HIPPOCAMPAL VASOPRESSIN 1B RECEPTORS AND THE NEURAL REGULATION OF SOCIAL BEHAVIOR

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by

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xv</td>
</tr>
<tr>
<td>CHAPTER 1: INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Social Behavior</td>
<td>1</td>
</tr>
<tr>
<td>Elements of Social Behavior</td>
<td>1</td>
</tr>
<tr>
<td>Social Perception</td>
<td>1</td>
</tr>
<tr>
<td>Social Motivation</td>
<td>4</td>
</tr>
<tr>
<td>Social Memory</td>
<td>5</td>
</tr>
<tr>
<td>Aggressive Behavior</td>
<td>7</td>
</tr>
<tr>
<td>Neuropeptides and Social Behavior</td>
<td>8</td>
</tr>
<tr>
<td>Vasopressin (Avp)</td>
<td>10</td>
</tr>
<tr>
<td>Structure</td>
<td>10</td>
</tr>
<tr>
<td>Distribution</td>
<td>11</td>
</tr>
<tr>
<td>Receptors</td>
<td>13</td>
</tr>
<tr>
<td>The Avpr1b</td>
<td>14</td>
</tr>
</tbody>
</table>
Avp and Social Behavior ................................................................. 16
Avp and Social Motivation .............................................................. 16
Avp and Social Memory ................................................................. 18
Avp and Aggression .................................................................... 19
The Avpr1b and Social Behavior ..................................................... 21
The Avpr1b and Social Motivation ................................................... 22
The Avpr1b and Social Memory ...................................................... 22
The Avpr1b and Aggression ........................................................... 24
The Avpr1b and Olfaction .............................................................. 26
Is the Avpr1b within the CA2 region of the hippocampus important? ................................................................. 27
Scope of Dissertation .................................................................. 29

CHAPTER 2: IMMEDIATE EARLY GENE ACTIVATION IN THE CA2 REGION OF
THE HIPPOCAMPUS AFTER A SOCIAL INTERACTION ...................... 34
Introduction .................................................................................. 32
Materials and Methods ................................................................. 37
Results ......................................................................................... 42
Discussion ................................................................................. 50
CHAPTER 3: LESIONS TO THE CA2 REGION OF THE HIPPOCAMPUS ALTER
SOCIAL BEHAVIORS IN MICE................................................................. 54
  Introduction................................................................................. 54
  Materials and Methods............................................................. 57
  Results....................................................................................... 69
  Discussion................................................................................. 73

CHAPTER 4: THE EFFECTS OF shRNA KNOCKDOWN OF THE AVPR1B IN THE
CA2 REGION OF THE HIPPOCAMPUS ON AGGRESSIVE BEHAVIOR AND
SOCIAL RECOGNITION................................................................. 79
  Introduction................................................................................. 79
  Materials and Methods............................................................. 81
  Results....................................................................................... 90
  Discussion................................................................................. 93

CHAPTER 5: LOCALIZATION OF THE VASOPRESSIN 1B RECEPTOR IN THE
SYRIAN HAMSTER................................................................. 95
  Introduction................................................................................. 95
  Materials and Methods............................................................. 97
LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGURE 1</td>
<td>A diagram of the mammalian accessory olfactory system.</td>
<td>3</td>
</tr>
<tr>
<td>FIGURE 2</td>
<td>A schematic of the oxytocin and vasopressin genes, preprohormones, and amino acid structure.</td>
<td>12</td>
</tr>
<tr>
<td>FIGURE 3</td>
<td>Photomicrographs of coronal sections of a mouse hippocampus showing the subdivisions (lightfield) and Avpr1b receptor binding within the CA2 region (darkfield).</td>
<td>15</td>
</tr>
<tr>
<td>FIGURE 4</td>
<td>Location of the CA2 region of the hippocampus in a mouse coronal brain section.</td>
<td>28</td>
</tr>
<tr>
<td>FIGURE 5</td>
<td>Number of c-Fos immunoreactive cells per brain region in male and female Avpr1b +/+ and Avpr1b −/− mice after an aggressive encounter.</td>
<td>43</td>
</tr>
<tr>
<td>FIGURE 6</td>
<td>Photomicrographs of c-Fos immunoreactive cells in the ventral lateral septum of maternal aggression tested versus untested female mice.</td>
<td>44</td>
</tr>
<tr>
<td>FIGURE 7</td>
<td>Photomicrographs of c-Fos immunoreactive cells in the dorsal bed nucleus of the stria terminalis of maternal aggression tested versus untested female mice.</td>
<td>45</td>
</tr>
</tbody>
</table>
FIGURE 8: Photomicrographs of c-Fos immunoreactive cells in the ventral bed nucleus of the stria terminalis of maternal aggression tested versus untested female mice.

FIGURE 9: Photomicrographs of c-Fos immunoreactive cells in the medial preoptic area of maternal aggression tested versus untested female mice.

FIGURE 10: Photomicrographs of c-Fos immunoreactive cells in the medial amygdala of maternal aggression tested versus untested female mice.

FIGURE 11: Illustrations of coronal sections of hippocampus from -1.46 to -2.06 posterior to Bregma.

FIGURE 12: An illustration of the 2-trial social discrimination test.

FIGURE 13: Photomicrograph showing a coronal section of a mouse brain with bilateral lesions of the CA2 region of the hippocampus.

FIGURE 14: Graphs of time spent investigating the stimulus animals in Trial 1 and 2 of the 2-trial social discrimination test and all of the trials in the habituation/dishabituation social recognition test.

FIGURE 15: Graphs depicting the latency to find a hidden cookie and the time spent investigating the different odors in a habituation/dishabituation olfactory test.
**Figure 16:** Illustration of a coronal section of a mouse brain at -1.58 posterior to Bregma where the virus injection took place.

**Figure 17:** Schematic of control virus (LL 3.7) construct.

**Figure 18:** Graph depicting the time spent in olfactory investigation of the stimulus females in Trial 2 of the 2-Trial social discrimination test.

**Figure 19:** Schematic of the pGEM cloning vector into which the 572bp hamster Avpr1b sequence was cloned.

**Figure 20:** Alignment of a section of the mouse Avpr1b with that that was sequenced of the Syrian hamster Avpr1b.

**Figure 21:** Photomicrograph of the hippocampus of a male Syrian hamster labeled with sense probe.

**Figure 22:** Photomicrograph of neurons labeled with the antisense DIG-labeled probe for hamster Avpr1b.

**Figure 23:** Photomicrograph of a coronal section of a male Syrian hamster brain that highlights the indusium griseum labeled with a DIG-labeled in situ hybridization probe specific for the Avpr1b.

**Figure 24:** Photomicrograph of a coronal section of a male Syrian hamster brain that highlights the piriform cortex labeled with a DIG-labeled in situ hybridization probe specific for the Avpr1b.
Figure 25: Photomicrographs of a coronal section of a male Syrian hamster brain that highlights the hippocampus labeled with a DIG-labeled in situ hybridization probe specific for the Avpr1b.

Figure 26: Photomicrograph of a coronal section of a male Syrian hamster brain that highlights the thalamus labeled with a DIG-labeled in situ hybridization probe specific for the Avpr1b.

Figure 27: Photomicrograph of a coronal section of a male Syrian hamster brain that highlights the oculomotor and red nuclei labeled with a DIG-labeled in situ hybridization probe specific for the Avpr1b.

Figure 28: Photomicrograph of a coronal section of a male Syrian hamster brain that highlights the dorsal raphe and trochlear nucleus labeled with a DIG-labeled in situ hybridization probe specific for the Avpr1b.

Figure 29: Photomicrograph of a coronal section of a male Syrian hamster brain that highlights the median raphe nucleus labeled with a DIG-labeled in situ hybridization probe specific for the Avpr1b.

Figure 30: Photomicrograph of a coronal section of a male Syrian hamster brain that highlights the pontine nucleus labeled with a DIG-labeled in situ hybridization probe specific for the Avpr1b.
Figure 31: Schematics of proposed action of Avpr1b in the CA2 region of the hippocampus in the accessory olfactory-based social memory circuit in a social animal, such as the mouse.

LIST OF TABLES

TABLE 1: Number of c-Fos immunoreactive cells in female tested and untested Avpr1b +/+ and Avpr1b −/− mice. 49

TABLE 2: Behaviors measured in resident-intruder test in Experiment 1. 70

TABLE 3: Behaviors measured in the 2-trial social discrimination test. 92

TABLE 4: Behaviors measured in the resident-intruder test. 94
LIST OF ABBREVIATIONS

accessory olfactory bulb (AOB)
analysis of variance (ANOVA)
anterior hypothalamic area (AHA)
anterior hypothalamus (AH)
basal lateral amygdala (BLA)
bed nucleus of the stria terminalis (BNST)
c-FOS immunoreactive (Fos-ir)
central nervous system (CNS)
cerebral spinal fluid (CSF)
diaminobenzidine (DAB)
diethylpyrocarbonate (DEPC)
digoxigenin (DIG)
dorsal bed nucleus of the stria terminalis (BNSTD)
dorsal lateral septum (LSD)
etanol (EtOH)
extracellular signal-regulated kinase (ERK)
green fluorescent protein (GFP)
immediate early gene (IEG)
indusium griseum (IG)
in situ hybridization histochemistry (ISHH)
intraperitoneally (i.p.)
Kent State University (KSU)
knockout (−/−)
large dense-core vesicle (LDCV)
lateral habenular nucleus (LHb)
lateral septum (LS)
long-term potentiation (LTP)
medial amygdala (MeA)
medial preoptic area (MPOA)
N-methyl-D-aspartate (NMDA)
normal sheep serum (NSS)
oxytocin (Oxt)
paraventricular nucleus (PVN)
phosphate buffered saline (PBS)
phosphorylated cyclic AMP response element binding protein (pCREB)
post-partum day (PPD)
preoptic area (POA)
room temperature (RT)
suprachiasmatic nucleus (SCN)
tris buffered saline (TBS)
triton X-100 (T)
vapressin (Avp)
vapressin 1a receptor (Avpr1a)
vapressin 1b receptor (Avpr1b)
vapressin immunoreactive (Avp-ir)
ventral bed nucleus of the stria terminalis (BNSTV)
ventral lateral septum (LSV)
wildtype (+/+)
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CHAPTER 1

Introduction

1. Social Behavior

Social interactions are crucial to an animal's survival. There are several general classifications of social behavior, which include affiliative behavior, aggressive behavior, communicative (olfactory and vocal) behavior, parental behavior, and sexual behavior (Caldwell et al., 2008a). Although these categories seem distinct, they often overlap (i.e. affiliation and parental behavior), and are often both sex- and species-specific. Social behaviors have been widely studied in a variety of species, and are all regulated by the central nervous system (CNS) and modulated by neuropeptides. It is the neuroregulatory effects of the neuropeptide arginine vasopressin (Avp) on social behaviors, such as aggression, that will be the focus of this dissertation. This section describes some of the essential elements of social behavior and specifically, aggressive behavior.

1.1 Elements of Social Behavior

1.1.1 Social Perception

Social behavior is dependent on the ability to perceive social cues, process them, and elicit the appropriate behavioral response. In rodents, social perception
depends largely on chemical cues (Popik et al., 1991a). The main olfactory system and accessory olfactory system work together to gather this vital social information (Johnston, 1985). Unlike the main olfactory system, which detects a large number of volatile odorants, the accessory olfactory system detects only a limited variety of non-volatile pheromones. Pheromones are unique from other chemical cues in that they are used to transfer information exclusively between animals of the same species. They provide sex- and species-specific information about social status, gender, reproductive state, location, and territory. In mice, pheromones are produced by a variety of specialized glands, such as the preputial gland, lachrymal gland, and harderian gland (Albone, 1984). Male mice also produce large quantities of major urinary proteins (Robertson et al., 1996) that have been implicated in playing an important pheromonal role (Bocskei et al., 1992).

Initially, pheromones are detected by the vomeronasal organ, which sends a single, unbranched axon to the accessory olfactory bulb (AOB) (Barber and Raisman, 1974). Mitral cells, output neurons of the AOB, then send projections to the medial amygdala (MeA) and posterior medial cortical nucleus, which are collectively called the vomeronasal amygdala (Kevetter and Winans, 1981). The MeA then sends signals to the bed nucleus of the stria terminalis (BNST), the medial preoptic area (MPOA), ventromedial hypothalamus, and the arcurate nucleus to effect behavior (Figure 1) (Lehman and Winans, 1983).

The detection of pheromones by the accessory olfactory system provokes rapid changes in behavior and physiological state. In rodents, these changes include
Figure 1: Illustration of the mammalian accessory olfactory system. Adapted from Dulac and Torello (2003) with copyright permission from Nature Publishing Group.

1.1.2 Social Motivation

Once social cues are perceived, an animal must be motivated to interact with other animals (i.e. affiliative behavior) in order to participate in normal social behaviors. Most rodents are highly social and prefer to interact with a social stimulus rather than an inanimate object because the interaction is more rewarding. Unlike the inanimate object, the interaction with a social stimulus could lead to finding a mate, procreation, or the insured survival of offspring. This preference to engage in affiliative behavior is known as social motivation and is essential to all social behaviors.

Affiliation is the social bonding between individuals, such as between mates or parents and offspring. Pair bonding, sex behavior, and parental care are all examples of affiliative behavior that are all extremely motivating for most animals. For example, in an operant conditioning test, studies showed that postpartum female rats will bar press to gain access to pups (Lee et al., 1999) and that male rats
will bar press for access to an estrous female (Everitt, 1990). Further, postpartum female rats show preference for a cage associated with pups over a cage associated with cocaine (Mattson et al., 2001), suggesting that social interaction is more rewarding, and thus more motivating, than addictive drugs.

Social motivation can be determined in a variety of ways, but is commonly tested in mice using a bedding preference test or an operant conditioning test (Everitt, 1990, Lonstein and Fleming, 2002, Wersinger et al., 2004, Panksepp and Lahvis, 2007). In a bedding preference test, a male mouse is allowed to investigate the following pairings of bedding materials: 1) female-soiled bedding vs. male-soiled bedding, 2) female-soiled bedding vs. clean bedding, and 3) male-soiled bedding vs. clean bedding. It is expected that socially motivated individuals would spend more time investigating the female-soiled bedding over the male-soiled bedding and either soiled bedding over clean bedding. An operant conditioning test asks the animal to perform a task, such as a nosepoke or bar press, in order to gain access to a social stimulus. Both tests allow the observer to gauge the level of social motivation of an animal as well as the strength of the stimulus as a motivator.

1.1.3 Social Memory

In order to display normal social behavior, an animal must be able to recognize and remember conspecifics by forming social memories. To do this an animal can engage in two different forms of learning: imprinting and social recognition. Imprinting is a permanent form of social memory that is used to identify kin and species-specific phenotypes. This type of social memory is primarily
used to avoid incest. It is unlike any other learning in that it is fast, requires minimal trials, has an obvious sensitive period (just after birth), and is irreversible in a natural setting. Social recognition, on the other hand, is a temporary form of social memory used to recognize conspecifics in adulthood (as reviewed in Insel and Fernald, 2004). Both forms of social learning provide a foundation upon which all social relationships are built.

In rodents, social memory is dependent on processing olfactory cues and can be measured by a social recognition test (Dantzer et al., 1987). This test measures the ability of an individual to remember a familiar conspecific over a set period of time. There are two commonly used variations of this test: the social discrimination test and the habituation/dishabituation social recognition test. In the social discrimination test, the experimental animal is given a choice between investigating a previously encountered stimulus animal and a novel conspecific (Engelmann et al., 1995, Macbeth et al., 2009). Social recognition is assessed by comparing the amount of time spent in chemoinvestigation of the novel animal versus the familiar. The habituation/dishabituation recognition test exposes the test animal to a novel stimulus animal repeatedly for 1 minute with 5-minute inter-trial intervals to allow the animal to habituate to the stimulus female. In the last trial, the test animal is dishabituated to the stimulus by exposure to a novel female. Social recognition is measured by a decrease in investigation on each trial of habituation and then an increase in investigation in the last trial (Ferguson et al., 2002).
Pheromonal signals encode vital information about each individual conspecific that the experimental animal must process, form memories of, and recall. Rodents have a natural preference to investigate a novel stimulus over a familiar one and will, therefore, spend more time investigating a novel conspecific over a familiar one (Thor, 1982). However, it is unknown whether an animal investigating an intruder is comparing the encounter to imprinted memories of kin or rather just recognizing individual characteristics of the intruder. Thus, it is possible that social memory could be a combination of both imprinting and social recognition (Insel and Fernald, 2004).

1.2 Aggressive Behavior

All social behaviors are characterized by approach and withdrawal behaviors. Approach behaviors, which include affiliative behaviors, serve to bring animals together. Conversely, withdrawal behaviors, such as aggression, serve to keep animals apart. Aggressive behavior is the intent to inflict damage or pain on another individual, and is imperative for the survival of most mammalian species; allowing for better access to vital resources, such as food, shelter, and mates (Moyer, 1968, Moyer, 1971). While there are numerous ways to classify aggression, the system developed by Miczek and colleagues (2001) uses five classifications, which include maternal aggression, offensive aggression, defensive aggression, play fighting, and predatory aggression. While some of these are social forms of aggression, i.e. maternal, offensive, defensive, and play fighting, others are not, i.e., predatory aggression.
Rodents are the primary mammalian models used to study aggressive behavior because they display robust and reliable territorial aggression. The most common way of testing aggression in rodents is the resident-intruder test. This is due to the fact that mice predominantly attack in defense of territory (Miczek et al., 2001). In this test a resident animal defends its territory against an intruder. In a laboratory setting, adult males are forced to cohabitate (Council, 1996), which is not conducive to the formation of a natural social organization in this species. Males housed in a laboratory setting are often seen huddling and seldom display aggressive behavior without several experiences with a conspecific (King, 1957). For this reason, male mice are often isolated for a period of time before a resident-intruder test in order to increase isolation-induced aggression (Malick, 1979). Once the animals engage in aggression some typical behaviors observed in rodents include: attack biting, tail rattling (rapid vibration of the tail), chasing, pushing/shoving, and mounting (Miczek et al., 2001).

2. Neuropeptides and Social Behavior

Neuropeptides, such as Avp, can cause changes in displays of social behavior. Peptide hormones are hormones that are composed of proteins and constitute most of the hormones in vertebrate species. Peptide hormones that are produced by neurons are referred to as neuropeptides and have the ability to act as neurotransmitters if released in a synapse, or as neurohormones, if activating receptors distant from the site of release (Ludwig and Leng, 2006). Many neuropeptides act on brain areas rich in peptide receptors, but that lack direct
innervation by the neuropeptide releasing neurons. Like classical neurotransmitters (e.g. glutamate), neuropeptides are released in response to action potentials and are packaged in secretory vesicles, specifically, large dense-core vesicles (LDCV). However, unlike classical neurotransmitters, that also activate G-protein coupled receptors, neuropeptides bind to their receptors with higher affinity and activate intracellular signaling cascades that cause long lasting effects. They also have long half-lives, unlike classical neurotransmitters which usually degrade within seconds, which allow them to travel great distances within the brain (Mens et al., 1983). In this manner, neuropeptides can act like neurohormones by travelling long distances and evoking enduring changes in behavior.

Due to the their release from LDCVs, which are rarely found in synaptic terminals, neuropeptides are released from all parts of the neuron, and most often from the dendrites (Morris and Pow, 1991). Most peptidergic neurons produce not only neuropeptides, but also neurotransmitters (Torrealba and Carrasco, 2004). Neuropeptides are thought to augment the release of neurotransmitters by acting at presynaptic terminals to retrogradely modulate neurotransmitter release, or by acting on the cell of origin to exert autoregulatory effects (Ludwig and Pittman, 2003). It is the autoregulatory effects that are thought to be responsible for the hormone-like signal that is lingering and self-sustaining.

Neuropeptides acting on their producing neurons perpetuate the release of that neuropeptide causing it to increase in concentration in the cerebral spinal fluid (CSF) (Ludwig and Leng, 1997, Gouzenes et al., 1998). Many neuropeptides are
found in high enough concentration in the CSF to bind to high-affinity receptors discretely located in specific brain regions. Therefore, the behavioral effects of these neuropeptides are dependent on the distribution of their receptors and not the distribution of their releasing neurons (Ludwig and Leng, 2006). For example, monogamous prairie voles have a highly differentiated pattern of expression of Avp 1a receptors (Avpr1a) compared to that of the polygamous Montane voles (Phelps and Young, 2003). This is evidence that individual variations in social behavior within a species are associated with individual differences in the expression of receptors in the brain (Hammock et al., 2005, Hammock and Young, 2005) rather than being due solely to species-specific differences in Avp release.

3. Avp

3.1 Structure

Avp is a cyclic, nine amino acid neuropeptide. It was first described as an antidiuretic compound that acted on the kidneys and blood vessels to control water balance and vasoconstriction (as reviewed in Brownstein et al., 1980, Liard et al., 1982, Strange and Spring, 1987, Young and Gainer, 2003). Although Avp is primarily found in mammals, its analogs, vasotocin and conopressin, can be found in vertebrate and invertebrate species, respectively. In addition, its protein structure is highly similar to that of oxytocin (Oxt), another neuropeptide, differing by only two amino acid residues, specifically those in the third and eighth positions (as reviewed in Hara, 1990, Burbach et al., 2001, Young and Gainer, 2003, Caldwell et al., 2008a, Donaldson and Young, 2008, Lee et al., 2009). Their similarities are thought to be
the result of a gene duplication of the ancestral vasotocin gene (Acher and Chauvet, 1995).

In addition to having a similar sequence, the gene structure of Oxt and Avp are also similar and are oriented in opposing transcriptional direction on the chromosome (Figure 2) (as reviewed in Stevenson et al., In Press). Within a species, the Oxt and Avp genes are located on the same chromosome (i.e. chromosome 2 in mice, 20 in humans, and 3 in rats) and contain three exons and two introns. A region of DNA referred to as the intergenic region separates the genes (Gainer et al., 2001, Young and Gainer, 2003). Oxt and Avp are each synthesized as part of a larger preprohormone, which contains a signal peptide, the biologically active peptide, a neurophysin, and a glycoprotein. Avp is produced from the hydrolysis of the prohormone, propressophysin, into Avp, neurophysin II, and a short glycoprotein (Norris, 2007). Alone, neurophysin II is not biologically active, but its association with Avp is thought to play a role in protecting Avp from enzymatic degradation (Legros and Geenen, 1996, de Bree, 2000). Avp is considered less biologically stable than Oxt and it is thought that the glycoprotein portion of the Avp preprohormone may be important for folding of the Avp precursor (Barat et al., 2004).

3.2 Distribution

Avp is primarily synthesized in the magnocellular neurons of the paraventricular (PVN) and supraoptic nucleus of the hypothalamus. The axons of these neurons project to the posterior pituitary and terminate near the capillaries where Avp is ultimately released into the bloodstream where its peripheral effects
Figure 2: A schematic of the oxytocin and vasopressin genes (top), preprohormones (middle), and amino acid structure (bottom) (from Caldwell et al., 2008a with copyright permission from Elsevier).
can be exerted. In most species, neurons that produce Avp whose axons project centrally are parvocellular and originate in the hypothalamus, similar to Oxt (Swanson and Sawchenko, 1980, Castel and Morris, 1988). However, unlike Oxt, central Avp is produced in a variety of extrahypothalamic brain areas. In all mammals studied to date, there are Avp-producing neurons found in the preoptic area (POA) and anterior hypothalamus (AH), and in many species there are Avp-immunoreactive (Avp-ir) cells in the BNST and the MeA. These latter two sites send projections to the lateral septum (LS) and the lateral habenular nucleus (LHb). The suprachiasmatic nucleus (SCN), the primary mammalian circadian clock, is also an area rich in Avp-producing neurons (Buijs et al., 1978, Castel et al., 1990). In most mammalian species, Avp-ir fibers can be found in the POA, anterior (AHA) and lateral hypothalamic areas, midbrain tegmentum, periaqueductal grey, locus coeruleus, LS, LHb, nucleus of the solitary tract, and area postrema (Moore and Lowry, 1998). The Avp found in these subcortical regions is thought to be involved in the regulation of social behaviors and will be discussed in section 4.

3.3 Receptors

Three receptor subtypes mediate the effects of Avp: the Avpr1a, the Avp 1b receptor (Avpr1b), and the Avp 2 receptor. All three receptor subtypes can be found in the periphery (Jard et al., 1987, Arsenijevic et al., 1994, Knepper, 1997, Thibonnier et al., 2002, Koshimizu et al., 2006), but only the centrally expressed Avpr1a and Avpr1b are known to mediate the effects of Avp on social behavior (Antoni, 1984, Ostrowski et al., 1992, Johnson et al., 1993, Lolait et al., 1995, Foletta
et al., 2002, Young et al., 2006). All three of the receptors have seven transmembrane domains and are G protein-coupled receptors. The Avpr1a and Avpr1b activate $G_{a_{q/11}}$ GTP binding proteins, which in turn activate phospholipase C with the help of $G_{\beta\gamma}$ while the Avpr2 activates $G_s$ and acts through the cyclic AMP system (Michell et al., 1979, Jard et al., 1987).

3.3.1 The Avpr1b

The Avpr1b is expressed in a variety of tissues, including the pancreas, where it has been linked to insulin secretion, and the adrenal gland, where it has been linked to catecholamine release. It is also heavily expressed in the corticotrophes of the anterior pituitary gland (Antoni, 1984, Jard et al., 1986), but can also be found within the CNS. In the rat brain, Avpr1b transcripts and immunoreactive cell bodies are localized to the cerebellum, cerebral cortex, hippocampus, olfactory bulb, PVN, piriform cortical layer II, red nucleus, septum, and SCN (Lolait et al., 1995, Saito et al., 1995, Barberis and Tribollet, 1996, Vaccari et al., 1998, Hernando et al., 2001, Stemmelin et al., 2005). However, a more recent in situ hybridization study, in which more specific riboprobes and more stringent wash conditions were utilized, found that the Avpr1b of mice, rats, and humans is more discretely localized than previous studies suggested, with prominence in the dorsal one-third of pyramidal cells of the CA2 region of the hippocampus (Figure 3), and in a few cells within the anterior amygdala and the PVN (Young et al., 2006). The study of the distribution of the Avpr1b in the CNS is further complicated by the lack of antibodies in species such as mice and humans, as well as the lack of specific radiolabeled ligands. To date there
Figure 3: A) A brightfield photomicrograph of a coronal section of the mouse hippocampus with arrows indicating the borders of the CA2 region. B) A darkfield photomicrograph, which highlights the presence of the Avpr1b transcripts within the CA2 region of the hippocampus (from Young et al., 2006 with copyright permission from Elsevier).
are no published studies using receptor autoradiography to map the central
distribution of the Avpr1b; thus, in humans and mice the presence of Avpr1b
protein is inferred from the in situ hybridization studies.

4. Avp and Social Behavior

Avp has been implicated in the neural regulation of a variety of social
behaviors, including aggression (Griebel et al., 2002, Wersinger et al., 2002,
Blanchard et al., 2005, Wersinger et al., 2007, Caldwell et al., 2010), social memory
(Engelmann et al., 1994, Everts and Koolhaas, 1997, Wersinger et al., 2002, Bielsky
et al., 2004, Bielsky et al., 2005, Wersinger et al., 2008, Choleris et al., 2009, DeVito
et al., 2009), social motivation (Wersinger et al., 2004, Yang et al., 2007, DeVito et al.,
2009), pair bonding (Winslow et al., 1993, Insel and Hulihan, 1995, Lim et al., 2004),
and maternal behavior (Bosch and Neumann, 2008, Nephew and Bridges, 2008a). As
previously mentioned, it modulates social behaviors via its two centrally expressed
receptors: the Avpr1a and the Avpr1b (Antoni, 1984, Ostrowski et al., 1992, Johnson
et al., 1993, Lolait et al., 1995, Foletta et al., 2002, Young et al., 2006). However, as
evidenced by Avp administration, this neuropeptide has varying effects on different
species, sexes, and hormonal states. Much of the data supporting the importance of
Avp in the neural regulation of social behavior has been seen in pharmacological
studies and those utilizing knockout mice. This section will review those studies and
the effects of Avp on social motivation, social memory, and aggression.

4.1 Avp and Social Motivation
There is compelling evidence that Avp plays a role in the regulation social motivation. In a bedding preference test using Avpr1b wildtype (+/+) and Avpr1b knockout (−/−) mice, null mutants fail to exhibit a preference for urine soiled bedding over clean bedding. Rather, Avpr1b −/− mice spend similar amounts of time inter-acting with all of the bedding choices and less time overall interacting with the bedding than Avpr1b +/+ mice (Wersinger et al., 2004). This suggests that the Avpr1b is needed for normal social motivation and will be discussed further in Section 5.1.

Another measure of social motivation is the ability to form social attachments. The monogamous male prairie vole shows increased partner preference when given an intracerebroventricular injection of Avp before mating (Winslow et al., 1993). Conversely, an Avp receptor antagonist injected into this species prior to mating reduces partner preference (Cho et al., 1999). Interestingly, polygamous montane voles given central injections of Avp do not show the same increase in affiliative behavior (Young et al., 1999). Studies looking at Avp receptor densities in these two differing species find that prairie voles have a higher density of Avpr1a in the amygdala and less in the LS than montane voles (Insel et al., 1993, Insel et al., 1994). Increased expression of the Avpr1a delivered via viral vector to the ventral forebrain of montane voles can increase the incidence of affiliative behavior (Lim et al., 2004, Young and Wang, 2004). Transgenic mice that have a prairie vole-like Avpr1a transgene have Avpr1a binding patterns resembling that of prairie voles and show increased affiliative behavior (Young et al., 1999).
4.2 Avp and Social Memory

Social memory, like social motivation, is facilitated by the peripheral and central administration of Avp, its analogs, and selective metabolites (Dantzer et al., 1987, Le Moal et al., 1987, Popik et al., 1991b, Popik and Van Ree, 1992). A social recognition test performed in Brattleboro rats, a naturally occurring mutant that is unable to synthesize the biologically active form of Avp (Schmale and Richter, 1984, Schmale et al., 1989), showed that these rats display reduced social memory that can be rescued by central administration of Avp (Engelmann and Landgraf, 1994). Conversely, male rats given Avp antagonists directly into the septum fail to recognize a familiar male conspecific (Peele and Vincent, 1989). Avp given immediately after exposure to a social stimulus facilitates social memory as evidenced by the decreased amount of time spent investigating an animal upon second exposure at a time period when placebo rats show no recognition (Dantzer and Bluthe, 1992). It has also been demonstrated that Avp administration produces not only a short-term effect, lasting several hours, but also a long-term effect in which social recognition is maintained 24 hours after the first exposure (Popik and Van Ree, 1992).

Pharmacological agents can also alter social memory. Injections of Avp antisera, Avpr1a antagonists, or Avpr1a antisense DNA into the LS inhibits social recognition that cannot be rescued by administration of Avp into the LS (Dantzer et al., 1988, Bluthe and Dantzer, 1992, Landgraf et al., 1995, van Wimersma Greidanus and Maigret, 1996, Everts and Koolhaas, 1999, Appenrodt et al., 2002).
Administration of an Avpr1a antagonist to the hippocampus in males also inhibits social recognition (van Wimersma Greidanus and Maigret, 1996). Another study found that injection of Avp into the main olfactory bulb facilitated social recognition while an Avpr1a antagonist injected into the same area did not alter the behavior (Dluzen et al., 1998). However, a more recent study using a nonpeptide Avpr1a antagonist and siRNA against the Avpr1a injected into the main olfactory bulb was found to inhibit social recognition (Tobin et al., 2010).

Knockout studies have also given evidence of a role for Avp and its central receptors in the regulation of social memory. Avpr1a knockout (−/−) mice have impaired social recognition that can be rescued by overexpression of the receptor in the LS (Bielsky et al., 2004, Bielsky et al., 2005). Male Avpr1b −/− mice also show impairments in social memory (Wersinger et al., 2002), which will be discussed further in Section 5.2. In females, social memory is often tested using the Bruce effect as an indicator of recognition. This is when a pregnant female will terminate the pregnancy in response to an interaction with an unfamiliar male (Bruce, 1959). It has been observed that Avpr1a −/− females show a normal Bruce effect, while Avpr1b −/− females do not terminate their pregnancies in the presence of an unfamiliar male (Wersinger et al., 2008), showing an inability to recognize their mate.

4.3 Avp and Aggression

Several studies have implicated Avp in the neuroregulation of aggression in a variety of rodent species, such as mice (Bester-Meredith and Marler, 2001,
Wersinger et al., 2002), rats (Ferris, 2008, Veenema and Neumann, 2009), and prairie voles (Winslow et al., 1993). However, the most compelling evidence comes from studies in hamsters. These animals are a solitary species that display robust and reliable territorial aggression (Garrett and Campbell, 1980), as well as, stereotypic flank marking when presented with another male in a resident-intruder test (Siegel, 1985). Microinjection of Avp into the medial preoptic-anterior hypothalamic area results in an increase of flank marking behavior (Ferris et al., 1984). Avpr1a antagonists injected into this area or given orally block Avp-facilitated flank marking behavior (Ferris et al., 1988, Ferris et al., 1993, Caldwell and Albers, 2003, Ferris et al., 2006). In male Syrian hamsters (Mesocricetus auratus), microinjections of Avp into the AH increases aggression and injection of specific Avpr1a antagonists into the same area attenuates aggression (Ferris and Potegal, 1988, Potegal and Einon, 1989, Caldwell and Albers, 2004, Gobrogge et al., 2007). Similarly, Avp injected into the ventrolateral hypothalamus in males also increases aggressive behavior (Delville et al., 1996). There is also evidence of a role for the Avpr1b in the regulation of aggression in hamsters and will be further discussed in Section 5.3.

Comparable data has been found in other species, but some of the neuroanatomical areas involved differ from that of hamsters. Mated prairie voles that received injections of Avp into the AH show increased offensive aggression (Winslow et al., 1993). These animals also show increased c-Fos activation within the AH after high levels of aggression towards intruders (Gobrogge et al., 2007). In
mice bred for short attack latency, there is less Avp-ir innervation in the LS and fewer Avp-ir neurons in the BNST than long attack latency mice (Compaan et al., 1993). In rats, there is also a negative correlation between Avp and aggression in the LS (Everts et al., 1997). However, in female pigs, a contradictory relationship has been observed with Avp mRNA being significantly higher in the LS and MeA in highly aggressive animals versus non-aggressive animals (D'Eath et al., 2005).

The role of Avp in the regulation of female offensive aggression has only recently been investigated. Female Syrian hamsters injected with Avp into the AH display attenuated aggression, while Avpr1a antagonists seem to facilitate aggression (Gutzler et al., 2010) suggesting an inhibitory role for Avp in female hamster aggression. Maternal aggression, another form of female aggression, is often displayed by lactating postpartum mothers towards an intruder (Lonstein and Gammie, 2002). Central injections of Avp decreased and an Avpr1a antagonist increased maternal aggression in female rats (Nephew and Bridges, 2008b, Nephew et al., 2010). However, further studies are needed to elucidate the brain regions involved in Avp's regulation of female aggression.

5. The Avpr1b and Social Behavior

To date, much of the work in rodents implicating Avp in the regulation of social behavior has focused on its action via the Avpr1a. However, there is mounting evidence that the Avpr1b also plays a significant role in Avp's modulation of social behavior. Knockout and pharmacological studies implicate the central Avpr1b as an important neuromodulator of social behavior (Griebel et al., 2002, Wersinger et al.,
2002, Wersinger et al., 2004, Blanchard et al., 2005, Wersinger et al., 2007, Caldwell et al., 2008a, Caldwell and Young, 2009). This section will focus on those studies and how the Avpr1b regulates social motivation, social memory, and aggression.

5.1 The Avpr1b and Social Motivation

As previously mentioned, in an olfactory-based social motivation test, Avpr1b −/− mice fail to exhibit a preference for urine soiled bedding over clean bedding. Rather, Avpr1b −/− mice spend equal amounts of time interacting with either of the choices and less time overall interacting with the bedding (Wersinger et al., 2004). In a social preference test, Avpr1b −/− mice prefer a novel mouse over an inanimate object, suggesting that in the presence of myriad sensory cues Avpr1b −/− mice are still interested in social stimuli (Yang et al., 2007). Though, the amount of time spent interacting with another mouse tends to be much lower in Avpr1b −/− mice compared to controls (DeVito et al., 2009). Taken together, these data suggest that Avpr1b −/− mice have deficits in their motivation to interact with social stimuli.

5.2 The Avpr1b and Social Memory

In addition to deficits in social motivation, Avpr1b −/− mice also have mild impairments in social recognition memory (for review see Caldwell et al., 2008a, Caldwell et al., 2008c). In both an 11-trial habituation/dishabitation social recognition test and a 2-trial social discrimination test, Avpr1b −/− mice display deficits in social memory. Specifically, Avpr1b −/− males spend more trials of an 11-trial habituation/dishabitation task (with 5-minute intertrial intervals) investigating a familiar mouse than their Avpr1b +/+ counterpart. The impairment
is considered mild as Avpr1b −/− males do eventually decrease the amount of time spent investigating a familiar mouse over the 11 trials, which indicates recognition of the familiar animal. However, following either a 30-minute or 24-hour delay, Avpr1b −/− males are unable to discriminate between a novel female and a familiar female or a littermate and a novel animal, respectively (Wersinger et al., 2002, DeVito et al., 2009). Avpr1b −/− mice also have difficulty associating objects with odors in a specific sequence when the training sessions and testing sessions are separated by 24 hours during an object-trace-odor test, suggesting that Avpr1b −/− mice have impaired temporal memory in which they cannot integrate and/or recall information after a substantial time delay (DeVito et al., 2009). This may explain why Avpr1b −/− males are able to recognize a novel animal when there is a short time delay between presentations, such as in the 11-trial habitation/dishabituation test where there is only a 5-minute interval between trials, but not in a test with a longer time delay, such as 30 min (Wersinger et al., 2002).

Since the standard tests used to measure social recognition memory are generally less robust in females, Wersinger et al. (2008) measured the Bruce effect in Avpr1b −/− females. While social recognition and the Bruce effect are likely mediated by different neural pathways, both require the detection and processing of olfactory cues. The removal of the vomeronasal organ, but not the main olfactory epithelium, eliminates pregnancy block, indicating that the Bruce effect is mediated by the accessory olfactory system (Bellringer et al., 1980). As previously mentioned, unlike controls, female Avpr1b −/− mice fail to terminate their pregnancies when
presented with an unfamiliar male (Wersinger et al., 2008). These data suggest that, if the time interval is long enough, female Avpr1b −/− mice, like males, cannot discriminate between novel and familiar conspecifics.

5.3 The Avpr1b and Aggression

Avpr1b −/− mice show deficits in social aggression by displaying longer attack latencies and fewer attacks in a resident-intruder and neutral-arena aggression test compared to Avpr1b +/+ controls (Wersinger et al., 2002, Wersinger et al., 2007). Further, in a reversal of a resident–intruder test, where the experimental mice are intruders rather than residents, Avpr1b −/− mice display normal defensive avoidance behaviors (i.e., boxing stance and protection of their flanks) when attacked by a stimulus animal, but are less likely to initiate retaliatory attacks (Wersinger et al., 2007). The Avpr1b antagonist, SSR149415, shows results consistent with the Avpr1b −/− mice. Syrian hamsters given SSR149415 orally have reductions in the frequency and duration of offensive attacks, chase behaviors, flank marking, and in the olfactory investigation that often precedes and accompanies an offensive attack (Blanchard et al., 2005). In addition, mice that are orally administered SSR149415, display fewer defensive bites when forced to encounter a threatening predator and reductions in the duration of offensive aggression in a resident–intruder test (Griebel et al., 2002).

While repeated aggression testing or food competition can increase aggressive behaviors in Avpr1b −/− mice, they never reach the levels observed in control animals (Wersinger et al., 2007), which may reflect the importance of the
Avpr1b for normal displays of aggressive behavior under social conditions or could be an artifact resulting from developmental compensation in Avpr1b −/− mice. In tests of social dominance, male Avpr1b −/− mice are able to form dominance hierarchies, but they do so by employing alternative strategies and fewer displays of aggressive behaviors. Specifically, in early hierarchy formation, socially dominant Avpr1b −/− mice display more mounting behavior than Avpr1b +/+ mice, and non-socially dominant Avpr1b −/− mice engage in fewer attacks and have shorter attack durations compared to controls (Caldwell et al., 2010). The reduced aggression phenotype observed in Avpr1b −/− mice does not appear to be strain specific, as Avpr1b −/− mice crossed with the more “wild” outbred strain, Mus musculus castaneus, have reduced aggressive behaviors relative to controls (Caldwell and Young, 2009).

The deficits in aggressive behaviors observed in Avpr1b −/− mice are not limited to males. Following parturition, female Avpr1b −/− mice have reductions in maternal aggressive behaviors, compared to control mice, as measured by longer attack latencies and fewer attacks, directed toward a male intruder (Wersinger et al., 2007). Interestingly, the disruption of the Avpr1b does not affect all forms of aggressive behavior. In a non-social context, such as the predation of a cricket, Avpr1b −/− and Avpr1b +/+ mice have similar attack latencies (Wersinger et al., 2007). These data are important because they demonstrate that Avpr1b −/− mice are capable of detecting and attacking a stimulus. Interestingly, in tests of juvenile play behaviors, which include play soliciting behaviors such as “push/crawl” and
investigative behaviors such as nose-to-nose sniff, Avpr1b −/− mice and controls spend similar amounts of time engaged in sniffing, pushing, crawling over and following of littermates. The only measureable differences in behavior at this time in development are that Avpr1b −/− mice spend less time huddling with littermates when compared to controls (Yang et al., 2007). While juvenile play behaviors do not include aggressive behaviors per se, there are aspects of agonistic behaviors that, at least in some rodents, appear to morph into adult aggressive behaviors (Wommack et al., 2003, Delville, 2005). Thus, the lack of genotypic differences in predatory aggression and juvenile play behaviors, as well as, the other impairments in social behavior suggest that disruption of the Avpr1b does not result in a global disruption of aggressive behavior, but rather affects aggressive behavior only in specific social contexts.

5.4 The Avpr1b and Olfaction

As a functional olfactory system is critical for normal displays of social behaviors in rodents, many of the behavioral deficits observed in Avpr1b −/− mice could be explained if they were to have an impaired olfactory system. However, in a simple test of olfactory ability Avpr1b −/− mice are as adept at finding a hidden cookie as Avpr1b +/+ mice (Wersinger et al., 2002). In a more refined olfactory discrimination test using an olfactometer, Avpr1b −/− mice are able to discriminate between male and female urine (Wersinger et al., 2004). Further, when compared to Avpr1b +/+ mice, Avpr1b −/− mice display a similar elevation in the number of c-Fos containing neurons in the main and accessory olfactory bulbs after a social
encounter with another male (Wersinger et al., 2002). These data suggest that the chemosensory neural circuit is not compromised in Avpr1b −/− mice.

5.5 Is the Avpr1b within the CA2 region of the hippocampus important?

While it is clear that Avp and the Avpr1b are important to the neural regulation of social behavior, the specific molecular mechanisms and brain regions involved are unknown. Though, the prominent expression of Avpr1b in the CA2 region of the hippocampus (Figure 4) in mouse, rat, and human is intriguing and suggests conservation across species. A possible role for the CA2 region in the regulation of social behaviors is revealed in hippocampal lesion studies in rodents that include the CA2 region. When the CA2 region is a part of the lesion, rodents show a phenotype similar to that found in Avpr1b −/− mice. Specifically, reduced aggression and impaired social recognition (Ely et al., 1977, Maaswinkel et al., 1996, Uekita and Okanoya, 2011). Based on these data and the function of the hippocampus, it has been hypothesized that the Avpr1b within the CA2 field may aid in the formation and/or recall of memories related to social encounters and in particular those that are accessory olfactory system-based (Young et al., 2006, Caldwell et al., 2008c).

The CA2 region is quite distinct from the CA1 and CA3 regions, receiving input from the posterior hypothalamus, particularly the supramammillary nucleus (Borhegyi and Leranth, 1997, Bartesaghi et al., 2006) and the perforant pathway, which connects the entorhinal cortex to the hippocampal formation, bypassing the granule cell layer (Bartesaghi and Gessi, 2004). While Avp is often found in the
Figure 4: Illustration of a coronal section of the mouse brain. "⋆" indicates the CA2 region of the hippocampus (Paxinos and Franklin, 2001).
dorsal hippocampus (Landgraf et al., 1991), there is no known direct Avp innervation to the CA2 region. Possible sources of Avp to this part of the hippocampus, and ultimately to the Avpr1b, may be from extracellular and cerebral spinal fluid via distant axonal release and long distance diffusion (Herkenham, 1987, Landgraf and Neumann, 2004), nearby axonal release from the entorhinal cortices, or from the pyramidal cells of the CA1 and CA3 fields of the hippocampus (Hallbeck et al., 1999). It is also possible that the prominence of the Avpr1b in the CA2 region does not reflect its importance in the neural regulation of social behavior, and that one, or several, of the other brain areas that express the Avpr1b are also important.

6. Scope of Dissertation

The goal of this dissertation is to elucidate the role of the Avpr1b within the dorsal third of the CA2 region of the hippocampus in social behavior. We used a mouse model in order to be able to compare animals with hippocampal lesions or site-specific knockdown of the Avpr1b to Avpr1b +/+ mice and Avpr1b −/− mice. Mice are very social animals that display stereotypical social behaviors that have been thoroughly studied (Banks, 1962, Scott, 1966). Methods to reliably measure social behavior in these animals have are also well developed, thus allowing us to easily observe the changes in social behavior that resulted from the lesions, knockdown, and knockout of the Avpr1b receptor. There is a large body of evidence that Avp plays a critical role in the regulation of social behavior in mice, as well as, other species (Goodson and Bass, 2001, Caldwell et al., 2008a, Albers, 2012). The mounting evidence that the Avpr1b is also playing a role in the neural modulation of
social behavior spearheaded this body of work. The fact that the Avpr1b is prominent in the CA2 region of the hippocampus of mice, rats, and humans and that animals lacking this receptor have social deficits lead to the theory that the Avpr1b within the CA2 region must be a factor in the receptor's regulation of social behavior.

The focus of Chapter 2 was to see if there was a difference in neuronal activation in the brains of male and female Avpr1b +/+ mice and Avpr1b −/− mice after a social interaction. Specifically, we chose to look at the effects of aggression on neuronal activation using the immediate early gene, c-Fos, as an indicator. We focused on areas of the brain known to be important in the neural regulation of aggression in both males and females, as well as, the CA2 region of the hippocampus. It was hypothesized that there would be a measurable genotypic difference in neuronal activation after a resident-intruder test. A previous study had shown that male Avpr1b +/+ mice and Avpr1b −/− mice do not show a genotypic difference in c-Fos expression in areas of the brain known to effect social behavior, but do have increased c-Fos expression compared to animals that did not encounter an intruder (Wersinger et al., 2002).

To follow up the results of Chapter 2, we conducted a study in Chapter 3 in which we site-specifically lesioned the dorsal third of the CA2 region of the hippocampus of male C57BL/6J mice and then looked at the effects on aggression, social memory, and olfaction. Earlier studies had shown that animals with lesions to the hippocampus that included the CA2 region had a similar social behavioral
phenotype to that of Avpr1b −/− mice, specifically reduced aggression and impaired social recognition (Ely et al., 1977, Maaswinkel et al., 1996, Wersinger et al., 2002, McHugh et al., 2004, Wersinger et al., 2004, Wersinger et al., 2007, Caldwell et al., 2008c, Caldwell et al., 2010, Uekita and Okanoya, 2011, Stevenson and Caldwell, 2012). This current study was unique in that it was focused solely on the contributions of the CA2 region of the hippocampus to aggression and social recognition memory. It was hypothesized that animals with bilateral lesions to the CA2 region would show deficits in aggression and impaired social memory. We predicted that this would come in the form of longer attack latencies and fewer attacks than controls and the inability to discriminate between a novel and a familiar stimulus animal in a social recognition test. In regards to olfaction, there have been no reports of impaired olfaction in either the Avpr1b −/− mice or hippocampal lesioned animals in which the CA2 region was included (Bunsey and Eichenbaum, 1995, Burton et al., 2000, Wersinger et al., 2002, Wersinger et al., 2004). However, since olfaction plays such an important role in the social behavior of rodent species we tested this ability in our CA2 lesioned animals. We hypothesized that, like Avpr1b −/− mice, there would be no deficits in olfaction observed.

In Chapter 4, to further elucidate the role of the Avpr1b within the CA2 region of the hippocampus on the neural modulation of social behavior we aimed to site-specifically knockdown the receptor within the CA2 region and look at the effects on aggression and social memory. To do this we injected a lentiviral vector
containing siRNA against the Avpr1b into the CA2 region and then tested the animals in a resident-intruder test and a social discrimination test. This study would tell us if, specifically, the Avpr1b within the CA2 region are responsible for the behavioral deficits observed in Avpr1b −/− mice. It was hypothesized that adequate knockdown of the receptor would result in reduced aggression and impaired social memory. Further, we predicted that animals that received site-specific injections of the siRNA against Avpr1b would have longer attack latencies and fewer attacks than controls and would not be able to discriminate between novel and familiar stimulus females in a test of social memory.

Finally, in Chapter 5 we wanted to localize the Avpr1b in the brain of another aggressive rodent species, the Syrian hamster. Syrian hamsters, like mice, display territorial aggression (Garrett and Campbell, 1980) that is known to be affected by Avp (Albers et al., 2002) and regulated, in part, by the Avpr1b. To do this we cloned a region of the hamster Avpr1b and used it as a template to make a DIG-labeled probe for use in in situ hybridization histochemistry. Previous studies have shown that hamsters given the Avpr1b antagonist, SSR149415, show behaviors similar to that of Avpr1b −/− mice (Blanchard et al., 2005). This suggests a conserved role for the receptor in social behavior. In addition, as previously stated, the receptor has been localized in similar regions in the brains of mice, rats, and humans, suggesting a conserved distribution of the receptor. For this reason, we hypothesized that the Avpr1b would be localized in the hamster in many of the same brain regions that it
is found in mice. The results of this study could lead to the innovation of new scientific tools that could be used to study this receptor further.
Chapter 2

Immediate early gene activation in the CA2 region of the hippocampus after a social interaction

Introduction

Aggression is the intent to inflict damage or pain on another individual (Moyer, 1968, Moyer, 1971). In a social context aggression can be used to maintain social status, possibly providing access to mates or food, and is important for the survival of most mammalian species. Breeding males defend their territory by excluding or dominating intruder males with displays of offensive aggression (Crowcroft and Rowe, 1963, Poole and Morgan, 1976, Hurst, 1987). In mice, aggressive behaviors include attack biting, tail rattling, pushing/shoving, mounting, and chasing (Miczek et al., 2001). Male mice are typically more aggressive than female mice, however, females do show elevated aggression around the time of parturition. While the neural regulation of aggression is complex, gonadal steroids, monoamines, such as serotonin, and neuropeptides have all been implicated in its regulation.

In particular, the neuropeptide, arginine vasopressin (Avp), has been identified across species as playing a significant role in the regulation of aggressive
behavior (for review see Goodson and Bass, 2001, Caldwell et al., 2008a, Albers, 2012). While there has been a substantial amount of work implicating the centrally expressed Avp 1a receptor (Avpr1a) (Young et al., 1999, Hammock and Young, 2002, Bielsky et al., 2004, Walum et al., 2008), more recent work in Avp 1b receptor knockout (Avpr1b −/−) mice has shown that the centrally expressed Avpr1b is also essential for normal displays of offensive aggression (for review see Caldwell et al., 2008a, Caldwell et al., 2008c, Stevenson and Caldwell, 2012). Null mutation of the Avpr1b results in deficits in aggression, in the form of longer attack latencies and fewer attacks than Avpr1b wildtype (+/+ ) controls in a resident-intruder test (Wersinger et al., 2002, Wersinger et al., 2007). In a reversal of a resident-intruder test, where the experimental animal acts as the intruder, Avpr1b −/− mice display normal defensive behaviors when attacked by the resident animal, but are less likely to initiate a retaliatory attack (Wersinger et al., 2007). Female Avpr1b −/− mice also display reductions in maternal aggression following parturition compared to Avpr1b +/+ controls. The deficits in aggression observed in Avpr1b −/− mice does not seem strain specific, as evidenced by the persistence of reduced aggression in Avpr1b −/− mice crossed with the more “wild” outbred strain, Mus musculus castaneus (Caldwell and Young, 2009). Interestingly, the absence of the Avpr1b does not affect all forms of aggression. For example, Avpr1b −/− mice display normal predatory aggression, which is considered a non-social form of aggressive behavior (Wersinger et al., 2007). However, despite deficits in social aggression, Avpr1b −/− mice are still able to form dominance hierarchies, but use fewer displays of
aggressive behavior and more frequent bouts of mounting behavior (Caldwell et al., 2010). What remains unknown is where in the brain Avp is acting through the Avpr1b to modulate aggressive behavior.

The Avpr1b has been localized in the corticotrophes of the anterior pituitary, several extrapituitary brain regions, and in the periphery (Arsenijevic et al., 1994, Lolait et al., 1995, Young et al., 2006). Recently, a study using in situ hybridization histochemistry found that the Avpr1b is abundant in the CA2 region of the hippocampus in the mouse, rat, and human (Young et al., 2006). This region is of interest to our lab as there is evidence that animals with lesions to the hippocampus that include the CA2 region show deficits in social behavior similar to that of Avpr1b −/− mice; specifically, reduced aggression and impaired social recognition (Ely et al., 1977, Maaswinkel et al., 1996, McHugh et al., 2004, Uekita and Okanoya, 2011). Due to the restricted expression of the Avpr1b, we as well as others have hypothesized that the Avpr1b receptor within the CA2 region may play a critical role in Avp's regulation of social behavior. Further, it has been suggested that the CA2 region may form a link between olfactory cues and social interactions (Young et al., 2006, Caldwell et al., 2008c) and that its presence within the CA2 region may help to form and/or recall accessory olfactory-based memories (Caldwell and Young, 2009).

The use of immediate early genes (IEGs) as an indicator of neuronal activation is one tool that has previously been used to identify the neural circuitry underlying social behavior, including aggression (Morgan and Curran, 1989, Kollack-Walker and Newman, 1995, Gammie and Nelson, 2001, Hasen and Gammie,
Immediate early genes, such as c-Fos, are upregulated in response to depolarization of a neuron and cause changes in gene transcription within that cell (Sheng and Greenberg, 1990, Ghosh et al., 1994). Thus, changes in IEG expression, when correlated with aggressive behavior, may indicate changes in neuronal activity important to the display of aggressive behavior. In order to elucidate the possible contributions of the CA2 region of the hippocampus to aggressive behavior we investigated the expression of the IEG, c-Fos, in male and female Avpr1b +/+ and Avpr1b −/− mice after either a resident-intruder test or a maternal aggression test. We hypothesized that an aggressive encounter would increase IEG expression in Avpr1b +/+ mice compared to Avpr1b −/− mice.

**Materials and Methods**

*Animals and Housing*

Adult male and female Avpr1b +/+ and Avpr1b −/− mice were bred from mating heterozygotes in our animal colony. The animals were kept on a 12:12 light:dark cycle and provided food and water *ad libitum*. At the time of weaning (18-21 days post-partum), tails were clipped to extract DNA and PCR was performed as previously described (Caldwell et al., 2010). All subjects were 2 to 6 months of age at time of testing and all experiments were conducted in accordance with the protocol approved by the Kent State University Institutional Animal Care and Use Committee.
**General Aggression Testing**

To acclimate animals to the testing space, all animals were moved to the behavioral testing room at least 1 hour prior to testing and left undisturbed. Testing commenced approximately 1 hour after lights out under dim red light illumination during the dark phase of the light: dark cycle. All sessions were recorded using an infrared camera. Group-housed male Balb/c mice (as previously used in Caldwell and Young, 2009, Dhakar et al., 2012), between the ages of 2 and 5 months, were purchased from The Jackson Laboratory (Bar Harbor, ME) and used as intruder animals. At the initiation of testing, a male intruder mouse was added to the home cage of the resident and the animals were allowed to interact for 5 minutes. Following the initial attack, the observation continued for another 2 minutes. Therefore, the test could be as short as 2 minutes or as long as 7 minutes. Animals that had a latency score of 300 seconds were classified as not initiating an attack and those that did not as initiators of an attack. At the conclusion of testing, the intruder was removed and placed back into its home cage. The experimental animals were euthanized via cervical dislocation 60 minutes following the testing session to allow time for the transcription and translation of c-Fos. The brains of the animals were then immediately removed and fixed in 4% paraformaldehyde. The fixed tissue was then cut into 4 series of 50μm free-floating sections using a vibratome (Pelco® 102 vibratome sectioning system, Ted Pella, Inc., Redding, CA) and stored at -20°C in an ethylene glycol based cryoprotectant (30% sucrose, 30%
ethylene glycol, 1% polyvinyl pyrrolidine in 0.1M PBS) (Watson et al., 1986) prior to immunohistochemical staining.

**Resident-Intruder Test**

Prior to testing, male Avpr1b +/+ (n = 12) and Avpr1b −/− (n = 10) mice were single housed for 2-4 weeks, with no bedding/cage change in the week leading up to testing. The purpose of this was to increase isolation-induced aggression in our experimental animals and establish them as residents for the resident-intruder test. All animals then underwent the general aggression testing procedure as described above.

**Maternal Aggression Test**

Adult female Avpr1b +/+ (n = 10) and Avpr1b −/− (n = 13) mice were randomly assigned to a group that was either tested (Avpr1b +/+ n = 5, Avpr1b −/− n = 6) or untested (Avpr1b +/+ n = 5, Avpr1b −/− n = 7) in a maternal aggression test. All experimental mice were housed in single-sex groups (up to four per cage) for two weeks to synchronize estrous cycles. Three days prior to mating, male bedding was added to the females’ cages to induce the Whitten effect, where male odours induce oestrus and synchronize oestrus cycles among females (Whitten, 1956, 1958). An experienced adult breeder male, from one of our transgenic lines, was placed in the cage for 1 week to impregnate the females. One week prior to estimated parturition, the females were single-housed. Litters were culled to 4 pups
on post-partum day (PPD) 2 and animals were either tested or untested on PPD4. Cage changes were not permitted during the isolation period. The tested animals underwent the general aggression testing procedure as described above. Untested animals underwent the same procedure omitting the intruder being placed into the resident’s cage.

*Immunohistochemistry for c-Fos*

c-Fos immunostaining was performed as described in Dhakar et al. (2012). Briefly, sections were washed in 1X phosphate buffered saline (PBS) 6 times for 10 minutes each, then incubated in 1.5% hydrogen peroxide for five minutes, and washed again in 1XPBS 2 times for 5 minutes each. Using Power Block™ Universal Blocking Reagent (BioGenex, San Ramon, CA), the sections were blocked for 30 minutes. The sections were then incubated overnight in rabbit anti-c-Fos primary antibody (Santa Cruz Biochemicals, Santa Cruz, CA, USA, sc-52) at a dilution of 1:5000 in antisera diluent (1XPBS + 1% normal goat serum + 0.3% Triton X-100) at 4°C. The sections were then washed in 1XPBS 3 times for 5 minutes each to remove excess primary antibody. The sections were then incubated for 1 hour in biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:500 in antisera diluent. Following incubation, the sections were washed in 1XPBS 3 times for 5 minutes each and exposed to an avidin-biotin complex for 1 hour (Vector Laboratories, Burlingame, CA). The sections were then washed in 1XPBS 3 times for 5 minutes each and staining was visualized using
diaminobenzidine (DAB) for 2-10 minutes until staining was sufficient. Sections were then washed in 1XPBS twice for 5 minutes to inactivate the DAB, mounted onto gel-subbed slides, dried overnight, counterstained with 0.2% methyl green, and coverslipped.

Quantification of c-Fos Immunoreactivity

c-Fos immunoreactive (Fos-ir) cells were counted at 100X magnification by a single observer blind to testing groups. iVision software (BioVision Technologies, Exton, PA) was used to capture the images and immunoreactive cells were manually counted within each neuroanatomical area. Three sections per area, with sections being 100μm apart, were bilaterally counted and averaged using set box sizes for each area (box sizes from Hasen and Gammie, 2005). Areas measured included the dorsal and ventral aspects of the lateral septum, the LSD (732 x 754 pixels) and the LSV (380 x 338 pixels), respectively, the dorsal and ventral aspects of the bed nucleus of the stria terminalis, the BNSTD (600 x 870 pixels) and the BNSTV (600 x 435 pixels), respectively, the medial preoptic area (MPOA) (465 x 870 pixels), the paraventricular nucleus (PVN) (350 x 600 pixels), the medial amygdala (MeA) (990 x 870 pixels) and the CA2 region of the hippocampus (300 x 300). These areas were identified based upon Paxinos and Franklin (2001) mouse brain atlas.

Statistical Analysis
Comparisons were made between genotypes and treatment groups (females) using a two-way ANOVA (SPSS 16.0 for Mac, IBM, Armonk, NY). Since there were no significant differences between the c-Fos expression in untested female Avpr1b +/+ and Avpr1b −/− mice, these two groups were combined into one group that served as controls. A result was considered statistically significant if $p \leq 0.05$.

Due to the fact that Avpr1b −/− mice rarely fought during testing, only animals that did not engage in fighting behavior were used (males $n = 22$, females $n = 23$). This also allowed us to avoid confounds due to the induction of neuronal activation that resulted from the physical act of fighting. It also helped assure that any changes that were observed in IEG expression could be attributed to the detection and/or processing of social cues rather than the behavioral output.

**Results**

*Fos-ir*

There was no significant genotypic difference seen within either sex in any of the brain regions examined (Figure 5A and 5B). There was however a significant increase in the number of Fos-ir cells seen between females exposed to a male intruder (tested) and those that were not (untested). Those differences were found in the following brain regions: the LSV, BNSTD, BNSTV, MPOA, and MeA (Figures 6-10, respectively, and Table 1). Fos-ir was not induced by the social stimulus in the LSD, PVN, or CA2 region of the hippocampus.
A c-Fos expression in male Avpr1b +/+ and Avpr1b −/− mice after a resident-intruder test

B c-Fos expression in female Avpr1b +/+ and Avpr1b −/− mice after a maternal aggression test

Figure 5: A) Graph depicting the number of c-Fos labeled cells in various brain regions of male Avpr1b +/+ and Avpr1b −/− mice after a resident-intruder test. B) Graph depicting the number of c-Fos labeled cells in various brain regions of female Avpr1b +/+ and Avpr1b −/− mice after a maternal aggression test. Abbreviations: (LSD) dorsal lateral septum, (LSV) ventral lateral septum, (BNSTD) dorsal bed nucleus of the stria terminalis, (BNSTV) ventral bed nucleus of the stria terminalis, (MPOA) medial preoptic area, (PVN) paraventricular nucleus, (MeA) medial amygdala, (CA2) CA2 region of the hippocampus.
Figure 6: Photomicrographs of the ventral lateral septum (LSV) showing c-Fos staining of female mice that were either untested (A) or tested (B) in a maternal aggression test. C) Illustration of the corresponding coronal section of the mouse brain (Paxinos and Franklin, 2001).
Figure 7: Photomicrographs of the dorsal bed nucleus of the stria terminalis (BNSTD) showing c-Fos staining of female mice that were either untested (A) or tested (B) in a maternal aggression test. C) Illustration of the corresponding coronal section of the mouse brain (Paxinos and Franklin, 2001).
Figure 8: Photomicrographs of the ventral bed nucleus of the stria terminalis (BNSTV) showing c-Fos staining of female mice that were either untested (A) or tested (B) in a maternal aggression test. C) Illustration of the corresponding coronal section of the mouse brain (Paxinos and Franklin, 2001).
Figure 9: Photomicrographs of the **medial preoptic area (MPOA)** showing c-Fos staining of female mice that were either untested (A) or tested (B) in a maternal aggression test. C) Illustration of the corresponding coronal section of the mouse brain (Paxinos and Franklin, 2001).
Figure 10: Photomicrographs of the **medial amygdala (MeA)** showing c-Fos staining of female mice that were either untested (A) or tested (B) in a maternal aggression test. C) Illustration of the corresponding coronal section of the mouse brain (Paxinos and Franklin, 2001).
Table 1: Number of c-Fos immunoreactive cells in female tested and untested Avpr1b +/+ and Avpr1b −/− mice. There was a significant increase in Fos-ir found in animals exposed to an intruder male in the LSV, BNSTD, BNSTV, MPOA, and MeA.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Avpr1b +/+</th>
<th>Avpr1b −/−</th>
<th>Untested</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSD</td>
<td>80 ± 4</td>
<td>94 ± 19</td>
<td>85 ± 12</td>
</tr>
<tr>
<td>LSV</td>
<td>58 ± 4</td>
<td>58 ± 4</td>
<td>45 ± 3*</td>
</tr>
<tr>
<td>BNSTD</td>
<td>125 ± 13</td>
<td>105 ± 10</td>
<td>81 ± 6*</td>
</tr>
<tr>
<td>BNSTV</td>
<td>58 ± 8</td>
<td>48 ± 6</td>
<td>31 ± 3*</td>
</tr>
<tr>
<td>MPOA</td>
<td>167 ± 26</td>
<td>139 ± 20</td>
<td>88 ± 8*</td>
</tr>
<tr>
<td>PVN</td>
<td>124 ± 23</td>
<td>117 ± 5</td>
<td>96 ± 9</td>
</tr>
<tr>
<td>MeA</td>
<td>255 ± 42</td>
<td>225 ± 30</td>
<td>127 ± 11*</td>
</tr>
<tr>
<td>Ca2</td>
<td>24 ± 1</td>
<td>26 ± 3</td>
<td>21 ± 2</td>
</tr>
</tbody>
</table>

* Effect of aggression testing P<0.05
Discussion

In this study, an increase in Fos-ir was observed in the LSV, BNSTD, BNSTV, MPOA, and MeA of female Avpr1b +/+ and Avpr1b −/− mice exposed to an intruder male compared to those that were not exposed (Table 1). Fos-ir was also observed in these brain regions in males, however no comparison could be made between tested and untested males because only tested males were used in this study. All of these areas have previously been implicated in the neural circuitry of aggression in rodents (as reviewed in Nelson and Trainor, 2007) and are known to have increases in Fos-ir after both inter-male (Kollack-Walker and Newman, 1995, Delville et al., 2000) and maternal aggression (Hasen and Gammie, 2005). Increases in IEG expression in these areas upon exposure to an intruder male suggest that these animals are able to detect the social stimulus and recognize that the appropriate response is to behave aggressive. The pathway for the transduction of these social signals is initiated in the vomeronasal organ. From there the information is sent to the accessory olfactory bulbs, which in turn the sends information to the MeA. The MeA then relays information to the MPOA and LS through the BNST in order to facilitate changes in behavior, such as aggression (Delville et al., 2000). When there is a lack of an appropriate aggressive response, this suggests that there is a breakdown in the system, somewhere between processing behavioral cues and the initiation of a behavioral response.

We were somewhat surprised that we found no genotypic differences in c-Fos expression in either sex in any of the brain regions examined, including the CA2
region. Though, these results are consistent with a study by Wersinger et al. (2002) in Avpr1b −/− mice that looked primarily at inter-male aggression but did not explore the effects of the aggressive stimulus on the CA2 region of the hippocampus. Like the current study, they found that exposure to an intruder male increased Fos-ir in the MeA and BNST, but despite this increase between tested and untested animals, there were no genotypic differences in neuronal activation.

Possibly, the absence of the Avpr1b in null mutants is affecting neuronal activation somewhere downstream of the CA2 region. Perhaps if we had measured Fos-ir in the CA1 region of the hippocampus, where the CA2 region makes strong excitatory connections (Chevaleyre and Siegelbaum, 2010), rather than the CA2 region itself, we may have found a genotypic difference. Although the CA2 region is generally assumed to only be a bridge between the CA1 and CA3 regions (Sekino et al., 1997), in fact, it is the only region of the hippocampus to receive inputs from the perforant pathway, which links the entorhinal cortex to the hippocampal formation (Bartesaghi and Gessi, 2004). The entorhinal cortex processes individual social odors and is a major site for memory formation and consolidation (Petrulis et al., 2005, Bartesaghi et al., 2006). These inputs are then passed on to the CA1 region, which is the main site of hippocampal output (Chevaleyre and Siegelbaum, 2010), followed by the CA3 region (Bartesaghi and Gessi, 2004), so the CA2 region acts as a link between the entorhinal cortex and the CA1 region.

It has been hypothesized that the CA2 region may play a role in the retrieval and/or formation of memories of social encounters (Caldwell and Young, 2009). For
example, both Avpr1b −/− mice and animals with lesions that include the CA2 region display impaired social memory (Ely et al., 1977, Maaswinkel et al., 1996, Maaswinkel et al., 1997, Wersinger et al., 2002, Uekita and Okanoya, 2011). It is also possible that the CA2 region may aid in general memory formation. Avpr1b −/− mice have difficulty associating objects with odors in a specific sequence when the training sessions and testing sessions are separated by 24 hours during an object-trace-odor test, suggesting deficits in temporal memory (a non-social form of memory) after a significant time delay (DeVito et al., 2009). However, Avpr1b −/− mice do display normal spatial memory as evidenced by escape latencies in a Morris water maze similar to that of Avpr1b +/+ mice (Wersinger et al., 2002). This suggests that the CA2 region may be more selective in the types of information that it processes.

It may also be that an immediate early gene other than c-Fos might have been better at detecting differences in neuronal activation between Avpr1b +/+ and Avpr1b −/− mice. Other studies looking at IEG activation resulting from an aggressive encounter have looked at extracellular signal-regulated kinase (ERK) (Trainor et al., 2010) and phosphorylated cyclic AMP response element binding protein (pCREB) (Gammie and Nelson, 2001). These IEGs are unique in that both ERK and pCREB can be quickly phosphorylated by estrogens (Abraham et al., 2004, Zsarnovszky et al., 2005, Szego et al., 2006). It has been shown that estrogens can facilitate aggression in male Peromyscus mice exposed to short days (Trainor et al., 2007, Trainor et al., 2008). Also, Avp gene transcription is facilitated by activation of
estrogen receptors, alpha and beta (Pak et al., 2005, Pak et al., 2007), and aggressive behavior in males is facilitated by an increase in AVP (as reviewed in Goodson and Bass, 2001, Caldwell et al., 2008a, Albers, 2012). Perhaps, estrogens are acting in areas of the brain in male mice to rapidly increase Avp, which in turn primes the system for aggressive behavior. The IEGs, ERK and pCREB, could be an indicator of this mechanism. Future studies in this lab will likely pursue these avenues.
CHAPTER 3

LESIONS TO THE CA2 REGION OF THE HIPPOCAMPUS ALTER SOCIAL BEHAVIORS IN MICE

Introduction

Our laboratory is interested in the neuropeptide vasopressin (Avp), which has been implicated in the neural regulation of a variety of social behaviors, including aggression (Griebel et al., 2002, Wersinger et al., 2002, Blanchard et al., 2005, Wersinger et al., 2007, Caldwell et al., 2010), social memory (Engelmann et al., 1994, Everts and Koolhaas, 1997, Wersinger et al., 2002, Bielsky et al., 2004, Bielsky et al., 2005, Wersinger et al., 2008, Choleris et al., 2009, DeVito et al., 2009), social motivation (Wersinger et al., 2004, Yang et al., 2007, DeVito et al., 2009), pair bonding (Winslow et al., 1993, Insel and Hulihan, 1995, Lim et al., 2004), and maternal behavior (Bosch and Neumann, 2008, Nephew and Bridges, 2008a). Avp modulates social behaviors via its two centrally expressed receptors, the vasopressin 1a receptor (Avpr1a), which is widely distributed throughout the brain, and the vasopressin 1b receptor (Avpr1b), which is more discretely distributed (Antoni, 1984, Ostrowski et al., 1992, Johnson et al., 1993, Lolait et al., 1995, Foletta et al., 2002, Young et al., 2006).
Unfortunately, where in the brain Avp signaling through the Avpr1b modulates social behavior remains unknown. Though, in situ hybridization has localized the Avpr1b to the pyramidal cells of the CA2 region of the hippocampus in mice, rats, and humans (Young et al., 2006). The prominence of the Avpr1b in this part of hippocampus was somewhat surprising as the CA2 region is quite unique from the CA1 and CA3 regions. For instance, unlike the adjacent fields, the CA2 region does not receive rich mossy fiber inputs from the dentate gyrus and lacks the thorny excrescences that are characteristic of mossy fiber synapses (Lorente de No, 1934, Tamamaki et al., 1988). The CA2 region is also neurochemically distinct from CA1 and CA3; creating distinct boundaries between the CA2 region and the rest of the hippocampus (Lein et al., 2004, Lein et al., 2005). Specifically, fibroblast growth factor 2 (Williams et al., 1996), adenosine A1 receptor (Ochiishi et al., 1999), epidermal growth factor receptor (Tucker et al., 1993), neurotrophin-3 (Vigers et al., 2000), and Purkinje cell protein 4 (Zhao et al., 2001) are all exclusively expressed in CA2 region, with no expression in CA1 and CA3 fields. The CA2 region is also the only part of the hippocampus that receives input from the posterior hypothalamus, particularly the supramammillary nucleus (Borhegyi and Leranth, 1997, Vertes and McKenna, 2000, Bartesaghi et al., 2006) and from the perforant pathway, which connects the entorhinal cortex to the hippocampal formation, bypassing the granule cell layer (Bartesaghi and Gessi, 2004). Interestingly, the CA2 region also shows resistance to pyramidal cell loss following head trauma (Maxwell et al., 2003), intractable epilepsy (Fried et al., 1992, Mathern et al., 1995b), and in
some models of ischemia (Kirino and Sano, 1984, Sadowski et al., 1999). Combined, these characteristics suggest that the CA2 region is distinct both in form and function from the rest of the hippocampus.

There is also some evidence that the Avpr1b in the CA2 region may be important to social behavior since rodents with hippocampal lesions, which include the CA2 region, show a phenotype similar to that of Avpr1b knockout (−/−) mice, specifically, reduced aggression and impaired social recognition (Ely et al., 1977, Maaswinkel et al., 1996, Wersinger et al., 2002, McHugh et al., 2004, Wersinger et al., 2004, Wersinger et al., 2007, Caldwell et al., 2008c, Caldwell et al., 2010, Uekita and Okanoya, 2011, Stevenson and Caldwell, 2012). Based on these data, and the general function of the hippocampus, we as well as others have hypothesized that the Avpr1b within the CA2 region of the hippocampus may aid in the formation and/or recall of accessory olfactory-based memories (Young et al., 2006, Caldwell et al., 2008c, Stevenson and Caldwell, 2012).

Unfortunately, the aforementioned lesion studies were generally focused on damaging other areas of the hippocampus and were therefore too large to elucidate the specific contributions of the CA2 region to the regulation of social behavior. For this reason we set out to lesion the dorsal third of the CA2 region of the hippocampus, where the Avpr1b is most abundant, and examine the effects on aggression, social recognition, and olfaction. We hypothesized that bilateral lesions to the CA2 region would affect aggression and social recognition memory. We predicted that lesions would result in reduced aggression as measured by longer
attack latencies and fewer attacks relative to controls in a resident-intruder test. We also predicted that lesions to the CA2 region would impair social recognition as measured by the inability to discriminate between a novel and a familiar stimulus animal. Lastly, we hypothesized that lesions of the CA2 region would have no effect on olfaction.

**Methods and Materials**

*Animals and Housing*

Adult male C57BL/6J were bred in the Kent State University vivarium (Exp. 1 and 2) or ordered from The Jackson Laboratory (Bar Harbor, ME) (Exp. 3, 4, and 5) and kept on a 12:12 light: dark cycle. At the time of weaning (postnatal day 18-21), animals were housed in single-sex sibling groups until surgery. Food and water were given *ad libitum*, except during testing and prior to the hidden cookie test. All subjects were 2 to 6 months of age at time of testing. Three sets of surgical animals were generated for behavioral testing; the first set for Exp. 1, the second set for Exp. 2, and the third set for Exp. 3-5. All experiments were conducted in accordance with the protocol approved by the Kent State University Institutional Animal Care and Use Committee.

*Lesion Surgery*

Prior to surgery the animals were randomly assigned to one of the following surgical groups: 1) bilateral excitotoxic lesion of the CA2 region or 2) sham surgery
to the CA2 region (surgical control). At time of surgery animals were anesthetized using a ketamine/xylazine cocktail at a dose of 81mg/kg ketamine and 16.2mg/kg xylazine (for Exp. 1 only) or a 2% isoflurane/oxygen mixture (for all other experiments). Using an Ultraprecise stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) and a 2μl Hamilton syringe (Hamilton Company, Reno, NV), groups of C57BL/6J male mice either underwent bilateral, site-specific microinjections of 10mg/ml N-methyl-D-aspartate (NMDA) solution or a bilateral sham operation in which the needle was inserted but no injection administered. A total of 6 separate injections (0.02μl of NMDA per injection) were performed, bilaterally, in order to localize the lesion to the dorsal third of the CA2 region (Figure 11). The coordinates used were: from Bregma (a) ML = ±1.3, AP = −1.3, DV = −1.45 (b) ML = ± 1.5, AP = −1.5, DV = −1.5 (c) ML = ± 1.9, AP = −1.8, DV = −1.6 (from top of the brain). Burr holes were made using a hand-held drill (Dremel®, Racine, WI) with an engraving cutter bit (model #105, Dremel®, Racine, WI). Once the burr hole was produced, the needle tip was placed at the appropriate depth and allowed to sit for 2 minutes prior to injection to allow the brain to reposition. Each injection took place over ~30 seconds and then the needle remained in place for another 2 minutes to allow the NMDA to diffuse away from the needle tip. The needle was then slowly removed, the skin brought back together over the skull, and closed with a wound clip. Following surgery, animals were administered 0.3ml of warm saline (9%) intraperitoneally (i.p.) to aid in recovery and returned to their home cage. Animals receiving bilateral lesions of the CA2 region were given chlordiazepoxide i.p. at a dose of 10mg/kg
Figure 11: Illustrations of coronal sections of hippocampus from -1.46 to -2.06 posterior to Bregma (modified from Paxinos and Franklin, 2001). (⋆) indicates injection target.
immediately after surgery to prevent seizures. All animals were allowed to recover for at least 1 week before behavioral testing and wound clips removed at the end of that week.

Experiment 1: Resident-Intruder Test

Twenty-three surgical animals were single housed for 2-4 weeks, with no bedding/cage change in the week prior to testing; single housing increased isolation-induced aggression and established the mice as residents. Group-housed male Balb/c mice (as previously used in Caldwell and Young, 2009), between the ages of 2 and 5 months, were purchased from The Jackson Laboratory (Bar Harbor, ME) and used as intruder animals. To acclimate animals to the testing space, all animals were moved to the behavioral testing room at least 1 hour prior to testing and left undisturbed. Testing commenced approximately 1 hour after lights out under dim red light illumination during the dark phase of the light: dark cycle. All sessions were recorded using a camera equipped with infrared. At the initiation of testing, the intruder Balb/c mouse was added to the home cage of the resident and the animals were allowed to interact for 5 minutes. Following the initial attack, the observation continued for another 2 minutes. Therefore, the test could be as short as 2 minutes or as long as 7 minutes. Animals that did not initiate attacks were given a latency of 300 seconds. At the conclusion of testing the intruder was removed and placed back into its home cage. Testing occurred over 5 sessions with 72 hours between testing sessions. Only testing sessions that included an attack were scored.
The behaviors that were analyzed were: latency to attack, duration of aggression (which included chasing, pushing, mounting of the intruder, and the lunge-bite-roll attack sequence), attack frequency (defined as a lunge followed by a bite), tail rattle frequency, duration of non-social behavior (the time the resident spent investigating the cage and grooming), and contact (the time the resident spent with its nose within 1cm of the intruder’s body) (as previously described in Dhakar et al., 2012).

**Experiment 2: 2-Trial Social Discrimination Test**

One week before testing, the stimulus animals (intact female Swiss Webster mice ages 2-4 months) were single-housed while the 28 surgical animals remained group housed. On the day of testing, all animals were moved to the testing room at time of lights out, individually housed in clean cages, and allowed to habituate to their new environment for 30 minutes. After the 30-minute habituation period, 2 clean wire corrals were placed on either side of the cage and the animals were allowed to habituate for another 30 minutes (Figure 12A). Testing commenced 1 hour after the start of the dark phase of the light: dark cycle. At the start of Trial 1, one of the corrals was removed and a female stimulus animal was placed in the remaining corral (Figure 12B). The experimental animal was observed investigating, which is defined as any time the experimental animal would insert their nose and/or forepaw through the bars of the corral or sniff any portion of the stimulus animal that was outside of the corral, the stimulus female over a period of 5 minutes. After that time, the stimulus animal was removed, returned to its home
Figure 12: The 2-Trial social discrimination test. A) An illustration of the placement of the corrals in the test cage during the habituation period. B) An illustration of Trial 1 in which the experimental animal is exposed to a single corralled female. C) An illustration of Trial 2 in which the experimental animal is exposed to both the “familiar” female (white) from Trial 1 and the “novel” female (grey).
cage, and the second corral placed in the experimental animal’s test cage (Figure 12A). During Trial 2, the “familiar” female from Trial 1 was placed back into its original corral, which had been moved to the opposite side of the cage, while a second “novel” female was placed into the remaining corral (Figure 12C). The experimental animal was observed for 5 minutes while being allowed to investigate both females. Both trials were videotaped and continuously scored for the amount of time spent investigating each stimulus female, duration of non-social behavior (as described above), and tail rattle frequency.

**Experiment 3: 11-Trial Habituation/Dishabituation Social Recognition Test**

One week prior to testing, the stimulus animals (intact female Swiss Webster mice ages 2-4 months) were single-housed while the 19 surgical animals were kept group housed. On the day of testing, all animals were moved to the testing room at the time of lights out, individually housed in clean cages with a clean wire corral placed in the center, and allowed to habituate to their new environment for 1 hour. At the time of testing, a randomly assigned stimulus female was placed into the corral for one minute (Trial 1) and the experimental animal was observed for olfactory investigation of the female. After 1 minute the stimulus animal was removed and placed back into its home cage for 5 minutes. This series of events occurred 9 more times with the same “familiar” stimulus female for a total of 10 1-minute trials with an inter-trial interval of 5 minutes (habituation). On the eleventh trial, a “novel” stimulus female was placed into the wire corral for 1 minute
(dishabituation). All 11 trials were videotaped and scored using a continuous scoring method for time spent investigating the stimulus animal (as described above), time spent in non-social behavior (as described above), and tail rattle frequency.

**Experiment 4: Hidden Cookie Test**

On the day prior to testing, the 19 surgical animals were weighed and food-restricted, but not water-restricted, overnight (approximately 10 hours). One hour before testing commenced the animals were moved to the testing room and allowed to acclimate to their new surroundings. Prior to testing the mice were again weighed. A small piece of Nabisco Nutter Butter™ cookie (5mm cube) was randomly placed on either the left or right side of an empty mouse box and covered with approximately 4mm of woodchip bedding. The subject was then placed into the center of the cage and allowed to investigate for up to 5 minutes or until the cookie was found. Finding the cookie was defined as having both front paws on the cookie piece. The latency to find the hidden cookie was recorded with a stopwatch.

**Experiment 5: Habituation/Dishabituation Olfactory Test**

Prior to testing, urine was collected from male and female mice that were a mixture of C57BL/J6 and 129/SvJ by placing 4-5 mice of each sex (at separate times) in an open field, in which the floor was covered with aluminum foil, for 15 minutes and then pipetting the drops of urine into a microcentrifuge tube. The urine was
then diluted to 1:100 with sterile water, aliquoted, and frozen at -20°C until used for testing. On the day of testing the 19 surgical animals were moved to the testing location at the time of lights out, singly-housed in clean cages, and allowed to acclimate to their new environment for 1 hour. Immediately before use, a cotton-tipped applicator was soaked in the appropriate odorant. Each cotton swab was presented to the subject by suspension from the cage lid, approximately 4cm above the bedding. The odorants (in order: water, almond extract (Market Pantry®, Target Brands, Inc., Minneapolis, MN), vanilla extract (Market Pantry®, Target Brands, Inc., Minneapolis, MN), male urine, and female urine all at a 1:100 dilution) were thawed immediately before use and kept on ice. A fresh microcentrifuge tube of each odorant was used for each animal. The odorants were presented for 2 minutes with an intertrial-interval of 1 minute for 3 trials apiece (habituation) before switching to the next novel odorant (dishabituation). The trials were videotaped and continuously scored for the amount of time spent in olfactory investigation (<1cm proximity) of the cotton swab.

Immunohistochemistry for NeuN

At the conclusion of behavioral testing all experimental animals were euthanized via cervical dislocation, their brains removed, and fixed in 4% paraformaldehyde. The brains remained in fixative at least 24 hours before being cut into two series of 50μm sections using a vibratome (Pelco® 102 vibratome sectioning system, Ted Pella, Inc., Redding, CA) and placed into scintillation vials
containing 1X phosphate buffered saline (PBS). Sections were washed in 1XPBS 2 times for 5 minutes each before blocking with 1X Power Block™ Universal Blocking Reagent (BioGenex, San Ramon, CA) at room temperature (RT) for 30 minutes and washing again in 1XPBS for 5 minutes. The sections were then incubated overnight at 4°C in primary antibody (Millipore™, Temecula, CA, mouse anti-NeuN, A60) diluted to 1:500 with antisera diluent (PBS + 1% normal goat serum + 0.3% Triton X-100). Following incubation in primary antibody the sections were washed in 1XPBS for 5 minutes and then incubated for 1 hour in secondary antibody (goat anti-mouse 1:500) from a Mouse Vectastain ABC kit from Vector Laboratories (Burlingame, CA, USA, PK-4002). Following incubation, the tissue was washed in 1XPBS for 5 minutes, incubated in 3% hydrogen peroxide for 20 minutes, and washed again in 1XPBS 2 times for 5 minutes. During this time the ABC complex was prepared according to kit instructions and allowed to sit for 30 minutes. The sections were then incubated in the ABC complex for 1 hour at RT, washed in 1XPBS for 5 minutes, and incubated with diaminobenzidine (DAB) solution for 2-10 minutes (Vector Laboratories, Burlingame, CA, USA, DAB substrate kit, SK-4100). Lastly, sections were washed in 1XPBS 2 times for 5 minutes, mounted onto gel-subbed slides, air dried, and coverslipped.

Lesion Quantification

Once stained for NeuN, the number of sections containing bilateral lesions of the CA2 region were counted from Bregma -0.94 to -2.30 (the dorsal third) and the
percentage of lesioned sections determined (number of lesioned sections/12 * 100 = percent lesioned). There was an average hit rate of 80% for bilateral CA2 lesions. For all experiments, only animals with >25% bilateral lesions were included in the statistical analyses (Figure 13).

Statistical Analysis

The mean group differences for the cumulative data from the resident-intruder test were analyzed using a Kruskal Wallis test (SPSS 16.0 for Mac, IBM, Armonk, NY). For the day-to-day measurements in the resident-intruder test a repeated measures analysis of variance was used. The number of animals attacking in the resident-intruder test was analyzed using a Fisher’s exact test. For the 2-trial social discrimination test and the 11-Trial habituation/dishabitation social recognition test an analysis of variance (ANOVA) was used to analyze Trial 1 data and each individual trial (treatment x behavior), respectively. A 1-tailed paired-sample Student’s t-test (as previously used in Macbeth et al., 2009) was run within each group to directly compare time spent during Trial 2 with “familiar” and “novel” females and the changes between trials, respectively. For the measurements in the habituation/dishabituation olfactory experiment a repeated measures analysis of variance was used for each odorant (trial x treatment). The data from the hidden cookie experiment were analyzed using an ANOVA. For all statistical tests a result was considered statistically significant if $p \leq 0.05$. 
Figure 13: Photomicrograph showing a coronal section of a mouse brain with bilateral lesions of the CA2 region of the hippocampus.
Results

Experiment 1: Resident-Intruder Test

Sham surgery controls (n = 10) and animals with lesions to the CA2 region (n = 13) were tested in a resident-intruder test. Animals with lesions <25% (n = 4) and missed targets (n = 2) were removed from the statistical analysis (control n = 8, lesion n = 9). Lesions to the CA2 region did not cause a detectable effect on aggression levels. However, aggression levels were lower in both treatment groups than what is typically observed. This is evident by the low percentage of animals attacking across testing days in both groups (Table 2). Duration of aggression, non-social behavior, and contact time, latency to attack, frequency of attack, and number of tail rattles were not significantly different between treatment groups (Table 2). There were also no significant differences observed across days within treatment groups.

Experiments 2 and 3: Social Memory Tests

Sham surgery controls (n = 13) and animals with lesions to the CA2 region (n = 14) were tested in a 2-Trial social discrimination test, while a second set of animals (control n = 11, lesion n = 8), were tested in an 11-trial habituation/dishabituation social recognition test. Two animals were removed from the 11-Trial test because the stimulus females used escaped their corrals in one or more of the 11 trials (control n = 11, lesion n = 6). In both tests we found that social recognition was impaired in animals with bilateral lesions to the CA2 region.
Table 2: Behaviors measured in resident-intruder test in Experiment 1 where there were no statistically significant differences observed.
compared to controls. Lesioned animals did not show the same pattern of chemoinvestigation as controls in either test for social memory (Figure 14A-14C). In the first trial of the 2-Trial social discrimination test the control animals spent significantly more time with the novel female than the CA2 lesioned animals \((F_{1,26} = 9.904 * p = 0.004)\) (Figure 14A). This suggests a decrease in social motivation in lesioned animals. In the second trial, in which the experimental animal was presented with the familiar female from Trial 1 as well as a novel female, the control animals spent significantly more time with the novel female than the familiar \((*p = 0.029)\), indicating the ability to socially discriminate, while the lesioned animals spent a similar amount of time sniffing both stimulus females \((p = 0.134)\) (Figure 14B). When the mice were presented with the same stimulus female over a series of ten trials, the lesioned animals spent significantly more time with the stimulus female than control animals only in the 10th trial \((F_{1,16} = 6.885 * p = 0.019)\) and significantly less time investigating the novel female in the 11th trial \((F_{1,16} = 4.940 * p = 0.042)\) (Figure 14C) showing an inability to discriminate between stimulus animals.

*Experiments 4 and 5: Olfaction Tests*

The animals from the 11-trial social recognition test were then tested in a hidden cookie test and a habituation/dishabituation olfactory test. There was a significant difference between treatment groups in the hidden cookie test \((F_{1,18} = 4.930 * p = 0.040)\) with CA2 lesioned animals having a longer latency to find the
Figure 14: A) Graph depicting the time spent in olfactory investigation of the familiar female in Trial 1 of the 2-trial social discrimination test. The control group spent significantly more time in chemoinvestigation than the lesion group (*p = 0.004). B) Graph depicting the time spent in olfactory investigation of the stimulus females in Trial 2 of the 2-trial social discrimination test. The control group showed discrimination by spending significantly more time with the novel vs. familiar female (*p = 0.029) while the lesion group did not show discrimination (p = 0.134). C) Graph depicting the time spent in olfactory investigation of the stimulus female in each trial of an 11-trial habituation/dishabituation social recognition test. There was a significant decrease in the time spent sniffing the familiar female by the control group between Trials 9 and 10 (*p = 0.003). The control group also showed discrimination by significantly increasing the time spent sniffing between Trial 10 (familiar female) and Trial 11 (novel female) (*p = 0.002), while the lesioned animals did not show discrimination.
hidden cookie than controls (Figure 15A). However, there were no significant differences observed between treatment groups in the amount of olfactory investigation of the 5 odorants in the habituation/dishabituation olfactory test (Figure 15B). The only odorant that the mice did not habituate to was the almond extract.

Discussion

In the current study we looked at the effects of excitotoxic lesions of the CA2 region of the hippocampus on social behavior, specifically aggression and social recognition. Animals that received bilateral lesions showed no reduction in aggression, though overall levels of aggression were fairly low, as seen by the low percentage of animals attacking per day as well other measures of aggression (Table 2). This was perhaps due to the use of ketamine as an anesthetic, which can bind opioid receptors, causing long-lasting reductions in aggression (Cook, 2000, Tordjman et al., 2003, Wolff and Winstock, 2006). It is also possible that the environment the animals were housed in was not conducive to this type of behavioral testing. The vivarium at KSU is extremely quiet and the colony is not frequently intruded upon during the dark phase, we have noticed low levels of aggression in a variety of other studies as well (E.L.S. and H.K.C. personal observations).

Impairment in social recognition was detected in CA2 lesioned animals. Unlike surgical controls, lesioned animals were unable to discriminate between a
Figure 15: A) Graph depicting the latency to find a piece of hidden food. Mice with lesions to the CA2 region took significantly more time to find the hidden cookie than control animals (*p = 0.040). B) Graph depicting the time spent in olfactory investigation of 5 different odorants in a habituation/dishabituation test of olfaction. In this test, animals with lesions to the CA2 region showed no significant differences in olfaction compared to controls.
novel and a familiar stimulus animal in both tests for social memory (Figure 14A-14C). These data are consistent with previous studies in rodents, which have found impaired social recognition in animals with lesions of the hippocampus that included the CA2 region (Maaswinkel et al., 1996, Uekita and Okanoya, 2011). However, our study is the first to specifically focus on the CA2 region of the hippocampus and its possible contributions to social behavior. The specificity of our lesions and their effects on social behavior suggests that the CA2 region of the hippocampus is important for normal displays social memory.

Since social behaviors in rodents rely heavily on the ability to discern olfactory cues and it is for this reason that we chose to investigate olfaction following bilateral lesions to the CA2 region (Bronson, 1971, Schultz and Tapp, 1973). Avpr1b −/− mice, despite having social behavior deficits, display normal olfaction (Wersinger et al., 2002, Wersinger et al., 2004). Total hippocampal lesions also have not been found to disrupt olfaction (Bunsey and Eichenbaum, 1995, Burton et al., 2000). However, as the CA2 region of the hippocampus receives inputs from the entorhinal cortex, lesions to this region could cause a disconnection between olfactory cues and the formation and/or recall of accessory olfactory-based memories. In this study, it was observed that olfaction (i.e., hidden cookie test) in the lesioned animals was impaired, and consequently had a significantly longer latency to find the hidden cookie. However, it must be noted that the hidden cookie test is not only a test of olfaction, but to also tests the animal’s ability to find it. Therefore, it is possible that CA2 lesioning may have compromised the animal’s
innate navigation system. This conclusion seems to be supported by reports that indicate that animals with hippocampal lesions that also target the CA2 region have impaired spatial memory (Uekita and Okanoya, 2011). However, since our mice did not have prior knowledge of where the cookie would be located, and were not required to remember its location, it seems unlikely that the longer latencies in lesioned animals were due to impaired spatial memory. Rather, a behavioral test that directly assesses this type of memory would be required.

There is also the possibility that lesions to the CA2 region reduce the animal's social motivation, resulting in the animals being less motivated to investigate novel odors, including those that transmit social cues (i.e. male and female urine). The CA2 lesioned animals showed significantly reduced amounts of investigation of the familiar female in Trial 1 of the 2-Trial social discrimination test, which indicates that the CA2 region may be important for social motivation. As social motivation is essential to normal displays of social behavior, impairment in the CA2 lesioned animals might also explain the unusual up and down pattern observed in this group in the habituation/dishabituation social recognition test. Their interest in the familiar stimulus female was high initially, then decreased, then increased, then decreased again, and finally increased again around Trial 10 when they should have been habituating to the stimulus female, and thus have their lowest level of investigation. An argument could be made that the sporadic behavior by the CA2 lesioned animals could be due to increased anxiety. However, past studies of hippocampal lesioned animals report a reduction in anxiety-like behavior rather
than an increase (Deacon et al., 2002). For this reason, it seems unlikely that lesions of the CA2 region increased anxiety-like behavior, but to know for sure it would be necessary to test the anxiety-like behavior of these animals. However, the most likely explanation for the unusual behavior is a reduction in the animal’s social motivation.

In summary, our hypothesis that the CA2 region of the hippocampus is important to normal displays of social recognition was substantiated, but we did not observe the reduced aggression that we expected. The deficits in social recognition, along with reduced social motivation, are also found in mice lacking the Avpr1b (for review see Caldwell et al., 2008a, Caldwell et al., 2008c, Stevenson and Caldwell, 2012). Interestingly, the Avpr1b is found to be prominent in the CA2 region suggesting that this receptor, in this region, is playing a role in the regulation of some social behaviors. The behavioral deficits exhibited by Avpr1b −/− mice are considered to be strictly affecting social behaviors because these animals show normal non-social forms of aggression, such as predatory aggression, and display normal memory of inanimate objects (Wersinger et al., 2007, DeVito et al., 2009). Avpr1b −/− mice do, however, still show a preference for a novel mouse over an inanimate object, suggesting that Avpr1b −/− mice are still interested in social stimuli (Yang et al., 2007). In this study, we have demonstrated that animals with lesions to the CA2 region of the hippocampus have some phenotypic similarities to that observed in Avpr1b −/− mice. This suggests that Avp acting through the Avpr1b in this region could be regulating these types of social behaviors. Future work will
need to be done to demonstrate that it is specifically the expression of the Avpr1b in this region that is responsible for the observed behavioral deficits.
CHAPTER 4

THE EFFECTS OF SHRNA KNOCKDOWN OF THE AVPR1B IN THE CA2 REGION OF THE HIPPOCAMPUS ON AGGRESSIVE BEHAVIOR AND SOCIAL RECOGNITION

Introduction

Arginine vasopressin (Avp) is a cyclic nine amino acid neuropeptide known to regulate social behaviors, such as aggression and social recognition (as reviewed in Goodson and Bass, 2001, Caldwell et al., 2008a, Albers, 2012). Avp's behavioral effects are mediated by its two centrally expressed receptors (Lolait et al., 1995, Foletta et al., 2002, Young et al., 2006), the Avp 1a receptor (Avpr1a) and the Avp 1b receptor (Avpr1b). The Avpr1a is widely distributed throughout the central nervous system (Johnson et al., 1993, Tribollet et al., 1997, Young et al., 2000), but the Avpr1b is more discretely localized, with prominence in the corticotrophes of the anterior pituitary, the dorsal third of the CA2 region of the hippocampus, and to a lesser extent the anterior amygdala and the paraventricular nucleus (Antoni, 1984, Young et al., 2006).

While the Avpr1a’s role in the neural regulation of social behavior has been well characterized, data from studies utilizing pharmacological agents specific to the Avpr1b, as well as, Avpr1b knockout (−/−) mouse studies suggest a significant role
for the Avpr1b receptor antagonist, SSR149415, have reductions in the frequency and duration of offensive attacks (Griebel et al., 2002, Blanchard et al., 2005). The aggression data from Avpr1b−/− mouse studies are consistent with those using pharmacological agents (as reviewed in Caldwell et al., 2008a, Caldwell et al., 2008c, Stevenson and Caldwell, 2012). In addition to reductions in aggression, Avpr1b−/− mice also show mild impairments in social recognition and social motivation (Wersinger et al., 2002, Wersinger et al., 2008).

Unfortunately, while it is clear that the Avpr1b is important for the regulation of social behavior, it is still unknown as to where in the brain Avp acting via the Avpr1b is affecting behavior. While the Avpr1b is not widely distributed centrally, its prominent expression within the CA2 region of the hippocampus of the mouse, rat, and human is intriguing and suggests evolutionary conservation across species. Lesion studies support a possible role for the CA2 region in the regulation of social behaviors. Animals with lesions to the hippocampus that include the CA2 region show deficits in aggression and social recognition similar to that of Avpr1b−/− mice (Ely et al., 1977, Maaswinkel et al., 1996, Uekita and Okanoya, 2011). Data from Chapter 3, in which it was observed that specific lesions to the CA2 region cause deficits in social recognition and olfaction, but not aggression, also suggest that the CA2 region is important for some aspects of social interactions.

However, not much is known about the mechanisms whereby the CA2 region regulates social behavior. The CA2 region is unique from other parts of the
hippocampus as it is the region that receives input from the posterior hypothalamus, in particular the supramammillary nucleus (Borhegyi and Leranth, 1997, Vertes and McKenna, 2000, Bartesaghi et al., 2006) and from the perforant pathway, which connects the entorhinal cortex to the CA1 and CA3 regions (Bartesaghi and Gessi, 2004). Therefore, the CA2 region acts as a link between a region that is receiving input from the olfactory system for memory formation and consolidation and a major site of hippocampal output (Bartesaghi et al., 2006, Chevaleyre and Siegelbaum, 2010). Based on these data and the abundance of the Avpr1b in the CA2 region, it has been hypothesized that the CA2 region may aid in the formation and/or recall of memories related to social encounters, in particular, those that are accessory olfactory-based (Young et al., 2006, Caldwell et al., 2008c).

We know that the CA2 region of the hippocampus is involved in some aspects of social behavior, but the specific contributions of the Avpr1b within this region have yet to be determined. Thus, we hypothesized that site-specific knockdown of the Avpr1b in the dorsal third of the CA2 region of the hippocampus would result in a phenotype similar to that found in Avpr1b −/− mouse and those with lesions to the hippocampus that included the CA2 region. Specifically, we predicted that knockdown of the Avpr1b in the CA2 region would result in reductions in aggressive behavior and impairments in social recognition memory.

**Methods and Materials**

**Animals and Housing**
Adult male mice from a mixed background of C57BL/6J and 129/SvJ were bred in the Kent State University vivarium and kept on a 12:12 light: dark cycle. At the time of weaning (post-natal day 18-21), animals were housed in single-sex sibling groups until surgery. Food and water were given *ad libitum* except during testing. All subjects were 2 to 6 months of age at time of testing. All experiments were conducted in accordance with the protocol approved by the Kent State University Institutional Animal Care and Use Committee.

*Stereotaxic Surgery*

Prior to surgery, the thirty-five adult male mice, which were a mixture of C57BL/6J and 129/SvJ, were randomly assigned to one of the following surgical groups: Avpr1b-siRNA into the CA2 (2192/CA2) (n = 11), control virus into the CA2 (LL3.7/CA2) (n = 8), Avpr1b-siRNA into the basolateral amygdala (BLA) (2192/BLA) (n = 8), control virus into the BLA (LL3.7/BLA) (n = 8). At time of surgery animals were anesthetized using a 2% isoflurane/oxygen mixture. Using an Ultraprecise stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) and a 25 gauge, 2μl Hamilton syringe (Hamilton Company, Reno, NV), the mice underwent site-specific microinjections into either the CA2 region or the BLA. A total of 2 separate injections (1μl of virus per injection) were performed, bilaterally, in order to localize the viral infection to the dorsal third of the CA2 region (Figure 16). When the injection target was the dorsal third of the CA2 region of the hippocampus, the coordinates used were: from Bregma ML = ±1.50, AP = −1.50, DV = −1.50 (from top
Figure 16: Illustration of a coronal section of the mouse brain. “★” indicates the CA2 region of the hippocampus in which either control virus or virus containing siRNA against Avpr1b was injected (Paxinos and Franklin, 2001).
of the brain). For BLA injections the coordinates used were: from Bregma ML = ±2.85, AP = −1.25, DV = −4.50 (from top of the brain). Burr holes were made using a hand-held drill (Dremel®, Racine, WI) with an engraving cutter bit (model #105, Dremel®, Racine, WI). Once the burr hole was created, the needle tip was placed at the appropriate depth and allowed to sit for 2 minutes prior to injection to allow the brain to reposition. Each injection took place over a 10 minute time period in order to limit mechanical damage to the brain. Then the needle remained in place for another 5 minutes to allow the fluid to diffuse away from the needle tip. The needle was then slowly removed, the skin brought back together over the skull, and closed with a wound clip. Following surgery, animals were administered 0.3 ml of warm saline (9%) intraperitoneally (i.p.) to aid in recovery and returned to their home cage. Following surgery, the animals were quarantined for 3 days as a safety precaution to prevent infecting other animals in the colony due to shedding of the virus. All experimental animals then remained in their home cages for at least 21 days post-op to allow adequate infection and knockdown to occur (observation made by H. K. Caldwell in preliminary studies).

2-Trial Social Discrimination Test

One week before testing, the stimulus animals (intact female Swiss Webster mice ages 2-4 months) were single-housed while the surgical animals remained group housed. On the day of testing, all animals were moved to the testing room at time of lights out, individually housed in clean cages, and allowed to habituate to
their new environment for 30 minutes. After the 30-minute habituation period, 2 clean wire corrals were placed on either side of the cage and the animals were allowed to habituate for another 30 minutes (Figure 12A). Testing commenced 1 hour after the start of the dark phase of the light: dark cycle. At the start of Trial 1, one of the corrals was removed and a female stimulus animal was placed in the remaining corral (Figure 12B). The experimental animal was observed investigating, which is defined as any time the experimental animal would insert their nose and/or forepaw through the bars of the corral or sniff any portion of the stimulus animal that was outside of the corral, the stimulus female over a period of 5 minutes. After that time, the stimulus animal was removed, returned to its home cage, and the second corral placed in the experimental animal’s test cage (Figure 12A). During Trial 2, the “familiar” female from Trial 1 was placed back into its original corral, which had been moved to the opposite side of the cage, while a second “novel” female was placed into the remaining corral (Figure 12C). The experimental animal was observed for 5 minutes while being allowed to investigate both females. Both trials were videotaped and continuously scored for the amount of time spent investigating each stimulus female, duration of non-social behavior (as described above), and tail rattle frequency.

*Resident-Intruder Test*

Following social discrimination testing, the surgical animals were single housed for 2 weeks, with no cage change and using a microisolator lid in the week
prior to testing; single housing increased isolation-induced aggression and established the mice as residents. Group-housed male Balb/c mice (as previously used in Caldwell and Young, 2009, Dhakar et al., 2012), between the ages of 2 and 5 months, were purchased from The Jackson Laboratory (Bar Harbor, ME) and used as intruder animals. To acclimate animals to the testing space, all animals were moved to the behavioral testing room at least 1 hour prior to testing and left undisturbed. Testing commenced approximately 1 hour after lights out under dim red light illumination during the dark phase of the light: dark cycle. All sessions were recorded using an infrared camera. At the initiation of testing, the intruder Balb/c mouse was added to the home cage of the resident and the animals were allowed to interact for 5 minutes. Following the initial attack, the observation continued for another 2 minutes. Therefore, the test could be as short as 2 minutes or as long as 7 minutes. Animals that did not initiate attacks were given a latency of 300 seconds. At the conclusion of testing the intruder was removed and placed back into its home cage. Testing occurred over 3 sessions with 72 hours between testing sessions. Only testing sessions that included an attack were scored. The behaviors that were analyzed were: latency to attack, duration of aggression (which included chasing, pushing, mounting of the intruder, and the lunge-bite-roll attack sequence), attack frequency (defined as a lunge followed by a bite), tail rattle frequency, duration of non-social behavior (the time the resident spent investigating the cage and grooming), and contact (the time the resident spent with its nose within 1cm of the intruder's body) (as previously described in Dhakar et al., 2012).
Injection Site Confirmation and Statistical Analysis

Once the animals completed aggression testing their brains were removed, fresh frozen on dry ice, and kept at −80°C. Infection was to be localized by staining the tissue for green fluorescent protein (GFP), which is incorporated into the Avpr1b-siRNA and control siRNA (Figure 17). Histology for GFP resulted in minimal labeling of cells surrounding the injection site. This led us to believe that insufficient infection by the virus occurred. To visualize the injection sites, the slides were stained with cresyl violet and observed with a microscope under 50X and 100X magnification.

The mean group differences for the cumulative data from the resident-intruder test were analyzed using a Kruskal Wallis test (SPSS 16.0 for Mac, IBM, Armonk, NY). For the day-to-day measurements in the resident-intruder test a repeated measures analysis of variance was used. The number of animals attacking in the resident-intruder test was analyzed using a Fisher’s exact test. For the 2-trial social discrimination test an ANOVA was used to analyze Trial 1 data (genotype x behavior), while a 1-tailed paired-sample Student’s t-test (as previously used in Macbeth et al., 2009) was run within each group to directly compare time spent during Trial 2 with “familiar” and “novel” females. A discrimination percentage was calculated from \[\text{novel}/(\text{novel} + \text{familiar}) \times 100\]. For all statistical tests a result was considered statistically significant if \(p \leq 0.05\).

Immunohistochemistry for GFP
Figure 17: Schematic of control virus (LL 3.7) construct. Green fluorescent protein (GFP) insert produces GFP for use in visualizing extent of infection. Avpr1b-siRNA would be inserted just after the CMV insert in the experimental virus construct (2192). The entire construct is packaged into a lentivirus, which acts as the delivery and replication system.
Ten of the thirty-five brains (that did not initiate attacks) were cut at a thickness of 12μm at -20°C on a cryostat (CM1950, Leica, Buffalo Grove, IL) and thaw-mounted onto Superfrost® Plus slides (Fisher Scientific, Pittsburgh, PA). On the first day of staining the tissue was warmed to room temperature, fixed in 4% formaldehyde for 5 minutes and then rinsed 4 times with 1X PBS before washing for 5 minutes. The tissue was then blocked for 10 minutes with 1X Power Block™ Universal Blocking Reagent (BioGenex, San Ramon, CA) at RT followed by a rinse and a wash for 5 minutes in 1X PBS. The tissue was then incubated overnight at 4°C in primary antibody (Molecular Probes, Invitrogen, Carlsbad, CA, rabbit anti-GFP, A6455) diluted to 1:20,000 with 1XPBS and 1% bovine serum albumin. After incubation was completed, the slides were brought up to RT, rinsed with 1X PBS, and then washed 3 times for 3 minutes each in 1X PBS. The tissue was then treated with 1.5% hydrogen peroxide for 20 minutes at RT followed by 4 washes in 1X PBS for 3 minutes each. After removing any excess fluid, 100μl of anti-rabbit poly HRP conjugate was applied to each slide (Super PicTure, Life Technologies, Grand Island, NY), and allowed to incubate for 30 minutes at RT. The tissue was then washed 2 times in 1X PBS for 3 minutes each and then once in 0.1M Tris, pH 8.0 for 3 minutes. Staining was visualized using diaminobenzidine (DAB) for 2-10 minutes until staining was sufficient, washed 2 times for 5 minutes each in 1X PBS, allowed to dry overnight, and then coverslipped.

Cresyl Violet Stain
Twenty-five of the thirty-five frozen brains (6 that initiated attacks and 19 that did not) were cut at a thickness of 20μm at -20°C on a cryostat (CM1950, Leica, Buffalo Grove, Il) and thaw-mounted onto Superfrost® Plus slides (Fisher Scientific, Pittsburgh, PA). At time of staining, the tissue was warmed to room temperature, fixed in 4% formaldehyde for 5 minutes, and then rinsed 4 times with 1X PBS before washing for 5 minutes. Then the slides were dehydrated in a series of alcohols and rehydrated with distilled water before staining with cresyl violet. The stain was then lifted in a series of alcohols and the tissue defatted with xylenes. The slides were then immediately coverslipped.

Results

2-Trial Social Discrimination test

Both CA2 region surgical groups showed a significant difference in the time spent with the novel stimulus female versus the familiar female in the second trial (LL3.7/CA2 *p = 0.005 and 2192/CA2 *p = 0.013) (Figure 18). This data suggests that both treatment groups were able to socially discriminate. There were no statistical differences found for time spent investigating the familiar female, time spent investigating the novel female in Trial 2, duration of non-social behavior, and frequency of tail rattle in either Trials 1 or 2 (Table 3).

Resident-Intruder Test
Figure 18: Graph depicting the time spent in olfactory investigation of the stimulus females in Trial 2 of the 2-Trial social discrimination test. Both groups showed discrimination by spending significantly more time with the novel vs. familiar female (LL3.7/CA2 *p = 0.005 and 2192/CA2 *p = 0.013).
<table>
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<tr>
<th></th>
<th>LL3.7/BLA</th>
<th>LL3.7/CA2</th>
<th>2192/BLA</th>
<th>2192/CA2</th>
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<tr>
<td>Trial 1 Familiar</td>
<td>62.05 ± 0</td>
<td>44.56 ± 10.73</td>
<td>79.01 ± 10.50</td>
<td>67.37 ± 8.54</td>
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<tr>
<td>Trial 1 Non-social</td>
<td>238.01 ± 0</td>
<td>255.51 ± 10.73</td>
<td>221.04 ± 10.51</td>
<td>232.73 ± 8.54</td>
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<td>Trial 1 Tail Rattle</td>
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<td>1.17 ± 0.48</td>
<td>0.25 ± 0.25</td>
<td>1.55 ± 0.79</td>
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<tr>
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<td>11.92 ± 2.23</td>
<td>41.29 ± 8.08</td>
<td>19.38 ± 3.30</td>
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<tr>
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<td>Trial 2 Non-social</td>
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<td>244.69 ± 6.23</td>
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<td>Discrimination %</td>
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<td>77.12 ± 6.10</td>
<td>42.96 ± 11.77</td>
<td>62.95 ± 5.83</td>
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</tbody>
</table>

Table 3: Behaviors measured in the 2-trial social discrimination test where there were no statistically significant differences observed.
Animals that did not have bilateral injection sites in either the CA2 region or the BLA were not included in the statistical analysis (LL3.7/BLA n = 5, LL3.7/CA2 n = 2, 2192/BLA n = 4, 2192/CA2 n = 0). Two animals, both in the LL3.7/BLA group, were removed from the study due to insufficient tissue collection and therefore a lack of histology. This left the BLA groups with too little statistical power to analyze. For this reason only the data for the LL3.7/CA2 and 2192/CA2 groups were analyzed.

Observed levels of aggression across all groups were much lower than typically observed. This was evident by the fact that only 3 of the 22 animals in the CA2 region surgical groups attacked. Cumulative duration of aggression, non-social behavior, and contact time, latency to attack, frequency of attack and number of tail rattles were also not significantly different between treatment groups (Table 4).

Discussion

Of the 2 surgical groups that we were able to include in the statistical analysis we found no differences in either aggression or social memory. We think this is due to the viral vector not infecting the neurons sufficiently. In a pilot study we saw greater than 90% knockdown of the Avpr1b in an in vitro assay and greater than 50% knockdown in vivo. However, due to the fact that the GFP immunostaining had worked previously and there is some evidence that minimal staining was visible in some sections near the injection site, it seems likely that the GFP immunostaining is working, but insufficient infection took place, likely due to insufficient viral titers. For this reason, this study will need to be attempted again in the future.
Table 4: Behaviors measured in the resident-intruder test where there were no statistically significant differences observed.

<table>
<thead>
<tr>
<th></th>
<th>LL3.7/BLA</th>
<th>LL3.7/CA2</th>
<th>2192/BLA</th>
<th>LL3.7/CA2</th>
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</thead>
<tbody>
<tr>
<td>Duration of Aggression</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>63.22 ± 38.81</td>
<td>13.35 ± 0</td>
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<tr>
<td>Duration of Non-social</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>56.02 ± 47.76</td>
<td>19.22 ± 0</td>
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<tr>
<td>Duration of Contact</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.85 ± 0.85</td>
<td>0.18 ± 0</td>
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<tr>
<td>Latency to Attack</td>
<td>900 ± 0</td>
<td>900 ± 0</td>
<td>692.18 ± 167.75</td>
<td>835.71 ± 0</td>
</tr>
<tr>
<td>Frequency of Attack</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>12.50 ± 8.50</td>
<td>1.64 ± 0</td>
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<tr>
<td>Number of Tail Rattles</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>1.50 ± 1.50</td>
<td>0.82 ± 0</td>
</tr>
</tbody>
</table>
CHAPTER 5

LOCALIZATION OF THE VASOPRESSIN 1B RECEPTOR IN THE SYRIAN HAMSTER

Introduction

In a variety of species, the neuropeptide arginine vasopressin (Avp) has been implicated in the neural regulation of social behavior (as reviewed in Goodson and Bass, 2001, Caldwell et al., 2008a, Albers, 2012). Avp acts through two centrally expressed receptor subtypes in order to affect social behavior (Lolait et al., 1995, Foletta et al., 2002, Young et al., 2006). These receptor subtypes are the widely distributed Avp 1a receptor (Avpr1a) and the more discretely localized Avp 1b receptor (Avpr1b) (Jard et al., 1987, Arsenijevic et al., 1994, Thibonnier et al., 2002). While the role of the Avpr1a in social behavior has been extensively studied in several different species (Young et al., 1999, Hammock and Young, 2002, Bielsky et al., 2004, Walum et al., 2008), there is mounting evidence of a role for the Avpr1b in the neural regulation of social behavior (as reviewed in Caldwell et al., 2008a, Caldwell et al., 2008c, Stevenson and Caldwell, 2012).

The Avpr1b has been localized in several different tissues, but is most heavily expressed in the corticotrophes of the anterior pituitary gland (Antoni, 1984, Jard et al., 1986). In the rat brain, Avpr1b mRNA and immunoreactive cells have been found
in the cerebellum, cerebral cortex, hippocampus, olfactory bulb, paraventricular nucleus, piriform cortical layer II, red nucleus, septum, and suprachiasmatic nucleus (Lolait et al., 1995, Saito et al., 1995, Barberis and Tribollet, 1996, Vaccari et al., 1998, Hernando et al., 2001, Stemmelin et al., 2005). More recently, Avpr1b mRNA has been found to be prominent in the dorsal third of the CA2 region of the hippocampus, and to a lesser extent in the paraventricular nucleus and anterior amygdala of mice, rats, and humans (Young et al., 2006).

Unfortunately, neither the Avpr1b protein nor mRNA has yet been localized in any other species due to the lack of specific antibodies, radiolabeled ligands, and sequence information. However, there is data suggesting that the Avpr1b plays a role in the regulation of social behavior in species other than in those in which it has been localized. One such species is the Syrian hamster (*Mesocricetus auratus*). Syrian hamsters display robust and reliable territorial aggression similar to mice and rats (Garrett and Campbell, 1980). However, unlike mice and rats, hamsters are solitary animals. In fact, some of the most compelling evidence implicating a role for Avp in the regulation of social behavior has been seen in the Syrian hamster (Ferris et al., 1997, Cooper et al., 2005, Albers et al., 2006, Ferris et al., 2006, Caldwell et al., 2008b, Bolborea et al., 2010). For example, hamsters given oral antagonists specific to the Avpr1b show reductions in aggressive behavior similar to those observed in Avpr1b null mutant mice (Blanchard et al., 2005). It is for this reason that it would be beneficial to determine the distribution of the Avpr1b in Syrian hamsters.
In order to elucidate the similarities between the mouse and hamster Avp/Avpr1b system we cloned and sequenced a portion of the Avpr1b in the Syrian hamster. We then used *in situ* hybridization histochemistry (ISHH) to localize Avpr1b mRNA in the brains of male and female Syrian hamsters. We hypothesized that the distribution of the Avpr1b would be conserved between the Syrian hamster and other rodent species, such as mice and rats.

**Methods and Materials**

*Animals and Housing*

Male (n = 14) and female (n = 12) Syrian hamsters were obtained from the Glass lab at Kent State University. All animals were 6 months of age at the time of tissue collection. Animals were lightly anesthetized with pentobarbital and then decapitated using a guillotine. Brains were removed and immediately frozen on powdered dry ice. All tissue was stored at -80°C until cut on cryostat. Also, the pituitaries of 4 male Syrian hamsters were removed for RNA isolation using a RiboPure™ Kit (Life Technologies, Grand Island, NY) according to its directions. All experiments were conducted in accordance with the protocol approved by the Kent State University Institutional Animal Care and Use Committee.

*Hamster Avpr1b cloning, sequencing, and amplification*

cDNA was made (ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis, Life Technologies, Grand Island, NY) using the RNA isolated from the
pituitaries of the male hamsters. The cDNA was then used in PCR using primers based on the consensus sequence of the Avpr1b gene in mouse and rat (forward primer: 5’TTTTTGTCCCAATGCCACCG3’ and reverse primer: 5’TGAGCCCTGGATCACCTCT3’). This yielded a 572bp sequence that was sent out for sequencing (Eurofins, Huntsville, Alabama) while the remainder was cloned into the cloning vector pGEM (pGEM-T EZ Vector System II, Promega, Madison, WI) (Figure 19). The plasmid was then transformed into E. coli (One Shot Stbl3 cells, Life Technologies, Grand Island, NY) and saved as glycerol stocks at -80°C.

In order to amplify the Avpr1b plasmid DNA, 100μl of glycerol stock was added to LB broth plus ampicillin (40μg/ml). The broth was then incubated overnight (16-24 hours) at 37°C and agitating at ~300 rpm. The cells were then collected and the DNA isolated using a Maxiprep system (NucleoBond®Xtra Maxi EF, MACHERY-NAGEL, Inc., Bethlehem, PA) according to kit directions. The Avpr1b plasmid DNA was then stored at -20°C for future use.

*Digoxigenin (DIG)-labeled Riboprobe Production*

The Avpr1b plasmid DNA was linearized using restriction enzymes; either Ncol to use with the SP6 polymerase to generate the sense strand or SpeI to use with the T7 polymerase to generate the antisense strand. A master mix that included the restriction enzyme (7.5units per μg of DNA plasmid), the appropriate buffer, 2μg of Avpr1b DNA plasmid, diethylpyrocarbonate (DEPC) water, and RNase OUT was incubated at 37°C for 2 hours. To analyze extent of linearization 1μl of reaction mix
Figure 19: Schematic of the pGEM cloning vector into which the 572bp hamster Avpr1b sequence was cloned. The entire construct was then transformed into *E. coli*.
was mixed with 0.1μl of loading buffer, loaded onto a 1% agarose gel with GelGreen™ (Biotium, Inc., Hayward, CA) and run by electrophoresis. To stop the reaction the sample was heated to 65°C (for NcoI) or 80°C (for SpeI) for 20 minutes. The linearized Avpr1b DNA plasmid was then purified using a Wizard® SV gel and PCR clean-up system (Promega, Madison, WI) according to kit instructions. The DIG-labeled riboprobes were made from a master mix containing 1μg of purified linearized Avpr1b DNA plasmid, either SP6 or T7 polymerase (depending on the restriction enzyme used), DIG-labeled RNA mix 10X (Roche, Indianapolis, IN), and 10X transcription buffer that was incubated at 37°C for 3 hours. Following incubation TURBO DNase and RNase OUT were added to the sample and incubated at 37°C for 15 minutes. Adding 2μl of 0.2M EDTA (pH 8.0) stopped the reaction. The DIG-labeled riboprobe was then precipitated with 4M lithium chloride and prechilled (-20°C) 100% ethanol (EtOH) for at least 30 minutes at -80°C. The probes were then centrifuged at 13,000 x g for 15 minutes at 4°C, the EtOH decanted, and pellet washed with prechilled 70% EtOH. Finally the probes were centrifuged again at 13,000 x g for 15 minutes at 4°C, EtOH decanted, dried in a speed vacuum on low (no heat), and pellet dissolved in 50μl of RNase-free water with 1μl RNase OUT. The DIG-labeled riboprobes were then aliquoted and stored at -80°C until used in ISHH.

In Situ Hybridization Histochemistry

The frozen tissue was cut on cryostat into 25μm sections in 3 serial series, thaw-mounted onto Superfrost® Plus slides (Fisher Scientific, Pittsburgh, PA), and
stored at -80°C until used. At time of staining, one set of slides was brought to room temperature and then dried in a 55°C incubator for 15 minutes. Slides were then outlined with a hydrophobic pen (ImmEdge™ Hydrophobic Barrier Pen, Vector Laboratories, Burlingame, CA) and dried for another 5 minutes in a 55°C oven. The sections were then fixed in fresh, cold 4% paraformaldehyde for 20 minutes, washed 2 times for 5 minutes in sterile 1X phosphate buffered saline (PBS) made with 0.1% DEPC water, and treated with protein kinase buffer (2μg/ml) for 10 minutes. The tissue was then washed again 2 times for 5 minutes in 1XPBS before being acetylated with 0.25% acetic anhydride for 15 minutes and washed again 2 times for 5 minutes in 1XPBS. A pre-hybridization solution was then applied to the slides and incubated for 2 hours in a humid chamber at 55°C. The hybridization solution containing 10ng/μl of probe was then applied to the slides and incubated overnight (20-24 hours) in a humidity chamber at 57°C. The following day, the slides were washed 2 times for 5 minutes in 2XSSC, 4 times for 15 minutes in 2XSSC at 55°C, 4 times for 15 minutes in 1XSSC at 55°C, and then 3 times for 5 minutes in room temperature 2XSSC. The tissue was then treated with RNase A (20μg/ml)/2XSSC for 30 minutes at 37°C. The slides were then washed again 3 times for 5 minutes in room temperature 2xSSC, 2 times for 30 minutes in 0.1XSSC at 37°C, and then 3 times for 5 minutes with 0.05M tris buffered saline (TBS) with 0.3% Triton X-100 (T). The sections were then blocked with 2% normal sheep serum (NSS) in TBS-T for 1 hour and then incubated in anti-DIG Fab Fragments from sheep (1:5000) (Roche Applied Science, Indianapolis, IN) in 2% NSS/TBS-T for 3
days at 4°C. Following incubation, the slides were washed 2 times for 5 minutes with TBS-T and then warm (37°C) BM Purple AP substrate precipitating solution (Roche Applied Science, Indianapolis, IN) was applied. The slides were then placed in a dark drawer at room temperature for 8-16 hours and then washed 2 times for 5 minutes in TBS. Then they were fixed in cold 4% paraformaldehyde for 5 minutes, washed again 2 times for 5 minutes with distilled water, and coverslipped with glycerol jelly (7.7% gelatin/54% glycerol) heated to 50°C.

Results

A 572bp stretch of the Avpr1b sequence was isolated from the pituitaries of Syrian hamsters. There was approximately 90% identity with the corresponding consensus sequence of mice (Figure 20). This sequence was then used as a template to produce the sense and antisense forms of the DIG-labeled RNA probes for in situ hybridization. The sense probe produced no specific hybridization (Figure 21), demonstrating the specificity of the antisense probe that did show specific binding. The antisense mRNA was visualized as dark blue deposits in the cytoplasm of neurons (Figure 22) and used to map the presence of the Avpr1b in the adult male Syrian hamster brain. To date, the Avpr1b has been localized in the indusium griseum (IG) (Figure 23), piriform cortex (Pir) (Figure 24), hippocampus (Figure 25), thalamus (Figure 26), oculomotor nucleus (Figure 27), red nucleus (Figure 27), dorsal raphe (Figure 28), trochlear nucleus (Figure 28), median raphe nucleus (Figure 29), and pontine nucleus (Figure 30).
Figure 20: Alignment of a section of the mouse Avpr1b (purple) with that was sequenced of the Syrian hamster Avpr1b (blue). Nucleotides in red indicate sequence differences.
Figure 21: A) Brightfield photomicrograph of a coronal section of the **hippocampus** in a male Syrian hamster after *in situ* hybridization using the sense probe for the Avpr1b. B) Illustration of the corresponding coronal section of the hamster brain (Morin and Wood, 2001).
Figure 22: Brightfield photomicrograph of neurons labeled with the antisense DIG-labeled probe for hamster Avpr1b. The antisense mRNA was visualized as dark blue deposits in the cytoplasm of neurons.
Figure 23: A) Brightfield photomicrograph of a coronal section of a male Syrian hamster brain that highlights the **indusium griseum** labeled with a DIG-labeled *in situ* hybridization probe specific for the Avpr1b. B) Illustration of the corresponding coronal section of the hamster brain (Morin and Wood, 2001).
Figure 24: A) Brightfield photomicrograph of a coronal section of a male Syrian hamster brain that highlights the **piriform cortex** labeled with a DIG-labeled *in situ* hybridization probe specific for the Avpr1b. B) Illustration of the corresponding coronal section of the hamster brain (Morin and Wood, 2001).
Figure 25: A, B) Brightfield photomicrographs of a coronal section of a male Syrian hamster brain that highlights the hippocampus labeled with a DIG-labeled in situ hybridization probe specific for the Avpr1b. C, D) Illustrations of the corresponding coronal section of the hamster brain (Morin and Wood, 2001).
Figure 26: A) Brightfield photomicrograph of a coronal section of a male Syrian hamster brain that highlights the thalamus labeled with a DIG-labeled *in situ* hybridization probe specific for the Avpr1b. B) Illustration of the corresponding coronal section of the hamster brain (Morin and Wood, 2001).
Figure 27: A) Brightfield photomicrograph of a coronal section of a male Syrian hamster brain that highlights the oculomotor and red nuclei labeled with a DIG-labeled in situ hybridization probe specific for the Avpr1b. B) Illustration of the corresponding coronal section of the hamster brain (Morin and Wood, 2001).
Figure 28: A) Brightfield photomicrograph of a coronal section of a male Syrian hamster brain that highlights the **dorsal raphe** and **trochlear nuclei** labeled with a DIG-labeled *in situ* hybridization probe specific for the Avpr1b. B) Illustration of the corresponding coronal section of the hamster brain (Morin and Wood, 2001).
Figure 29: A) Brightfield photomicrograph of a coronal section of a male Syrian hamster brain that highlights the median raphe nucleus labeled with a DIG-labeled in situ hybridization probe specific for the Avpr1b. B) Illustration of the corresponding coronal section of the hamster brain (Morin and Wood, 2001).
Figure 30: A) Brightfield photomicrograph of a coronal section of a male Syrian hamster brain that highlights the **pontine nuclei** labeled with a DIG-labeled *in situ* hybridization probe specific for the Avpr1b. B) Illustration of the corresponding coronal section of the hamster brain (Morin and Wood, 2001).
Discussion

Our data supports the hypothesis that the Avpr1b is conserved in sequence and, in some brain regions, distribution across various rodent species. To date, the Avpr1b has been localized in mouse, rat and human by in situ hybridization (Young et al., 2006) and now we add the Syrian hamster to this list. We found that there is a 90% identity between mouse and hamster in the Avpr1b gene, suggesting a conserved sequence and thus, similar protein confirmations.

Another interesting finding was the presence of the Avpr1b in the thalamus. This area is known to function as a relay station for the sensory systems, excluding the olfactory system, sending signals to the corresponding primary cortical area. It is also functionally connected to the hippocampus (Stein et al., 2000), which suggests that it plays a role in memory formation. In fact, there is evidence that the thalamic-hippocampal connections aid in the formation of episodic and spatial memory (Burgess et al., 2002, Aggleton et al., 2010). There is support for the hypothesis that thalamic regions connecting to particular parts of the mesio-temporal lobe provide differentiation of the functioning of recognition and familiarity memory (Carlesimo et al., 2011).

The Avpr1b was also found in the IG, which is considered the supracommissural portion of the hippocampal continuation, although it is unknown as to which portion of the hippocampus the IG eventually becomes (Shipley and Ennis, 1996). This region receives laminar specific afferents from the entorhinal
cortex, the piriform cortex, and area that also contains the Avpr1b, and the supramammillary region (Kunzle, 2004). Also, a few fibers originate from the olfactory bulb as well (Kunzle, 2004). These connections to the IG are similar to those that connect to the CA2 region suggesting that the IG may form part of the CA2 region as it moves posteriorly.

This study also localized the Avpr1b within the CA2 region of the hippocampus. Its presence in the CA2 region in hamsters, as well as mice, rats and humans fits the suggested role for the CA2 region in social behavior. This region of the hippocampus, though at first regarded as just a transition area between the CA1 and CA3 regions, has unique characteristics. Several substances are either concentrated or totally absent from the CA2 region, causing abrupt boundaries between the CA2 region and the CA1 and CA3 regions (for examples see Lein et al., 2004, Lein et al., 2005). This region also shows resistance to pyramidal cell damage following traumatic brain injury (Maxwell et al., 2003), temporal lobe epilepsy (Fried et al., 1992, Mathern et al., 1995a), and some forms of ischemia (Kirino and Sano, 1984, Sadowski et al., 1999). Unlike the adjoining regions, the CA2 region does not receive rich mossy fiber inputs from the dentate gyrus and the pyramidal neurons of this region lack the characteristic thorny excrescences of the CA3 region (Lorente de No, 1934, Tamamaki et al., 1988).

The major input into the CA2 region comes from the perforant pathway, which connects the entorhinal cortex to the hippocampal formation, bypassing the granule cell layer (Bartesaghi and Gessi, 2004) and from the posterior
hypothalamus, particularly the supramammillary nucleus (Borhegyi and Leranth, 1997, Bartesaghi et al., 2006). These inputs are then passed on to the CA1 region and then the CA3 region (Bartesaghi and Gessi, 2004). This allows the CA2 region to act as a link between the entorhinal cortex, an important site for memory formation and consolidation that also receives inputs from the olfactory system, and the CA1 region, the site of major hippocampal output (Bartesaghi et al., 2006, Chevaleyre and Siegelbaum, 2010). It is this link that suggests that this region plays a role in the formation and/or recall of accessory olfactory-based memories, such as those that are needed for displays of normal social behaviors.

Although the Avpr1b is present in the CA2 region of the hippocampus and there is often Avp found in the dorsal hippocampus (Landgraf et al., 1991), to date there is no known direct innervation by vasopressin-producing neurons to the CA2 region. Some possible sources of Avp to this region of the hippocampus may be from long-distance diffusion through extracellular and cerebral spinal fluid (Herkenham, 1987, Landgraf and Neumann, 2004) or from nearby axonal release either from the entorhinal cortex or the CA1 or CA3 regions (Hallbeck et al., 1999).

From the results of Chapter 3, we know that the CA2 region is playing a role in the regulation of social behavior in mice. It is also known that the Avpr1b plays a role in the regulation of social behavior in the Syrian hamster. Hamsters given a specific Avpr1b antagonist show deficits in social behavior similar to that of mice lacking the Avpr1b. Syrian hamsters show reductions in aggressive behaviors such as frequency of attacks, duration of offensive aggression, flank marking, chase
behaviors, and olfactory investigation that normally precedes and accompanies attacks (Blanchard et al., 2005). The findings of this study along with the knowledge that the Avpr1b plays a role in social behavior in hamsters as well as three other species, suggests that there is a possible role for the Avpr1b within the CA2 region in the regulation of social behavior and that it is evolutionarily conserved.
CHAPTER 6

GENERAL DISCUSSION

The studies conducted and described in this dissertation have contributed to elucidation of the role of the Avpr1b in social behavior. Specifically, how the Avpr1b within the CA2 region of the hippocampus is involved in the regulation of social behavior. Further, it determined that the sequence and distribution of the Avpr1b gene was conserved across several rodent species.

Chapter 2 provided valuable information about the neuronal activation of male and female Avpr1b −/− mice after an aggressive encounter. The findings from this study supported the findings from Wersinger et al. (2002) who found that an aggressive encounter increased c-Fos immunoreactivity (Fos-ir) in both Avpr1b +/+ and Avpr1b −/− mice in brain regions known to be involved in aggressive behavior. Looking specifically at the Fos-ir in the CA2 region of the hippocampus, we were able to determine that the lack of the Avpr1b did not affect neuronal activation in this region after an aggressive encounter. Actually, there were no genotypic differences observed in any of the brain regions studied in either sex. This suggests that neuronal activation in Avpr1b −/− mice is either affected somewhere downstream of the CA2, perhaps in the CA1 region, or the lack of the receptor in this
area has no effect on neuronal activation after an aggressive encounter. It is also possible that the change in neuronal activation could be better detected by another immediate early gene, such as early growth response protein 1, and this may be explored in the future.

Despite the lack of genotypic differences seen in c-Fos expression in the CA2 region between Avpr1b +/+ and Avpr1b −/− mice, we still wanted to examine the importance of the CA2 region in relation to changes in social behavior. In Chapter 3, the role of the CA2 region in the regulation of social behavior was investigated using site-specific excitotoxic lesions. Male C57BL/J6 mice underwent bilateral lesions to the dorsal third of the CA2 region of the hippocampus and were then tested for aggression, social memory, and olfaction. The data showed that specific lesions to the CA2 region cause impaired social recognition compared to controls. This is consistent with other lesion studies in which the CA2 region was not the primary target, but was included in the lesion (Maaswinkel et al., 1996, Uekita and Okanoya, 2011). However, these studies also found reductions in aggressive behavior, which was something that we did not observe in our site-specific CA2 lesioned animals. This was possibly due to the low levels of aggression observed in both treatment groups. We also found deficits in olfaction that were not previously observed in any other study (Bunsey and Eichenbaum, 1995, Burton et al., 2000). There are several possible explanations for this finding, but we hypothesize that lesions to the CA2 are either causing deficits in the animal’s social motivation, or are causing a break in the
system between detection of social cues and the formation and/or recall of social memories. Both of which would ultimately affect their social behavior.

The experiments in Chapter 4 were performed in order to investigate the role of the Avpr1b within the CA2 region in the regulation of social behaviors. Male mice from a C57BL/J6 and 129/SvJ mixed background were injected with either control virus or virus containing siRNA against the Avpr1b into either the basolateral amygdala or the CA2 region of the hippocampus. The virus containing the siRNA was supposed to cause site-specific knockdown of the Avpr1b. Following surgery, the animals were tested for offensive aggression and social memory. Unfortunately, our findings did not support our hypothesis that site-specific knockdown of the Avpr1b in the CA2 region would cause deficits in aggression similar to those found in Avpr1b −/− mice. The aggression levels were so low in all treatment groups that we did not have enough data to discern a statistical significance. The social recognition memory data was also contradictory to our hypothesis with all animals receiving injections into the CA2 region being able to discriminate between a novel and familiar female stimulus animal. When attempting to quantify the extent of infection we found that either we could not get the stain to work or insufficient infection took place. We tend to think that it is the latter of the two scenarios since pilot studies showed that the green fluorescent protein (GFP) staining was working when adequate infection occurred and there was evidence of a small amount of GFP staining visible around the site of injection. This was the first time to date that site-specific knockdown of the Avpr1b using siRNA against the
receptor delivered via viral vector *in vivo* with observation of behavior post-infection was attempted. Had this experiment not been plagued with so many technical issues we may have been able to see if the Avpr1b within the CA2 region was playing a role in the regulation of social behavior.

Chapters 2-4 focused on the role of the Avpr1b in the regulation of social behavior in the common laboratory mouse. In Chapter 5 we set out to localize the Avpr1b in another aggressive rodent species, the Syrian hamster. To do this, we cloned a stretch of the Avpr1b sequence to use as a template to make a probe for digoxigenin (DIG)-labeled *in situ* hybridization histochemistry. We found that the Avpr1b was localized in the CA2 and CA3 regions of the hippocampus, thalamus, indusium griseum, piriform cortex, oculomotor nucleus, red nucleus, pontine nucleus, trochlear nucleus, dorsal raphe, and median raphe nucleus. This finding was interesting because the Avpr1b is also found in the CA2 region of the mouse, rat, and human (Young et al., 2006). All three species investigated in that study are considered social species, while the Syrian hamster is a solitary species. This suggests that the differences observed in the distribution of the Avpr1b amongst these species have to do with its role in the regulation of social behaviors in animals with different social structures. Perhaps the Avpr1b acts to facilitate social behavior in different areas of the brain depending on if the animal lives in social groups or is a mainly solitary species.

When considering the data of this dissertation in its entirety, it can be deduced that the CA2 region of the hippocampus, in respects to the regulation of
social behavior, is an important neuroanatomical area. It is still unknown as to what role the Avpr1b plays within the CA2 region, but when the CA2 region itself is taken out of the equation, social behavior deficits are observed.

One mechanism that might be responsible for the behavioral changes seen in animals lacking the Avpr1b and those that have lesions to the hippocampus that include the CA2 region may be that hippocampal output from the CA1 region may be weakened by inadequate inputs from the CA2 region either due to the absence of the Avpr1b or the CA2 region itself. Rats that undergo prolonged social isolation just after weaning show reduced long-term potentiation (LTP) in the CA1 in the subiculum pathway (Roberts and Greene, 2003), a structure that is imperative for the flow of information from the hippocampus to other brain regions (Amaral et al., 1991). This suggests that the absence of social stimuli has profound effects on the synaptic plasticity of the CA1 region. With the CA2 receiving major inputs from the entorhinal cortex and its major output being to the CA1 region of the hippocampus, we can hypothesize that the CA2 region plays an important role in linking the perception of social cues and the formation and/or recall of accessory olfactory-based memories. This may be possible by activation of the Avpr1b within the CA2 region causing long lasting changes in synaptic plasticity of the CA2 region, possibly through epigenetic mechanisms. This would thus strengthen the connection between the CA2 and CA1 regions, increasing the formation and/or recall of social memories by increasing the LTP of the CA1 region to other parts of the brain (Figure 31).
Figure 31: A) Schematic of proposed action of the Avpr1b in the CA2 region of the hippocampus in the accessory olfactory-based social memory circuit in a social animal, such as the mouse. B) Schematic of the accessory olfactory-based social memory circuit without the Avpr1b in the CA2 region.
The body of work described in this dissertation has provided a basis for future work in the neural regulation of social behavior by the CA2 region of the hippocampus, as well as, the Avpr1b. The use of site-specific lesions in particular has given us evidence of the importance of the CA2 region in the regulation of social behavior and shed some light on the possible function of this mysterious region. Though the attempt to site-specifically knockdown the Avpr1b in vivo and then observe the subsequent behavior was not successful, and it is likely that the Avpr1b within the CA2 region is important to the regulation of social behavior. While some research has been done on the behavioral effects of the Avpr1b on species other than mice and rats, the work can really go no further until we improve our approach. Our hope is that the sequencing and localization of the Avpr1b in a species that has a more robust behavioral repertoire, such as the Syrian hamster, could help to develop tools such as RNA interference or overexpression vectors that can be site-specifically delivered in these animals in order to further the study of Avp’s effects on behavior through the Avpr1b.
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