, it is thought that parental behavior is mediated by the AVP system. Injection of a V1aR antagonist prevents the expression of paternal care. Infusions of AVP into the lateral septum of male prairie voles increases time spent crouching over and licking and grooming pups (Wang, Ferris, & De Vries, 1994). Similar to the relationship between OTR levels in females and maternal behavior patterns, expression patterns of the V1aR corresponds to paternal behavior, in the form of licking and grooming of pups (Hammock & Young, 2006).

In Humans

Reduced social motivation is one of the defining and most debilitating features of ASD. The expression of social withdrawal is a dimension of diagnostic instruments, like the Aberrant Behavior Checklist. This makes characterization of the neurobiological mechanisms underlying

this behavior central to developing novel treatment strategies for autism. Normal social motivation in humans, like in rodents, has been studied in mother infant interactions. It has been suggested, though not directly shown, that OT influences some aspects of human maternal behavior. OT is released peripherally during the initiation of labor, the early post partum period and breast-feeding in human mothers (Carmichael et al., 1987; McNeilly, Robinson, Houston, & Howie, 1983; Nissen, Lilja, Widstrom, & Unvas-Moberg, 1995; Vasicka, Kumaresan, Han, & Kumaresan, 1978). About of third of women also see a spike in peripheral OT levels late in their third trimester of pregnancy, which interestingly has been correlated with higher ratings of maternal-infant bonding (Levine, Zagoory-Sharon, Feldman, & Weller, 2007). Maternal behavior in humans, though, is not entirely mediated by hormonal changes as spontaneous parental behavior can be seen in families with adopted children. Despite this correlational evidence, precise role of OT in modulating maternal responsiveness in humans remains unknown.

Social Information Processing

After the initiation of interaction, social recognition and social memory are critical for stable functioning in social groups. Social memory, which enables an individual to distinguish between family, friends and foes and behave accordingly, is dependent on the processing of social information. The social impairments of autism are associated with deficits in face identity recognition and facial expression perception (Schultz, 2005). The ability to recognize individuals in animal models, referred to as social recognition, has been used to examine the neural mechanisms underlying social memory and social information processing.

In Rodents

Humans and other non-human primates primarily depend on visual and auditory cues to recognize conspecifics. Rodents, however, while employing similar mechanisms for discrimination, use an alternative modality; relying on olfactory and phermonal cues detect and recognize familiar individuals. Research into this field has shown that both OT and AVP are

involved in the encoding and recall of olfactory-cued social memories. Social recognition can be modeled experimentally in rodents by utilizing their natural interest in novelty. Rats and mice will generally spend more time investigating a novel object or animal than a familiar one. To experimentally assay social recognition, an animal is allowed to investigate a conspecific for a period of time, thus becoming familiar with the animal. The animal is then re-exposed to either the same familiar animal or a novel animal and the amount of time the test animal spends sniffing the stimulus animal is measured (Ferguson, Young, Hearn, Insel, & Winslow, 2000). Typically over multiple exposures to the same animal the amount of investigatory time will decrease and this is interpreted as recognition of the stimulus animal. If a novel animal is then introduced, the test animal should return to their original levels of investigation. Manipulations that disrupt social recognition will be reflected in a constant investigation time over multiple trials. OT enhances social recognition. OT infused into the olfactory bulbs (OB), lateral septum (LS) and medial preoptic area (MPOA) of male rats prolongs the duration of the social recognition response (Dluzen, Muraoka, Engelmann, & Landgraf, 1998). The generation of oxytocin knock-out (OTKO) mice has provided an eloquent model for assessing the role of OT in social memory. Male OTKO mice are unable to recognize familiar conspecifics following a prolonged encounter (Ferguson et al., 2000). In OTKO mice, a novel female stimulus mouse elicits the same amount of olfactory investigatory behavior, as does a familiar mouse after multiple exposures, whereas control mice show a decrease in olfactory investigation of familiar individuals (See Figure 5). This deficit does not represent an impairment in either non-social sensory or memory processes, as the OTKO mice are able to discriminate between non-social scents. This suggests that the impairments of OTKO mice are specific to the recognition of socially important cues (Ferguson et al., 2000). Anatomical studies of c-Fos expression, a marker of neuronal activity, show that OTKO mice have decreased activation in the medial amygdala following a social encounter (See Figure 6). Furthermore, OT injection into the medial amygdala (MeA), but not into the olfactory bulb of OTKO mice restores social recognition. Complementarily, application of an OTR

antagonist only to the MeA inhibits social recognition in wildtype mice (Ferguson, Aldagm, Insel, & Young, 2001). The rescue effect of OT in OTKO mice is only effective if it is injected prior to initial encounter. This suggests OT may function in the processing of social information or memory acquisition rather than in social memory recall.

The AVP system has also been shown to play a significant role in mediating social recognition. Intracerebroventricular injection of AVP prolongs social recognition by activating the V1aR in the lateral septum (LS) and in the OB. Site-specific injection of AVP into the LS enhances social recognition, while V1aR antagonist administration inhibits social recognition (Dantzer, Koob, Bluthe, & Moal, 1988). Down regulation of the V1aR through the use of antisense oligonucleuotides reduces social recognition in normal rats (Landgraf et al., 1995). Conversely, up regulation of the V1aR using viral vector gene transfer enhances the duration of the animals' social recognition memory. Recently, the V1b receptors have also been implicated in mediating social recognition. Both V1aR and V1bR knock-out mice show impairments in social memory. Characterization of the deficits in social recognition of V1bRKO mice, though, suggests they are more mild than in the V1aRKO and may be primarily due to deficits of social motivation. The mice can differentiate between male and female urine, but do not spend more time investigating the scent of the opposite sexed animal, thus behaving in a highly species atypical fashion. Thus they can discriminate the odors but are not motivated to further investigate scents of socio-sexual importance (Caldwell et al., 2008).

In Humans

The significant body of evidence linking OT with social recognition in rodents has spurred a number of studies looking at the effects of OT on face processing in humans. A number of caveats exist in translating research on social behavior in rodents to humans, primarily related to how social information is differentially processed between the species. Social cognition in humans involves far more cortical processes than in rodents. Human social behavior is based on

an integration of the visual and auditory systems with executive cortical regions, while in rodents the sensory systems are primarily connected to the limbic system. It is possible that the neuropeptides play a strong modulatory role in mediating olfactory or limbic responses and consequently may have less of an effect on human social behavior (Hammock & Young, 2006). However, it is likely that OT and AVP modulate the circuitry involved in processing of visual and auditory social information in humans analogously to its role in rodents.

Research into the function of OT and AVP in the brains of humans is in its infancy and is limited in the types of experiments that can be undertaken. As a result scientists are just coming to understand the role of neuropeptides in human social cognition. Currently, the most effective technique for studying the direct functional effects of the neuropeptides in the brain is through intranasal administration of the peptides. OT and AVP have low penetrance across the blood-brain barrier and consequently have limited efficacy when administered peripherally. However, it is thought that inhaled peptides are transported directly across the nasal mucosa to the cerebrospinal fluid, into the brain parenchyma thus allowing them to have central effects (Bartz & Hollander, 2008). To date, two studies have physiologically demonstrated the efficacy of this route of administration. In humans, AVP was shown to increase in CSF within ten minutes after intranasal administration (Born et al., 2002). In the second study, an increase in OT in the cerebrospinal fluid of rhesus monkeys was shown after intranasal administration of the peptide (Ebitz, Watson, & Platt, 2008).

Several recent studies have linked the functional effects of the neuropeptides with cognitive processes associated with social recognition and social information processing. Intranasal OT has been shown to enhance face recognition and interpretation of facial expression (Domes, Heinrichs, Michel, Berger, & Herpertz, 2007; Rimmele, Hediger, Heinrichs, & Klaver, 2009). OT administration preferentially enhanced memory of faces over memory of non-social stimuli (Rimmele et al., 2009). OT may also play a role in social memory consolidation as it has been

shown to enhance memory for previously seen faces when administered after viewing the faces (Savaskan, Ernhardt, Schulz, Walter, & Schachinger, 2008). OT has even been shown to enhance emotional perception by as seen in a test of "mind reading", a paradigm which requires individuals to infer the mental states of others by looking at images of only the eye region (Domes et al., 2007). These findings are likely due to OT's ability to enhance facial and emotional recognition by increasing the amount of time an individual spends looking at the eye region of a conspecific (Guastella, Mitchell, & Mathews, 2008). OT also regulates social stimuli perception by modulating amygdala function in humans. Functional imagining studies have shown that strong amygdala activation is induced by fear inducing visual stimuli. Administration of intranasal OT potently reduces the activation of the amygdala in response to these stimuli (Kirsch et al., 2005). Interestingly, fMRI studies have shown that application of intranasal OT specifically decreases the activation of the right amygdala typically associated with emotional facial recognition (Domes et al., 2007).

Individuals with ASD appear to have deficits in social recognition that that are reminiscent to those seen in OTKO mice. Individuals with autism can identify faces in relation to basic objects, however they do show impairments when asked to match unfamiliar faces on tests like the Benton Face Recognition Test (Barton, 2003; Davies, Bishop, Manstead, & Tantam, 1994). They also show deficits in perceiving facial expression, age and sex (Hobson, 1987; Tantam, Monaghan, Nicholson, & Stirling, 1989). Patients with Turner's syndrome, which has a high co-morbidity with autism, also show deficits in recognizing faces and facial expressions (Lawrence et al., 2003). This deficit may in part be attributed to decreased activation in the amygdala and the fusiform face area, the areas typically involved in face recognition, and increased activation of cortical regions when viewing images of human faces compared to normal subjects (Critchley et al., 2000; Pierce, Müller, Ambrose, Allen, & Courchesne, 2001; Schultz et al., 2000). Individuals with autism also fail to show amygdala activation typically seen in control subjects, when asked

to interpret the expressions viewing human eye regions (Baron-Cohen et al., 1999). Interestingly, OTKO mice show a similar pattern of decreased amygdala activation and increased cortical recruitment in social recognition tasks (Ferguson et al., 2001).

Currently only a limited number of studies have looked at the effects of AVP on human social information processing. Intranasal AVP modulates the perception of facial expression in a sexually dimorphic fashion. In animals, AVP modulates social communication, particularly the production of social signals involved in courtship and aggression. In human males, intranasal AVP modulates facial expressions in response to social stimuli. AVP administration in men decreases the perception of friendliness in unfamiliar male faces and stimulates the production of antagonistic facial expressions. However in females, the opposite effect is observed; AVP increases the perception of friendliness in female faces and stimulates affiliative facial motor patterns in response to those faces (Thompson, George, Walton, Orr, & Benson, 2006). It is possible that this dimorphic effect is due to the opposing strategies adopted by men and women to handle stressful social situations.

Social Attachment

Social bonding is a complex social behavior that requires social motivation and social information processing to develop an enduring attachment. A social bond is apparent when individuals demonstrate a preference for interaction with a specific conspecific. Impairments in social motivation and social information processing in individuals with autism likely contribute to difficulty in forming normal social relationships. The study of social attachment in animal models allows for the investigation of the neurobiological mechanisms underlying the attachment process and may be relevant to many types of social relationships. Two animal models have contributed significantly to our understanding of the neurobiological mechanisms underlying attachment, pair bonding in the prairie vole and maternal-infant bonding in sheep. Each of these models is discussed in detail below.

In Voles

The genus *Microtus* (voles) is an ideal model for the comparative study of social attachment as it contains both monogamous (prairie and pine voles) and polygamous (meadow and montane voles) species. Prairie and pine voles are highly social, monogamous animals that form long lasting social bonds with a specific partner. Contrastingly, meadow and montane voles are asocial and polygamous, with males and females coming together only to mate. One difference between these species that contributes to their highly divergent social systems is the organization of their OT and AVP systems.

Because of the important role that OT plays in mother-infant relationships, the peptide was hypothesized to mediate pair bonding in prairie voles. OT infusions into the brains of female prairie voles induces pair bonds as measured by the development of a partner preference (See Figure 7) under conditions non-conducive to bond formation (Williams, Harbough, & Carter, 1994). Complementarily, application of an OTR antagonist intracerebroventricularly prevents the formation social bonds even after long co-habitation periods with mating (Williams et al., 1994). Central OT receptor manipulation was able to directionally modulate social bond formation without having any impact on sexual behavior. These data suggest that the species differences in the ability to form a social bond between mates may be attributed to differences in the OT system. The distribution of OT fibers throughout the brain is well conserved throughout mammalian species and both prairie and meadow voles have similar patterns of OT in the brain. There is, however, striking variation in the distribution of the OTR between the prairie and the meadow vole. Prairie voles have a significantly higher density of OTR in the caudate putamen and the nucleus accumbens than do the polygamous species. Application of OTR antagonists directly to the nucleus accumbens inhibits mating induced partner preference, implicating the accumbens particularly as a site necessary pair bonding (Young, Lim, Gingrich, & Insel, 2001).

In males, OT plays a lesser role in pair bond formation (Liu, Curtis, & Wang, 2001). Instead AVP has primarily been implicated in mediating pair bonding. AVP's effect on male pair bonding parallels the role of OT in females, in that centrally infused AVP facilitates partner preference (without mating) while a V1aR antagonist inhibits mating induced partner preference (Winslow, Hastings, Carter, Harbaugh, & Insel, 1993). Like OT, vasopressin innervation is consistent across mammalian species with dramatic differences in receptor densities apparently mediating inter and intra-species variation (Wang, Zhou, Hulihan, & Insel, 1996). Considerable variation in the distribution of V1aR in the brain is seen between prairie and meadow voles, with higher densities of the receptor in the ventral pallidum in the monogamous voles. The ventral pallidum is a major efferent of the nucleus accumbens, and like the accumbens, mediates reward and reinforcement. Interestingly, the difference in distribution between monogamous and polygamous species of voles is mirrored in other organisms that have similar social organizations of other species of rodents and even in some non-human primates. For example, the monogamous marmoset has higher densities of V1aR in the ventral forebrain reward areas than do non-monogamous rhesus macaques (See Figure 8) (Young, 1999). Despite the variation in receptor densities between the two microtine species, there are no species differences in the binding kinetics or second messenger coupling of the V1aR between the motane and prairie voles and the coding region of the V1aR gene (*avpr1a*) is 99% genetically identical (Insel, Wang, & Ferris, 1994; Young, Nilsen, Waymire, MacGregor, & Insel, 1999). This implicated the promoter region of the gene encoding the V1aR (*avpr1a*) in the differential expression of the receptor and the subsequent differences in both paternal care and social bonding. Transgenic mice containing 2.2kb of the 5' regulatory region and coding region of prairie vole *avpr1* show a receptor expression profile very similar to that of prairie voles (Young et al., 1999). Furthermore, if these mice are given an injection of AVP they showed an increase in affiliative behavior. This led to comparison of the regulatory regions between prairie voles and montane voles. The prairie vole promoter region at the *avpr1a* locus contains a microsatellite element that consists of a repetitive

di and tetranucleutide sequence between 720 and 1,150 bp upstream of the transcriptional start site that is largely absent from the meadow vole. Within prairie voles there is also great degree of variation in the density of V1aR binding in specific brains regions, including the olfactory bulb, extended amygdala, thalamus, cingulated cortex and the ventral pallidum. Furthermore, there is significant variation in the length of the repetitive microsatellite among individual prairie voles. To determine whether variation in the microsatellite contributed to variation in V1aR expression and social behavior, two lines of prairie voles were generated, one with a long microsatellite repeat and one with the short repeat (Hammock & Young, 2005). The resulting animals had robust differences in V1aR binding in the brain, with the long-alleled animals showing greater binding in olfactory bulb and the lateral septum but with lower levels in the amygdala and the hippocampus than the short-alleled animals. Long-alleled male prairie voles also displayed a shorter latency to approach and investigate a novel juvenile male and developed partner preferences more quickly than short-alleled animals (Hammock & Young, 2005). These data suggest that the microsatellite polymorphism in the 5' regulatory region of the *avpr1a* gene alters the expression of the receptor throughout the brain and that the distribution of the receptor influences social behavior. The relationship between receptor density in the ventral pallidum and prosocial behavior was further tested by over-expressing the V1aR in the ventral pallidum of non-monogamous meadow voles. Upregulation of the V1aR in the ventral pallidum enhances partner preference in prairie voles and can even induce partner preferences in non-monogamous meadow voles (See Figure 9) (Lim, Murphy, & Young, 2004).

In Sheep

Maternal-infant bonding in sheep has been used as a model to identify the mechanisms involved in social attachment as well. Unlike rodents, which are typically promiscuously maternal, ewes form a strong selective bond for their own lamb. Sheep live in large social groups and are constantly moving while they graze. They are synchronous breeders, which means many young

are born in the herd at the same time, and they birth precocial young who are able to stand and run shortly after birth. These features require that ewes form a strong selective bond shortly after birth to prevent the lamb from being lost (See Figure 10) (Kendrick et al., 1997). Bond formation, like maternal behavior, in ewes is induced by the hormonal changes associated with pregnancy and the physiological feedback associated with labor and delivery. Ewes become maternal immediately after birth, and within a couple of hours postpartum they become selectively maternal towards one lamb and will only allow their own lamb to nurse. Vagino-cerival stimulation (VCS) can induce an ewe to accept an unfamiliar lamb even after she has bonded with her own lamb. Epidural anesthesia prevents VCS from inducing maternal bonding (Levy, Kendrick, Keverne, Piketty, & Poindron, 1992). OT infusion alone induces acceptance of an unfamiliar lamb even in a non-pregnant ewe (Kendrick, Keverne, & Baldwin, 1987). It is thought that in sheep VCS, which occurs naturally during parturition, sends sensory signals through the spinal cord to the PVN, which then releases large amounts of OT into the circulation and throughout the brain. OT release in limbic structures facilitates the onset of maternal nurturing behavior, while OT infusion in the olfactory bulb induces a reorganization resulting in selective responsiveness to their own lamb's odor. The specific odor of the lamb is encoded through plasticity changes in the olfactory bulb that are permitted by OT and mediated by the neurotransmitters GABA and glutamate (Kendrick et al., 1997). In humans, selective kin recognition is primarily mediated by visual input, but remarkably it has also been reported that women are able to recognize the smell of their infant within hours of giving birth suggesting a potentially conserved mechanism (Porter, Cernock, & McLaughlin, 1983).

In Humans

Preliminary research in humans is consistent with the animal studies mentioned above and suggests that the OT and AVP systems may also be involved in human social attachments. Functional magnetic resonance imaging has shown there is a high degree of overlap between the

distribution of OT receptors in the human brain and the regions of brain activation when viewing images of romantic partners or mothers viewing images of their infants (Bartels & Zeki, 2004). Plasma levels of OT have also been shown to increase in both males and females during sexual activity and intercourse (Blaicher et al., 1999; Carmichael et al., 1987; Uvnas-Moberg, 1998). As the most intense form of social contact, sexual intercourse is also likely to be the stimulus for OT release in the brain (Neumann, 2008). Women with high levels of OT report non-sexual tactile interaction between partners, like massage or hugging, at greater frequency than those with lower levels (Light, Grewen, & Amico, 2005). Perceived partner support has also been associated with higher levels of OT in both men and women (Grewen, Girdler, Amico, & Light, 2005). Conversely negative social experiences, like early life abuse or neglect, result in decreased levels of OT in the CSF. OT levels are negatively correlated with the number and duration of abuses (Heim et al., 2008).

A negative relationship was associations between reports on relationship quality and levels of OT. Basal plasma levels of OT were negatively correlated with living situations, marriage quality, physical affection and communication (Taylor et al., 2006; Turner, Altemus, Enos, Cooper, & McGuienss, 1999). However, it should be noted that plasma levels of neuropeptide should be interpreted with caution since peptides are released into the periphery and into the brain independently, and circulating peptides do not cross the blood-brain barrier.

A recent study demonstrated an association between a microsatellite element in the 5' flanking region of the human *avpr1a* gene, similar to the microsatellite seen in prairie voles, and several traits indicative of pair bonding behavior in men. One particular allele was associated with marital status, perceived marital problems and even marital quality as perceived by their spouses. As would be predicted by the animal research, no association was found between women and this locus (Walum et al., 2008). This study provides remarkable evidence of a conserved mechanism regulating social cognition in rodents and man.

In humans, both neuropeptides may also promote interpersonal relationships by increasing perceptions of trust and altruism. The aforementioned microsatellite polymorphism of the human *avpr1a* gene has also been linked to prosocial behavior. Individuals with the short allele (308-325bp) are less generous in the Dictator game, an economic game that looks whether subjects make altruistic decisions on money sharing that deviate from simple profit maximization, than those with the long allele (Knafo et al., 2008). In a similar game, intranasal OT was found to promote trust behavior, in that subjects that had received the peptide shared more money with their social partner than those that had not, even after being cheated by their social partner (Kosfeld, Heinrichs, Zak, Fischbacher, & Fehr, 2005).

Evidence for Disregulation of Neuropeptides Systems in Autism Spectrum Disorders

Genetic Evidence

It is clear that OT and AVP play a central role in the regulation of complex social behaviors. This coupled with the high heritability of autism has led many researchers to look at the possible link between mutations of the neuropeptides systems and ASD. To date several genetic association studies have implicated the OTR and V1aR in the etiology of autism. The human OTR is encoded by a 19 kbp gene located on chromosome 3p25 containing three introns and four exons (Inoue et al., 1994). Two genome wide scans have highlighted the 3p25 region as a linkage site for ASD (Lauritsen et al., 2006; McCauley et al., 2005). Four studies to date have found a positive association of the OTR gene with autism in three distinct populations. A study of the Chinese Han trios demonstrated an association between ASD and two single nucleotide polymorphisms (rs2254298 and rs53576) and several haplotypes involving one of the sites (Wu et al., 2005). A second study by Jacob et al. (Jacob et al., 2007) looked at whether these associations replicated in a Caucasian population and verified the association at the rs2254298 site. The two studies found overtransmission of opposing alleles in the affected population, the G allele in the Caucasian population and the A allele in the Chinese population. A third

comprehensive association study was undertaken by Israel et al. (Israel et al., 2008) of an Israeli population looking at all of the known labeled SNPs across the OTR gene region and found a strong association between a five locus haplotype block including the site found in both previous studies and ASD. This group also showed an association with IQ and daily living skills with SNPs in the OTR gene, suggesting this gene may play a role in other disorders and non-clinical populations (Israel et al., 2008). These findings reinforced the work done by Lerer et al. (Lerer et al., 2008) which showed similar association of ASD with specific loci in the OXTR gene in the Israeli population, as well as associations with IQ and the Vineland Adaptive Behavior Scales, a clinical measure of personal and social skills. The association of the OXTR gene with ASD found in aforementioned targeted studies, was also seen by Yrigollen et al. in a study of genes associated with affiliative behavior as candidate genes for autism (Yrigollen et al., 2008).

Single subject cases have also implicated the OTR gene in the etiology of autism. A unique case study of a nine-year old boy with pervasive developmental disorder was found to have a duplication of the chromosome region containing the OTR gene resulting in a two-threefold increase of OTR expression compared to control subjects (Bittel, Kibiryeva, Dasouki, Knoll, & Butler, 2006). The oxytocin gene has also been implicated in autism in a case study in which a child with Asperger's syndrome was found to have a 1.1Mb deletion of 20p13 a region with involves ~27 genes including the OT peptide gene (Bittel et al., 2006; Sebat et al., 2007).

Associations have also been made between the *avpr1a* gene and autism. In humans, the *avpr1a* gene located on chromosome 12q14-15, contains three microsatellite regions in the 5' flanking and one in the single intron, analogous to but different from the vole polymorphisms. Two microsatellites, RS1 and RS3, have been the focus of much investigation due to their highly polymorphic number of repeats. Three independent groups have found a possible link between polymorphisms in the *avpr1a* gene and autism. Studies have shown linkage disequilibrium between autism and the RS3 microsatellite in the *avpr1a* gene (Kim, 2001; Wassink et al., 2004;

Yirmiya et al., 2006). The microsatellites in humans, like in voles, have been shown to have functional consequences. The number of microsatellite repeats in both the RS1 and RS3 sites is shown to have an effect on amygdala activity in response to fear stimuli. The long allele of the RS3 site is associated with extremely high levels of amygdala activation in response to the stimuli (Meyer-Lindenberg et al., 2008), which is particularly interesting as autistic individuals have been shown to have abnormal amygdala activation in fMRI studies (Dalton et al., 2005). The long RS3 allele has also been associated with higher levels of the *avpr1a* mRNA in human post mortem hippocampal tissue than the short RS3 allele, similar to what is seen in the vole literature (Knafo et al., 2008). Despite these numerous studies, there is no evidence that genetic variation in the OTR or V1aR genes are a major contributor to ASD. Rather, they suggest that variation in these genes may be one of many contributing factors in some small fraction of ASD cases.

Peripheral Evidence

Children with autism have been observed to have lower levels of peripheral OT in plasma than age matched controls. Within the control group levels of OT were found to be positively correlated with measures of social behavior including socialization, social coping and interpersonal relationships. Unexpectedly, in the autistic group the lower baseline levels of OT were negatively correlated with these same measures of social behavior (Modahl et al., 1998). This difference could be the result of differential processing of OT in the brains of children with autism, as plasma samples of autistic children were also found to have higher levels of OT precursors as well as a higher ratio of OT precursor to OT (Green et al., 2001). These findings were replicated in a second independent study that also found autistic children to have lower peripheral levels of vasopressin (Al-Ayadhi, 2005). In adults the findings appear to be reversed as individuals with ASD have higher plasma levels of OT compared to controls (Jansen et al., 2006). There is no apparent reason to account for the discrepancy in findings between the groups suggesting there may be a developmental compensatory mechanism. However, it is important to

note that peripheral levels of oxytocin and vasopressin do not necessarily reflect central levels (Bartz & Hollander, 2006).

Implications for developing pharmacological therapies

Given the role of OT and AVP in modulating social motivation, social information processing, and social attachment, these systems may be viable targets for treating the social deficits associated with ASD. Indeed, some very preliminary studies have suggested that OT infusion may have positive effects in ASD patients, but more work is needed to draw any conclusions. Hollander et al. (Hollander et al., 2003) investigated the role of OT in repetitive behavior in a double-blind placebo controlled cross-over study in which Pitocin was intravenously administered to adults with ASD, in which it was seen that OT reduces the repetitive behaviors associated with autism over time. A similar procedure was used to look at the effect of OT on social cognition as assayed by a test of affective speech comprehension. Subjects who received the peptide prior to the first trial were shown to have enhanced retention of speech comprehension two weeks later compared to those who received it prior to the second trial. The study did, however, fail to observe a direct effect of OT on performance. Collectively these studies suggest that OT may have a therapeutic value for treating two of the three primary deficits of autism. One point of concern, though, is the issue of peripheral administration and brain penetration. Endogenous plasma OT is unable to efficiently cross the blood brain barrier (Landgraf & Neumann, 2004), which calls into question how intravenously administered OT can have a direct effect on behavior. It is hypothesized that high levels of exogenous administration allows for low levels of OT to be transported across the blood-brain barrier. As an alternative, intranasal OT has been proposed as a route of administration, as it has been used in many of the human behavioral studies. However, due to the relative inefficiency in the transmission of peripherally administered OT and the unknown efficacy of intranasaly delivered OT, the development of small molecule OTR agonists that more easily cross the blood brain barrier is

needed to explore the viability of peripherally administered OT drugs for the treatment of social deficits in autism.

Conclusions

OT and AVP are important regulators of complex social behaviors. These neuropeptides modulate three major components of social interaction: social motivation, social information processing and social attachment. Individuals with ASD show impairments in each of these three sub-domains, with reduced social interest, reduced perception of non-verbal social cues and reduced ability to form reciprocal social relationships. Social motivation to care for infants in many species is dependent on central release of OT. The proper processing of social information necessary for social recognition requires OT and AVP neurotransmission. Social attachment in monogamous species and the mother-infant bond are modulated by these neuropeptides. Even in humans it appears that OT system may mediate complex social behaviors such as attachment and interpersonal trust.

It is important to note that while this chapter has focused on the effects of OT and AVP directly, it is possible that disregulation in these systems may stem from other interacting molecules. Recently, the CD38 gene has been shown to play a role in mediating social behavior by regulating the release of OT. CD38 knock-out (CD38KO) mice show impaired maternal behavior and deficits in social recognition similar to those seen in OTRKO mice (Jin et al., 2007). Thus deficits in social behavior that might be dependent on neuropeptides systems may have both direct and indirect causes. Genetic associations studies have linked an array of genes related to synapse formation and development, like those for neurexins and neuroligins to autism. It is possible that these mutated genes may indirectly interfere with social behavior circuits mediated by neuropeptides, resulting in impairments similar to those seen by direct manipulation of the OT and AVP system.

The extensive evidence both in animal models and in human behavioral studies demonstrates the importance of continued research into the role OT and AVP and its potential role in the social impairments of autism. While it is unlikely that any of the genes in the neuropeptides systems are a monogenic cause of the disorder, it is a definite possibility that disregulation of the system may be underlying the at least a subset of the impairments associated with it. Regardless of whether there is a direct role for these neuropeptides in the etiology of autism, these neuropeptide systems should be considered as potential pharmacological targets for enhancing the social cognitive domain in ASD. Further work must be done to investigate both the basic neurobiological mechanisms of autism spectrum disorders and potential treatments that target those mechanisms and thus far, it seems that both the OT and AVP systems are viable targets for both.

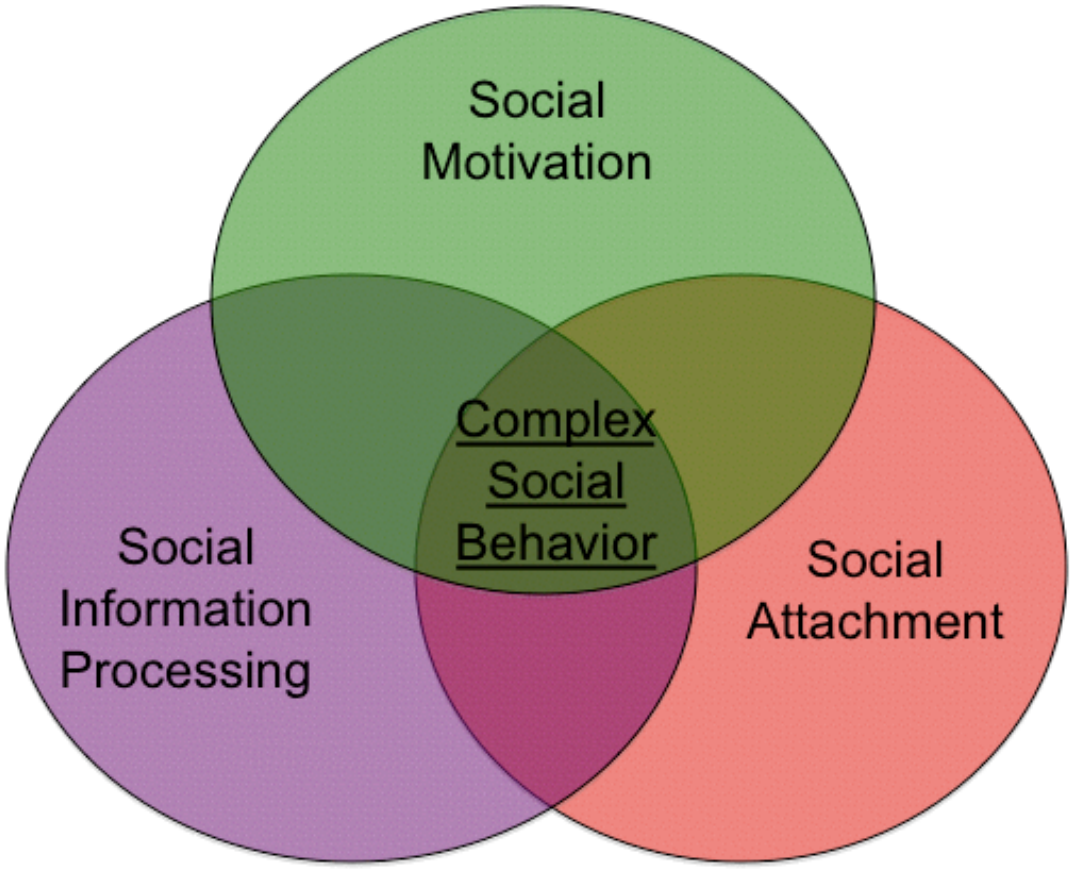

Figure 1. A conceptual model of complex social behavior. Social behavior is a broad term that encompasses any interaction between two conspecifics. To be able to study the biological mechanisms governing this behavior, it must be broken down into discrete subgroups of behavior that can be measured in a laboratory. Affiliative social behavior is comprised of three subdomains: social motivation, social information processing and social attachment. Each of these domains contributes to the complexity of social behavior through separate but converging systems. The study of each of these components independently allows for the design and implementation of experiments that can more directly address the mechanisms. Social motivation can be studied through experiments of maternal motivation, social information processing through social recognition paradigms and social attachment through pair bonding or maternal bonding.

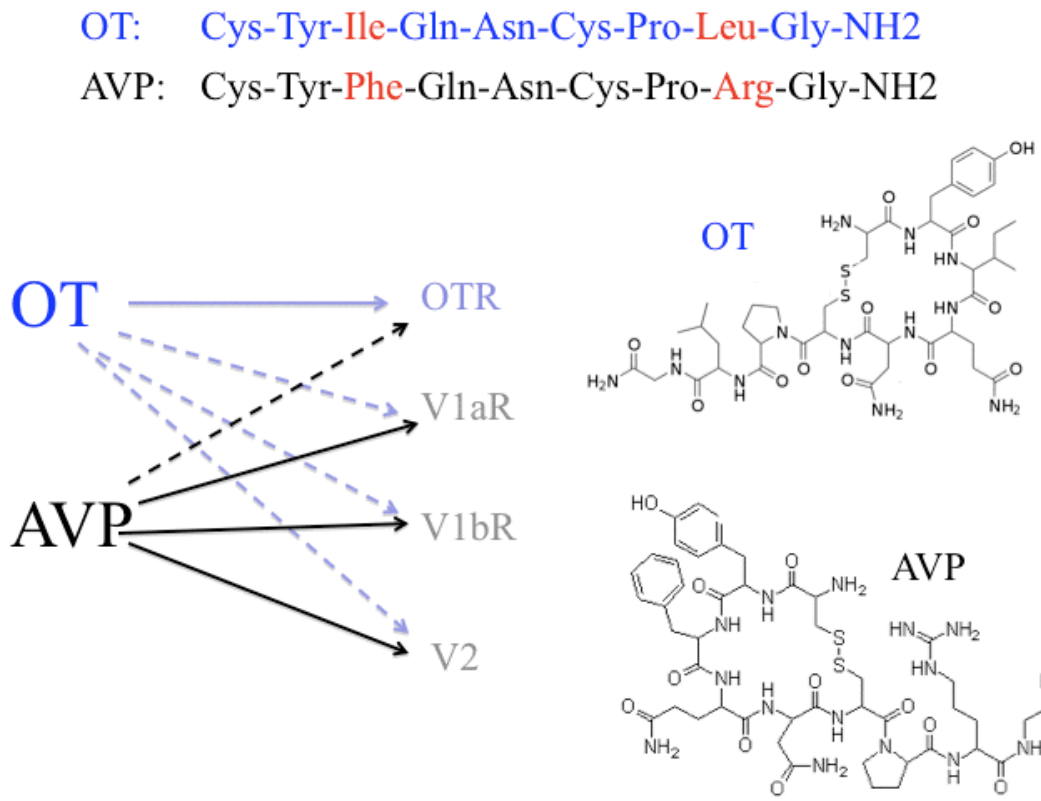

Figure 2. Oxytocin and Vasopressin. OT (oxytocin) and AVP (vasopressin) are closely related nine amino acid peptides. OT has only one known receptor, while AVP binds to three known receptors AVP-R1a, AVP-R1b and AVP-R2. However, as the peptides differ at only two amino acids there is some binding of each peptide at the other's receptor(s). Thus there is some degree of cross-reactivity of both endogenous peptides and in pharmacological studies.

Figure 3. Maternal Behavior as a Model for Social Motivation. Maternal behavior for rodents, including rats, consists of nest building (upper left panel), pup retrieval (upper right panel), crouched nursing (lower left panel), and licking and grooming (lower right panel). Female rats only express maternal behavior after giving birth to their first litter of pups; prior to this experience female rats are indifferent towards pups. This switch in behavior demonstrates the extremes of social motivation.

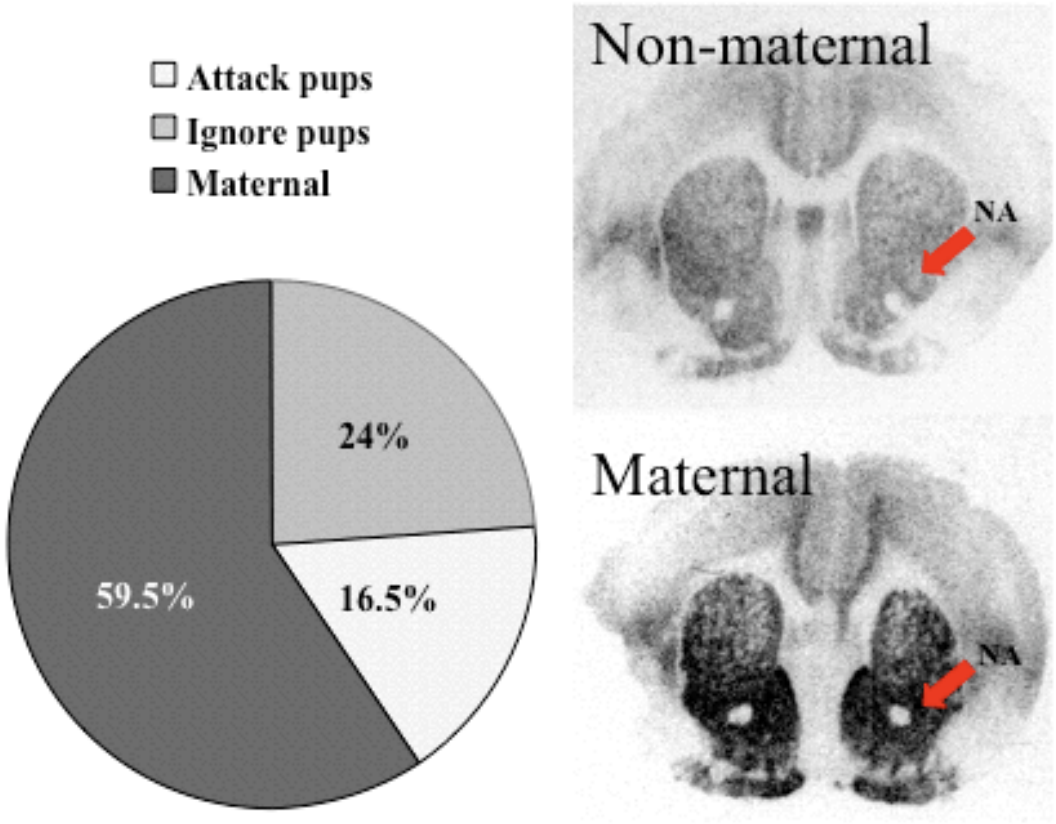

Figure 4. Variation in Maternal Behavior in Prairie Voles. Unlike mice or rats, about half of all virgin female prairie voles show spontaneous alloparental behavior. The expression of this behavior is positively correlated with the density of OTR in the nucleus accumbens (NA). Autoradiographic binding of brain sections shows high levels of receptors in maternal animals (lower panel). *Adapted from (Olazabal & Young, 2006a).*

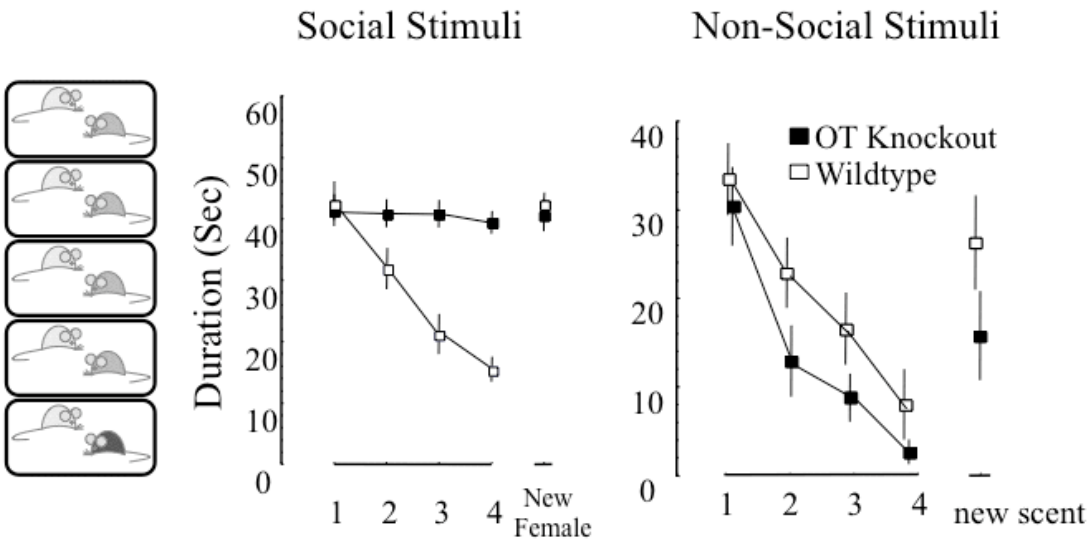

Figure 5. Social amnesia in OTKO mice. In wild-type mice, repeated exposure to the same stimulus animal results in decreased duration of olfactory investigation. When presented with a novel stimulus animal, the amount of olfactory investigation returns to the same levels as seen during the first trial of investigation of the original animal. In the OTKO mice there is no decrease in investigation of the stimulus animal over multiple trials. In olfactory investigation of a non-social, citrus scent, though, both the wild-type and the OTKO mice both show decreased investigation of the odor, indicating that the deficits of the OTKO mice are specific to the processing of social stimuli. *Reprinted from (Ferguson et al., 2000).*

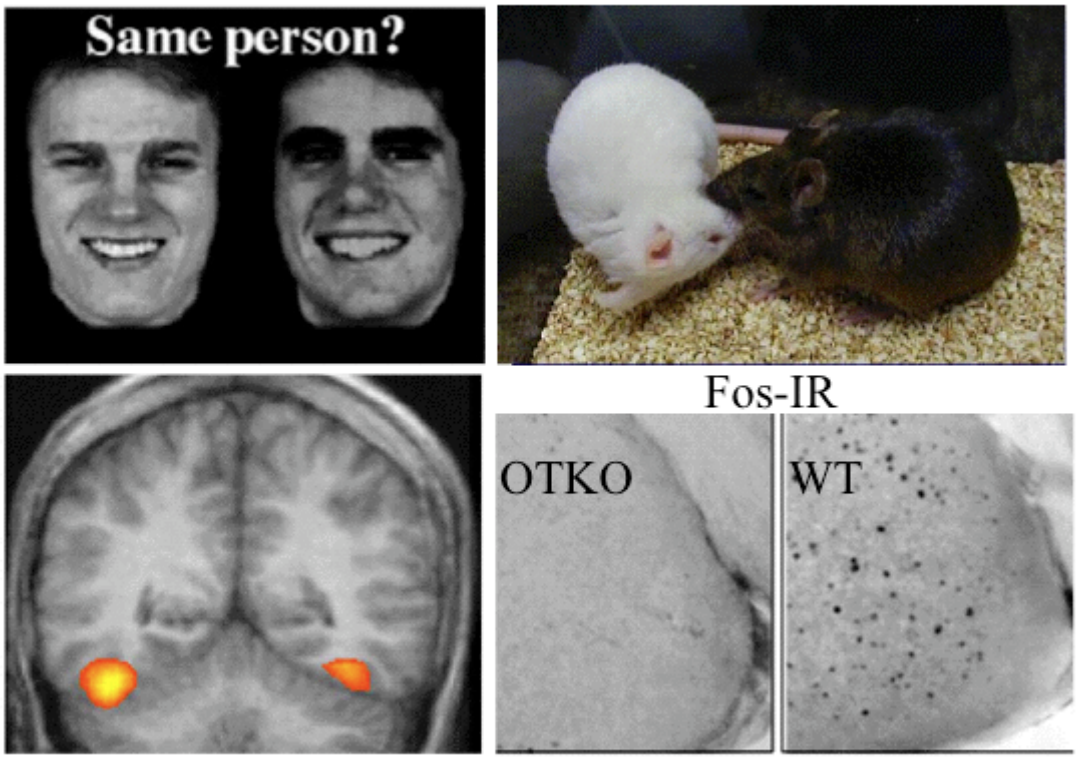

Figure 6. Social Information Processing in Humans and Rodents. Individuals with autism show reductions in the activation of the fusiform face area, a structure regulated by amygdala activation, as measured by fMRI, when viewing human faces compared to controls (Left Panel). Analogously, OTKO mice show reduced activity of the medial amygdala in response to olfactory investigation, as evidenced by the expression of c-Fos (a neuronal marker of activity), compared with wild-type mice (Right Panel). *Adapted from (Schultz, 2005) and (Ferguson et al., 2001).*

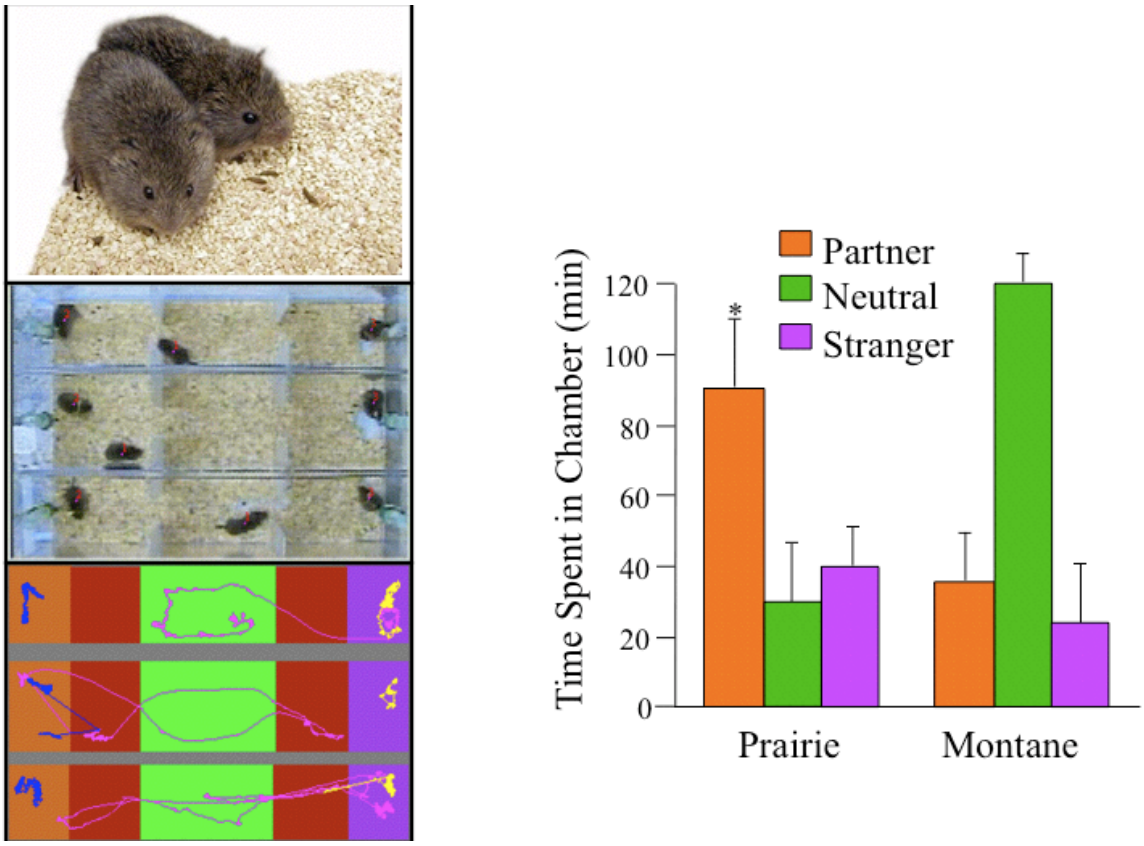

Figure 7. Partner Preference Test. Social attachment in prairie voles is measured using the partner preference test. In this behavioral test a male is paired with an unfamiliar female vole and allowed to co-habitate for a period of time (Upper Panel). The pair is then separated and the partner female is tethered in the partner preference apparatus along with a stranger female. The male is then placed in the apparatus and allowed to wander freely for three hours (Middle Panel). The amount of time the male spends with either the partner female or the stranger female is recorded and analyzed using behavior analysis software (Lower Panel). If the male is shown to have spent twice as much time with he partner female than with the stranger female, he is said to have formed a partner preference. Partner preference is a marker of social attachment. Prairie voles easily form a partner preference after about 24 hours of cohabitation. Montane voles, however, fail to show a preference for their partner after as much as two weeks of cohabitation.

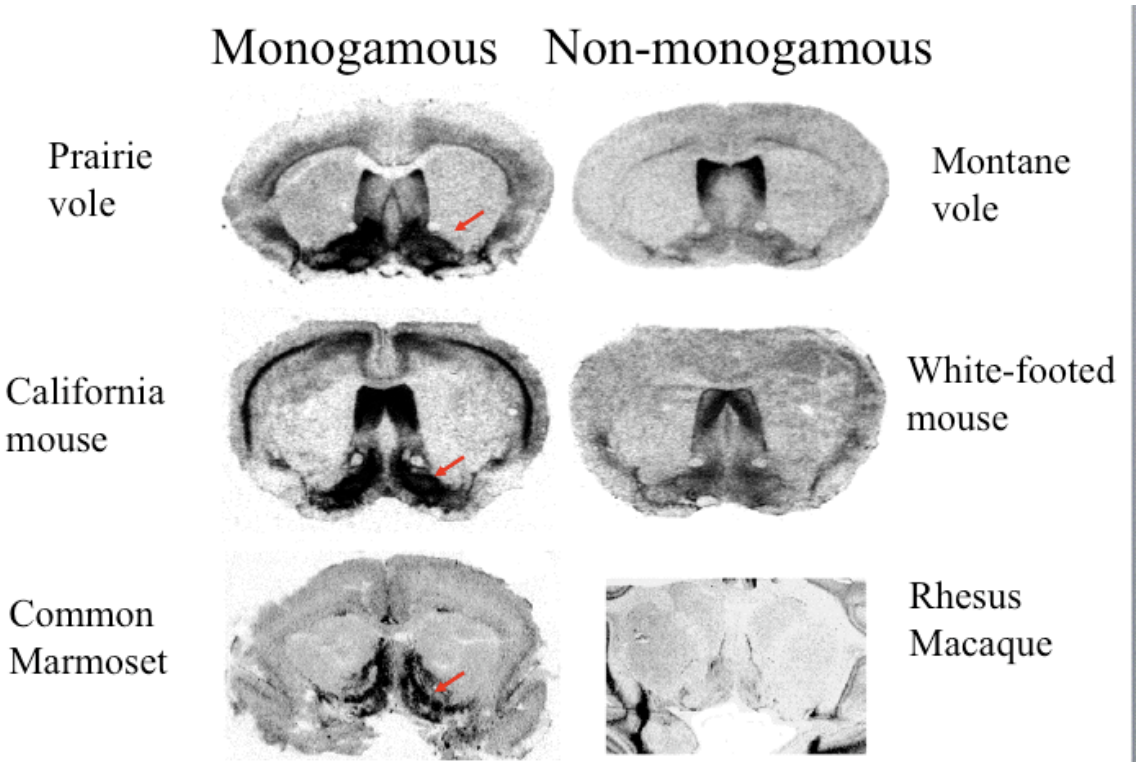

Figure 8. Distribution of V1aR in Monogamous and Non-Monogamous Species. The pattern of higher V1aR expression in the ventral pallidum of the prairie vole than in the montane vole is mirrored in several other species, including the monogamous California mouse and the closely related promiscuous white-footed mouse. This pattern is even seen in some primate species with the monogamous marmoset showing higher levels of V1aR in the ventral forebrain than the non-monogamous rhesus macaque. *Reprinted from (Young, 1999).*

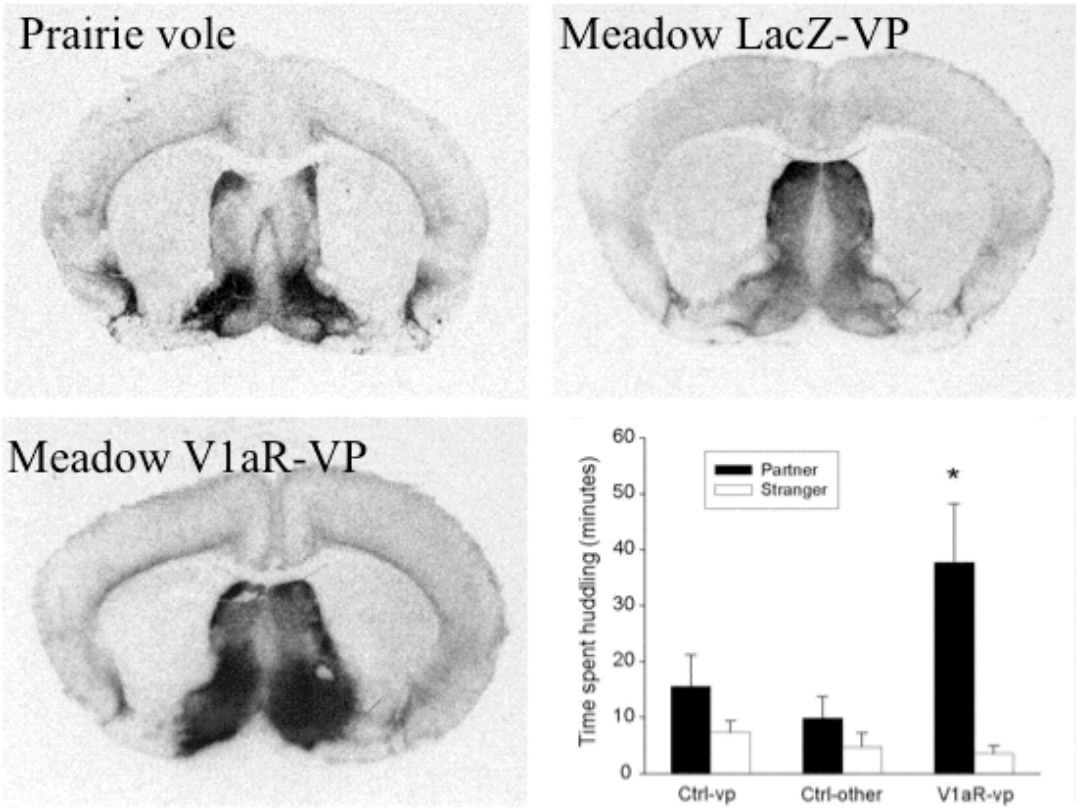

Figure 9. V1aR Gene Transfer in Meadow Voles. The monogamous male prairie vole shows high expression of the V1aR in the ventral pallidum (Upper Left Panel), which corresponds to high levels of partner preference. To induce the ability to form social attachments in the polygamous male meadow vole the V1aR was upregulated by the injection of a viral vector containing the *avpr1a* gene. When injected into a specific brain region the viral vector induces the production of its gene product, in this case causing the increased expression of the V1aR (Lower Left Panel). Upregulation of the V1aR but not a control molecule, LacZ (Upper Right Panel), induced partner preference in the male meadow vole (Lower Right Panel). *Reprinted from (Lim, Wang et al., 2004).*

Figure 10. Maternal Infant Bonding in Sheep. Maternal bonding in sheep is a model of social attachment. Ewes form a strong selective bond specific for their own lamb soon after parturition. It is particularly important that the ewe learn to recognize their lamb, as sheep are synchronous breeders that give birth to precocial young, which prevents kin identification by proximity. The formation of this selective bond is dependent on OT. *Photo courtesy of Keith Kendrick*

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