ABSTRACT

Vasopressin Decreases Excitability in Rat Lateral Amygdala Neurons through Inhibition of Hyperpolarization-Activated Cationic Current

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The amygdala is a critical part of the limbic system with important roles in social behavior. Abnormal activity in the lateral amygdala nucleus (LA) has been implicated in several disorders, including autism spectrum disorder (ASD) in which abnormal social functioning is a primary symptom. The peptide hormones arginine-vasopressin (AVP) and oxytocin (OT) are strongly implicated in social behavior, and may also be involved in the pathophysiology of ASD. Here, we show that AVP causes an increase in excitability, through eliciting a decrease in action potential accommodation and hyperpolarization-activated current (I_h) amplitude in LA pyramidal cells. OT exerts complementary effects, causing an increase in action potential accommodation and I_h amplitude, resulting in decreased excitability. These results suggest AVP and OT may modulate social behavior by controlling excitability in the amygdala.

Vasopressin Decreases Excitability In Rat Lateral Amygdala Neurons
Through Inhibition Of Hyperpolarization-Activated Cationic Current

by

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A Dissertation

Approved by the Department of Psychology and Neuroscience

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Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of
Doctor of Philosophy

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Accepted by the Graduate School
May 2011

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LIST OF ABBREVIATIONS

4-AP	4-aminopyridine
aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
ANS	autonomic nervous system
ASD	autism spectrum disorder
AVP	arginine-vasopressin
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
cAMP	cyclic adenosine monophosphate
CeA	central amygdala
CeL	lateral capsule of CeA
CeM	medial capsule of CeA
CNS	central nervous system
DAG	diacylglycerol
EPSC	excitatory post-synaptic current
EPSP	excitatory post-synaptic potential
GABA	γ-aminobutyric acid
GluR	glutamate receptor
HPA	hypothalamic pituitary axis
I_h	hyperpolarization-activated current
I_i	instantaneous current

IP_s	inositol triphosphate
I_{ss}	steady-state current
I/V	current/voltage
LA	lateral amygdala
LTP	long-term potentiation
OCD	obsessive compulsive disorder
OT	oxytocin
PLCβ	phospholipase C-β
PKC	protein kinase C
RMP	resting membrane potential
TEA	tetraethylammonium
TTX	tetrodotoxin
$V_{1/2}$	voltage of half-activation

ACKNOWLEDGMENTS

I would like to thank Dr. N. Bradley Keele for his direction and assistance on this project as well as the Baylor University Department of Psychology and Neuroscience for their continued support.

DEDICATION

To two very important people in my life

To my mother, whose injury and recovery initiated my interest in the mysteries of the brain and showed me what perseverance looks like.

And to my husband, Bryan, without whose unfaltering support I never would have been able to accomplish this.

CHAPTER ONE

Background and Significance[1]

Autism Pathology

Autism spectrum disorders (ASD) are a class of developmental disorders affecting approximately 1 out of 166 children (Fombonne, 2003). The first symptoms generally appear in early childhood, typically between the age of one and two (DiCicco-Bloom, et al. 2006). ASD affects between four and five times more males than females, and is characterized by symptoms ranging across three domains: social interaction deficits, communication deficits, and stereotyped or repetitive behaviors, interests, or activities (*DSM-IV*).

Social interaction deficits may consist of decreased eye contact, as well as less facial expression and body language. Communication deficits include a delay or lack of language acquisition. Stereotyped behaviors are heterogeneous, and may manifest in such disparate ways as an obsessive interest in a particular topic, repeatedly lining up objects, or repetitive body movements such as rocking back and forth (DiCicco-Bloom, et al. 2006). Despite these relatively defined symptom domains, the diversity of phenotypes in ASD has made it a remarkably difficult disorder to study. Thus endophenotypic animal models of select symptomatic behaviors and cellular processes can make important contributions to understanding neural underpinnings of complex disorders such as ASD.

[1] Portions of chapter one appeared previously in Blakeley, H. J. (2007). Functional roles of arg-vasopressin and oxytocin on cellular excitability in neurons of the rat lateral amygdala. Retrieved from http://hdl.handle.net/2104/5127.

The development of animal models of ASD is further complicated by individual differences in the timeline of symptom manifestation, which are generally quite inconsistent across individuals (DiCicco-Bloom, et al. 2006). For example, some autistic children experience an improvement in their social activity during preschool years, while at the same time their stereotyped behaviors may become much more pronounced. Alternatively, a child may gain some use of language, regress several months later, then regain the ability again later on. This variability not only in symptoms but in progression of the disorder is a major obstacle in the study of ASD. The diversity of phenotypes of ASD is further complicated by many symptoms that are present in a smaller subset of those with ASD. For instance, approximately 30% of autistic individuals develop epilepsy (Gillberg & Billstedt, 2000).

Obviously there is not a simple developmental or anatomical culprit in ASD, but because of the extreme deficits in social interactions, the peptide hormones arginine-vasopressin (AVP) and oxytocin (OT) have been a target of much investigation. AVP and OT have been closely linked to the pathology of ASD largely due to their role in social behavior such as pair bonding and mating, as well as fear, stress, maternal, and paternal roles. AVP pathways in the central nervous system (CNS) have been shown to be sexually dimorphic; they are androgen dependent and more abundant in males, thus providing a possible explanation of the much higher incidence of autism in males than females (Insel, O'Brien, & Leckman, 1999).

As arguably the primary symptom of ASD, social dysfunction suggests that there may be abnormalities in brain regions associated with normal social behavior. While there are several brain areas that could be involved because of their involvement in social

behavior (such as the hypothalamus, hippocampus, and thalamus), the amygdala is a particularly interesting candidate as it is known to regulate many aspects of social behavior and may play an important role in ASD. Several lines of evidence have been explored that implicate the amygdala in the pathology of ASD (Baron-Cohen at al., 2000; Gaigg & Bowler, 2007, Moldin, Rubenstein, & Hyman, 2006), which will be discussed in a later section.

One important piece of evidence that implicates the role of the amygdala in ASD is the relationship between temporal lobe epilepsy and ASD. The amygdala is a well-known locus of temporal lobe epilepsy, and approximately 30% of autistic patients develop epilepsy (Gillberg & Billstedt, 2000). Hyperexcitability in the amygdala similar to that seen in temporal lobe epilepsy could account for the increased fear and decreased social interactions seen in ASD. Since cellular mechanisms controlling the amygdala are important in seizure activity, neuronal excitability may also be important in the pathophysiology of ASD.

In this project, we aimed to elucidate a possible mechanism at work in ASD pathology: the effects of OT and AVP on the lateral amygdala (LA), specifically the modulation of hyperpolatization-activated current (I_h).

Amygdala

The amygdala is an almond-shaped structure located deep in the temporal lobe involved in many emotional and behavioral processes including fear, anxiety, emotional memory, and aggression. Disturbed amygdala function is implicated in anxiety disorders, mood disorders, epilepsy, and autism. The role of the amygdala in autism is particularly

intriguing based on the normal role of the amygdala in social behavior and the deficits seen in ASD (Sweeten, Posey, Shekhar, McDougle, 2001; Baron-Cohen, et. al, 2000).

The amygdaloid complex is comprised of thirteen nuclei which are divided into three groups: the basolateral, cortical, and centromedial nuclei. There are many connections between these nuclei (Sah, Faber, Lopez de Armentia, & Power, 2003). This project was specifically looking at the lateral amygdala (LA) which, with the basolateral amygdala (BLA) makes up the basolateral complex. The LA is a main target for incoming sensory information from the thalamus and cortex. From there, information is projected to the central amygdala (CeA) which functions as the main output of the amygdala. CeA output is directed to many areas of the brain, including the hypothalamic-pituitary axis (HPA), the bed nucleus of the stria terminalis (BNST), and the reticular formation (Sah et al., 2003). Because of these connections, the amygdala has the ability to control the HPA and consequent stress-related endocrine and hormonal activity as well as behavior.

There are two main cell types within the LA, pyramidal neurons and interneurons. Pyramidal cells, also called Class I or projection neurons, comprise 90-95% of the cells in the LA (Faber, Callister, & Sah, 2001; Rainnie, et al., 1993; Sah et al., 2003) and are glutamatergic. Distinguishing characteristics of these cells include the presence of 3 to 5 primary dendrites, moderate spine density, broad action potentials, and spike frequency adaptation in the continued presence of a depolarizing stimulus (Faber et al., 2001; Rainnie, Asprodini, Shinnick-Gallagher, 1991a). Interneurons make up 5-10 % of the cells present in the LA. These cells are GABAergic and are spine-sparse or aspiny, exhibit faster action potentials than pyramidal cells, and show no spike frequency

accommodation. Interneurons are generally utilized in local circuitry to maintain tonic inhibition of pyramidal cells (Rainnie, Asprodini, & Shinnick-Gallagher, 1991b; Sah et al., 2003).

The amygdala is involved in determining the emotional salience of environmental stimuli, especially dangerous or fear evoking stimuli. Fear conditioning studies have been used to further investigate the neural mechanisms involved in fear behavior. Strength of thalamic inputs to the LA increase after fear conditioning, and therefore fear conditioning has been used to study the role of long-term potentiation and synaptic plasticity in learning (Mahanty & Sah, 1999; McKernan & Shinnick-Gallagher, 1997). While long-term potentiation (LTP) and the increase of the excitability of the LA is a very adaptive mechanism, it is possible for synapses to become hyperexcitable, which could lead to many behavioral problems such as pathological fear and anxiety, or inappropriate social behavior, both of which are associated with ASD. Hyperexcitability in the LA may be mediating some of the behavioral abnormalities in ASD, therefore finding a mechanism that increases excitability in the LA could elucidate some or the underlying pathology in ASD.

Normal plasticity in the amygdala allows for fear learning and LTP as adaptive mechanisms. These functions involve strengthening synapses through up-regulation of glutamate receptors (GluRs), increasing LA excitability. However, in pathological conditions, cells in the LA may become hyperexcitable, leading to epilepsy. The amygdala has been found to be involved in the genesis of temporal lobe epilepsy, as it has one of the lowest seizure thresholds in the brain. Complex partial seizures, which

often originate in deep structures of the temporal lobe, can occur as a result of amygdala hyperexcitability (Keele, 2005).

While excessive hyperexcitability of LA neurons may lead to temporal lobe epilepsy or seizures, sub-seizure hyperexcitability in LA neurons may result in a number of nonadaptive social and emotional disturbances without the behavioral symptoms of epilepsy. This is one hypothesis for the pathophysiology of interictal violence and aggression, fear and anxiety disorders, and impulsive or aggressive behavior (Keele, 2005). Some epileptics experience bouts of aggression between seizures, suggesting that the increase in neural activity experienced during seizure is affecting their behavior. The LA, having a very low seizure threshold, is often a focal point of temporal lobe epilepsy and clearly plays a role in normal fear and aggression. Together, these data implicate hyperexcitability in the LA as a possible mechanism for interictal violence. Specifically, if the LA is hyperexcitable, even a small stimulus could evoke burst firing. This burst firing would then cause a chain of reactions: projections to the MeA would then travel to the HPA axis which would cause an increase in cortisol release then driving the autonomic nervous system (ANS) to increase heart rate and blood pressure, and behavioral effects such as fear or aggression.

This hypothesis is further supported by the finding that many antiepileptic drugs are also mood-stabilizers, thus suggesting related mechanisms in neuronal hyperexcitability and emotional disturbances (Rogawski & Loscher, 2004), and that amygdala kindling increases fear behavior in rats (Kalynchuk, Pinel, & Meaney, 2005; Adamec, 1990). The effects of antiepileptics on social deficits such as those seen in ASD

are unknown. However, if those symptoms are modulated by hyperexcitability in the LA, it would be expected that antiepileptics would be effective in treating them.

Clinical and behavioral studies have contributed to the understanding of the role of the amygdala in many pathologies, and fear conditioning studies conducted on subjects with ASD provide evidence supporting the role of the amygdala in ASD (Gaigg & Bowler, 2007). It was found that these subjects could acquire a fear response to a conditioned stimulus (based on measurements of skin conductance responses), but these responses were no different than their reactions to non-conditioned stimuli. This shows that the impairment may not be in fear acquisition, but fear discrimination, which would suggest a problem with fear learning circuits that likely involve the amygdala.

Neuroanatomical studies of individuals with ASD have revealed several results that point to amygdala involvement in ASD pathology (Amaral, Schumann, & Nordahl, 2008). In young children, a 13-16% enlargement of the amygdala was reported (Sparks, et al. 2002), followed by abnormal amygdala enlargement throughout late childhood (Sparks, et al. 2002, Schumann et al. 2004). Amygdala enlargement has also been found to correlate with more severe ASD symptoms, such as more severe anxiety and worse social and communication skills (Juranek et al. 2006, Munson et al. 2006; Schumann, Barnes, Lord, & Courchesne, 2009). In addition, there have been conflicting reports on the number and density of cells in the amygdala in individuals with ASD, with some reporting an increase in cell density (Bauman & Kemper, 1994) and others reporting a decrease (Schumann & Amaral, 2006).

These anatomical differences in amygdala size and growth seem to be correlated with many of the symptoms of ASD as well. One common feature of ASD is gaze

avoidance; whereas most people look to the eye region of a face to gather emotionally salient information, those with ASD tend to avoid making eye contact, or even looking at the eye region of a picture. In a case of bilateral amygdala lesion, the well-known patient S.M. was found to have a very specific deficit in the ability to read fear, anger, and other emotions from facial expressions (Adolphs, Tranel, Damasio, & Damasio, 1994; Adolphs, 2008), a problem also common in those with amygdala lesions (Vuilleumier, Richardson, Armony, Driver, & Dolan, 2004). Upon further investigation, it was found that this deficit was due to a decrease in time spent looking to the eye region of the face, which is where the most potent indicators of emotion can generally be found (Adolphs, et. al, 2005; Spezio, Huang, Castelli, & Adolphs, 2007; Adolphs, et. al, 1994), and more time spent fixating on the mouth (Spezio, Adolphs, Hurley, & Piven, 2007; Adolphs, et. al, 2005). A similar pattern has been discovered in those with ASD (Dalton, et. al, 2005). In addition, these patterns hold true for siblings of those with autism (Dalton, Nacewicz, Alexander, & Davidson, 2007). It has even been found that those with amygdala damage leads to an impaired ability to recognize emotion in music (Gosselin, Peretz, Johnsen, & Adolphs, 2007).

In a similar vein, it was found that amygdala size was correlated with ability to discriminate emotion from faces, with smaller amygdalae correlated with worse performance (Nacewicz, et al. 2006). Small amygdala size has also been correlated with more severe childhood social impairment and more gaze avoidance (Nacewicz, et al. 2006). In another approach it was found that, while those with ASD spend less time making eye contact, when eye contact was made, the time spent in gaze fixation was

positively correlated with amygdala activation in ASD subjects, but not control subjects (Dalton, et al. 2005).

There are many other lines of research that point to the involvement of the amygdala in ASD. For example, Kleinhans, et. al (2009) found reduced habituation in the amygdala of those with ASD in response to repeated exposure to faces – in control subjects, the amygdala is highly activated when first shown a face, but upon repeated exposure, amygdala activation decreases, whereas in ASD subjects, the amygdala continued to activate despite the repeated presentations. This suggests the amygdala may be more excitable in those with ASD, especially in response to social stimuli. Non-human primate studies also point to the importance of the amygdala in social behavior. For example, amygdala lesioned monkeys exhibited marked deficits in social interactions, failing to respond to their social group either aggressively or peacefully (Kling &Cornell, 1971). They also exhibit changes in foraging patterns, accepting previously unpalatable foods (Machado, Emery, Mason, & Amaral, 2010). Similarly, human subjects with amygdala lesions often exhibit ASD-like symptoms, such as impaired social interactions (Adolphs et al., 1994).

Taking into account the normal function of the amygdala, its dysfunction could result in many of the symptoms in ASD. The amygdala normally modulates the hypothalamic-pituitary axis (HPA) and therefore has the ability to modulate release of OT and AVP, which are thought to play a role in ASD pathology. Additionally, the amygdala is normally involved in learned fear and anxiety, as well as the behavioral response of the organism; therefore the reduced social interaction and increased fear and anxiety in ASD could suggest amygdala involvement (Moldin et al., 2006).

Many lines of research have pointed to the involvement of the amygdala in ASD, and clearly the amygdala plays an important role in the regulation of normal social behavior. It follows that abnormal amygdala function may lead to disturbances in those behaviors. By elucidating cellular mechanisms in the amygdala that may play a role in ASD pathology, we may be able to more fully understand the disorder and develop more efficient treatments.

Hyperpolarization-Activated Current

The hyperpolarization-activated cyclic nucleotide dependent non-selective cationic current (I_h) is present in many areas of the CNS, including the LA (Herman & Keele, 2007). While most currents are activated through depolarization, I_h is activated through hyperpolarization, which results in a slow inward current carried by sodium (Na^+) and potassium (K^+) ions. This inward current upon hyperpolarization functions to bring the cell back to a more depolarized state where another action potential is possible. This cycle provides an effective pacemaker not only in the sino-atrial node of the heart where it was first identified (DiFrancesco, 1993; Verkerk, van Ginneken, & Wilders, 2009), but also in areas of the CNS such as the thalamus and hippocampus (Luthi & McCormick, 1998; Luthi & McCormick, 1999; McCormick & Bal, 1997; Magee, 1998). It is also important in the modulation of network oscillation, temporal synaptic summation, and to the maintenance of resting potential.

The location and distribution of I_h has not been fully examined throughout the CNS, however several areas have been studied extensively. In hippocampal CA1 neurons, I_h channels are distributed throughout cell bodies and dendrites, but the density of I_h channels is approximately six to seven times higher in distal dendrites than in the

soma (Magee, 1998). This allows I_h to play a role in the integration of synaptic potentials as well as the modulation of retrograde propagation of activity from the soma (Magee, 1998; Poolos, Migliore, & Johnston, 2002; Luthi & McCormick, 1999). I_h has also been characterized in the LA (Womble & Moises, 1993).

The overall conductance of I_h tends to be relatively small compared to total membrane conductance, and is voltage-activated, generally first activated near -60 mV, although it often remains tonically active at rest (Magee, 1998). I_h also exhibits sensitivity to external cesium, which blocks the current.

The h-current is carried through hyperpolarization-activated cyclic nucleotide gated (HCN) channels, which are made up of a combination of several subunits, numbered 1-4. Slightly different characteristics result from different combinations of the subunits. For instance, HCN2 and 4 subunits are very sensitive to intracellular cyclic adenosine monophosphate (cAMP), whereas HCN1 and 3 are relatively insensitive. Previous studies in this lab have suggested that the LA most likely expresses HCN1 subunits in combination, perhaps, with others. With the large amount of variability in the data, however, it seems much too soon to make a definitive claim as to the conformation of the HCN channel subunits in the LA.

I_h plays a role in modulating neuronal excitability in several different areas of the brain. In the hippocampus it has been shown to reduce excitability due to a suppressive effect on excitatory dendritic input as well as reducing input resistance and temporal summation (Poolos, et al., 2002; Magee, 1998; George, Abbott, & Seigelbaum, 2009). However, some of these inhibitory actions are counteracted by the role of I_h in raising the resting potential of the cell closer to threshold for firing (Poolos, et al., 2002). I_h has been

implicated in seizure activity as well; it was found that the anticonvulsant drug lamotrigine reduced action potential firing in conjunction with dendritic depolarization by acting selectively on I_h channels, suggesting a role for I_h in epileptogenesis (Poolos, et al., 2002). In the LA it has been found that larger I_h amplitudes are correlated with stronger action potential accommodation, suggesting a role for I_h in amygdala excitability (Herman & Keele, 2007).

Different neurotransmitters may have the ability to modulate these I_h properties, affecting either I_h amplitude or activation kinetics, and it follows that they may play an important role in the modulation of excitability in the LA and in the pathology of disorders such as ASD. Amplitude changes would be expected to modulate action potential accommodation, thus increasing or decreasing excitability of the cell. If activation kinetics are modulated, and the voltage of half-activation ($V_{1/2}$, where the probability of any given HCN channel being open is 50%) becomes more hyperpolarized, it may suggest that I_h requires even more hyperpolarization to be induced.

Vasopressin and Oxytocin

Arg-Vasopressin (AVP) and oxytocin (OT) are human peptide hormones widely reported to be critically involved in a wide range of functions including pair bonding, kidney function and water retention, uterine contractions, and lactation. AVP and OT are nonapeptides produced in the hypothalamus (paraventricular nucleus and supraoptic nucleus) and released either from the pituitary into the bloodstream, where they elicit peripheral hormonal effects, or into the CNS from centrally projecting hypothalamic axons into such diverse regions as the limbic system, the brainstem, and the spinal cord (Raggenbass, 2001). Both AVP and OT function as neurotransmitters as well, generally

modulating distinct neuronal populations throughout the CNS (Huber, Veinante, & Stoop, 2005). In brainstem motoneurons and spinal cord neurons, excitation by either AVP or OT is accomplished through the opening of nonspecific cationic channels or the closing of potassium channels (Suzue, et al., 1981; Raggenbass, 2001).

Specific receptor types for AVP and OT have been identified in the diverse areas of the CNS, including high numbers of AVP receptors in the central amygdala (Huber et al., 2005), where they are G_q -protein linked to second messenger systems (Raggenbass, 2001). There are three types of AVP receptors, V1a, V1b, and V2, and one OT receptor type. The V1a receptor is the most abundant of the three AVP receptors in the CNS, and V2 receptors have only been identified in the periphery, primarily the kidneys. AVP V1a and V1b as well as the OT receptor are coupled to phospholipase C-β (PLCβ) and their activation increases hydrolysis of membrane phospholipid resulting in the activation of PKC and increased intracellular calcium concentration. The AVP V2 receptor is positively coupled to adenylyl cyclase, increasing intracellular concentration of cAMP (Insel, et al. 1999; Raggenbass, 2001). In facial motoneurons, activation of V1a receptors causes a persistent inward current that is dependent on sodium and insensitive to tetrodotoxin (TTX) (Raggenbass, et al., 1991). Activation of OT receptors in motoneurons also causes a persistent inward current that is insensitive to TTX (Raggenbass & Dreifuss, 1992). However, there is some variability in the effects of AVP and OT depending on the brain area being investigated (Raggenbass, 2001). For instance, in many areas, including the CeA, some cross-reactivity has been found between AVP and OT, with AVP sometimes binding to OT receptors, especially at relatively high concentrations (Huber, et al. 2005). Although AVP binding sites are

reported in the LA (Dorsa et al., 1984), the mechanism of action of AVP and OT receptors in the lateral amygdala has not been investigated.

While AVP and OT are often discussed together because of their physical similarities, they each elicit specific effects, especially in terms of anxiety, social behavior, and learning. AVP enhances aggression, anxiety, and stress (Huber et al., 2005); consolidates fear memory as well as social memory (Huber et al., 2005; Insel, 1999); facilitates learning of active and passive avoidance behavior (Insel, 1999); and is implicated in paternal behavior (Wang, Ferris, & De Vries, 1994) and other stereotypical male behaviors such as aggression, scent marking, and courtship (Young & Wang, 2004). AVP is largely androgen-dependent (Herman et al., 2005), and as such, may be a key factor in the sexually dimorphic etiology of ASD. Specific AVP receptor subtypes also mediate several distinct effects; the V1a receptor is important in social bonding (Winslow et al., 1993), and in some cases may facilitate social behaviors (Cho et al., 1999). The V1b receptor regulates responses to stress, and blocking the V1b receptor has been shown to reduce defensive behaviors (Serradeil-Le Gad et al., 2005). The V2 receptor is most often seen in the kidneys and carries out the antidiuretic action of AVP. The role of V2 in the CNS is largely unknown (Ring, 2005).

In contrast, OT decreases anxiety and stress, facilitates social behavior, maternal behavior, and mother/infant bonding (Young & Wang, 2004). OT administration also promotes extinction of conditioned avoidance behavior (Huber et al., 2005) and a decrease in repetitive behaviors in adult humans with ASD (Hollander, et al., 2003). OT administration has also been shown to increase the ability of adults with ASD to

determine the emotional valence of otherwise neutral statements, a task that ASD patients are generally very poor at performing (Hollander, et al., 2007).

The best known behavioral effects of AVP and OT are their roles in pair bonding and mating behavior. These behaviors were first investigated by Winslow, Hastings, Carter, Harbaugh, and Insel (1993) in two strains of voles; the monogamous prairie vole and the promiscuous montane vole. Monogamous prairie vole males exhibit strong pair bonding with their mate, strong paternal behavior, and aggression toward intruders or in defense of their mate and nest. Montane voles do not exhibit any of these traits and tend to live in solitary burrows. It was found that the prairie voles had a much higher density of AVP V1a receptors in the forebrain than did the montane voles (Winslow, Hastings, Carter, Harbaugh, & Insel, 1993; Hammock & Young, 2005). The genetic difference of interest between these two species involves the Avpr1a gene, which codes for the V1a receptor. The gene itself is homologous in prairie voles and montane voles, but it is preceded by a microsatellite sequence that is expanded in prairie voles but not in montane voles. This expanded microsatellite may be responsible for higher levels of transcription, leading to the higher density of AVP receptors seen in the prairie vole (Young & Wang, 2004). It was also found that the pathways for AVP are sexually dimorphic and are very important in paternal behavior (Winslow, et al., 1993; Insel, Wang, & Ferris, 1994).

Many additional experiments have been performed that uphold the importance of AVP and OT in social behavior. It has been found that, in rats, OT receptor mRNA increases in the presence of another healthy individual, and AVP receptor mRNA was increased in the presence of odor from a sick conspecific (Arakawa, Arakawa, & Deak, 2010); both OT and AVP increase prairie voles' preference for their partner (Cho, De

Vries, Williams, & Carter, 1999); an AVP V1b antagonist reduces aggression and stress in rats (Serradeil-Le Gal, et al. 2005; Greibel, et al. 2002); AVP release in the medial amygdala (MeA) increases maternal aggression, while a V1a antagonist decreases it (Bosch & Neumann, 2010); and OT knock-out mice are unable to recognize familiar conspecifics, which can be reversed upon OT administration (Ferguson, Aldag, Insel, & Young, 2001). All of this evidence points to the clear roles OT and AVP play in social behavior.

In addition to their role in the social aspects of ASD, AVP and OT are also implicated in the other two main symptom domains: communication and repetitive rituals. Administration of central OT, before isolation of infant rat pups from their parents and litter, reduced the distress call of isolated infant (6-8 day old) rat pups in a dose-dependent manner (Insel & Winslow, 1991). This suggests increased levels of the peptide may induce some of the same communication deficits seen in ASD. Also, both AVP and OT were shown to induce stereotypic behaviors such stretching, startling, repetitive grooming, and squeaking in mice with intracerebroventricular (ICV) injections (Insel et al., 1999), suggesting a role for AVP and OT in stereotyped or repetitive behaviors.

In humans, OT reduces repetitive behaviors (Hollander et al. 2003), increases ASD patients' ability to comprehend affective speech, a measure of social cognition (Hollander, et al. 2007). OT also reduces amygdala activation in response to fear-inducing images in healthy controls (Kirsch, et al. 2005), and inreases gaze fixation in normal controls – a piece of evidence that supports the work done with gaze fixation and

the amygdala as well – which suggests that OT plays a very important role in human social interactions (Guastella, Mitchell, & Dadds, 2008).

Further, abnormal levels of OT and AVP have been linked to multiple psychopathologies such as depression and other affective disorders, obsessive compulsive disorder (OCD), schizophrenia, Prader-Willi Syndrome, and Alzheimer's Disease, as well as autism spectrum disorder (ASD) (Insel, 1999; Hammock & Young, 2006). This is not surprising considering the wide range of behavioral effects these peptides normally modulate.

Summary and Significance

The amygdala is a critical part of the limbic system with important roles in many social behaviors. Abnormal activity in the lateral amygdala nucleus (LA) has been implicated in several disorders, including autism spectrum disorder (ASD) in which abnormal social functioning is a primary symptom. The peptide hormones arginine-vasopressin (AVP) and oxytocin (OT) are important in social behavior, and may also be involved in the pathophysiology of ASD; however, the functional role of AVP and OT in the amygdala had been largely unknown. Autism spectrum disorders include a wide array of symptoms, including increased anxiety and decreased social functioning, both of which have been linked to amygdala dysfunction.

Preliminary studies showed that AVP has an overall excitatory effect on LA neurons by decreasing hyperpolarization-activated current (I_h) and decreasing accommodation in LA pyramidal cells. OT administration, on the other hand, exerts inhibitory control over LA excitability by increasing I_h and promoting action potential accommodation. These complementary roles suggest that AVP and OT may work to

maintain a stable level of excitability in the LA under normal circumstances, and an imbalance of AVP and OT may lead to hyperexcitability in the LA, contributing to the psychiatric symptoms associated with ASD.

The primary focus of this project was to investigate the mechanisms affecting the excitability of the LA that may also be involved in the pathology of ASD. AVP and OT have been implicated in the pathology of ASD, and it was known that AVP and OT modulate excitability in the CeA (Huber, 2005), but the ionic mechanism mediated by these peptides in the LA had not been investigated. In addition to determining the roles of AVP and OT in the LA, the hyperpolarization-activated cyclic nucleotide dependent non-selective cationic current (I_h) was investigated. I_h is also known to modulate excitability in the LA, but the interaction of I_h and AVP/OT in the LA had yet to be investigated.

CHAPTER TWO

Materials and Methods

Animals

Male Sprague-Dawley rats were obtained from Harlan Laboratories (Houston, TX), weights upon delivery ranged from 45-60g. Animals were used within approximately two weeks of delivery (weights at use ranged from 45-150g). Animals were group housed, maintained on a 12-hour light/dark photoperiod cycle with food and water available ad libitum. Electrophysiological recordings were performed using procedures modified from those described previously (Blakeley, 2007; Herman & Keele, 2007; Keele, Arvanov & Shinnick-Gallagher, 1997; Magee 1998; Mahanty & Sah, 1999).

Tissue Preparation

Animals were decapitated and their brains quickly removed and placed in cold (4° C) low calcium artificial cerebrospinal fluid (low-Ca^{2+} aCSF) containing (in mM): NaCl (104), KCl (4.7), $MgCl_2$ (6), NaH_2PO_4 (1.2), $CaCl_2$ (0.5), glucose (11.5), and $NaHCO_3$ (25) , aerated with a 95% O_2, 5% CO_2 mix. Block cuts were then made, one just rostral to the cerebellum, one rostral to the optic chiasm, one removing the superior portion of the cortex, and one midsaggital to separate the hemispheres. Slices (500 μm) containing the lateral amygdala (LA) were made using a vibrotome. Slices remained in low Ca^{2+} aCSF for at least one hour to equilibrate to room temperature before recording.

Electrophysiological Recording

Recording Rig Setup

Brain slices containing the lateral amygdala were placed in a recording chamber and perfused with control aCSF containing (in mM): NaCl (117), KCl (4.7), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), $CaCl_2$ (2.5), glucose (11.5), and $NaHCO_3$ (25), which was aerated with a 95% O_2, 5% CO_2 mix.

Recording electrodes were made from borosilicate glass capillary tubing pulled in a Flaming-Brown puller. Recording electrodes had a tip resistance of 3-7 MΩ when filled with potassium gluconate internal solution containing (in mM): potassium-gluconate (122), NaCl (5.0), $MgCl_2$ (2.0), $CaCl_2$ (0.3), EGTA (1.0), HEPES (10.0), Na_2ATP (5.0), Na_3GTP (0.4).

Whole-cell voltage clamp recordings were obtained using the ~~–b~~lind" recording method (Blanton et al., 1989). Minimum seal resistance for each cell was at least 1 GΩ. Once ruptured, cells were held at -60 mV while running a 10mV hyperpolarizing step for at least 3 minutes to determine baseline properties. Voltage clamp experiments were accomplished using a patch clamp amplifier with pClamp software for experimental control and data acquisition.

Experimental Protocols

Current/voltage protocol. While in voltage clamp at a holding potential of -60 mV (V_h= -60 mV), the cell was given a series of 300 ms voltage steps ranging from -40 mV to -120 mV and the resulting current traces are recorded. A graph of the current/voltage relationship was then constructed for each cell and the conductance in the

different drug conditions was found by fitting a linear region of the I/V relationship to determine slope.

H-Current protocol. In voltage clamp held at -40 mV (V_h= -40 mV), the cell is given a series of 2 s hyperpolarizing voltage steps from -40 mV to -130 mV to assess the characteristics of I_h present in the cell. I_h was calculated by subtracting instantaneous current from steady state current (I_{ss} - I_i= I_h). Instantaneous current was measured immediately following the decay of the capacitive transient current, approximately 20 ms after the onset of the step. Steady state current was measured at the end of each trace, within 50 ms of the end of the current step.

When using the h-current protocol, cells were first tested in control aCSF, then in low calcium (0.5 mM) aCSF containing Ba^{2+} (1mM), tetrodotoxin (TTX, 1μM), tetraethylammonium (TEA, 20mM), and 4-aminopyridine (4-AP, 2mM) to block depolarization-activated sodium, calcium, and potassium currents, thus isolating the hyperpolarization-activated current, and then tested again. Subsequent testing conditions were all carried out in the presence of the low-calcium aCSF and the voltage-gated channel blockers.

Accommodation protocol. In current clamp, LA neurons are held at -60 mV, then 600 ms depolarizing current steps (50-600 pA) were given until action potentials were evoked. Three to four additional depolarizing steps were given to allow comparisons of spiking and accommodation properties in all conditions. This protocol was run in control aCSF without the presence of the voltage-gated channel blockers. Spike frequency was calculated in each cell by counting the number of spikes present in the step after the first action potentials were evoked. This stimulus intensity was then compared in all drug

conditions for each cell, and frequencies were calculated by dividing number of spikes by the length of the current step (600 ms).

Baseline Data

All LA cells were first assessed for initial physiological properties in normal aCSF containing 2.5 mM Ca^{2+} and no voltage-gated channel blockers. Upon obtaining whole-cell recording mode, the resting membrane potential (RMP) and action potential accommodation were recorded in current clamp. RMP was measured as the membrane potential where there is no current (V_m where I=0). Action potential accommodation was determined by delivering a series of 600ms depolarizing current steps (200-400 pA in 50pA steps) and recording the resulting action potentials.

Voltage clamp protocols were then used to collect data on the current/voltage (I/V) relationships, and the resulting current traces were recorded and used to determine slope conductance. An initial I_h test was also run before superfusion of low-calcium aCSF, voltage-gated channel blockers, and any other drugs.

All additions to the bath were allowed to superfuse over the tissue for approximately 12 minutes before testing for that condition began.

Agonist Pharmacology

Concentration-response relationships were constructed for OT- and AVP-mediated effects on I_h in LA-containing brain slices from naïve control animals. This protocol was run in low calcium (0.5 mM) aCSF containing Ba^{2+} (1mM), tetrodotoxin (TTX, 1μM), tetraethylammonium (TEA, 20mM), and 4-aminopyridine (4-AP, 2mM) unless otherwise noted, to block depolarization-activated sodium, calcium, and potassium currents, thus isolating the hyperpolarization-activated current. The superfusing solution

was then changed to aCSF containing either AVP or OT, and I_h was measured again, as in control. I_h was then measured repeatedly following the same protocol as progressively higher concentrations of AVP or OT were superfused. Concentrations of AVP used: 10^{-10}M, 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M; concentrations of OT used: 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M.

Drugs

AVP, OT, TEA, and Ba^{2+} were obtained from SigmaAldrich; TTX was obtained from Tocris. 4-AP was obtained from Acros.

Data Analysis

Analysis of variance (ANOVA) was used to compare the amplitudes of I_h in the absence (control) and presence (AVP or OT) of drugs. ANOVA was also used to compare the spike frequency before and after superfusing AVP or OT. Measures of membrane conductance were calculated from a linear fit of graphs of the current/voltage (I/V) relationships of individual cells.

Concentration-response relationships were constructed by determining the percent decrease (in AVP) or increase (in OT) of I_h at the -120 mV step compared to pre-drug control, then plotting I_h amplitude (in pA) as a function of the log of agonist concentration. Data were fit by the Hill equation to find the IC_{50} and EC_{50} of AVP and OT, respectively.

To determine drug-dependent changes on physiological properties of I_h , (I/V) data collected in control and during drug superfusion were analyzed to determine the voltage- and time-dependence of I_h (as in Figure 2). Using data collected during pharmacological experiments, I_h amplitudes at test potentials between -40 and -120 mV

(I_0) are normalized to I_h at -120 mV (I_{max}) in control and I_0 / I_{max} is plotted against holding potential (V_h). The data are fit by the Boltzmann equation (see Methods) to determine the voltage of half-activation ($V_{1/2}$) of I_h.

CHAPTER THREE

Results

Current and voltage clamp recordings were performed on 148 pyramidal cells from the lateral amygdala (LA) nucleus with average input resistance of 122.4 ± 3.9 MΩ.[2]

Effects of Oxytocin

Accommodation is Increased in Oxytocin

The effect of oxytocin (OT) on action potential accommodation was examined using current clamp by delivering depolarizing current steps (50-600 pA, 600 ms) to evoke repetitive action potential firing. In 4 of 8 neurons, OT increased accommodation; OT had no effect on the other four (Fig. 1). Figure 2 shows a representative LA neuron where OT increased accommodation. In control aCSF (Fig 2A), delivering a 200 pA depolarizing stimulus elicited 3 action potentials that accommodate by the end of the step. In the same cell in the presence of OT (1μM) superfused in the aCSF, the 200 pA stimulus elicited no action potentials. Further depolarization by a 400 pA stimulus elicited only a single action potential (Fig 2B). The effect of OT on spike accommodation occurred over a range of input current intensities (Fig. 2C). The summary of the effect of OT on neurons that responded by increased accommodation is

[2] The data presented here replicate and extend preliminary findings that were reported previously in Blakeley, H. J. (2007). Functional roles of arg-vasopressin and oxytocin on cellular excitability in neurons of the rat lateral amygdala. Retrieved from http://hdl.handle.net/2104/5127.

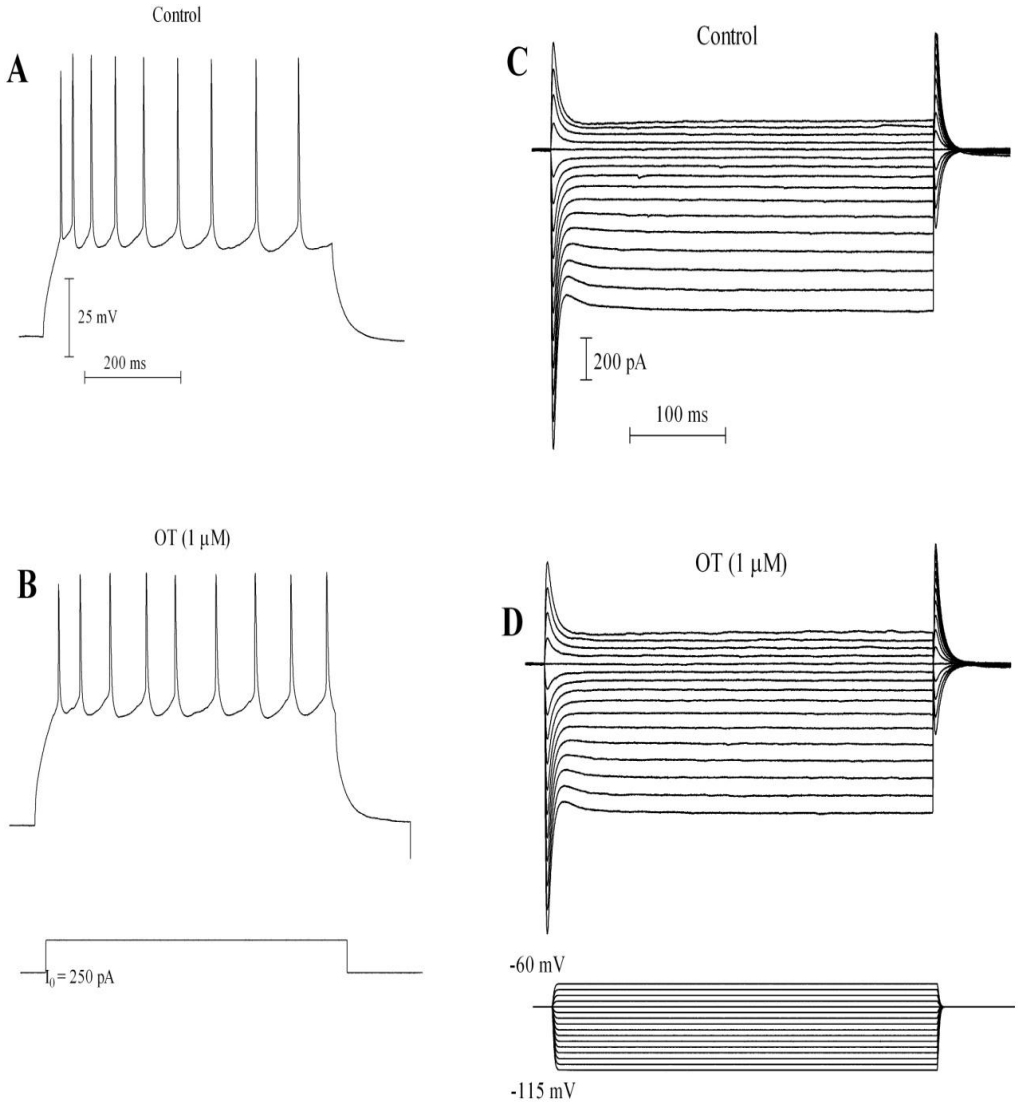

Figure 1. Population of OT unresponsive cells. In 4 of 8 LA neurons, oxytocin (OT) had no effect on repetitive firing or Ih amplitude . A and B show current clamp recordings of membrane potential in a representative LA neuron in response to a 250 pA depolarizing current step. In control aCSF (A) and oxytocin,1 µM (B) the 250 pA depolarizing current step elicits similar repetitive firing patterns. Representative voltage clamp recordings (Vh=-60 mV) are shown in C (control) and D (OT, 1 µM). Voltage steps (350 ms duration) elicit an instantaneous (ionic) current, Ii, followed by a slow inward rectification that reaches steady state (Iss) by the end of the step. Scale in A is the same in B. Scale in C is the same in D.

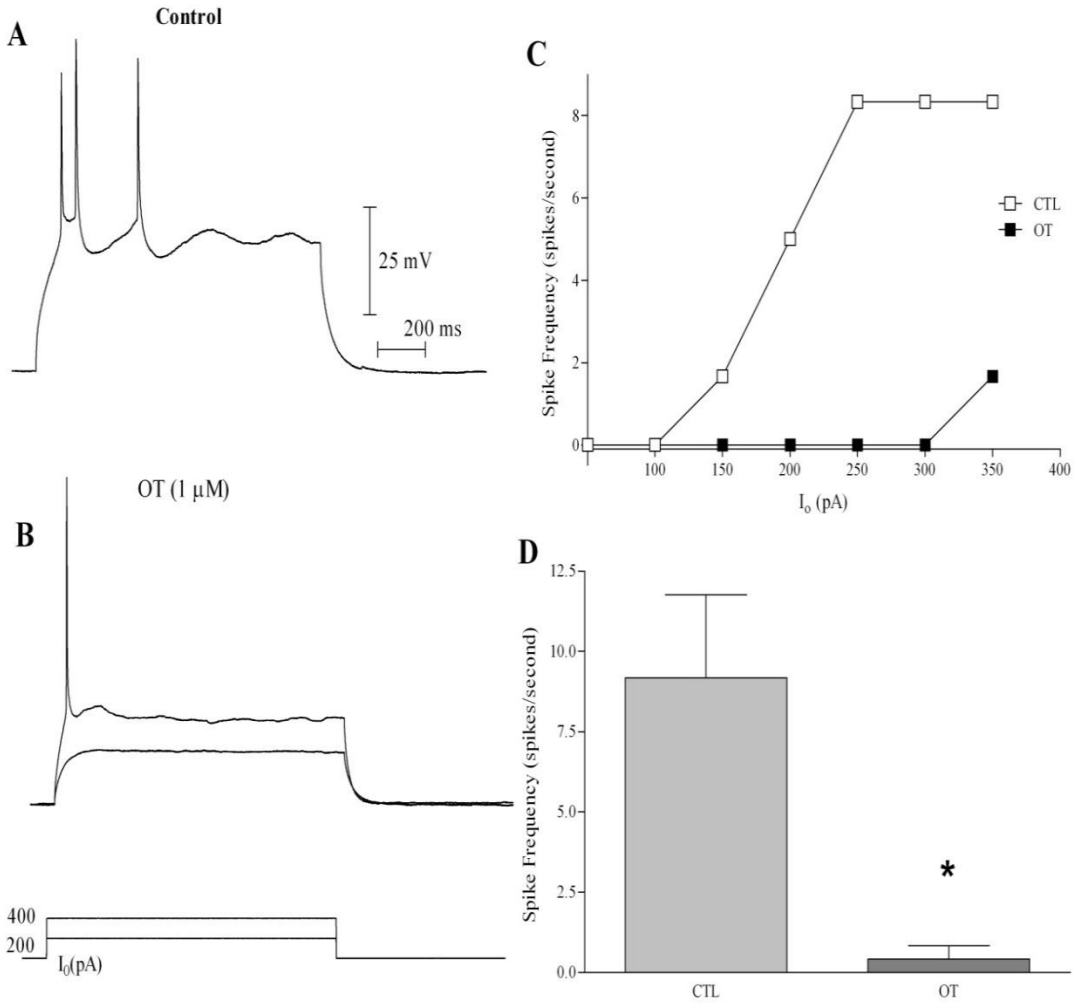

Figure 2. Oxytocin increases accommodation in the LA. A and B show current clamp recordings of membrane potential in a representative LA neuron. C. Summary of firing frequency in response to all current injections of the neuron shown in A and B. Spike frequencies for representative cell at increasing stimulus intensities D. Cumulative spike frequency averages; OT decreases the firing frequency of LA neurons from 9 ± 5 spikes/s in control (CTL) to 1 ± 1 in the presence of OT (n=4; p=0.016). * p<0.05.

shown in Figure 2D. The firing frequency of LA neurons was decreased from 9 ± 5 spikes/s in control aCSF (CTL) to 1 ± 1 in the presence of OT (n=4; p=0.016).

Hyperpolarization-Activated Current is Increased in OT

Based on the relationship between accommodation and I_h amplitude shown in LA neurons (Herman & Keele, 2007), wherein increased I_h amplitude is correlated with increased accommodation, the effect of OT on I_h amplitude was also investigated. Cells in the LA were voltage-clamped to a holding potential of -40 mV (V_h=-40 mV) and given 2 s hyperpolarizing voltage steps from -50 to -120 mV. Hyperpolarization beyond -70 mV revealed a slowly activating inward sag that resembled hyperpolarization-activated current (I_h). Here, I_h was elicited in control CSF containing barium (1mM) and TTX (1μM) (Fig. 3A), and again in the added presence of OT (1uM). I_h amplitude was tested again to determine effects of OT on I_h amplitude (Fig 3B).

Figure 3C shows a conductance increase in the linear instantaneous current, I_i, as well as increased conductance of the rectifying current, I_{ss} in the presence of OT. The I/V relationship of the subtraction current ($I_h = I_i - I_{ss}$) (Fig 3D) shows OT increases I_h amplitude. I_h was increased from -186 ± 45 pA in control to -215 ± 60 pA in OT (1 μM).

The voltage dependence of activation of I_h was analyzed by constructing activation curves for I_h in control aCSF with barium and TTX, and again in the presence of control aCSF with barium, TTX, and OT (n=7) (Fig 3E). The data was fit with the Boltzmann equation to determine the voltage dependence of I_h. In control, the voltage of half-maximal activation ($V_{1/2}$) was -113 ± 3 mV. OT (1 μM), had no effect on I_h voltage dependence ($V_{1/2}$= -113 ± 3 mV).

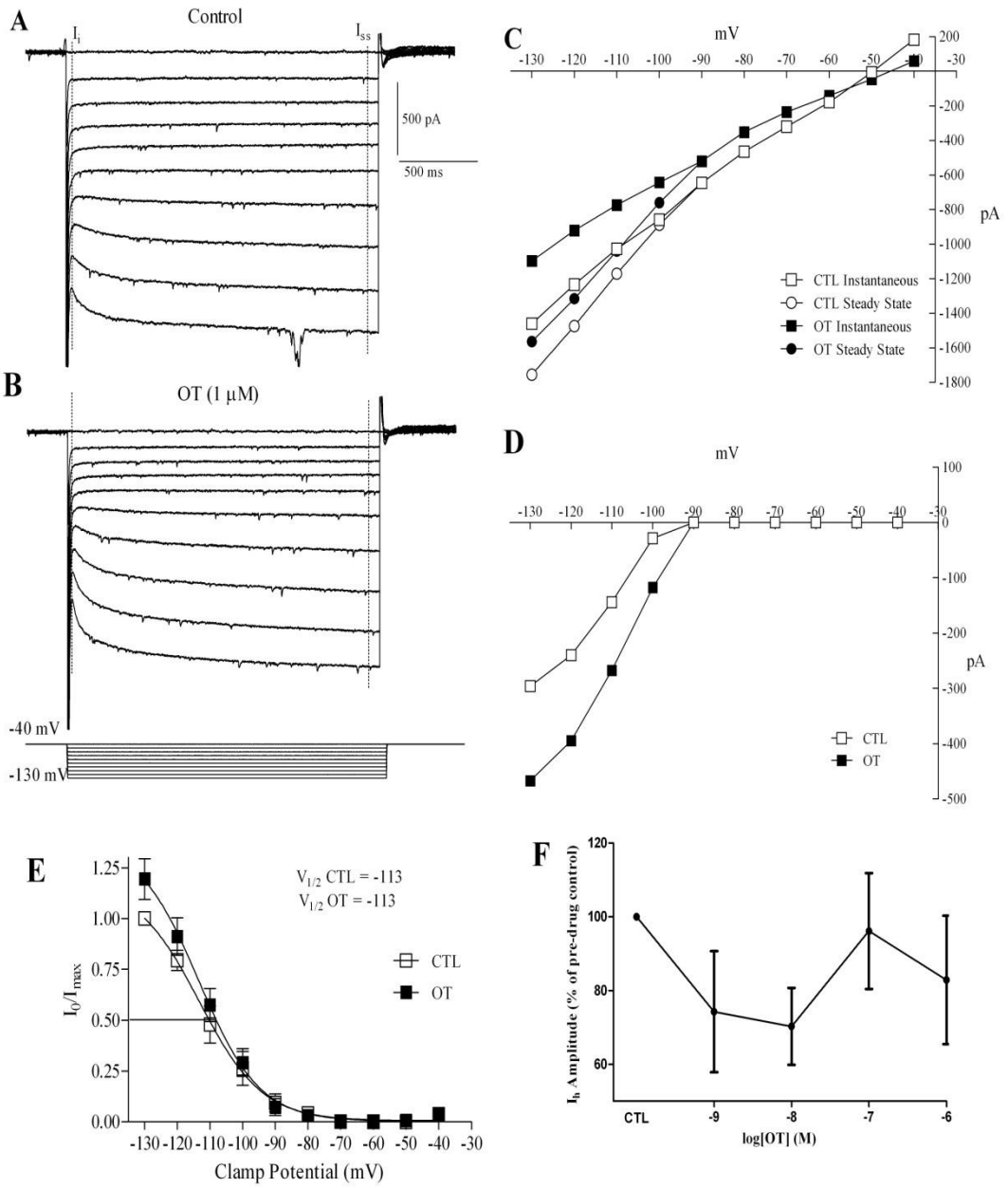

Figure 3. OT increases Ih in LA cells. A-B show representative voltage clamp recordings (Vh=-40 mV) in control (A) and OT (B). Voltage steps elicit an instantaneous (ionic) current, Ii, followed by a slow inward rectification (Iss). C. Current-voltage relationships of Ii and Iss in control and OT from the neuron shown in A and B. D. The subtraction (Ii-Iss)/V relationship shows specific increase of Ih in OT, increasing amplitude from -186 ± 45 pA in control to -215 ± 60 pA (n=7). E. Ih activation curve in control and OT (n=7). F. Concentration-dependent relationship of Ih amplitude with increasing OT concentration (n=5).

In separate experiments, a concentration-response curve was constructed to analyze the pharmacology of OT receptor-elicited increases in I_h amplitude (Fig 3F). For concentration-response experiments, control currents were elicited in a low-calcium aCSF containing barium (1mM) and TTX (1μM), and, unlike earlier experiments, 4-AP (2mM), and TEA (20mM) were added as well. As the concentration of OT increased, I_h amplitude decreased, then at higher concentrations increased again to near control levels (n=5).

Effects of Vasopressin

Accommodation is Decreased in AVP

Figure 4 shows a recording from a representative LA neuron. In current clamp, neurons were held at -60 mV with direct current injection. Depolarizing current steps (50-600 pA, 600 ms) were delivered to evoke repetitive action potential firing. In control aCSF (Fig 4A), delivering a 300 pA depolarizing stimulus elicited 5 action potentials that accommodate by the end of the step. In the same cell in the presence of AVP (3μM) added to the aCSF, the 300 pA stimulus elicited a response with reduced accommodation (Fig 4B). Figure 1C shows the effect was reversible on return to control aCSF in the same cell. The effect of AVP on spike accommodation occurred over a range of stimulus intensities (Fig. 4D). The summary of the effect of AVP on all neurons that responded by decreased accommodation is shown in Figure 4E. The firing frequency of LA neurons was increased from 7 ± 2 spikes/s in control (CTL) to 14 ± 3 in the presence of AVP (n=4; p=0.06).

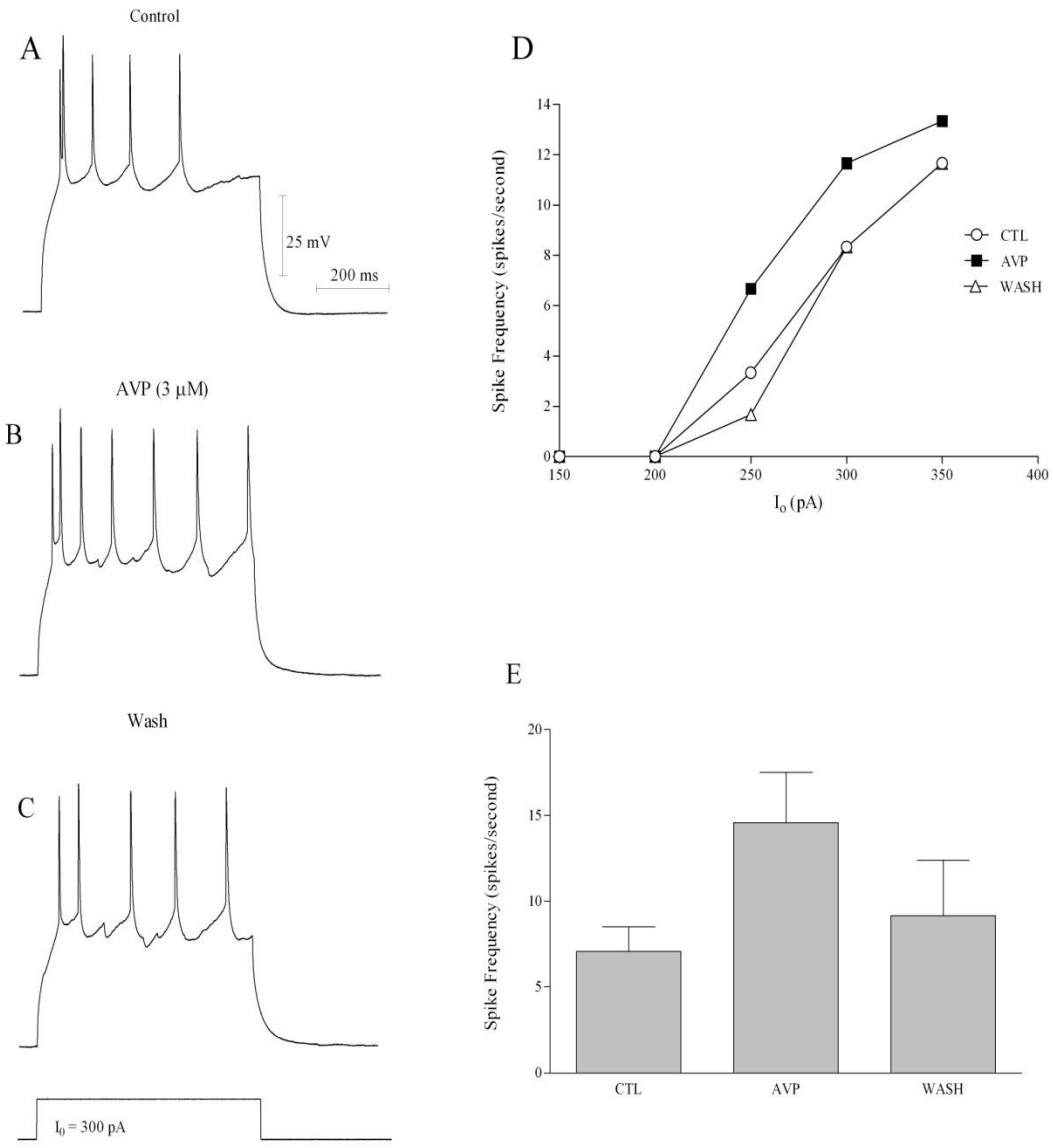

Figure 4. AVPdecreases accommodation in the LA. A-C show current clamp recordings of membrane potential in a representative LA neuron in response to a 300 pA depolarizing current step. A. In control, the depolarizing current step reveals an accommodating neuron with spike frequency adaptation.B. In arginine-vasopressin (AVP, 3µM) the accommodation is decreased. C. The effect of AVP on accommodation reverses on return to control ACSF (wash, 10 minutes). D. Summary of firing frequency in response to all current injections of the neuron shown in A-C. E. Cumulative spike frequency averages in control, and AVP (1 to 3µM) (n=4). Scale in A is the same for B and C. All recordings performed at Vm= -60mV.

Hyperpolarization-Activated Current is Decreased in AVP

Previous studies (Herman & Keele, 2007) have shown that the hyperpolarization-activated current, I_h, is an important mechanism controlling spike accommodation. Therefore, voltage clamp recordings were performed to determine the effect of AVP on I_h.

LA neurons were voltage-clamped at -40 mV (V_h=-40 mV). Hyperpolarizing steps were delivered between -40 and -130 mV (2s duration). Hyperpolarization beyond -70 mV revealed a slowly activating inward sag that resembled the hyperpolarization-activated current, I_h (Fig 5A). After the decay of the capacitive transient current, an instantaneous ionic current (I_i) was followed by a slow inward relaxation that reached steady state (I_{ss}) by the end of the step and resembled I_h ($I_h = I_i - I_{ss}$). Following superfusion of AVP (1 to 3 μM), the hyperpolarization protocol was used again to measure I_h amplitude in the presence of AVP (with Ba^{2+} and TTX) (Fig 5B).

Current-voltage (I/V) relationships were constructed to examine the change in membrane conductance mediated by AVP. Figure 5C shows a conductance decrease in the linear instantaneous current, I_i, as well as decreased conductance of the rectifying current, I_{ss}. The I/V relationship of the subtraction (I_i-I_{ss}) current (Fig 5D) shows I_h for the same cell, where AVP (1-3μM) inhibits I_h amplitude. Cumulative I_h amplitude upon hyperpolarization to the -130 mV step was reduced to 83 ± 12% of control amplitude (n=6).

The voltage dependence of activation of I_h was analyzed by constructing activation curves from the I/V relationship for I_h in control and in the presence of AVP

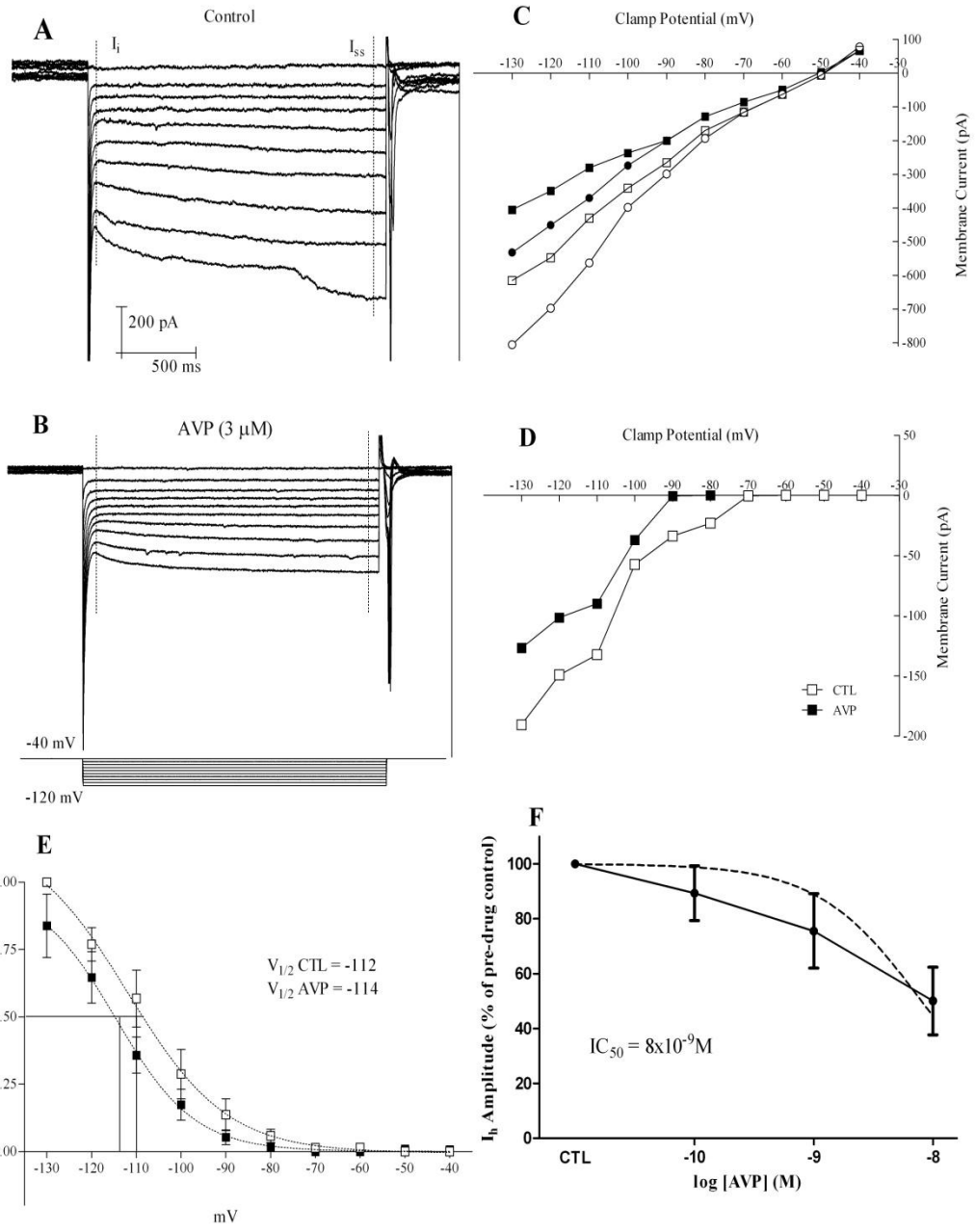

Figure 5. AVP decreases Ih in the LA. Representative voltage clamp recordings shown in A (control) and B (AVP, 1 µM). Hyperpolarizing voltage steps elicit an instantaneous (ionic) current, Ii, followed by a slow inward rectification that reaches steady state (Iss) by the end of the step. C. Current-voltage relationships of Ii and Iss in control and AVP from the neuron shown in A and B. D. Subtraction I/V relationship (Ii - Iss). E. Voltage-dependent activation curves for Ih in CTL and AVP (1 and 3 µM) (n=7). F. Concentration-dependent decrease of Ih amplitude with increasing AVP concentration, data was fit with a nonlinear regression (n=6).

(n=7). The data was fit to the Boltzmann equation to determine the voltage of half-maximal activation ($V_{1/2}$). In control, $V_{1/2}$=-112 ± 4 mV, in AVP (1-3μM), $V_{1/2}$ was shifted slightly in the hyperpolarizing direction to $V_{1/2}$=-114 ± 4 mV. This suggests that the effect of AVP on I_h does not involve a change in the voltage-dependence of the conductance.

The AVP concentration-response relationship was constructed to analyze the pharmacology of the AVP-mediated decrease in I_h amplitude (Fig 5F). As concentration increase, I_h amplitude decreased to 50 ± 12% of control at a concentration of 10^{-8}M. As concentrations increased even further, however, I_h amplitude began to increase again, perhaps because of off-target effects of AVP on OT receptors at higher concentrations. IC_{50}, determined from the fit of data collected in the presence of AVP concentrations 10^{-8} and below was $8x10^{-9}$ (n=6).

CHAPTER FOUR

Discussion

In the present study, we analyzed the pharmacological and physiological mechanisms of two peptide transmitters, Arg-Vasopressin and oxytocin, in LA neurons. The data show that both AVP and OT affect excitability and I_h in the LA, and that their effects were complementary. AVP decreased I_h amplitude, whereas OT increased I_h amplitude, resulting in an increase and decrease in excitability, respectively. In attempting to determine the mechanism by which these peptides exert their effects, the voltage-dependence of the I_h was investigated and it was found that neither AVP of OT significantly alter the voltage-dependence. Concentration-response experiments revealed that AVP decreased I_h in a dose-dependent manner.

Oxytocin

Our first experiments with OT found that OT increased action potential accommodation in LA cells and increased I_h amplitude, resulting in decreased LA excitability (see Fig. 2 and 3). These results support our overall hypothesis of the effects of decreasing excitability in the LA. Behavioral effects of OT, decreased stress and anxiety, are consistent with a decrease in amygdala excitability (Young & Wang, 2004).

It was also found that some cells in the LA were insensitive to the presence of OT (see Fig. 1). In these cells, there was no change in accommodation or I_h amplitude in response to OT. This suggests that there may be two distinct populations of pyramidal cells in the LA that can be distinguished based on their sensitivity to OT. Similarly, there

also appear to be two populations of cells in the CeA, where 21% showed increased firing after application of OT, while over 50% showed a decrease in firing (Huber et al., 2005). As in the CeA, the effects of AVP and OT in the LA are also complementary, although the effects in the LA are likely dependent on direct modulation of cellular excitability rather than through the mediation of inhibitory circuitry.

As shown in the OT concentration-response relationship, the OT-mediated increase in I_h amplitude was blocked by the addition of the potassium channel blockers, 4-AP and TEA, which had not been used in previous experiments. In the presence of TEA and 4-AP, the I_h amplitude was decreased in the presence of OT, rather than increased. Further increases in OT concentration caused I_h amplitude to return to near control levels. To investigate this possibility further, it is worth looking at how I_h works and what compounds it is sensitive to elsewhere in the brain.

In mouse hippocampal CA1 cells, I_h produced a dual excitatory and inhibitory effect on excitatory post-synaptic potentials (EPSPs), by depolarizing the peak voltage of weak EPSPs, but hyperpolarizing the peak voltage of stronger EPSPs (George, Abbot, & Siegelbaum, 2009). The inhibitory effect in the CA1 region was found to be modulated by M-type K^+ currents (George, et al. 2009), a type of current that could be blocked by the concentrations of 4-AP used in our experiments. This may be the mechanism through which OT affects I_h amplitude in the LA, hence the disruption of the concentration-response experiments in the presence of potassium channel blockers, especially considering the effect of I_h in OT is inhibitory, just as in CA1 neurons.

Another possible explanation of the OT data is that the mechanism by which OT exhibits its effects is presynaptic, as in rat olfactory neurons (Osako, Otsuka, Taniguchi,

Oka, & Kaba, 2000; 2001). OT is generally reported to have a post synaptic mechanism, but a presynaptic mechanism could serve to explain the block of OT-mediated increase in I_h amplitude. To further investigate the mechanisms reported to be involved in the effects of OT, we now look to OT research conducted in other areas of the CNS.

OT has been reported to have any number of effects in disparate regions of the CNS. Some areas are inhibited by the action of OT, including the hypoglossal motor nucleus (Wrobel, Reymond-Marron, Dupre, & Raggenbass, 2010). However, unlike the decrease in excitability in response to OT that we see in the LA, OT has also been shown to increase excitability in certain areas of the CNS, including vagal motoneurons (Raggenbass & Dreifuss, 1992) and the lateral division (CeL) of the central amygdala (CeA) (Huber, et al., 2005). In vagal motoneurons, OT induces a non-inactivating inward current leading to an overall increase in excitability very similar to that seen with AVP in facial motoneurons (Raggenbass & Dreifuss, 1992; Raggenbass, Goumaz, Sermasi, Tribollet, & Dreifuss, et al. 1991). In the CeA, there are two reported classes of cells, one of which is inhibited by OT, the other is excited by OT. The overall effect of OT in the CeA is inhibitory, as it excites GABAergic neurons and inhibits excitatory CeA neurons (Huber, et al., 2005). In the case of the CeA, OT works by inhibiting cells that are excited by AVP through excitation of different groups of cells. The results of this project in the LA are similar in that AVP and OT exhibited complementary effects, although OT showed direct inhibitory effects on cells as opposed to working through an inhibitory network as is seen in other brain regions. Additionally, there are many areas where AVP and OT can either both increase or both decrease excitability (Raggenbass,

2001). For example, both AVP and OT increase excitability in the CA1 region of the hippocampus (Muhlethaler, Dreifuss, & Gahwiler, 1982).

Clearly, the effects of OT are diverse. The commonality between all of these effects, however, is in the mechanism of action of the OT receptor (OTR). All OT effects are elicited through the activation of a single OT receptor, OTR. These receptors are G_q-coupled to phospholipase-Cβ (PLCβ), and subsequently increase intracellular calcium and activation of PKC. The downstream actions of OT depend on the area of the brain, the native circuitry therein, and the target effectors modulated by OTR-mediated changes in second messengers.

It is this OT receptor mechanism that is thought to modulate I_h amplitude as well. While I_h is, intuitively, a current that would lead to an increase in excitability - through allowing positive ion influx – it also has the ability to cause an inhibitory reaction, primarily through an increase in conductance. This conductance increase at hyperpolarized potentials leads to both a decrease in resting membrane potential and a shunting effect, which decreases the effect that any one EPSP can elicit. Based on this mechanism, an increase in I_h would be expected to increase cell accommodation, resulting in decreased excitability, congruent with results in the current study.

The question, then, rests with the connection between activation of the OT receptor and alteration of I_h amplitude. The next step would be to test the effect of OT in the presence of kinase inhibitors to see what is necessary to elicit the change in I_h amplitude. The role of specific kinases remains to be determined. To the best of our current knowledge, OT decreases excitability in the LA, shown by the increase in accommodation (Fig. 2D), but the way this is controlled may involve other mechanisms

in addition to the increase in I_h amplitude. However, the present results provide evidence for the overall inhibitory effect of OT on LA neurons, which is consistent with the behavioral effects of decreased LA excitability and the social and behavioral effects of OT.

Vasopressin

In the LA, AVP decreased action potential accommodation and decreased I_h amplitude, both of which contribute to increased excitability. These results are complementary to those of OT, which decreased accommodation and I_h amplitude. As mentioned above, it is fairly common to see these complementary effects of OT and AVP in different areas of the CNS. Unlike OT, however, which tends to result in either an increase or decrease in excitability, AVP has been shown in a majority of cases to increase excitability.

Vasopressin has been shown to increase excitability in diverse areas of the CNS including the spinal cord, facial motoneurons, and hypoglossal motoneurons (Raggenbass et al., 1991; Raggenbass, 2001). In spinal cord neurons, AVP activates V1a receptors to induce a prolonged depolarization through suppression of a K^+ current as well as increasing an inward nonspecific cationic current (Kolaj & Renaud, 1998). The effects of AVP in facial motoneurons is very similar, where there is a persistent inward nonspecific cation current that results in enhanced excitability and a reduction of firing threshold (Alberi, Dubois-Dauphin, Dreifuss, & Raggenbass,1993; Widmer, Dreifuss, & Raggenbass, 1992). AVP also induces an inward current in hypoglossal motoneurons that reverses around -15 mV, suggesting that it is mediated by both Na^+ and K^+ (Palouzier-Paulignan, Dubois-Dauphin, Tribollet, Dreifuss, & Raggenbass, 1994), and

increases excitability in the subfornical organ of rats (Washburn, Beedle, & Ferguson, 1999). AVP was found, however, to decrease EPSCs in the rat parabrachial nucleus (Chen & Pittman, 1999), but this remains one of the few instances of AVP activation resulting in decreased excitability.

The results of the current study are similar to most AVP research, in that AVP induces an increase in excitability in the LA, but the mechanism through which it appears to work—the modulation of I_h—has not been previously reported. Together, these data show that AVP increases neuronal excitability, although a variety of cellular mechanisms may be employed. The current results suggest a novel mechanism of AVP-mediated excitability, namely the inhibition of I_h.

It has been shown that larger I_h amplitudes are correlated with increased cell accommodation in the LA (Herman & Keele, 2007). The increased excitability seen in the current experiments in the LA after AVP application could be due to the effect of AVP on I_h. LA cells superfused with AVP showed a decrease in I_h amplitude as well as a decrease in accommodation, although a causal link between the actions of AVP, I_h amplitude, and accommodation has yet to be established. There have been many connections between I_h and excitability in other studies. It was found that CA1 dendrites exhibited a long-term increase in I_h with a depolarized voltage of half-activation after febrile seizures were induced (Dyhrfjeld-Johnsen, Morgan, Foldy, & Soltesz, 2008), and I_h decreases excitability in CA1 pyramidal neurons (Magee, 1998; Fan et al. 2005). I_h has even been suggested as a pharmacological target for treatment of seizure disorders (Poolos et al., 2002). In retinal ganglion cells, I_h is an important mechanism through which dopamine modulates excitability (Chen & Yang, 2007). Overall, I_h is thought to be

a very flexible mechanism, meaning that it can work differently in diverse areas of the brain, depending on the surrounding environment, circuitry, and other activity occurring in the vicinity (Luthi & McCormick, 1998; Accili, Proenza, Baruscotti, & DiFrancesco, 2002).

The amplitude and activation kinetics of I_h can be modulated by several compounds. Many studies have found that I_h amplitude and activation can be modulated by cyclic adenosine-monophosphate (cAMP) (Momin, Cadiou, Mason, & McNaughton, 2008; DiFrancesco & Tortora, 1991). Activation of I_h via DA application has also been identified as cAMP- and PKA-dependent (Chen & Yang, 2007). This activity of cAMP is somewhat unexpected, given the current understanding of AVP receptors and the effects of their activation.

The effects of AVP are mediated by three distinct receptor subtypes, V1a, V1b, and V2. V1a and V1b are Gq coupled to PLCβ, which increases the hydrolysis of membrane phospholipids, resulting in the activation of protein kinase C (PKC) and increased intracellular calcium concentration. V2 receptors are positively coupled to adenylyl cyclase and an increase in cAMP formation, but have only been reported in the periphery.

The most widely reported AVP receptor in the CNS is the V1a receptor. Other areas that exhibit V1a receptors include the CeA (Huber, et al. 2005), the olfactory bulb, nucleus accumbens, hypothalamus, MeA, and cortex of the common marmoset (Schorscher-Pectu, Dupre, & Tribollet, 2009). The V1b receptor has been found in the hippocampus, cerebral cortex, and hypothalamus of the Chinese hamster (Lolait, et al.

1995). The specific subtype of AVP receptor coupled to the decrease in I_h amplitude in LA neurons remains to be determined.

It seems likely, however, that the effects of AVP reported here occur through activation of a G_q-coupled receptor, either V1a or V1b, since V2 receptors are reportedly not expressed in the CNS. Thus, the AVP-induced effects on I_h amplitude and activation kinetics are likely due to increases in intracellular calcium levels or other actions of the PLCβ system. The activation of the G_q-coupled V1a receptor leads to several changes within the cell. First, PLCβ is activated, leading to the formation of diacylglycerol (DAG) and inositol triphosphate (IP3). From there, IP3 acts to increase intracellular calcium through interactions with the endoplasmic reticulum, and DAG activates protein kinase C (PKC), which can then initiate a cascade of intracellular effects. So the effects seen on I_h amplitude are due either to the increase in intracellular calcium or the actions of PKC.

Recently, this well-accepted system has, to a certain extent, been called into question; Wrobel, Dupre, & Raggenbass (2010) re-assessed the signaling pathway associated with V1a receptors, and found that in almost all cells tested in rat facial motoneurons, the action of AVP was mediated by cAMP, suggesting V1a receptors behave more similarly to V2 receptors and may be coupled to adenylyl cyclase and not PLCβ. This is supported by other studies showing that the effects of V1a activation are carried out independent of intracellular calcium (Reymond-Marron, Tribollet, & Raggenbass, 2006).

Another argument for the activation of adenylyl cyclase by V1a receptors lies in the differential effects of AVP and OT in many areas of the brain. It is generally thought that V1a and OT receptors are both coupled to PKC. Based on the results from this

project and many other behavioral and cellular studies, AVP and OT have opposing effects. If AVP and OT were causing the same physiological reaction, it would follow that the data would look the same for OT and AVP, which it does not. If, however, the mechanism through which AVP elicited its effects was different than that for OT, the differences in effects could be explained.

In addition to their receptors, AVP and OT are very similar peptides. They differ in only two of nine amino acids in their sequence. It is well known that AVP can often bind to OT receptors at moderate to high concentrations (Wrobel, et al. 2010; Barberis, Mouillac, & Durroux, 1998; Huber, et al. 2005), which is why, we believe, the concentration-response relationship of AVP on I_h amplitude is biphasic. Lower concentrations of AVP bind to the V1a receptors and elicit the expected decrease in I_h amplitude, then at higher concentrations, AVP may begin to bind to OT receptors as well, leading to the subsequent increase in I_h amplitude back to near-control levels. However, this has not been shown.

Conclusion

The modulation of excitability in the LA by AVP and OT suggests a role for LA excitability in the pathophysiology of ASD. AVP exacerbates anxiety and stress, and facilitates the learning of avoidance behaviors, all of which occur in people with ASD (Huber et al., 2005; Insel, et al., 1999). This project reports that AVP increases excitability in the LA, suggesting that hyperexcitability in the LA may be involved in many of the symptoms seen in ASD such as anxiety, aggression, and fear. In addition, a subset of patients with ASD suffer from epilepsy (Gillberg & Billstedt, 2000), suggesting that hyperexcitability, possibly in the LA, is involved in the pathology of ASD.

In contrast, oxytocin decreases anxiety and stress and facilitates social behavior and pair bonding. In this study it was found that OT decreases neuronal excitability in the LA, which suggests that a decrease in excitability in the LA or the presence of tonic inhibition in the LA, possibly through the presence of OT, could decrease the severity of ASD symptoms. This interpretation is consistent with recent evidence showing that OT decreases symptom severity in ASD (Hollander, et al., 2003).

Altogether, the results of the present study suggest a novel mechanism whereby pituitary peptide hormones can affect amygdala function. Specifically, AVP and OT may control excitability of LA neurons by altering the physiological properties of I_h, specifically altering I_h amplitude through the activation of OT and perhaps V1a receptors. Therefore, controlling excitability, possibly through I_h modulation, in the amygdala may be an important therapeutic target for disorders that involve amygdala hyperexcitability. Since AVP and OT and the amygdala are important in anxiety and social behavior, these data suggest that the complementary roles of AVP and OT on amygdala excitability are important in ASD, and may provide a novel pharmacological target for treatment.

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