Progesterone regulation of vasopressin-dependent social behavior

by

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# Table of Contents

ACKNOWLEDGEMENTS.............................................................................................................. iii

ABSTRACT....................................................................................................................................... v

Chapter 1: Introduction .................................................................................................................. 1
  Introduction .................................................................................................................................. 2
  Social recognition paradigms ....................................................................................................... 3
  Two exposure task ...................................................................................................................... 3
  Discrimination task ..................................................................................................................... 4
  Habituation-dishabituation ........................................................................................................ 5
  Social recognition and AVP ....................................................................................................... 5
  Vasopressin system .................................................................................................................... 7
  Progesterone ............................................................................................................................. 9
  Progesterone in males ................................................................................................................. 9
  Distribution of PR in the male brain .......................................................................................... 10
  AVP and progesterone ............................................................................................................... 10

Chapter 2: Progesterone Impairs Social Recognition in Male Rats ............................................. 16
  Abstract ..................................................................................................................................... 18
  Introduction ............................................................................................................................... 18
  Methods ..................................................................................................................................... 20
  Animals ....................................................................................................................................... 20
  Drug treatments ......................................................................................................................... 20
  Behavioral testing and Statistical analyses .............................................................................. 20
  Experiment 1 - Habituation-Dishabitation, RU-486 .................................................................... 21
  Experiments 2 and 3 - Social Discrimination, RU486, R5020 .................................................. 21
  Experiment 4 - Olfactory tests, Preputial preference test .......................................................... 22
  Experiment 4 - Olfactory tests, Food-finding test ..................................................................... 23
  Experiment 5 - Object recognition test ...................................................................................... 23
  Results ....................................................................................................................................... 24
  Experiment 1 - Habituation-Dishabitation: RU-486 ................................................................. 24
  Experiment 2 - Social discrimination: RU-486 .......................................................................... 27
  Experiment 3 - Social discrimination: R5020 ........................................................................... 27
  Experiment 4 - Olfactory tests ................................................................................................. 27
  Experiment 5 - Object recognition: Progesterone vs. oil ......................................................... 28
  Discussion .................................................................................................................................. 33
  References .................................................................................................................................. 38

Chapter 3: Vasopressin infusion into the lateral septum of adult male rats rescues a progesterone induced impairment in social recognition ......................................................... 41
  Abstract ...................................................................................................................................... 42
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ABSTRACT

Progesterone regulation of vasopressin-dependent social behavior

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Compared to females, little is known about the effect of progesterone in the brain and on the behavior of males. It has been shown that progesterone treatment reduces vasopressin (AVP) in the bed nucleus of the stria terminalis (BST) and medial amygdala (MeA) of adult male rats, which means that progesterone might affect AVP-linked behaviors like social recognition.

We found that progesterone treatment impaired recognition in both the habituation-dishabituation and social discrimination paradigms and that this effect was blocked by pretreatment with the progestin receptor (PR) antagonist RU-486. We have also seen an impairment in social recognition with the synthetic progestin R5020, which only binds to PRs. Furthermore, progesterone appears to interfere with the formation of social recognition memory without interfering with general non-spatial memory or olfactory functioning. We believe that progesterone is influencing social recognition through the AVP system in the BST and MeA, probably via PR that are co-localized with AVP immunoreactive neurons. Additional evidence for the involvement of the AVP system comes from our finding that when AVP was infused into the lateral septum (a projection site of the BST and MeA) of rats treated with progesterone, they exhibited normal social discrimination, suggesting that AVP treatment rescued the impairment in social recognition caused by progesterone. Our results provide a strong link between the previous data from our lab showing that progesterone reduces AVP expression in the bed nucleus of the stria.
terminalis (BST) and medial amygdala (MeA), and the functional significance of this reduction: an impairment in social recognition behavior in adult male rats.
CHAPTER 1

Introduction

The content in the following chapter was written by myself with the help of my dissertation advisor, Dr. Catherine Auger.
**Introduction**

Social recognition allows animals to distinguish friend from foe and family member from stranger. Long-term social memories that last for days, weeks or months are necessary for kin recognition, pair-bond formation, and dominance hierarchies and appear to rely on extended social exposure (Ferguson et al., 2002). Also, an animal that can’t distinguish between a member of the same sex and a member of the opposite sex will have limited reproductive potential. Short-term social recognition is a way to differentiate between familiar, previously encountered conspecific individuals and unfamiliar ones (Ferguson et al., 2002). Different species use different senses for recognition. Humans mainly use vision to identify each other and there is evidence that different areas of the brain are responsible for recognition of faces as opposed to recognition of other objects. People with prosopagnosia, a selective impairment in the ability to recognize familiar faces, have lesions in the ventral occipitotemporal cortex that are usually bilateral (Haxby et al., 2002). It is interesting to note, however, that the amygdala (a brain region known to be important in rodent social behavior) is believed to make important contributions to higher-level social cognition, especially for aspects that rely on getting social information from faces (Adolphs, 1999) and the perception of emotion in another's face elicits activity in the amygdala (Haxby et al., 2002). Unlike humans, most other mammalian species recognize conspecifics through olfactory cues (Ferguson et al., 2002).

The information an individual gathers from the constituents of a conspecific’s olfactory signature is essential for an animal’s ability to recognize another individual. There is a large body of evidence to support the role of the neuropeptide vasopressin (AVP) in short-term social recognition (Ferguson et al., 2002), as AVP agonists enhance and antagonists block social recognition. Previous data from our lab indicate that the hormone progesterone can influence AVP in areas of the male
brain known to be associated with social recognition; in this dissertation I will examine how progesterone regulates this AVP-linked behavior, as well as how progesterone may be regulating AVP to influence this behavior. In this introduction I will start by discussing social recognition and the main behavioral paradigms used to study it. I will then cover the role of AVP in social recognition and provide details about the AVP system in the rat brain. Finally, I will discuss progesterone and progestin receptors (PRs) in the male brain and previous data concerning the interaction between progesterone and the AVP system.

**Social recognition**

As mentioned before, social recognition in rats relies on olfactory information. Though most researchers score any investigatory behavior that an adult directs toward a juvenile, close anogenital contact through licking or sniffing is the primary way in which animals obtain social identity information about conspecifics (Popik et al., 1991). This suggests that the odor cues that animals use in making a social recognition decision are non-volatile pheromone signals produced in the anogenital region (Carr et al., 1976). Additionally, a study in which the primary organ for detecting pheromonal information, the vomeronasal organ (VNO), was removed animals failed to socially recognize juvenile conspecifics (Bluthe and Dantzer, 1993). The VNO sends projections to the accessory olfactory bulb, which in turn projects to the BST and MeA (Bluthe and Dantzer, 1993; Ferguson et al., 2002).

**Social recognition paradigms**

*Two exposure task*

There are several different accepted paradigms for the study of short-term social recognition and they all involve the natural tendency of rats and mice to intensely investigate novel
individuals (Ferguson et al., 2002). The first social recognition paradigm developed involves the simple dual exposure of an intruder (usually a 20-35 day old juvenile) to a resident adult (Thor and Holloway, 1982). The amount of time the resident adult spends in olfactory investigation of the intruder juvenile is recorded during both the first and second 5 minute exposures (Thor and Holloway, 1982). The resident male is highly motivated to investigate the unfamiliar individual during the first trial, but on the second encounter the resident will not investigate the intruder individual as much; the reduction in investigation of a previously encountered animal is indicative of social recognition of that animal (Thor and Holloway, 1981). In male rats, if the interval between the exposure to the first juvenile and the re-exposure to that individual juvenile exceeds 30-60 minutes, the adult will not recognize the juvenile during the second trial (Thor and Holloway, 1982), and the adult will investigate this animal as if it had never encountered it before. This type of short-term memory is distinct from the long-term memory necessary for remembering mates and kin for weeks or months and probably also involves different neural mechanisms (Ferguson et al., 2002).

Discrimination task

Engelmann et al. developed a social discrimination paradigm based on the original Thor and Holloway social recognition test (Engelmann et al., 1995). The first trial of the social discrimination paradigm is identical that of the simple dual exposure paradigm (Engelmann et al., 1995). In the second trial of the social discrimination test, however, the adult is exposed to two juveniles simultaneously and is allowed to investigate them both (Engelmann et al., 1995). One juvenile is the same juvenile from trial one, while the other is a novel juvenile. The adult typically displays a reduced level of investigation of the familiar juvenile in trial two and a high level of investigation toward the novel juvenile. This is believed to indicate that the adult has discriminated between the
familiar and unfamiliar individuals and recognition has occurred (Engelmann et al., 1995). The social discrimination paradigm is one of the social recognition tests used in our lab. Another variant of the social recognition paradigm that we perform in our lab is a multi-trial task.

*Habituation-dishabituation*

In this task, social recognition is indicated by a steady reduction in investigation of a same-sex juvenile intruder by an adult resident rat over four short successive encounters with the same juvenile (Winslow and Camacho, 1995). Each trial is separated by a ten minute intertrial interval. The paradigm ends with a fifth trial in which the adult is exposed to a novel juvenile, to ensure that the decrease in investigation is not due to decreased exploratory drive (Winslow and Camacho, 1995). Again, the amount of time a rat spends investigating the stimulus rat is recorded in every trial, and this amount of time typically decreases steadily with each successive trial. When a novel stimulus animal is presented to the adult after the successive exposures to the "familiar" juvenile, the adult is highly motivated to investigate this novel intruder. In terms of outcome, this test is a combination of the simple two trial test and the juvenile discrimination test. That is, the adult will display a decrease in the amount of time it investigates the stimulus juvenile as it becomes more familiar with it over successive trials (outcome in trial two of the two-trial task), and when the adult is exposed to a novel juvenile in the last trial its motivation to investigate this animal is greatly increased (similar to the outcome in trial two of the discrimination task).

*Social recognition and AVP*

The role that neuropeptides play in the regulation of social recognition behavior has been investigated for over 30 years. The subcutaneous injection of AVP facilitates social recognition, even when the period between the first exposure to a juvenile and the second exposure is lengthened to 2 hours (Dantzer et al., 1987). Social recognition behavior is impaired by infusions of
AVP antagonists and is enhanced by infusions of AVP agonists into the lateral septum (LS), one of the projection sites of the AVP cells in the BST and MeA (Dantzer et al., 1988). It has also been shown that castration, which depletes AVP in the BST and MeA and the projections from these cells, blocks social recognition (Bluthe et al., 1990). Additionally, bilateral injection of an AVP receptor 1a (V1aR) antagonist into the LS of wild-type mice impairs social recognition in the habituation-dishabituation paradigm, while bilateral injection of V1aR antagonist into the medial amygdala (MeA) in wild-type mice does not affect social recognition (Bielsky et al., 2005). These studies outline the basic importance of AVP in social recognition.

Experiments in knock-out (KO) mice have been conducted to examine the role of specific AVP receptors in social recognition and other social behaviors. Bielsky et al. (2005) demonstrated that the V1aR is necessary and sufficient for normal social recognition (Bielsky et al., 2005). Additionally, V1aR re-expression in the LS with a viral vector completely rescued the impairment in social recognition in V1aRKO mice (Bielsky et al., 2005). In a two-trial social recognition paradigm, the V1aR over-expression enhanced social recognition memory over intertrial intervals of 2, 6 and 24 hours (Bielsky et al., 2005). Contrastingly, animals treated with a control vector could only successfully recognize a familiar female after an intertrial interval of 0.5 hours but not at 2, 6 or 24 hours (Bielsky et al., 2005). However, Wersinger et al. (2007) found that V1aRKO mice displayed normal social recognition (Wersinger et al., 2007). The V1aRKO mice did, however, have increased latency to find a hidden cookie, which indicates reduced main olfactory ability (Wersinger et al., 2007). They also had disrupted habituation/dishabituation to a series of different olfactory cues and reduced attack latency in the resident-intruder aggression test (Wersinger et al., 2007). These results, particularly the social recognition data, are surprising in light of V1aR antagonist studies (Wersinger et al., 2007).
This same group found that AVP 1b receptor (V1bR) KO mice display significantly reduced aggression and impaired social recognition (Wersinger et al., 2002). However, the V1bRKO mice did not show impairments in behavioral tests of olfaction, vision or other sensorimotor functions, implying that the aforementioned behavior effects were not due to a global deficit in sensory or motor function (Wersinger et al., 2002). The changes in aggression and social recognition were also not due to impaired olfactory pathways because Fos-like immunoreactivity following a social stimulus was the same in the accessory olfactory bulb, main olfactory bulb, MeA and BST of both V1bRKO and wild-type mice (Wersinger et al., 2002). The data on AVP receptor knock out animals and social recognition highlight the importance of the AVP receptor in modulating this behavior. The site of action in the brain of AVP in regulating social recognition has been examined, and data suggest that AVP receptors in the LS are of particular importance in modulating this behavior.

**Vasopressin system**

Buijs et al. examined intra- and extrahypothalamic pathways and found that brain regions including the PVN, SON, SCN, LS and paraventriculo-supraoptico-neurohypophysial tract were immunoreative for AVP (Buijs et al., 1978). Later autoradiographic studies confirmed the presence of AVP in the LS (Baskin et al., 1983; Van Leeuwen and Wolters, 1983). De Vries and Buijs demonstrated via retrograde tracing and lesions that the AVP fibers in the LS originated in the BST and the amygdala (De Vries and Buijs, 1983). Further study into the anatomy of the AVP system by Caffé et al. confirmed that AVP fibers from the BST projected to the LS and that AVP cells in the MeA project to the LS as well as to the ventral hippocampus (Caffé et al., 1987). Over the years, many experiments have examined the AVP system in the brain because it is believed to be important for various behaviors. AVP-producing cell bodies are located in a number of regions throughout the brain (Figure 1). There are five major cell groups in the brain that contain AVP, the paraventricular...
nucleus (PVN), the supraoptic nucleus (SON) and the suprachiasmatic nucleus (SCN) of the hypothalamus, as well as the bed nucleus of the stria terminalis (BST) and the MeA (Figure 1). The cells in the PVN and SON are primarily involved in physiological maintenance of homeostasis (e.g., thermoregulation and osmoregulation), while the cells of the SCN are implicated in circadian functioning (Ring, 2005). The cells in these three areas are large, neurosecretory neurons called magnocellular cells and are, for the most part, anatomically distinct from other AVP producing cells in the brain (Ring, 2005). The SCN AVP cells project to the medial preoptic area, periventricular area of the PVN, paraventricular nucleus of the thalamus and dorsomedial hypothalamic nucleus, while the AVP neurons in the PVN and SON are the principle sources of AVP to the peripheral system (Ring, 2005).

The AVP cell bodies in the BST and MeA that are of interest in this dissertation are parvocellular cells. They are highly sexually dimorphic; the number of cell bodies in these areas and the density of projections from these cells is considerably higher in males compared to females (De Vries and Panzica, 2006). Additionally, the AVP cells in the BST and MeA are exquisitely responsive to steroids (De Vries et al., 1985). Gonadectomy nearly abolishes the expression of AVP in the cell bodies of the BST and MeA, as well as in the projection sites of these cells (De Vries et al., 1985). Likewise, testosterone treatment restores the expression of AVP in these areas (De Vries et al., 1985). De Vries et al. (1986) also found that treatment with estrogen or estrogen plus dihydrotestosterone (DHT) restored AVP cell labeling in the BST and MeA as well as LS AVP fiber density in castrated adult male Wistar rats to levels seen in rats that received sham surgery (De Vries et al., 1986). By comparison, treatment with just DHT did not restore these cells or AVP fiber density in the LS and similar results were seen in the lateral habenular nucleus and the MeA (De Vries et al., 1986). In light of these results, it appears that estrogen and DHT probably work
synergistically through the estrogen and androgen receptors on the AVP cells in the BST and MeA to maintain AVP expression (Axelson and Leeuwen, 1990; Zhou et al., 1994).

**Progesterone**

*Progesterone in males*

A notable lack of knowledge exists in the understanding of the role of the hormone progesterone in males. The vast body of our knowledge on progesterone and progestin receptor (PR) function comes from studies in females (Priest and Pfaff, 1995; Blaustein, 2008). Although progesterone has always been considered a “female hormone”, adult male rats have circulating levels of progesterone around 1.5 - 2 ng/ml (Auger and Vanzo, 2006; Andersen et al., 2004) compared to a range of 3 - 35 ng/ml in females that is seen throughout the rat estrus cycle (Weisz and Ward, 1980). Also, depending on the type of stressful event encountered, progesterone levels in males can approach 6 ng/ml (Andersen et al., 2004). Recent studies demonstrate that progesterone and its receptor play an important yet understudied role in males.

During development, progesterone/PR seems to be important in the male brain. As early as embryonic day 20, there are a much greater number of PRs expressed in the medial preoptic nucleus of male brain compared to the female brain (Wagner et al., 1998). Studies show that these PRs in developing males are important for sexual differentiation because blocking these receptors reduces the sex difference in the size of certain brain nuclei (Wagner, 2006). Additionally, studies show that the lack of the PR through PR gene deletion can result in alterations in brain physiology and behavior in adult male mice (Schneider et al., 2005). For example, male mice with a targeted disruption of the PR gene (PRKO) show alterations in androgen receptor content within the preoptic area and the BST (Schneider et al., 2005). In addition, male PRKO mice exhibit aggression toward infants, increased sexual behavior and anxiety-related behavior (Schneider et al., 2003).
These data suggest that PRs are important for normal development of the male brain and that progesterone and/or the PR influence the normal development of brain areas associated with social behavior.

Distribution of PR in the male brain

PRs are found in discrete areas of the brain and many of these areas play a large role in social behavior. Areas classically associated with reproduction: the preoptic area and the ventromedial, dorsomedial and arcuate nucleus of the hypothalamus all contain PRs (Guerra-Araiza et al., 2001). In addition, PRs have been reported in the olfactory bulb, frontal cortex, hippocampus, cerebellum and brainstem (Guerra-Araiza et al., 2001). Areas that have been implicated in the neural basis of fear, stress, and anxiety also contain PRs (Walker et al., 2003). For example, the BST contains especially high numbers of PRs and the amygdala also contains PR immunoreactivity (Brinton et al., 2008; Auger and De Vries, 2002). The distinct distribution of PRs suggests that they can have a number of functional implications in the male system.

AVP and progesterone

It has recently been shown that AVP cells in the BST and MeA also contain another transcription factor that is responsive to estrogen, progestin receptors (PR) (Figure 2) (Auger and De Vries, 2002). These receptors are in virtually every AVP cell in the BST and MeA and although this co-localization is not influenced by hormonal status, the PR immunoreactivity in these AVP cells is diminished in intensity in gonadectomized rats compared to gonadectomized rats that had hormonal replacement (Auger and De Vries, 2002). It has since been found that this co-localization has functional implications for AVP regulation in the cells of the BST and MeA (Auger and Vanzo, 2006). Our laboratory found that progesterone treatment resulted in a decrease in AVP
immunoreactive labeling in the cells of the BST and MeA as well as in 2 of the projection sites of these cells, the LS and lateral habenula (LH), which lends support to the notion that the two systems interact (Figure 3) (Auger and Vanzo, 2006). In corroboration of our data, it has been reported that progestin receptor knock-out (PRKO) mice have higher levels of AVP immunoreactivity in the LH than wild type controls (Rood et al., 2008). Rood et al. suggest that the increased AVP immunoreactivity could be the result of a lack of progesterone induced inhibition in adulthood (Rood et al., 2008).

As AVP and social recognition are clearly linked (Ferguson et al., 2002), virtually all the AVP cells in the BST and MeA contain PR (Auger and De Vries, 2002), and progesterone treatment functions to suppresses AVP-immunoreactive expression (Auger and Vanzo, 2006), we set out in this dissertation to examine the effect of progesterone on social recognition, an AVP-dependent behavior, and to determine if the previously observed decrease in AVP-ir is due to decreased AVP mRNA levels in the BST and MeA.
Figure 1. Sagittal section of rat brain showing location of major AVP cell groups and simplified version of the pathway connecting the BST, MeA and lateral septum. Figure modified from DeVries et al. 1985

Figure 2. PR expression (black) in AVP cells (grey). (Auger and DeVries, 2002)

Figure 3. Progesterone reduces AVP cell number in the BST and MeA. (Auger and Vanzo, 2006)
References


CHAPTER 2

Progesterone Impairs Social Recognition in Male Rats.

The experiments in the following chapter were completed by myself. Experimental design, data analysis and conclusions were developed with the help of my dissertation advisor, Dr. Catherine Auger. These results have been published in Hormones and Behavior:

Abstract

The influence of progesterone in the brain and on the behavior of females is fairly well understood. However, less is known about the effect of progesterone in the male system. In male rats, receptors for progesterone are present in virtually all vasopressin (AVP) immunoreactive cells in the bed nucleus of the stria terminalis (BST) and the medial amygdala (MeA). This colocalization functions to regulate AVP expression, as progesterone and/or progestin receptors (PR)s suppress AVP expression in these same extrahypothalamic regions in the brain. These data suggest that progesterone may influence AVP-dependant behavior. While AVP is implicated in numerous behavioral and physiological functions in rodents, AVP appears essential for social recognition of conspecifics. Therefore, we examined the effects of progesterone on social recognition. We report that progesterone plays an important role in modulating social recognition in the male brain, as progesterone treatment lead to a significant impairment of social recognition in male rats. Moreover, progesterone appears to act on PRs to impair social recognition, as progesterone impairment of social recognition is blocked by a PR antagonist, RU-486. Social recognition is also impaired by a specific progestin agonist, R5020. Interestingly, we show that progesterone does not interfere with either general memory or olfactory processes, suggesting that progesterone seems critically important to social recognition memory. These data provide strong evidence that physiological levels of progesterone can have an important impact on social behavior in male rats.
Introduction

There is a notable lack of knowledge about the role of the hormone progesterone in males. The vast body of our knowledge on progesterone and progestin receptor (PR) function comes from studies in females (Priest and Pfaff, 1995; Blaustein, 2008). Although progesterone has always been considered a “female hormone”, adult male rats have circulating levels of progesterone around 1.5 - 2 ng/ml (Auger and Vanzo, 2006; Andersen et al., 2004), compared to a range of 3 - 35 ng/ml in females that is seen throughout the rat estrus cycle (Weisz and Ward, 1980). Also, depending on the type of stressful event encountered, progesterone levels in males can approach 6 ng/ml (Andersen et al., 2004), suggesting a potential functional significance of this hormone in males. Recent studies demonstrate that progesterone and its receptor play an important, yet understudied, role in male behavior and physiology (Wagner, 2006). It is also important to note, that as males have higher levels of steroid receptor coactivators, which enhance steroid hormone action in many brain regions (Bian et al., 2011), it is likely that lower levels of progesterone are sufficient to elicit a physiological response within the male brain.

It has been shown that PRs are found in virtually every AVP-immunoreactive (AVP-ir) cell within the bed nucleus of the stria terminalis (BST) and medial amygdala (MeA) (Auger and De Vries, 2002). Indeed, this co-localization has functional implications for AVP regulation in the BST and MeA cells, as progesterone treatment results in a suppression of AVP-ir labeling within these cells, and two of the projection sites of these cells, the lateral septum (LS) and lateral habenula (LH) (Auger and Vanzo, 2006). Taken together, these data suggest an important role for progesterone regulation of AVP expression; however, it is unclear from these data if progesterone can regulate AVP-dependent behaviors.
One behavior that is linked to AVP in the LS, a site that receives AVP projections from the BST and MeA, is social recognition behavior. Social recognition paradigms capitalize on an animal’s innate motivation to investigate unfamiliar conspecifics (Ferguson et al., 2002). An animal’s ability to recognize a conspecific after an initial exposure typically lasts only 30 minutes; however, subcutaneous injections of AVP in rats and mice can facilitate social recognition by lengthening this social memory to 2 hours after initial exposure to a conspecific (Dantzer et al., 1987; Bielsky and Young, 2004). Social recognition is also enhanced by site-specific infusions of an AVP agonist or impaired by infusions of AVP antagonists directly into the lateral septum (Dantzer et al., 1988). Furthermore, castration, which depletes AVP in the BST and MeA, and in projection sites of these cells, also impairs social recognition (Bluthe et al., 1990). These studies demonstrate the importance of AVP in social recognition. As social recognition behavior is clearly AVP-dependent, and progesterone treatment functions to suppresses AVP-ir expression within the BST and MeA (Auger and Vanzo, 2006), we hypothesized that progesterone treatment would impair social recognition within the male brain.
**Methods**

**Animals**

Adult male Sprague-Dawley rats were bred in our animal facility from breeding stock obtained from Charles River (Charles River Laboratories, Inc., Wilmington, MA). Juvenile male stimulus animals (between 20-30 days old) were purchased directly from Charles River. All animals were group housed in our animal facility, unless otherwise noted, on a 12h light/12h dark cycle with lights off at 11:00 am, and had free access to food and water. This research was approved by the University of Wisconsin Animal Care and Use Committee. Different cohorts of animals were used in each experiment described below unless otherwise noted.

**Drug treatments**

All of the experiments described in this paper involved drug treatment administration via subcutaneous injection. Injections occurred during the “lights on” portion of the light cycle for three consecutive days. RU-486 (Steraloids, Newport R.I.; 5mg) was dissolved in 0.4 mL of vehicle (5% benzyl alcohol, 15% benzyl benzoate, sesame oil, all from Sigma-Aldrich Co., St Louis, MO, USA). Progesterone (Steraloids, Newport R.I.; 1mg) was dissolved in 0.1 mL of sesame oil. R5020 (Perkin Elmer, Boston MA; 20μg) was dissolved in 0.1 mL of sesame oil. 0.4 mL of vehicle was used as a control for RU-486 and 0.1 mL of sesame oil was used as a control for progesterone and R5020.

**Behavioral testing and Statistical analyses**

Testing took place during the “lights off” portion of the light cycle under dim red light in our behavior room. All behavior was digitally recorded, unless otherwise noted, and then analyzed by a trained researcher blind to all treatments using The Observer (Noldus Information Technologies,
Leesburg, VA) behavioral observation software. Sigma Stat 3.5 was used to conduct all statistical analyses. For the habituation-dishabituation study, a two-way repeated measures ANOVA was used to compare treatment by trial. A one-way ANOVA was run on habituation scores, which were calculated by subtracting the amount of investigation in trial 4 from the amount of investigation in trial 5. In the food-finding test, groups were compared using a Student’s t test. Paired t tests were used to statistically analyze all other experiments.

Experiment 1 - Habituation-Dishabituation, RU-486

Adapted from Winslow and Camacho (Winslow and Camacho, 1995). 4 month old male rats (n=23) were pretreated with RU-486 or vehicle, and then 2 hours later treated with progesterone or oil, for 3 days. During the 3 days of injections, the animals were separated from their cage mates and singly housed. On the third day of pretreatment and treatment, animals underwent behavioral testing 4 hours after the last round of injections. Testing occurred in the home cages of the adult male subjects. The test for each subject involved five, 1 minute trials. During the first four trials, the same juvenile male rat was placed in the subject’s cage. On the fifth trial, a novel juvenile male rat was placed in the subject’s cage. A 10 minute intertrial interval occurred between each of the five trials. Adult investigation of the juvenile was scored to include direct contact between the nose of the adult and the body of the juvenile and close (within 1 cm) following behavior. Cage directed behavior was also scored as a measure of basic locomotor and investigatory activity.

Experiments 2 and 3 - Social Discrimination, RU486, R5020

The social discrimination paradigm was adapted from Engelmann et al. (Engelmann et al., 1995). In experiment 2, 3 month old male rats (n=40) received the same pretreatment with RU-486 or
vehicle, and treatment with progesterone or oil as described above in the methods for experiment 1. In experiment 3, a different set of 3 month old male rats (n=40) received injections of progesterone, R5020 or oil for 3 days. Again, during the 3 days of injections, the animals were separated from their cage mates and singly housed. On the third day, injections of progesterone or oil were administered 4 hours before behavioral testing, while injections of R5020 were given 2 hours before behavioral testing, as this time course is when R5020 was empirically found to be the most behaviorally effective. Testing occurred in the home cages of the adult male rats. In trial 1, a male juvenile rat was placed in the home cage of the adult rat and the adult was allowed to freely investigate for 5 minutes. After 5 minutes, the juvenile was removed and the adult was alone in its cage for 30 minutes. After the 30 minute intertrial interval, the juvenile from trial 1 plus a novel juvenile were placed in the adult's cage, and the adult was again free to investigate for 5 minutes. The juvenile rats were distinguishable to the researcher scoring the video by unique tail marks drawn with permanent marker. Adult investigation of the juvenile(s) was scored to include direct contact between the nose of the adult and the body of the juvenile and close following behavior. Cage directed behavior was also scored as a measure of basic locomotor and investigatory activity.

Experiment 4 - Olfactory tests, Preputial preference test

4 month old male rats (n=40) received the same pretreatment with RU-486 or vehicle, and treatment with progesterone or oil as described above in the habituation-dishabituation paradigm. During the 3 days of injections, the animals were separated from their cage mates and singly housed. This test utilized preputial glands that were surgically removed from sacrificed male rats about 20-30 days old. The preputial glands were homogenized in ice cold tris buffered saline (TBS) and then centrifuged (Thompson et al., 2007). The supernatant (preputial extract) was then
removed and stored at -80 degrees Celsius. On the day of behavior testing, the preputial extract was thawed and used in a preference test. While in its home cage, each adult male rat was exposed to two Nestlets (Ancare, Bellmore, N.Y.): one with 40 μL of preputial extract on it, the other with 40 μL of TBS. The subject rat was freely allowed to investigate both Nestlets for 5 minutes. Behavior was digitally recorded and scored for direct contact between the subject and the Nestlet. Animals used in this experiment were the same cohort as that used in experiment 2.

**Experiment 4 - Olfactory tests, Food-finding test**

This paradigm was adapted from (Mencio-Wszalek et al., 1992). 6.5 month old rats (n=20) were treated with either progesterone or oil for three days. On the third day, injections occurred 4 hours before behavioral testing. A piece of chocolate chip cookie (average weight of 7.6 g) was buried at the center of a clean cage under a 1 cm layer of fresh bedding. Subjects were placed in the corner of the test cage and given a maximum of 10 minutes to uncover the cookie. The latency until the subject uncovered the buried cookie was measured in seconds with a stop watch.

**Experiment 5 - Object recognition test**

This paradigm was adapted from (Bevins and Besheer, 2006). 6.5 month old rats (n=20) were treated with either progesterone or oil for three days. On the third day, injections occurred 4 hours before behavioral testing. Identical objects (either large Lego blocks or metal tea balls) were placed in the back right and left corners of a Plexiglas chamber (60 x 38 x 39 cm). The subject was placed at the mid-point of the wall opposite the objects in a position facing away from the objects. For 5 minutes, the subject was freely allowed to investigate the objects. After a 1 hour intertrial interval, the subject was placed back in the same apparatus, which this time contained one of the objects
from trial 1 and one novel object (if Lego blocks were used in trial 1, a tea ball was the novel object in trial 2 and vice versa). Again, the subject was allowed to freely investigate the objects for 5 minutes. Behavior was digitally recorded and scored for direct contact between the subject and the objects. Objects were counter balanced for location in chamber and for being the novel or familiar object across subjects.

**Results**

*Experiment 1 - Habituation-Dishabituation: RU-486*

Since it has previously been shown that progesterone treatment decreases AVP immunoreactivity in the BST and the MeA, it was our goal to determine if progesterone would also affect social recognition, an AVP-dependent behavior. The group designated “control” consisted of the animals that were treated with RU-486 then oil (RU-486 + oil), and animals that were treated with vehicle then oil (vehicle + oil). As there were no significant difference between the two control groups ($p > 0.05$), these control groups were combined. In the habituation-dishabituation paradigm, we found a significant effect of exposure trial ($F(4, 80) = 7.65, p< 0.001$), as well as a significant interaction between treatment and trial ($F(8, 80) = 2.4, p = 0.022$). Post-hoc testing indicates a significant decrease in investigation between trial 1 and trials 3 and/or 4 in our control animals ($p = 0.028$ and $p = 0.039$ for trials 1 vs. trial 3 and trial 1 vs. 4, respectively). There was also a decrease in investigation between trial 1 and trials 3 and/or 4 in animals that were pretreated with RU-486 and then treated with progesterone (RU-486 + progesterone; $p = 0.009$ and $p < 0.001$ for trials 1 vs. trial 3 and trial 1 vs. 4, respectively). In contrast, animals treated with vehicle and then progesterone (vehicle + progesterone) displayed impaired social recognition, as they did not show significantly decreased investigation between trial 1 and trials 3 and/or 4 ($p = 0.179$ and $p = 0.726$ for trial 1 vs. trial 3 and trial 1 vs. 4, respectively). A habituation score (investigation in trial 5
minus investigation in trial 4) was also calculated to compare the treatment groups. Animals treated with vehicle and then progesterone (progesterone) were significantly different ($F(2, 19) = 8.740, p = 0.002$) from both the control group (control) and the animals treated with RU-486 and then progesterone (RU + prog). In summary, the control animals demonstrated normal recognition in the habituation-dishabituation paradigm, while the vehicle + progesterone group demonstrated impaired social recognition. Progesterone impairment of social recognition was blocked by pre-treatment with the progesterone antagonist, RU486 (Fig. 1a and 1b).
Figure 1 - Effect of treatment with progesterone or oil and pretreatment with RU-486 or vehicle on social recognition in the habituation-dishabituation paradigm. (A) Anogenital investigation of juvenile rat. Post-hoc analysis showed that animals in the control group (vehicle + oil and RU-486 + oil) exhibited normal social recognition (* p = 0.028 and p = 0.039 for trials 1 vs. trial 3 and trial 1 vs. 4, respectively), as did the animals in the RU-486 + progesterone group (* p = 0.009 and p < 0.001 for trials 1 vs. trial 3 and trial 1 vs. 4, respectively). Normal social recognition is identified when there is a significant decrease in investigation on trials 3 and 4 compared to trial 1, as indicated by the shaded box. Rats in the vehicle + progesterone group demonstrated impaired social recognition by not decreasing investigation in trials 3 and 4 (p = 0.179 and p = 0.726 for trial 1 vs. trial 3 and trial 1 vs. 4, respectively). (B) Habituation score, which was calculated by subtracting the amount of anogenital investigation in trial 4 from the amount of anogenital investigation in trial 5. Animals in the progesterone group were significantly different (* p = 0.002) from both the control group and the RU-prog group. Error bars represent SEM.
Experiment 2 - Social discrimination: RU-486

In order to confirm our results with a different social recognition paradigm, we tested social recognition using the social discrimination paradigm. In this paradigm, control animals discriminated normally (significantly more investigation of familiar juvenile than novel juvenile in trial 2 (p = 0.026, p = 0.021, respectively; Fig. 2a and 2b) while the animals in the vehicle + progesterone group failed to discriminate normally (p = 0.983, Fig. 2c). Importantly, the PR antagonist, RU-486, blocked progesterone impairment of social recognition, as the amount of time an animal in this group (RU-486 + progesterone) spent investigating the novel juvenile as opposed to the familiar one was significantly different (p = 0.041 Fig. 2d).

Experiment 3- Social discrimination: R5020

In order to further demonstrate the specificity of progesterone induced impairment of social recognition through action on PR, we treated male rats with a synthetic progestin, R5020. Again, control animals treated with oil discriminated normally (p = 0.045, Fig. 3a); however, animals treated with either the non-metabolizable synthetic progestin, R5020, or progesterone both showed impaired social discrimination (p = 0.313 and p = 0.868, respectively; Fig. 3b and 3c).

Experiment 4 - Olfactory tests

In the preputial preference test, a test of preference for social vs. non-social odors, all treatment groups (vehicle + oil, vehicle + progesterone, RU-486 + progesterone and RU-486 + oil) preferred the preputial-treated Nestlet (preputial) compared to the TBS-treated Nestlet (control) (vehicle + oil, p = 0.008; vehicle + progesterone, p < 0.001; RU-486 + progesterone, p = 0.004; RU-486 + oil, p = 0.007; Fig. 4a – 4d). Similarly, in a test of food-finding ability, animals treated with
either oil or progesterone did not statistically differ from each other in latency to uncover a hidden piece of cookie (p = 0.783; Fig. 5). These data suggest that progesterone did not interfere with general olfaction.

Experiment 5 - Object recognition: Progesterone vs. oil

In the object recognition paradigm, oil and progesterone treated animals significantly preferred to investigate a novel object compared to a familiar object (p < 0.001 and p = 0.04, respectively; Fig. 6a and 6b). These data suggest that progesterone does not impair memory in general.
Figure 2 - Effect of treatment with progesterone or oil and pretreatment with RU-486 or vehicle in the social discrimination paradigm. Anogenital investigation of juvenile rats in trial 2. (A) Animals in the vehicle + oil group discriminated normally (significantly more investigation of familiar juvenile than novel juvenile, * p = 0.026). (B) Animals in the RU-486 + oil group discriminated normally (* p = 0.021). (C) Animals in the vehicle + progesterone group failed to discriminate normally (p = 0.983). (D) Animals in the RU-486 + progesterone group discriminated normally (* p = 0.041), indicating that the PR antagonist RU-486 blocked the effect of progesterone on social discrimination. Error bars represent SEM.
Figure 3 - Effect of treatment with progesterone, oil, or synthetic progestin R5020 in the social discrimination paradigm. Investigation of juvenile rats in trial 2. (A) Animals in the oil group discriminated normally (* p = 0.045). (B) Animals treated with R5020 showed impaired social discrimination (p = 0.313). (C) Animals treated with progesterone showed impaired social discrimination (p = 0.868). Error bars represent SEM.
Figure 4 - Effect of treatment with progesterone or oil and pretreatment with RU-486 or vehicle on preference for preputial-treated Nestlet vs. control Nestlet. (A-D) All treatment groups (vehicle + oil, RU-486 + oil, vehicle + progesterone and RU-486 + progesterone) preferred the preputial-treated Nestlet (preputial) compared to the TBS-treated Nestlet (control) (vehicle + oil, * p = 0.008; RU-486 + oil, * p = 0.007; vehicle + progesterone, * p < 0.001; RU-486 + progesterone, * p = 0.004; Fig. 4a – 4d). Error bars represent SEM.
Figure 5 - Effect of treatment with progesterone or oil on food-finding ability. There was no statistical difference between treatment groups in latency to uncover a hidden piece of cookie ($p = 0.783$). Error bars represent SEM.

Figure 6 - Effect of treatment with progesterone or oil on object recognition. (A) Animals treated with oil significantly preferred investigating the novel object over the familiar object (* $p < 0.001$). (B) Animals treated with progesterone significantly preferred investigating the novel object over the familiar object (* $p = 0.04$).
Discussion

Our data support the notion that physiological levels of progesterone, by acting on progestin receptors (PRs), can have a significant modulatory action on social recognition memory within the male brain. We also report that progesterone impairment of social recognition is not a result of impaired olfactory ability or general impairment of memory systems. Taken together, these data provide strong evidence that progesterone interferes with social recognition memory in adult male rats.

Although the effect of progesterone via PRs appears to be specific to social recognition memory, the presence of PRs in the olfactory bulb suggested the possibility that the impairment in social recognition following progesterone treatment could be caused by a general impairment of olfactory ability. We controlled for this possibility, in two different tests of olfactory functioning. In both paradigms, progesterone did not impair olfactory performance. This suggests that the impairment in social recognition following progesterone treatment is more likely due to interference with social memory, rather than an impairment of olfactory ability. These results are supported by a 1978 study by Soares and Kalberer, where progesterone treatment prevented a typical social odor preference in male mice (Soares and Kalberer, 1978), but it did not alter latency scores in a food-finding task. It appears that in male mice, as in our male rats, progesterone seems to only impair memory for social olfactory cues and not general olfactory processing (Soares and Kalberer, 1978).

We report that progesterone impaired social recognition memory; however, novel object memory was not impaired by progesterone treatment. In agreement with our data, another study examined the effect of progesterone on working memory. It was found that progesterone did not impair performance in a novel object task, but it did impair performance on a spatial task (Sun et
al., 2010). These data suggest that the mechanism by which progesterone influences memory systems may be task-specific. As we have previously reported that progesterone treatment in adult male rats decreases AVP expression within the BST and MeA (Auger and Vanzo, 2006), it is possible that progesterone impairs social recognition via suppression of AVP expression. This potential pathway would be consistent with the well known role that AVP in the BST and MeA plays in social recognition.

Data from numerous labs have indicated the importance of AVP in social recognition. When AVP antagonists are infused into the LS, one of the projection sites of the AVP cells in the BST and MeA, social recognition is impaired; conversely, LS infusions of AVP agonists enhance social recognition (Dantzer et al., 1988). Also, castration, which depletes AVP in the cells of the BST and MeA and decreases the density of fibers projecting from these cells to the LS (De Vries et al., 1985) blocks social recognition (Bluthe et al., 1990). Additionally, while one study showed that animals lacking the AVP V1A receptor show deficits in social recognition (Bielsky et al., 2004), another lab showed that mice lacking the V1A receptor did not have impairments in this behavior (Wersinger et al., 2007). However, this same lab showed that animals lacking AVP V1b receptors show deficits in social recognition (Wersinger et al., 2002); taken together these data suggest that mechanisms altering AVP levels or AVP signaling have a strong potential to influence this AVP-dependant behavior. We hypothesize that the impairment in social recognition memory that we observed is mediated through the suppression in AVP levels that are seen following progesterone treatment in the BST and MeA (Auger and Vanzo, 2006). This hypothesis is consistent with data from a study that examined the role of AVP in both social and non-social behavioral paradigms. Everts and Koolhaas, 1997, have shown that infusions of a V1A receptor antagonist into the LS are able to block social recognition in rats, but not novel object recognition (Everts and Koolhaas, 1997a). These
data suggest that these two different forms of memory occur via different pathways within the brain. While social recognition memory is dependent upon AVP transmission in the LS (Everts and Koolhaas, 1997a), perhaps non-social memory is dependent upon other, non-AVP dependent, mechanisms.

The notion that progesterone can influence memory or structures associated with memory is not new, as it has been shown that spine density in the hippocampus is regulated over the estrus cycle in female rats (Woolley and McEwen, 1993). Specifically, dendritic spine density declines as estrogen levels fall, but they decline even faster if progesterone is administered during the decline in estrogen levels (Woolley and McEwen, 1993). On a behavioral level, changes in progesterone levels are associated with memory impairments in rodents (Vallee et al., 2001). Also, progesterone induced impairment of social memories has been demonstrated in humans. Exogenous progesterone exposure impairs women’s ability to remember socially relevant stimuli (i.e., human faces) (van Wingen et al., 2007). In this study, progesterone treatment is associated with decreased activity in the amygdala and in the fusiform face area as measured by event-related fMRI (van Wingen et al., 2007). The authors suggest that the “social recognition” memory tested is impaired by elevated allopregnanolone, a neuroactive metabolite of progesterone, levels in subjects treated with progesterone (van Wingen et al., 2007). Although this study cannot rule out the possibility that progesterone itself is influencing social memories, it has been suggested that progesterone-induced memory impairments result from the activity of allopregnanolone in the brain.

Allopregnanolone potentiates the effects of GABA at the GABA_A receptor, thereby increasing its inhibitory effect, and it is through this effect that allopregnanolone influences memory as well as a number of other behaviors (Dubrovsky, 2005). Although the mechanism of progesterone/allopregnanolone action on memories is not clear, allopregnanolone does appear to
impair non-social learning and memory tasks in rodents. Previous data indicate allopregnanolone can impair non-social memory in male mice (Ladurelle et al., 2000; Johansson et al., 2002). In a Y-maze novelty discrimination task, control animals preferentially explored a novel arm; however, mice receiving infusions of allopregnanolone into the lateral ventricles for several days did not investigate a novel, previously unexplored, arm in the maze, suggesting a form of spatial memory impairment (Ladurelle et al., 2000). Allopregnanolone also impairs learning in the Morris water maze following i.v. injection for several days. Animals treated with allopregnanolone show an increased latency to find the platform in the maze compared to controls (Johansson et al., 2002). In both of these experiments, motor behavior was not impaired by allopregnanolone treatment, only the learning and/or memory performance. These data, along with our data, suggest that progesterone, or its metabolites, may differentially affect social and non-social memories. The mechanism by which this may occur is unclear, but our data concerning the effects of PR modulators on social recognition memory argue that progesterone is functioning to impair social recognition memory through classical interaction with PRs, rather than through action on other receptors after its conversion to neuroactive metabolites.

Our current data also appear to extend our previous findings regarding progesterone suppression of AVP (Auger and Vanzo, 2006). That is, progesterone suppression of AVP may underlie progesterone impairment of social recognition. The mechanism by which progesterone influences AVP is likely through PRs, and our RU-486 and R5020 results presented here support that notion. Interestingly, PRs are found in discrete areas of the male brain and many of these areas play a large role in social behavior. Not surprisingly, PRs within the male brain have been reported to be expressed in areas classically associated with reproduction (Guerra-Araiza et al., 2001). However, PRs have also been found to be expressed within the olfactory bulb as well as the
hippocampus (Guerra-Araiza et al., 2001). PRs are also found in areas implicated in fear, stress, and anxiety (Walker et al., 2003; Brinton et al., 2008; Auger and De Vries, 2002). The distribution of PRs in the male brain suggests that they can have a number of functional implications in the male system; however, few studies have addressed the functional role of PRs within these brain regions. We demonstrate a functional role for progesterone, as well as PRs, in regulating social recognition memory in male rats. The specific distribution of PRs in the male brain, along with our current data, indicate that progesterone action in the male brain may regulate a small, targeted, number of social and emotional processes.

As numerous factors regulate social recognition memory, such as vasopressin, oxytocin and dopamine (Keverne and Curley, 2004) it is not clear if progesterone action on one or a number of these systems influences social recognition. It is also important to note that progesterone might be acting to alter the expression of other steroid receptors that are important for regulating AVP expression and social recognition memory (De Vries et al., 1985) (Imwalle et al., 2002). For example, progesterone reduces estrogen receptor expression in the BST (DonCarlos et al., 1995), which could reduce AVP expression, and this may be the mechanism by which social recognition memory is impaired in progesterone treated animals. Future research will be necessary to elucidate the molecular pathways involved in the progesterone impairment of social recognition memory.

In conclusion, our data suggest that progesterone is an important molecule in regulating social recognition in the male brain. Progesterone appears to interfere with the formation of social recognition memory without interfering with general non-spatial memory or olfactory functioning. Nonetheless, the data presented here indicate that progesterone has an important function in the male system that may be instrumental in mediating social interactions.
References


CHAPTER 3

Vasopressin infusion into the lateral septum of adult male rats rescues a progesterone induced impairment in social recognition

The experiments in the following chapter were completed by myself. Jesus Mena performed the stereotaxic cannulation surgeries. Experimental design and conclusions were developed with the help of my dissertation advisor, Dr. Catherine Auger.

Abstract

It is well established that social recognition is an arginine vasopressin (AVP) linked behavior. This behavior is mediated by AVP produced by cells within the bed nucleus of the stria terminalis (BST) and medial amygdala (MeA); these cells send AVP-ergic projections to the lateral septum (LS). We have previously demonstrated that progesterone treatment decreases AVP immunoreactivity in the BST, the MeA and the LS, and that progesterone treatment impairs social recognition. In the present experiment, we hypothesized that infusions of AVP into the LS would rescue the progesterone induced impairment of social recognition in adult male rats. One week after adult male rats underwent cannulation surgery, they were given systemic injections of either a physiological dose of progesterone or oil control for three days. Four hours after the last injection, social recognition testing was performed using the social discrimination paradigm, a two-trial test that is based on the natural propensity for rats to be highly motivated to investigate novel conspecifics. Immediately after the first exposure to a juvenile, each animal received bilateral infusions of either AVP or artificial CSF (aCSF) into the LS. Our results show that, as expected, control animals that received either oil and aCSF or oil and AVP exhibited normal social discrimination. In corroboration with previous experimental results from our lab, animals given progesterone and aCSF showed impairment in social discrimination. Interestingly, animals treated with progesterone and AVP exhibited normal social discrimination, suggesting that AVP treatment rescued the impairment in social recognition caused by progesterone.
Introduction

Social recognition allows animals to distinguish friend from foe and family member from stranger. Short-term social recognition is a way to differentiate between previously encountered conspecific individuals (Ferguson et al., 2002). It is well documented that vasopressin (AVP) is necessary for normal social recognition in intact, adult male rats (Ferguson et al., 2002). Infusion of an AVP agonist into the lateral septum (LS) enhances social recognition memory, while infusion of an AVP antagonist impairs it (Dantzer et al., 1988). There are several widely accepted behavioral paradigms for studying social recognition, one of which is a two-trial social discrimination paradigm based on the original social recognition test described by Thor and Holloway (Engelmann et al., 1995; Thor and Holloway, 1982; Thor and Holloway, 1981). In the first trial, an intruder (usually a 20-35 day old juvenile) is introduced to the cage of a resident adult (Engelmann et al., 1995). These animals are separated for 30 minutes, and then reunited in a second trial, but in this trial an additional juvenile is also placed in the cage. That is, the adult is exposed to two juveniles simultaneously and is allowed to investigate them both (Engelmann et al., 1995). The adult typically displays a reduced level of investigation of the familiar juvenile in trial two and a high level of investigation toward the novel juvenile. This is believed to indicate that discrimination has occurred (Engelmann et al., 1995). The role of AVP in the regulation of this behavior has been studied for decades, and these studies indicate that AVP is heavily involved in social recognition (Ferguson et al., 2002).

In male rats, if the interval between the first and second trials of the social recognition test exceeds 30-60 minutes, the adult will not recognize the familiar juvenile when it is encountered in the second trial (Thor and Holloway, 1982). Indeed, Dantzer et al. found that this memory does not seem to be maintained 120 minutes after the initial encounter, as there was not a decrease in the
amount of time the adult investigated a familiar juvenile rat after this longer intertrial interval. However, when an adult rat is treated with AVP, it does demonstrate social recognition memory of a familiar juvenile even after a 120 minute intertrial interval. Conversely, administration of a V₁ AVP receptor antagonist impairs the social recognition memory for a familiar juvenile rat 30 minutes after the first exposure. Brattleboro rats are an inbred strain that spontaneously arose from Long-Evans rats and the homozygous animals do not synthesize biologically active AVP in their brains (Engelmann and Landgraf, 1994). Engelmann and Landgraf (1994) found that these Brattleboro rats demonstrated impaired social recognition in the simple recognition paradigm when the intertrial interval was 30 minutes (Engelmann and Landgraf, 1994). This impairment was rescued with microdialysis treatment of synthetic AVP in the LS, providing additional evidence for the importance of AVP in social recognition in male rats (Engelmann and Landgraf, 1994). Additionally, recent data indicate that AVP is released in the LS during the acquisition of a social recognition memory (Lukas et al., 2011). These data indicate that AVP released in the LS plays an important role in social recognition.

The LS, which contains AVP receptors (Tribollet et al., 1988), receives AVPergic projections from neurons located in the bed nucleus of the stria terminalis (BST) and the medial amygdala (MeA). We have shown that the AVP cells in the BST and MeA are virtually 100% co-localized with progestin receptors (Auger and De Vries, 2002). Also, treatment with progesterone functions to reduce AVP protein levels in male rats in the BST and MeA and AVP fiber density in the LS (Auger and Vanzo, 2006). More recently, our lab has demonstrated that progesterone treatment also impairs social recognition memory in the social discrimination task (Bychowski and Auger, 2012). Based on our previous data, we hypothesize that the mechanism by which this occurs is through reduction in AVP protein following progesterone treatment. In these experiments, we
aimed to determine if the impairment in social recognition seen in progesterone treated rats could be rescued by infusion of AVP into the LS. We replicated our previous findings that progesterone impairs social recognition memory but we extended these findings by demonstrating that infusions of AVP into the LS can rescue the impairments in social recognition induced by progesterone treatment.
Methods

Animals

Adult male Sprague-Dawley rats were bred in our animal facility from breeding stock obtained from Charles River (Charles River Laboratories, Inc., Wilmington, MA). Juvenile male stimulus animals (between 20-30 days old) were purchased directly from Charles River. All animals were group housed in our animal facility, unless otherwise noted, on a 12h light/12h dark cycle with lights off at 11:00 am, and had free access to food and water. This research was approved by the University of Wisconsin Animal Care and Use Committee.

Surgical Procedures

Rats were anesthetized with Isofluorane gas and secured in a Kopf stereotaxic frame. Before surgery commenced, rats were given subcutaneous injections of ampicillin (30 mg/kg; Phoenix Pharmaceuticals, St. Joseph, MO) and ketoprofin (200 µg/0.1 mL; MP Biomedicals, Inc. Aurora, OH). The toothbar was set at –4.0 mm below the interaural line for all surgeries. Bilateral stainless steel cannulae (10 mm long, 23 gauge) were implanted according to standard stereotaxic procedures. To target the lateral septum, cannulae were placed at an angle to avoid the lateral ventricle. Coordinates of the injection site were AP: -0.2 mm from bregma, ML: ±1.32 mm from midline, DV: -5 mm from skull surface with cannulae angled at 8 degrees from vertical. Self-made cannulae were fixed to the skull 2 mm above the target with cranial plastic (Esschem, Linwood, PA) and anchoring skull screws (McMaster-Carr, Chicago, IL). Wire stylets (10 mm long, 30 gauge) were placed in the cannulae to prevent blockage. Rats were returned to their home cages upon awakening and given a recovery period of no less than five days (with daily health checks) before behavioral testing began.
Acclimation procedure

In order to familiarize the rats with the handling that would occur during the microinfusion procedure, all animals were handled every day between surgery and behavioral testing. Additionally, on the first day of progesterone or oil injection (see below), while animals were handled, the wire stylets in their cannulae were removed and replaced, in order to acclimate the animals to a manipulation of their heads. On the second day of injections, animals received infusions of saline into the LS while they were handled so that the infusions of AVP or artificial cerebrospinal fluid during behavior would not be their first experiences with the infusion procedure.

Drug treatments

Subcutaneous injections occurred during the "lights on" portion of the light cycle for three consecutive days. Progesterone (Steraloids, Newport R.I.; 1 mg) was dissolved in 0.1 mL of sesame oil and 0.1 mL of sesame oil was used as a control for progesterone. (Deamino-Cys¹,D-Arg⁸)-Vasopressin (0.1 ng/0.5 µL; Bachem, Torrance, CA) or artificial cerebrospinal fluid (0.5 µL) was infused into the lateral septum (as described below) during the "lights off" portion of the light cycle.

Microinfusion Procedures

Intracerebral microinfusions occurred immediately after the test animal completed trial 1 of the social discrimination paradigm, and rats were gently held in an identical way to that of the daily handling during the acclimation procedure. Stylets were removed from the guide cannulae and stainless steel injectors, connected via polyethylene tubing (PE-10, Becton Dickinson, Sparks, MD) to 10 µL capacity Hamilton syringes (Hamilton, Reno, NV) on a Harvard microdrive pump, were
lowered through the cannulae to the site of infusion. The flow rate for infusions was 0.32 µL/min. The total infusate volume for the bilateral infusions was 0.5 µL/side. After infusions, injectors were left in place for an additional minute to allow for diffusion of the injectate into the tissue. Injectors were then removed, and wire stylets replaced. The entire infusion procedure took approximately six minutes per animal.

*Behavioral testing and Statistical analyses*

Testing took place during the “lights off” portion of the light cycle under dim red light in our behavior room. All behavior was digitally recorded and then analyzed by a trained researcher blind to all treatments using The Observer (Noldus Information Technologies, Leesburg, VA) behavioral observation software. Sigma Stat 3.5 was used to conduct all statistical analyses. Paired t tests were used to statistically analyze both experiments.

*Experiment 1 - Social Discrimination, progesterone and AVP*

The social discrimination paradigm was adapted from Engelmann et al. (Engelmann et al., 1995). 3-month-old male rats were treated with progesterone or oil for 3 consecutive days. On day 1 of treatment, animals were separated from their cage mates and singly housed. On the third day of progesterone or oil treatment, animals underwent behavioral testing 4 hours after the last round of injections. Testing occurred in the home cages of the adult male subjects. In trial 1, a male juvenile stimulus rat was placed in the home cage of the adult rat and the adult was allowed to freely investigate both the juvenile and the cage for 5 minutes. After the 5 minute investigation period, the juvenile was removed from the cage and the adult began a 30 minute intertrial interval. As soon as the intertrial interval began, the adult was removed from the cage to undergo the microinfusion
procedure, as described above, which lasted approximately 6 minutes. After the 30 minute intertrial interval elapsed, the juvenile from trial 1 plus a novel juvenile were placed in the adult’s cage, and the adult was again free to investigate both individuals as well as the cage for 5 minutes. The juvenile rats were distinguishable to the researcher scoring the video by unique tail marks drawn with permanent marker. Adult investigation of the juvenile(s) was scored to include direct contact between the nose of the adult and the body of the juvenile and close following behavior.

*Experimental Treatment Design*

There were 20 adult male rats in this study. In order to reduce the number of animals used, we employed a counterbalanced within subjects design that was carried out in 2 separate testing blocks. A testing block refers to: progesterone or oil treatment, trial 1 and trial 2 of social recognition testing paradigm, as well as AVP agonist or control infusions. In testing block 1, half of the animals were treated with progesterone and half of the animals with oil (described above). Immediately after trial 1 of social recognition testing, half of the progesterone and half of the oil treated animals received infusions of AVP agonist (described above). The other half of the progesterone and oil treated animals received infusions of aCSF (described above). After infusions, all animals were then tested in trial 2 of the social discrimination paradigm. Three weeks after testing block 1, the same animals that received progesterone was again treated with progesterone and the same animals that were treated with oil in block 1 were again treated with oil in test block 2. During social recognition testing, the animals that received aCSF after trial 1 in the first testing block, 3 weeks earlier, were infused with AVP agonist and the animals that received AVP agonist in block 1 received infusions of aCSF. For example, if an animal was treated with progesterone and AVP in test block 1, it received progesterone and aCSF in test block 2.
Experiment 2 - Social Discrimination, control

The social discrimination paradigm was adapted from Engelmann et al. (Engelmann et al., 1995). The animals that were treated with oil in experiment 1 were used as subjects in experiment 2. As in experiment 1, testing occurred in the home cages of the adult male subjects. In trial 1, a male juvenile rat was placed in the home cage of the adult rat and the adult was allowed to freely investigate for 5 minutes. After 5 minutes, the juvenile was removed and then the adult underwent the microinfusion procedure, as described above. After a 2 hour intertrial interval, the juvenile from trial 1 plus a novel juvenile were placed in the adult’s cage, and the adult was again free to investigate for 5 minutes. The juvenile rats were distinguishable to the researcher scoring the video by unique tail marks drawn with permanent marker. Adult investigation of the juvenile(s) was scored to include direct contact between the nose of the adult and the body of the juvenile and close following behavior.

Verification of placements

At the end the experiments, rats were deeply anesthetized with Isofluorane and perfused transcardially with a 4% formaldehyde solution. Brains were collected and stored in 4% formaldehyde. Coronal sections (60 µm) were cut through the infusion site on a cryostat microtome, collected on slides, stained with cresyl violet, and subsequently reviewed to verify correct placement of the injections. Images of representative sections were captured using Scion Image software on a computer interfaced with a microscope-mounted Hitachi HV-C20 CCD camera. All animals were found to have the tip of the injector positioned correctly within the lateral septum, as determined with the aid of the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1998).
**Results**

*Experiment 1 - Social Discrimination: progesterone and AVP*

We have previously shown that progesterone treatment impairs social discrimination, and we hypothesize that this effect of progesterone on behavior is due to its suppression of AVP levels, as we have seen previously. Therefore, it was our goal to determine if administration of AVP into the lateral septum could rescue this deficit. Using the typical social discrimination paradigm, control animals (oil + aCSF and oil + AVP) discriminated normally. That is, adult males spent significantly more time investigating the novel juvenile than familiar juvenile in trial 2 (p = 0.048, p = 0.003, respectively; Fig. 1a and 1b). Similarly to our previous data (Bychowski and Auger, 2012), animals in the progesterone + aCSF group failed to discriminate normally (p = 0.639, Fig. 1c), as progesterone appeared to impair social discrimination. Animals in the progesterone + AVP group demonstrated normal social discrimination (p = 0.045, Fig. 1d), indicating that AVP administration successfully rescued the deficit in social recognition memory seen in progesterone treated rats.

*Experiment 2 - Social discrimination: control*

In order to confirm the effectiveness of AVP, animals previously treated with oil in experiment 1 were used to determine if AVP infusion into the lateral septum would enhance social recognition memory. Our previous tests of social discrimination were performed with a 30 minute intertrial interval between exposures, as the short-term social memory is only thought to be maintained for 30 to 60 minutes (Ferguson et al., 2002). In another variation of the social discrimination paradigm, AVP can be used to lengthen the amount of time this social memory is maintained, so that animals can successfully discriminate between a novel and a familiar juvenile after a 2 hour intertrial interval (Dantzer et al., 1988). Control animals (those infused with aCSF)
failed to discriminate normally (p = 0.959, Fig. 2a) after a 2 hour intertrial interval, while animals treated with AVP demonstrated normal social recognition (p = 0.037, Fig. 2b). These results confirmed that the dose of AVP used was sufficient to enhance social recognition memory in our animals, replicating the findings of Dantzer et al. (1988).
Figure 1 - Effect of treatment with progesterone or oil and infusion of AVP or aCSF into LS on social recognition in the social discrimination paradigm. (A) Investigation of juvenile rats in trial 2. Animals in the oil + aCSF group discriminated normally (significantly more investigation of familiar juvenile than novel juvenile, (* p = 0.048). (B) Investigation of juvenile rats in trial 2. Animals in the oil + AVP group discriminated normally (* p = 0.003). (C) Investigation of juvenile rats in trial 2. Animals in the progesterone + aCSF group failed to discriminate normally (p = 0.639). (D) Investigation of juvenile rats in trial 2. Animals in the progesterone group discriminated normally (* p = 0.045), indicating that AVP administration successfully rescued the deficit in social recognition memory seen in progesterone treated rats. Error bars represent SEM.
Figure 2 - Effect of infusion of AVP or aCSF into LS on social recognition in the social discrimination paradigm with a 2 hour intertrial interval. (A) Investigation of juvenile rats in trial 2. Animals in the aCSF group failed to discriminate normally ($p = 0.959$). (B) Investigation of juvenile rats in trial 2. Animals in the AVP group discriminated normally (* $p = 0.037$). These results confirmed that the dose of AVP used was sufficient to enhance social recognition memory in our animals. Error bars represent SEM.

Figure 3 – Representative coronal slice of adult, male rat brain confirming localization of cannulae to LS.


**Discussion**

Our data provide evidence that progesterone, by acting on the AVP system, can influence social behaviors in male rats. We have shown in a previous study (Bychowski and Auger, 2012), that progesterone impairs social recognition. We confirm and extend these results by demonstrating that AVP infusion into the LS rescued this progesterone-induced impairment in social discrimination. Also, in confirmation of earlier studies (Bielsky et al., 2005; Dantzer et al., 1988; Engelmann and Landgraf, 1994), we show that AVP infusion into the LS extends social recognition memory over a 2 hour intertrial interval. These data provide a strong link between our previous data, that progesterone reduces AVP in the BST and MeA, and the functional significance of this reduction: impairment in social recognition behavior in adult male rats (Auger and Vanzo, 2006; Bychowski and Auger, 2012).

The role that neuropeptides play in the regulation of social recognition behavior has been investigated for over 30 years. The subcutaneous injection of AVP facilitates social recognition when the period between the first exposure to a juvenile and the second exposure is lengthened to 2 hours (Dantzer et al., 1987). Social recognition behavior is impaired by infusions of AVP antagonists and is enhanced by infusions of AVP agonists into the lateral septum, one of the projection sites of the AVP cells in the BST and MeA (Dantzer et al., 1988). It has also been shown that castration, which depletes AVP in the BST and MeA and in the projection sites of these cells, blocks social recognition (Bluthe et al., 1990). Additionally, rats without functional AVP in their brains (Brattleboro rats) demonstrate impaired social recognition in the simple recognition paradigm when the intertrial interval is 30 minutes and this impairment is rescued with synthetic AVP in the LS (Engelmann and Landgraf, 1994). These studies illustrate the basic importance of AVP in social recognition; in contrast, other studies have demonstrated that AVP is not involved in non-social investigatory behavior (Everts and Koolhaas, 1997b). Everts and Koolhaas (1997)
performed a simple recognition paradigm that used two objects instead of two juveniles (Everts and Koolhaas, 1997b). They found that control rats were able to recognize a familiar object after a 30 minutes intertrial interval (as evidenced by decreased investigation in trial 2) and this recognition ability was not impaired by an AVP antagonist (Everts and Koolhaas, 1997b). These findings are similar to previous data from our laboratory demonstrating that systemic progesterone treatment, which has been demonstrated to suppress AVP levels, did not affect object recognition, although the same treatment impaired social recognition memory (Bychowski and Auger, 2012).

It is likely that progesterone is influencing social recognition by directly acting on the AVP system, as progesterone treatment suppresses AVP levels in the BST and MeA as well as the projection sites of these cells. Because progesterone levels can be sensitive to changes in social and environmental conditions, these data suggest a molecular pathway by which the environment can influence social memory. For example, in a study by Andersen et al., animals sleep deprived for more than 24 hours all had significantly elevated serum levels of progesterone compared to control animals (Andersen et al., 2005). Progesterone levels did return back to normal following sleep recovery, but in a dose dependent manner that was based on the number of hours of sleep recovery (Andersen et al., 2005). Similarly, Persengiev, Kanchev and Vezenkova (1991) found that compared to controls, plasma progesterone levels were significantly elevated in adult Wistar rats stressed with 72 hours of sleep deprivation (Persengiev et al., 1991). Additionally, normal non-stress progesterone levels can vary over the course of the day in young, male rats (Simpkins et al., 1981). Repeated blood sampling from free-moving, unanesthetized Sprague Dawley rats showed that plasma progesterone levels increased 2.5-fold from lowest to highest levels in young (3-4 months old) rats (Simpkins et al., 1981). These data suggest that environmental changes, some very
common, can induce changes in levels of progesterone, and these changes could potentially alter AVP levels, resulting in altered social functioning. While these data suggest that stressful situations can elevate progesterone levels in the blood, it is important to note that inevitable biological processes unrelated to stress can also influence progesterone levels.

In addition to environmental factors that can induce alterations in progesterone levels, it has been shown that levels of progesterone and its metabolites can also change as animals age, which may also influence social recognition. Gruenewald et al. (1992) have shown that serum progesterone levels were significantly increased in intact 24 month old Fisher 344 rats compared to 3 or 12 month old rats (Gruenewald et al., 1992). Also, in old rats, Simpkins et al. (1981) found that plasma progesterone levels reached highs and lows at the same times of day as seen in young rats but a greater than 4-fold increase was seen between lowest and highest levels and peak progesterone levels were significantly higher in old rats compared to young rats (Simpkins et al., 1981). It is clear that levels of progesterone are elevated in aged animals, this change in hormonal profile may be important in our ability to identify early symptoms of pathologies of aging.

Similar to the notion that changes in progesterone can help identify pathologies associated with aging, it also appears that declines in social recognition and general olfactory function might be an indicator of pathological aging in rats and humans. This is of interest because a decrease in recognition ability might be an early indicator of age-related disorders such as Alzheimer’s disease before a pathological diagnosis is made (Prediger et al., 2006). It has been shown that compared to young rats, control aged rats are unable to successfully discriminate between familiar and unfamiliar chambers in a simple olfactory discrimination task and, more importantly, between novel and familiar animals in a social recognition task (Prediger and Takahashi, 2005). These data indicate that normal social and olfactory recognition abilities can decline with age, whether or not
this decline in ability is associated with impaired aging in rats is unclear. However, Hargrave, Maddock and Stone (2002) found that compared to control subjects and psychiatric control subjects, subjects with Alzheimer’s disease demonstrated significant impairment on tests of recognition of social stimuli, i.e. faces and emotions (Hargrave et al., 2002). It remains to be determined if a decline in social recognition is an early hallmark of disease course in Alzheimer’s patients, but these data suggest that impairments in social discrimination might be clinically relevant in human populations.
References


CHAPTER 4

**mRNA expression in the bed nucleus of the stria terminalis following progesterone treatment**

The experiments in the following chapter were completed by myself along with Robin Forbes-Lorman, who performed the ERα and PR PCR runs. Experimental design and conclusions were developed with the help of my dissertation advisor, Dr. Catherine Auger.

*Bychowski M, Forbes-Lorman R, Auger C. mRNA expression in the bed nucleus of the stria terminalis following progesterone treatment.*
Abstract

As our laboratory has previously demonstrated that progesterone treatment in male rats decreases vasopressin (AVP) immunoreactivity in the bed nucleus of the stria terminalis (BST) and medial amygdala (MeA), we wanted to examine whether progesterone’s influence on protein expression could be explained by a decrease in AVP mRNA. We performed quantitative PCR to determine the effect of progesterone treatment on AVP mRNA expression in the BST and in the paraventricular nucleus of the hypothalamus (PVN), a region rich in AVP neurons that are not co-localized with progestin receptors (PRs). We also examined mRNA expression of estrogen receptor α (ERα) and PR to determine the effectiveness of progesterone treatment. As expected, we did not observe an effect of progesterone treatment on AVP mRNA expression in the PVN, though progesterone treatment did decrease both ERα and PR mRNA expression in the BST, compared to oil control, demonstrating that our progesterone treatment was successful and had a physiological effect. We did not see an effect of progesterone treatment on AVP mRNA expression in the BST, but there are several possible explanations for this result, including the small number of AVP cells in the BST, and the possibility that slight variations in locations of punches introduced enough non-AVP cells into the samples that an effect of progesterone was obscured.
**Introduction**

The role that neuropeptides play in the regulation of social recognition behavior has been investigated for over 30 years. Social recognition behavior is impaired by infusions of vasopressin (AVP) antagonists and is enhanced by infusions of AVP agonists into the lateral septum (LS), one of the projection sites of the AVP cells in the bed nucleus of the stria terminalis (BST) and medial amygdala (MeA) (Dantzer et al., 1988). It has also been shown that castration, which depletes AVP in the BST and MeA and the projections from these cells, blocks social recognition (Bluthe et al., 1990). Previous data from our lab indicates that progesterone treatment in adult male rats decreases AVP protein expression in the BST, MeA and LS (Auger and Vanzo, 2006). We have also demonstrated that this reduction in progesterone has impairing effects on social recognition behavior (Bychowski and Auger, 2012). Additionally, we have shown that this deficit can be rescued by administering an AVP agonist. The molecular mechanisms that underlie progesterone’s regulation of AVP are unclear, and we therefore have incomplete knowledge of this hormone-behavior interaction.

Based on gene array results, and confirmed with real-time PCR, we have seen that progesterone regulates a number of genes in the male mediobasal hypothalamus (Auger et al., 2006). Of particular interest to us is the immediate early gene activity-regulated cytoskeleton-associated protein (Arc), which, after periods of neuronal activation, is transported to dendrites (Steward and Worley, 2002). It has been demonstrated by other laboratories that Arc expression is affected by behavioral experiences and memory is disrupted when Arc induction is blocked (Steward and Worley, 2002). Taken together with the data from our gene array results, these results support our belief that progesterone treatment can influence the mRNA expression of genes known to be involved in rodent behavior and memory.
Although we have examined progesterone regulation of AVP protein (Auger and Vanzo, 2006), it has not yet been established whether progesterone treatment also results in a corresponding decrease in AVP mRNA. We hypothesized that progesterone treatment would decrease AVP mRNA expression in the BST but not affect AVP mRNA expression in the PVN. We also examined mRNA expression of PR and ERα because both receptors are known to be regulated by progesterone (DonCarlos et al., 1995; Blaustein and Feder, 1979). In addition, our measure of ER and PR would provide us with a physiological assay of progesterone’s effect in the brain.

**Methods**

**Animals**

Adult male Sprague-Dawley rats were obtained from Charles River (Charles River Laboratories, Inc., Wilmington, MA). All animals were group housed in our animal facility on a 12h light/12h dark cycle with lights off at 11:00 am and had free access to food and water. This research was approved by the University of Wisconsin Animal Care and Use Committee.

**Drug treatments**

This experiment involved drug treatment administration via subcutaneous injection. Injections occurred during the “lights on” portion of the light cycle for three consecutive days. Progesterone (Steraloids, Newport R.I.; 1mg) was dissolved in 0.1 mL of sesame oil. 0.1 mL of sesame oil was used as a control for progesterone.

**Tissue Collection**

Animals were sacrificed via rapid decapitation. Brains were extracted and snap frozen in isopentane on dry ice and then kept at -80°C. Using a cryostat, brains were sectioned at 300µm and
regions of interest (BST, MeA and paraventricular nucleus of the hypothalamus (an AVP-rich control) were dissected out of the section using a tissue punch. The dissected brain regions were snap frozen in isopentane on dry ice and then kept at -80°C until homogenization.

**Quantitative PCR**

Methods were adapted from Jessen et al. and Auger et al. (Jessen et al., 2010; Auger et al., 2011). Total RNA was isolated from snap frozen tissue using the AllPrep DNA/RNA Mini Kit (Cat #80004, Qiagen, Valencia, CA). RNA concentrations were determined using the Qubit Quantification Platform (Cat #Q32857, Invitrogen, Carlsbad, CA). A StrataScript first-strand synthesis system kit (Cat #200420, Stratagene, La Jolla, CA) was used to reverse transcribe RNA to cDNA in a StratageneMx3000 real-time PCR system (Invitrogen). The cDNA was amplified in the same PCR machine using Platinum qPCR SuperMix-UDG (Cat #11730-017, Invitrogen). cDNA concentrations were determined via Qubit. Samples containing cDNA, 50 mM MgCl₂, Rox reference dye, and SuperMix-UDG from the Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen), underwent qPCR in a Stratagene Mx3000™ real-time PCR system. The relative levels of AVP and estrogen receptor α (ERα) mRNA in the samples was assessed using primers that were designed using the OligoPerfect tool (Invitrogen), and were purchased from Invitrogen or synthesized with standard purity by the Biotechnology center at the University of Wisconsin. Primers are as follows: AVP, accession number NM_016992.2, forward TGCCTGCTACTTCCAGAACTGC, reverse AGGGGAGACACTGTCTCAGCTC; ERα, accession number NM_012689.1, forward TCCGGCACATGAGTAACAAA, reverse TGAAGACGATGAGCATCCAG; PR, accession number NM_022847.1, forward GGTGGAGTCGTAAGCAT, reverse AGGCTTCCAAAGGAATTGT.

Expression of Ywahz, a housekeeping gene that has not been shown to be regulated by
progesterone, was measured within the same samples and was used as a control. The Real-Time PCR protocol was as follows: an initial denaturing step at 95 °C for 25 minutes followed by 40–45 cycles of a 95 °C denaturing step for 15 s, a 55 °C annealing step for 30 s, and a 72 °C elongation step for 30 s. Relative mRNA levels were calculated using the ΔΔCT method (Livak and Schmittgen, 2001).

Statistical Analyses

N-fold differences between the groups were compared by t-test, using the statistical software package Sigma Stat 3.5 (Systat). Significance was set at p < 0.05.

Results

AVP mRNA in BST

We did not see regulation of AVP mRNA by progesterone in our samples. There was not a statistically significant difference in mRNA fold-change between the progesterone and oil treatment groups (p = 0.233, Fig. 1).

ERα mRNA in BST

We observed a decrease in ERα mRNA expression in the BST in animals treated with progesterone, compared to those treated with oil (p = 0.016, Fig. 2). These results support previous data that indicate that progesterone treatment lowers ER expression (DonCarlos et al, 1995).

PR mRNA in BST

As was originally demonstrated in the guinea pig brain, treatment with progesterone decreases PR protein expression (Blaustein and Feder, 1979). Our examination of PR mRNA expression levels in
the BST of the rat indicate that animals treated with progesterone expressed decreased levels of PR mRNA compared to animals in the oil group (p = 0.015, Fig. 3).

**AVP mRNA in PVN**

We chose to examine AVP mRNA expression in the PVN because it is an area rich in AVP cells, and our lab has previously shown that AVP protein levels in this region were not affect by progesterone treatment (Auger and Vanzo, 2006), so we used it as a control region. Our data indicate that, as expected, progesterone treatment did not decrease AVP mRNA expression the PVN compared to the oil treatment group (p = 0.638, Fig. 4).
Figure 1 - Effect of progesterone treatment on AVP mRNA in BST. Relative AVP mRNA expression within the BST in each treatment group. AVP was not significantly affected by progesterone treatment ($p = 0.233; n \geq 9$). Error bars represent SEM.

Figure 2 - Effect of progesterone treatment on ERα mRNA in BST. Relative ERα mRNA expression within the BST in each treatment group. As expected, ERα was significantly decreased by progesterone treatment ($*p = 0.016; n \geq 9$). Error bars represent SEM.
Figure 3 – Effect of progesterone treatment on PR mRNA in BST. Relative PR mRNA expression within the BST in each treatment group. As predicted, PR was significantly decreased by progesterone treatment (*p = 0.015; n ≥ 9). Error bars represent SEM.

Figure 4 - Effect of progesterone treatment on AVP mRNA in PVN. Relative AVP mRNA expression within the PVN in each treatment group. As expected, AVP was not affected by progesterone treatment (p = 0.638; n ≥ 9). Error bars represent SEM.
Discussion

We examined AVP mRNA expression in the BST and PVN after progesterone treatment in order to determine if the decrease in AVP-ir after progesterone treatment previously seen in our lab was the result of a corresponding decrease in mRNA expression. While, as expected, we did not see an effect of progesterone treatment on AVP mRNA expression in the PVN, we also did not see a decrease in AVP mRNA expression in the BST. To ensure that lack of expected results in the BST was not due to a problem with progesterone treatment, we also examined ERα and PR mRNA expression in the BST. As expected, both ERα and PR mRNA expression was decreased in animals treated with vehicle + progesterone, compared to animals treated with vehicle + oil.

Many different laboratories have performed experiments to determine if there is a correlation between mRNA and protein levels in complex biological systems. Jonsdottir et al. (2008) found a significant correlation between mRNA and protein levels for only one of the six genes that they examined in tumor samples (Jonsdottir et al., 2008). In another study involving human samples, Sarro et al. (2010) found that levels of the protein CD20 were low in patients with chronic lymphocytic leukemia but that there was not a corresponding low level of CD20 mRNA (Sarro et al., 2010). A similar discrepancy between mRNA and protein expression has also been seen in bacteria. When Nie et al. (2006) examined the correlation between mRNA and protein expression using linear regression, they found that only 20-28% of the variation in protein levels can be explained by mRNA abundance (Nie et al., 2006).

While the advent of RT-PCR has allowed for the quantification of steady state measurement of mRNA, many reports only show a weak correlation between mRNA and protein expression (Maier et al., 2009). These results are likely not due to faulty statistical analysis because both Spearman rank coefficient and Pearson correlation coefficient analysis produce similar results.
when used to analyze mRNA-protein correlations (Maier et al., 2009). Both cis-acting and trans-acting mechanisms can enhance or repress the number of protein copies synthesized from a certain number of mRNA copies (Maier et al., 2009). Furthermore, adding another layer or variability, different events can affect transcription and translation either continuously or only under certain conditions (Maier et al., 2009). The factors that can influence mRNA-protein correlation include the following: physical properties of transcripts, regulatory proteins and sRNAs, codon bias (the differential frequency of codons that code for the same amino acid), translational efficiency, translation initiation, and protein half-life (Maier et al., 2009). It is unclear which of these factors, if any, could be responsible for our present results. One particular physical property of AVP transcripts, poly (A) tail length, has previously been shown to be responsive to physiological processes in the brain and to occur independently of changes in mRNA level (Carter and Murphy, 1991). Additionally, there is evidence that changes in poly (A) tail length of AVP transcripts can affect AVP protein expression in the rat brain (Maciejewski-Lenoir et al., 1993). Carter and Murphy (1993) have also demonstrated that poly (A) tail length of AVP mRNA in the BST can be steroid sensitive (Carter and Murphy, 1993). Taken together, these data suggest that changes in the poly (A) tail length of AVP transcripts could affect AVP protein levels without changing AVP mRNA expression and that this change in poly (A) tail length could potentially be responsive to our hormonal treatment paradigm.

Specific characteristics of the BST in the rat brain could also help to explain our AVP mRNA expression results. Magnocellular AVP neurons have been observed in the BST (unpublished observation, and De Vries personal communication) and as these cells do not contain PR and therefore would not be regulated by progesterone treatment, it is possible that the presence of their mRNA in our samples prevented us from observing a treatment effect. Additionally, the population
of AVP cells in the BST is very small, so it is possible that slight variations in locations of punches introduced enough non-AVP cells into the samples that an effect of progesterone on AVP mRNA expression was obscured.
References


Carter DA, Murphy D (1993) Regulation of vasopressin (VP) gene expression in the bed nucleus of the stria terminalis: gonadal steroid-dependent changes in VP mRNA accumulation are associated with alterations in mRNA poly (A) tail length but are independent of the rate of VP gene transcription. J Neuroendocrinol 5:509-515.


CHAPTER 5

Discussion & Conclusions

The content in the following chapter was written by myself with the help of my dissertation advisor, Dr. Catherine Auger.
Summary

Our data support the notion that physiological levels of progesterone, by acting on progestin receptors (PRs), can have a significant modulatory action on social recognition memory within the male brain. We confirm and extend these results by demonstrating that vasopressin (AVP) infusion into the lateral septum (LS) rescued this progesterone-induced impairment in social discrimination. We also report that progesterone impairment of social recognition is not a result of impaired olfactory ability or general impairment of memory systems. Taken together, these data provide strong evidence that progesterone interferes with social recognition memory in adult male rats.

Although the effect of progesterone via PRs appears to be specific to social recognition memory, the presence of PRs in the olfactory bulb suggested the possibility that the impairment in social recognition following progesterone treatment could be caused by a general impairment of olfactory ability. We controlled for this possibility, in two different tests of olfactory functioning. In both paradigms, progesterone did not impair olfactory performance. This suggests that the impairment in social recognition following progesterone treatment is more likely due to interference with social memory, rather than an impairment of olfactory ability. These results are supported by a 1978 study by Soares and Kalberer, where progesterone treatment prevented a typical social odor preference in male mice (Soares and Kalberer, 1978), but it did not alter latency scores in a food-finding task. It appears that in male mice, as in our male rats, progesterone seems to only impair memory for social olfactory cues and not general olfactory processing (Soares and Kalberer, 1978).

While we report that progesterone impaired social recognition memory, novel object memory was not impaired by progesterone treatment. In agreement with our data, another study
examined the effect of progesterone on working memory. It was found that progesterone did not impair performance in a novel object task, but it did impair performance on a spatial task (Sun et al., 2010). These data suggest that the mechanism by which progesterone influences memory systems may be task-specific. As we have previously reported that progesterone treatment in adult male rats decreases AVP expression within the bed nucleus of the stria terminalis (BST) and medial amygdala (MeA) (Auger and Vanzo, 2006), it is possible that progesterone impairs social recognition via suppression of AVP expression. This potential pathway would be consistent with the well known role that AVP in the BST and MeA plays in social recognition.

We confirmed and extended the results in Chapter 2 by demonstrating that AVP infusion into the LS rescued a progesterone-induced impairment in social discrimination. Also, in confirmation of earlier studies (Bielsky et al., 2005; Dantzer et al., 1988; Engelmann and Landgraf, 1994) we showed that AVP infusion into the LS extended social recognition memory over a 2 hour intertrial interval. These data provide a strong link between the knowledge that progesterone reduces AVP in the BST and MeA, and the functional significance of this reduction: an impairment in social recognition behavior in adult male rats (Auger and Vanzo, 2006; Bychowski and Auger, 2012).

As our laboratory has previously demonstrated that progesterone treatment in male rats decreases AVP immunoreactivity in the BST and MeA, in Chapter 4 we wanted to examine whether progesterone’s influence on protein expression could be explained by a decrease in AVP mRNA. We performed quantitative PCR to determine the effect of progesterone treatment on AVP mRNA expression in the BST and in the paraventricular nucleus of the hypothalamus (PVN), a region rich in AVP neurons that are not co-localized with progestin receptors (PRs). We also examined mRNA expression of estrogen receptor α (ERα) and PR to determine the effectiveness of progesterone treatment. As expected, we did not observe an effect of progesterone treatment on AVP mRNA
expression in the PVN, though progesterone treatment did decrease both ERα and PR mRNA expression in the BST, compared to oil control, demonstrating that our progesterone treatment was successful and had a physiological effect. We did not see an effect of progesterone treatment on AVP mRNA expression in the BST, but there are several possible explanations for this result, including the small number of AVP cells in the BST, and the possibility that slight variations in locations of punches introduced enough non-AVP cells into the samples that an effect of progesterone was obscured.

**Mechanism by which progesterone treatment could affect social recognition**

Data from numerous labs have indicated the importance of AVP in social recognition. When AVP antagonists are infused into the LS, one of the projection sites of the AVP cells in the BST and MeA, social recognition is impaired; conversely, LS infusions of AVP agonists enhance social recognition (Dantzer et al., 1988). Also, castration, which depletes AVP in the cells of the BST and MeA and decreases the density of fibers projecting from these cells to the LS (De Vries et al., 1985) blocks social recognition (Bluthe et al., 1990) and Brattleboro rats, which don’t synthesize biologically active AVP in their brains, demonstrated impaired social recognition in the simple recognition paradigm when the intertrial interval was 30 minutes (Engelmann and Landgraf, 1994). Additionally, while one study showed that animals lacking the AVP V₁A receptor show deficits in social recognition (Bielsky et al., 2004), another lab showed that mice lacking the V₁A receptor did not have impairments in this behavior (Wersinger et al., 2007). However, this same lab showed that animals lacking AVP V₁B receptors show deficits in social recognition (Wersinger et al., 2002). Taken together, these data suggest that mechanisms altering AVP levels or AVP signaling have a strong potential to influence this AVP-dependant behavior.
We hypothesized that the impairment in social recognition memory that we observed is mediated through the suppression in AVP levels that are seen following progesterone treatment in the BST and MeA (Auger and Vanzo, 2006). This hypothesis is consistent with data from a study that examined the role of AVP in both social and non-social behavioral paradigms. Everts and Koolhaas (1997) have shown that infusions of a $V_{1A}$ receptor antagonist into the LS are able to block social recognition in rats, but not novel object recognition (Everts and Koolhaas, 1997a). These data suggest that these two different forms of memory occur via different pathways within the brain. While social recognition memory is dependent upon AVP transmission in the LS (Everts and Koolhaas, 1997a), perhaps non-social memory is dependent upon other, non-AVP dependent, mechanisms.

The data presented in Chapter 2 also appear to extend previous findings from our lab regarding progesterone's suppression of AVP (Auger and Vanzo, 2006). That is, the suppression of AVP by progesterone may underlie the progesterone-induced impairment of social recognition. The mechanism by which progesterone influences AVP is likely through PRs, and our RU-486 and R5020 results presented in Chapter 2 support that notion. Interestingly, PRs are found in discrete areas of the male brain and many of these areas play a large role in social behavior. Not surprisingly, PRs within the male brain have been reported to be expressed in areas classically associated with reproduction (Guerra-Araiza et al., 2001). However, PRs have also been found to be expressed within the olfactory bulb as well as the hippocampus (Guerra-Araiza et al., 2001). PRs are also found in areas implicated in fear, stress, and anxiety (Walker et al., 2003; Brinton et al., 2008; Auger and De Vries, 2002). The distribution of PRs in the male brain suggests that they can have a number of functional implications in the male system; however, few studies have addressed the functional role of PRs within these brain regions. In Chapter 2, we demonstrated a functional
role for progesterone, as well as PRs, in regulating social recognition memory in male rats. The specific distribution of PRs in the male brain, along with our Chapter 2 data, indicate that progesterone action in the male brain may regulate a small, targeted, number of social and emotional processes.

As numerous factors regulate social recognition memory, such as AVP, oxytocin (OXT) and dopamine (Keverne and Curley, 2004) it is not clear if progesterone action on one or a number of these systems influences social recognition. It is also important to note that progesterone might be acting to alter the expression of other steroid receptors that are important for regulating AVP expression and social recognition memory (De Vries et al., 1985; Imwalle et al., 2002). AVP parvocellular neurons of the BST and MeA, in addition to containing PRs, have also been co-localized with estrogen receptors (ERs) (Axelson and Leeuwen, 1990). Progesterone reduces ER expression in the BST (DonCarlos et al., 1995), which could reduce AVP expression, and this may be the mechanism by which social recognition memory is impaired in progesterone treated animals. Also, male rats lacking estrogen receptor α (ERαKO) displayed impaired social recognition memory for an ovariectomized female conspecific (Imwalle et al., 2002). However, the ERαKO mice also investigated the female intruders less than the wild-type controls did, suggesting that the rats had decreased motivation for social contact, which could have contributed to impaired social recognition (Imwalle et al., 2002). Interestingly, female rats that received a shRNA against the ERα gene via a viral vector infused into the MeA demonstrated impaired social recognition but levels of cage-directed investigation indistinguishable from those of control rats (Spiteri et al., 2010). If progesterone is acting through ER to affect AVP and social recognition, it is unsurprising that in our experiments, vehicle + progesterone treated animals have demonstrated the same amount of total
investigation as the control animals (unpublished results), and therefore appear to behave more like the female rats with knocked down ER expression than the males with total abolition of ER. When considering our data, it is interesting to reflect on the evidence that some neurotransmitters have been shown to activate hormone receptors in a ligand-independent manner. In vitro, it has been demonstrated that dopamine can mimic the action of progesterone and activate PR to translocate from the cytoplasm to the nucleus, theoretically through an intracellular signaling pathway (Power et al., 1991). Studies have also been performed in vivo to determine if physiologically relevant stimuli can elicit a typical behavioral response in the absence of the usual hormonal milieu. Results from Auger et al. (1997) indicate that even in the absence of progesterone (due to ovariectomy and adrenalectomy) female rats displayed enhanced subsequent sexual receptivity in response to sexual stimulation and this effect was blocked by a PR antagonist, suggesting that the behavior was affected by PR activity (Auger et al., 1997). Because dopamine release from the VMH and nucleus accumbens is increased by mating stimuli, this is potentially an in vivo example of naturally released dopamine acting on PR in a ligand-independent manner (Auger et al., 1997). These data provide evidence for a mechanism by which sexual behavior in female rats is affected in a ligand-independent manner, so it is possible that social encounters could increase dopamine levels in the male brain, activating PR and decreasing AVP protein levels to affect social recognition in males.

Vasopressin and oxytocin and their roles in male and female social recognition

While several studies have demonstrated that AVP and its receptors are necessary for social recognition in males, other studies have shown that OXT also plays a roll. Additionally, OXT is believed to be necessary for normal social recognition in females. AVP and OXT are incredibly similar, differing by two amino acids (Insel and Young, 2000), so it is possible that progesterone
could also affect social recognition via the OXT system in males and females. Whereas AVP has consistently been shown to play a vital role in short-term social recognition memory in both rats and mice (Ferguson et al., 2002), various studies have produced contradictory data in regards to the role of OXT in male rat social recognition. Depending on the dose and route of administration, OXT has been shown to both enhance and impair social recognition in male rats (Ferguson et al., 2002). For example, low, physiological doses of OXT given via subcutaneous injections facilitate social recognition, while high, pharmacological doses impair normal social recognition (Ferguson et al., 2002). Similarly inconsistent results were found when OXT was infused intraventricularly (Ferguson et al., 2002). Because agonists are typically less receptor-specific than antagonists, it is possible that the contradictory data are the result of OXT agonists binding to V1aR rather than OXT receptors (Ferguson et al., 2002).

The effect of OXT on social recognition memory in males is much more straightforward in mice than it is in rats. Ferguson et al. (2000) performed a series of experiments in which male OXT knock-out (OTKO) mice demonstrated impaired social recognition memory in the habituation-dishabituation paradigm but behavior comparable to that of wild-type mice in tests of general olfactory ability and non-social olfactory habituation-dishabituation (Ferguson et al., 2000). Also, other forms of memory were not affected in OTKO mice, as evidenced by normal behavior in the Morris water maze and the two-trial Y-maze memory tasks (Ferguson et al., 2000). It was additionally shown that intraventricular treatment with OXT, but not AVP, rescued social recognition memory in the OTKO mice (Ferguson et al., 2000). The same research group showed that OXT infusion into the MeA, but not the olfactory bulb, rescued the impaired social recognition seen in OTKO mice (Ferguson et al., 2001). Similarly, an OXT antagonist impaired social recognition in wild-type mice when infused into the MeA, but not when targeted to the olfactory bulb (Ferguson
et al., 2001). In mice, the MeA is rich in OXT receptors, making it a logical site of action for OXT (Ferguson et al., 2001). Even among closely related species, there are considerable differences in OXT receptor distribution, which could play a role in the different effects of OXT on social recognition memory in male mice and rats (Insel and Young, 2000).

Before examining the potential effects of progesterone on social recognition in females, it is important to understand the similarities and differences in the pathways and mechanisms underlying social recognition in males and female rodents. For example, Bluthe and Dantzer (1990) found that in trial one of the simple social recognition paradigm, when an animal was exposed to a single intruder, males spent significantly more time investigating a juvenile intruder than females did (Bluthe and Dantzer, 1990). Additionally, females displayed normal social recognition memory over 30 and 120 minute intertrial intervals, while males recognized normally over 5 and 30 minute intervals (Bluthe and Dantzer, 1990). Also, females could no longer recognize a familiar juvenile after 180 minutes, while males could not recognize after 120 minutes (Bluthe and Dantzer, 1990). This could be interpreted to mean that juveniles are more salient stimuli to adult females than to adult males (Bluthe and Dantzer, 1990). Interestingly, a subcutaneous injection of AVP enhanced social recognition memory in females and allowed them to maintain a social memory over 180 minutes, but peripheral treatment with an AVP antagonist (that had previously been shown to impair social recognition memory in males) did not impair normal social recognition in females after a 30 minute intertrial interval (Bluthe and Dantzer, 1990). This indicates that while AVP can enhance social recognition memory in female rats, it is not necessary for it (Bluthe and Dantzer, 1990).

As mentioned earlier, there is evidence in the literature that OXT is involved in short-term olfactory memory in female rats (Engelmann et al., 1998). When female rats were tested in the
social discrimination paradigm, the ability to discriminate between a novel and a familiar juvenile was intact as long as the interval between the first and second trials was less than 180 minutes (Engelmann et al., 1998). However, when the rats were treated with intracerebroventricular (i.c.v.) infusions of an OXT receptor antagonist, they failed to demonstrate normal social discrimination (Engelmann et al., 1998). This was not the case when the female rats were treated in the same manner with a V1R antagonist (Engelmann et al., 1998). Additionally, if the rats were given an i.c.v. infusion of synthetic OXT right after trial one of the social discrimination paradigm, memory wasn't enhanced over a longer intertrial interval, suggesting that in females, the first exposure to a novel juvenile releases enough endogenous OXT that the receptors are saturated and therefore exogenous OXT does not have an effect (Engelmann et al., 1998).

In order to study the effect hormones on social recognition memory in female mice and rats, several laboratories have studied the effects of estrogen and progesterone on social recognition. The rat estrus cycle is four days long and contains four stages (Staley and Scharfman, 2005). For this discussion the two stages of most interest are estrus and proestrus. During the estrus phase, both estrogen and progesterone are at basal levels (Staley and Scharfman, 2005). During proestrus, there is a rise in estrogen levels, followed a few hours later by a spike in progesterone levels (Staley and Scharfman, 2005). Within twelve hours of the progesterone peak, levels or estrogen and progesterone return to basal levels (Staley and Scharfman, 2005). Markham and Juraska (2007) found that female rats successfully demonstrated social recognition memory when exposed to juvenile females and phase of estrus cycle did not affect their ability to recognize (Markham and Juraska, 2007).

It has also been found that both ovariectomized female rats treated with oil and ovariectomized females treated with estrogen and progesterone (simulating proestrus)
demonstrated social recognition when tested with the habituation-dishabituation paradigm (Spiteri and Agmo, 2009). There was, however, a significant treatment difference in juvenile investigation during the fourth trial of the habituation-dishabituation paradigm, suggesting that while the presence of estrogen and progesterone was not necessary for social recognition memory in adult female rats, they enhanced habituation compared to oil treated control animals (Spiteri and Agmo, 2009). As is clear from the data presented in this section, there is evidence for both the role of progesterone and OXT in female social recognition, but, as of yet, it does not appear that anyone has performed an experiment examining all of the components at the same time.

**Effect of progesterone on memory**

The notion that progesterone can influence memory or structures associated with memory is not new, as it has been shown that spine density in the hippocampus is regulated over the estrus cycle in female rats (Woolley and McEwen, 1993). Specifically, dendritic spine density declines as estrogen levels fall, but they decline even faster if progesterone is administered during the decline in estrogen levels (Woolley and McEwen, 1993). On a behavioral level, changes in progesterone levels are associated with memory impairments in rodents (Vallee et al., 2001). Also, progesterone induced impairment of social memories has been demonstrated in humans. Exogenous progesterone exposure impairs women’s ability to remember socially relevant stimuli (i.e., human faces) (van Wingen et al., 2007). In this study, progesterone treatment is associated with decreased activity in the amygdala and in the fusiform face area as measured by event-related fMRI (van Wingen et al., 2007). The authors suggest that the “social recognition” memory tested is impaired by elevated allopregnanolone, a neuroactive metabolite of progesterone, levels in subjects treated with progesterone (van Wingen et al., 2007). Although this study cannot rule out the possibility
that progesterone itself is influencing social memories, it has been suggested that progesterone-induced memory impairments result from the activity of allopregnanolone in the brain.

Allopregnanolone potentiates the effects of GABA at the GABAA receptor, thereby increasing its inhibitory effect, and it is through this effect that allopregnanolone influences memory as well as a number of other behaviors (Dubrovsky, 2005). Although the mechanism of progesterone/allopregnanolone action on memories is not clear, allopregnanolone does appear to impair non-social learning and memory tasks in rodents. Previous data indicate allopregnanolone can impair non-social memory in male mice (Ladurelle et al., 2000; Johansson et al., 2002). In a Y-maze novelty discrimination task, control animals preferentially explored a novel arm; however, mice receiving infusions of allopregnanolone into the lateral ventricles for several days did not investigate a novel, previously unexplored, arm in the maze, suggesting a form of spatial memory impairment (Ladurelle et al., 2000). Allopregnanolone also impairs learning in the Morris water maze following i.v. injection for several days. Animals treated with allopregnanolone show an increased latency to find the platform in the maze compared to controls (Johansson et al., 2002). In both of these experiments, motor behavior was not impaired by allopregnanolone treatment, only the learning and/or memory performance. These data, along with the data presented in this dissertation, suggest that progesterone, or its metabolites, may differentially affect social and non-social memories. The mechanism by which this may occur is unclear, but our data concerning the effects of PR modulators on social recognition memory argue that progesterone is functioning to impair social recognition memory through classical interaction with PRs, rather than through action on other receptors after its conversion to neuroactive metabolites.
Relevance of work to humans

Although humans rely primarily on vision to recognize each other, rather than through olfaction like rodents, some of the systems and mechanisms involved in rodent social recognition are applicable to humans. The difference in the sensory modality involved in social recognition in humans and rodents makes it difficult to compare the brain regions involved. While this dissertation has gone into significant detail about the anatomical regions involved in social recognition in rodents, particularly those involved in the AVP-ergic system, social recognition in humans appears to be dominated by two cortical regions, the lateral fusiform gyrus and the anterior middle temporal gyrus (Haxby et al., 2002). The former is involved in the perception of the unique identity of a face, while the latter is involved in the recognition of familiar faces (Haxby et al., 2002). It is interesting to note, however, that the amygdala is believed to make important contributions to higher-level social cognition, especially for aspects that rely on getting social information from faces (Adolphs, 1999) and the perception of emotion in another's face elicits activity in the amygdala (Haxby et al., 2002).

One important similarity between humans and rodents is the presence of AVP and AVP receptors in the brains of both species. There is evidence of AVP binding in the dorsal part of the lateral septal nucleus and the dorsolateral part of the basal amygdaloid nucleus in humans (Loup et al., 1991), which is similar to the location of AVP receptors in rats. AVP cells have also been found in the BST in humans, but unlike rodents, sex differences in AVP innervations have not been found in the human brain (Fliers et al., 1986).

In an attempt to elucidate the effect of AVP treatment on species-specific social stimuli in humans, Thompson et al. (2004) examined intranasal AVP administration and its effect on male college students' attention, heart rate, skin conductance and corrugator supercilii (the muscle
under the eyebrows) electromyograms (EMGs) in response to emotional facial expressions (Thompson et al., 2004). They only found an effect of AVP on EMGs in response to emotionally neutral facial expressions, enhancing them to the levels seen in control subjects in response to angry facial expressions (Thompson et al., 2004). Because corrugator supercili EMG activity is correlated both with anger expressed to other individuals and angry facial stimuli, this study provides evidence that AVP can influence facial muscles important for emotional communication in human males (Thompson et al., 2004).

There is also evidence that AVP can influence memory in humans. Young, unimpaired subjects were treated with an AVP agonist and demonstrated statistically significant increases on several measures of learning and memory, including serial learning, prompted free recall and recall of semantically related words (Weingartner et al., 1981). A different laboratory examined the effect of the same AVP analog on the encoding and retrieval of memories (Till and Beckwith, 1985). When subjects were given sentences to memorize after a nasal AVP and then participated in a recall session the next week, they recalled more sentences than subjects given a placebo (Till and Beckwith, 1985). The AVP analog also improved sentence recall when subjects were asked to recall sentences soon after they were first presented with them (Till and Beckwith, 1985). Although these experiments did not have a social component, they demonstrate that AVP can have an effect on memory in humans.

Now that the evidence for the presence of AVP in the brains of humans and its effect on memory has been demonstrated, we can turn to the experiments aimed at studying the presence of progesterone in the human brain and elucidating its functional role. The enzyme ($\beta$-hydroxysteroid dehydrogenase) that converts pregnenolone to progesterone is expressed in various regions of the human brain, indicating that progesterone can by synthesized in the human
brain, just as it is in rodents (Schumacher et al., 2004). Hammond et al. (1983) examined the concentrations of several hormones, including progesterone and testosterone, in certain brain regions of human cadavers (Hammond et al., 1983). They found that while testosterone levels were much higher in the samples taken from male brains, there was no sex difference in progesterone levels in any of the brain regions, one of which was the amygdala (Hammond et al., 1983). These results provide evidence that not only can progesterone be produced in the human brain, it is also present in a human brain region known to contain AVP receptors and be responsive to social stimuli.

Progesterone in humans has also been studied in the context of its relationship to behavior. Opstad (1994) studied male cadets during a five day military training course with continuous heavy physical activity corresponded that also involved an almost total lack of food and sleep (Opstad, 1994). These conditions lead the increased 24-hour means of both cortisol and progesterone levels in the blood during the training course (Opstad, 1994). Although no social tests were run on the subjects during the training course, mental performance was tested via the code test, which tested visual acuity, motor coordination and speed of association and a logical reasoning test to test higher-level cognitive processes (Opstad, 1994). Subjects demonstrated significantly decreased scores in mental performance over the course of training (Opstad, 1994). These data indicate that physiologically relevant conditions can lead to an increase in progesterone levels in human males and impair cognitive ability.
References


