ABSTRACT

Functional Roles of Arg-Vasopressin and Oxytocin on Cellular Excitability in Neurons of the Rat Lateral Amygdala

Hillary Joy Blakeley, M.A.

Thesis Chairperson: N. Bradley Keele, Ph.D.

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. AVP and OT increase excitatory responses in the brain, however their role in the LA is unknown. Here, the effects of AVP and OT on membrane properties of LA neurons were investigated using whole-cell recording of LA neurons *in vitro*. AVP decreased accommodation and hyperpolarization-activated current (I_h) in LA pyramidal cells, resulting in increased excitability. OT increased action potential accommodation and I_h resulting in decreased excitability. These results suggest AVP and OT may modulate social behavior by controlling excitability in the amygdala.

Functional Roles of Arg-Vasopressin and Oxytocin on Cellular Excitability in Neurons of the Rat Lateral Amygdala

by

Hillary Joy Blakeley, B.A.

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Jaime L. Diaz-Granados, Ph.D., Chairperson

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Approved by the Thesis Committee

N. Bradley Keele, Ph.D., Chairperson

Jaime L. Diaz-Granados, Ph.D.

Jim H. Patton, Ph.D.

Kevin G. Pinney, Ph.D.

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J. Larry Lyon, Ph.D., Dean

Page bearing signatures is kept on file in the Graduate School.

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aCSF	artificial cerebrospinal fluid
ASD	autism spectrum disorder
AVP	arginine-vasopressin
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CeA	central amygdala
CeL	lateral capsule of CeA
CeM	medial capsule of CeA
CNS	central nervous system
CS	conditioned stimulus
CSF	cerebrospinal fluid
EPSC	excitatory post-synaptic current
GABA	γ-aminobutyric acid
GluR	glutamate receptor
HPA	hypothalamic pituitary axis
ICV	intracerebroventricular
I_h	hyperpolarization-activated current
LA	lateral amygdala
LTD	long-term depression
LTP	long-term potentiation
MGN	medial geniculate nucleus

NMDA	N-methyl D-aspartate
NMDA-R	NMDA receptor
OCD	obsessive compulsive disorder
OT	oxytocin
PLC	phospholipase C
TTX	tetrodotoxin
US	unconditioned stimulus

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CHAPTER ONE

Background and Significance

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, such 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 domains of symptoms, 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.

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 patients develop epilepsy (Gillberg & Billstedt, 2000). In addition, the three classes of symptoms develop along different timelines, none of which are consistent

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. Or a child may gain some use of language, regress to nothing several months later, then regain 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. In this project, an animal model is used to elucidate characteristics of the basic physiology that may be involved in ASD as well as the roles hormones may play in the disorder.

The peptide hormones arginine-vasopressin (AVP) and oxytocin (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. Early life stress, thought to play a role in the development on ASD, reduces cerebrospinal fluid (CSF) levels of OT in male rhesus monkeys and causes deficits in social development similar to ASD (Winslow, 2005). AVP pathways in the 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).

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

peptides 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/OT in ritualistic behaviors.

Social dysfunction in ASD suggests that there may be abnormalities in brain regions associated with normal social behavior. While there are several brain areas that could be involved, 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).

Lesion studies also implicate the amygdala in ASD pathology. Amygdala lesions cause deficits in social interactions, with amygdala-lesioned monkeys failing to respond to their social group either aggressively or peacefully (Kling &Cornell, 1971). Similarly, human subjects with amygdala lesions often exhibit ASD-like symptoms, such as impaired social interactions (Adolphs et al., 1994). Additionally, one of the only animal models of autism in use involves the ablation of the amygdala (Bachevalier, 1991; Prather, et al., 2001).

The amygdala is also implicated in the finding that approximately 30% of autistic patients develop epilepsy (Gillberg & Billstedt, 2000). The amygdala is a well known locus of temporal lobe epilepsy, and hyperexcitability in the amygdala, perhaps modulated by increased levels of AVP, could account for the increased fear and decreased social interactions seen in ASD. In addition, since I_h modulates excitability in

the LA and has been implicated in seizure activity, it provides an intriguing candidate mechanism to be investigated regarding the neurophysiology of ASD.

Taking into account the normal function of the amygdala, its dysfunction could allow for 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 fear learning, anxiety, as well as the behavioral response of the organism; therefore the reduced social interaction and increased fear and anxiety in ASD also suggest amygdala involvement (Moldin et al., 2006). Higher cell density has also been reported in the amygdalae of autistic individuals post mortem (Bauman & Kemper, 1994). Clearly the amygdala plays an important role in the regulation of normal social behavior, and 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.

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.

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). The basolateral complex includes the basolateral amygdala (BLA) and the lateral amygdala (LA) nuclei. Sensory information from the thalamus and cortex is relayed to the LA. The central amygdala (CeA) 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.

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. Fear conditioning is a process that pairs an unconditioned stimulus (US, foot shock) with a conditioned stimulus (CS, tone or light). After several CS-US pairings, the animal learns that the CS predicts the US, resulting in a central fear state in the presence of the CS alone. Magnitude of the fear state can be quantified by measuring autonomic nervous system activity such as heart rate or blood pressure. Fear responses can also be quantified by measuring freezing or fear potentiated startle. 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 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 an increased fear response and anxiety, or decreased social behavior, both of which are seen in 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.

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 the pyramidal cells, and show no spike frequency accomodation. Interneurons are generally utilized in local circuitry to maintain tonic inhibition of pyramidal cells (Rainnie, Asprodini, & Shinnick-Gallagher, 1991b; Sah et al., 2003). While the interneurons in the LA are activated by serotonin, thus inhibiting amygdala output, a small subset of interneurons present at the border of the BLA and LA, the intercalated nuclei are modulated by dopamine. At rest they inhibit pyramidal cells in the amygdala, and when hyperpolarized by DA, the amygdala becomes disinhibited (Marowsky, Yanagawa, Obata, & Vogt, 2005).

Long-Term Potentiation and Fear Conditioning

Long-term potentiation (LTP), a possible cellular mechanism of learning, was first studied in the hippocampus. LTP is a long term strengthening of particular synapses after repeated stimulation. The presence of Ca^{2+} is necessary for LTP to occur, often occurring through the stimulation of NMDA receptors. NMDA receptors are necessary for LTP to occur in the CA1 region of the hippocampus (Morris, Anderson, Baudry, 1986). In the amygdala, it has been found that NMDA-R antagonists block the acquisition but not the expression of learned fear responses. This suggests that NMDA receptors are necessary for acquisition of fear responses but that they are not necessary for the expression of the responses. It has also been found that fear conditioning potentiates thalamic inputs to the LA and strengthens glutamatergic synapses (McKernan & Shinnick-Gallagher, 1997; Rogan, Staubli, & LeDoux, 1997). Upregulation of GluR1 results in LTP, and down-regulation of NMDA-R results in long-term depression (LTD). Since both LTP and fear conditioning require NMDA, many studies suggest LTP may be the mechanism underlying fear conditioning.

Several changes in receptor function occur following fear conditioning in the amygdala. There is an overall long-lasting increase in synaptic efficacy in the medial geniculate nucleus (MGN)-LA pathway (McKernan & Shinnick-Gallagher, 1997); the excitatory post-synaptic currents (EPSCs) in the LA exhibit a lower threshold and larger amplitude in fear conditioned animals as compared to controls; and there is a presynaptic increase in transmitter release (McKernan & Shinnick-Gallagher, 1997).

Based on this evidence, normal amygdala function allows for the formation of fear learning as an adaptive trait to protect against encountering the same harmful stimuli.

Abnormal function of the amygdala may overly-sensitize fear and anxiety circuits, leading to the maladaptive behaviors seen in patients with ASD.

Fear conditioning studies conducted on subjects with ASD provide further 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.

Kindling and Hyperexcitability

Normal plasticity in the amygdala allows for fear learning and LTP as adaptive mechanisms. These functions involve strengthening synapses through up-regulation of 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).

Using kindling, a well-established model of temporal lobe epilepsy (Goddard, 1969), an initially sub-convulsive stimulation of a particular brain area is repeated daily, eventually leading to spontaneous seizures. Following the induction of "fully kindled" seizures, there are long-term changes in receptor function (Keele, 2005; Keele, Zinebi, Neugebauer, & Shinnick-Gallagher, 2000; Neugebauer, Keele, & Shinnick-Gallagher, 1997; Rainnie, Asprodini, & Shinnick-Gallagher, 1992). Cells become hyperexcitable,

so that lower stimulation intensity is needed to evoke burst firing than before eliciting seizures. It has been found that kindled amygdala neurons are more easily excited and they produce burst firing upon stimulation. In control neurons, increasing stimulus intensities produce proportional increases in EPSC amplitude until a single action potential is evoked. In kindled neurons, however, increasing stimulus intensity does not produce proportionally larger EPSCs, the EPSCs tend to stay very small until the stimulus evokes burst firing (Keele, Neugebauer, & Shinnick-Gallagher, 1999).

While excessive hyperexcitability of LA neurons may lead to temporal lobe epilepsy or seizures, sub-seizure hyperexcitability in LA neurons may result in be 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 violonce. Specifically, if the LA is hyperexcitable, even a small stimulus could evoke burst firing that would drive the HPA and cause autonomic effects such as increased heart rate and blood pressure, cortisol release, 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 effectively treat them.

The focus for this project is the investigation of 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 is known that AVP and OT modulate excitability in the CeA (Huber, 2005), but the ionic mechanism mediated by these peptides in the LA has 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) will be investigated. I_h is also known to modulate excitability in the LA, but the interaction of I_h and AVP/OT in the LA has yet to be investigated.

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 lateral amygdala (LA). 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 heart where it was first identified (DiFrancesco, 1993), 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 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 modulating retrograde propagation of activity in the soma (Magee, 1998; Poolos, Migliore, & Johnston, 2002; Luthi & McCormick, 1999).

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

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 the reduction of input resistance and temporal summation (Poolos, et al., 2002; Magee, 1998). 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).

Changes in I_h could take place either as a change in overall amplitude of the current, or by the shifting of the activation kinetics ($V_{1/2}$) of the current. An increase in the amplitude of I_h would be expected to increase action potential accommodation, thus decreasing the excitability of the cell. A decrease in amplitude would cause an increase in excitability. Regarding activation kinetics, if the activation curve shifts in the negative direction ($V_{1/2}$ becomes more hyperpolarized), it suggests that I_h requires more hyperpolarization to be induced and therefore excitability would be increased. If AVP or OT have the ability to modify these properties, 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.

Vasopressin and Oxytocin

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). Excitation by either AVP or OT in brainstem motoneurons and spinal cord

neurons 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-protein linked to second messenger systems (Raggenbass, 2001). The AVP receptors V1a (the most abundant in the CNS) and V1b as well as the OT receptor are coupled to phospholipase C-β (PLC) and their activation increases hydrolysis of membrane phospholipid resulting in the activation of PKC and increased intracellular calcium concentation. The AVP V2 receptor is positively coupled to adenylyl cyclase, increasing intracellular concentration of cAMP (Insel, et al. 1999; Raggenbass, 2001). Activation of V1a receptors in facial motoneurons 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 the CeA, some cross-reactivity has been found between AVP and OT, with AVP sometimes binding to OT receptors (Huber, et al. 2005). The mechanism of action of AVP and OT receptors in the lateral amygdala has not been investigated.

While AVP and OT are often grouped together, they each have 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). In contrast, OT decreases anxiety and stress, facilitates social behavior, maternal behavior, and mother/infant bonding (Young & Wang, 2004). OT also promotes extinction of conditioned avoidance behavior (Huber et al., 2005).

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 motane vole. Monogamous prairie voles exhibit not only strong pair bonding with their mate, but also 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 receptors in the forebrain than did the montane voles. The genetic difference of interest between these two species involves the Avpr1a gene, which contains information for the V1a receptor. The gene itself is homologous in prairie voles and montane voles, but it is preceded by a microsatellite that is expanded in prairie voles as compared to 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).

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

Summary and Significance

ASD is a disorder primarily of abnormal social functioning including decreased social interaction and a lack of language development. The peptide hormones AVP and OT have been strongly implicated in both ASD pathology and the modulation of many social behaviors. Amygdala dysfunction is also implicated in ASD, specifically the LA, which is very important in normal social behavior as well as learning and memory. Hyperexcitability in the amygdala leads to abnormal social behavior, such as fear and aggression, and AVP and OT generally cause excitatory responses in the CNS, though affecting different cell groups resulting in opposing effects. The effects of AVP and OT in the LA are still unknown, and could provide new information regarding the underlying mechanisms responsible for ASD.

This project investigates the cellular effects of AVP and OT in the lateral amygdala. It was hypothesized that AVP would increase excitability in the LA, possibly through modulation of I_h. OT was hypothesized to have an opposing effect, decreasing excitability in the LA. Modulation of excitability in the LA in response to AVP and OT would indicate a role for amygdala dysfunction in the pathology of ASD, and would provide information for the development of more advanced animal models, as well as providing information for novel treatments of the disorder.

CHAPTER TWO

Materials and Methods

Animals

Male Sprague-Dawley rats that have been bred in-house were used between day P17 and P30; weights ranged from 40-110g. Animals were group housed with their littermates and weaned on day P21. Animals were maintained on a 12-hour light/dark photoperiod cycle with food and water available ad libitum.

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 "blind" 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.

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. 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).

Drug Application

After initial control testing for accommodation and I/V relationship, drugs (either AVP or OT, 1μM) were superfused across the tissue slice for no less than 5 minutes, after which the same tests were repeated to establish differences between control and drug conditions. At the end of the experiment, the slice was washed with control aCSF for no less than 10 minutes and tested to show the reversibility of the drug effects.

When using the h-current protocol, the cell was first tested in control aCSF, then the slice was superfused with barium (1mM) and TTX (1μM) to block a contaminating inward-rectifying potassium current (K_{ir}) and tested again. The slice was then superfused with barium (1mM), TTX (1μM), and OT or AVP (1μM) and tested a third time. The slice was then washed in CSF containing barium (1mM) and TTX (1μM) and tested again for washout effects.

AVP, OT, and Ba^{2+} were obtained from SigmaAldrich; TTX was obtained from Tocris.

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 I/V relationships of individual cells. Boltzmann curves were fitted to I_h activation curves to determine half-activation ($V_{1/2}$) and slope.

CHAPTER THREE

Results

Current and voltage clamp recordings were performed on 55 pyramidal cells from the lateral amygdala (LA) nucleus with average input resistance of 97.6 ± 3.5 MΩ.

Effects of Vasopressin

Accommodation is Decreased in AVP

Figure 1 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 4 of 7 neurons, AVP decreased accommodation; AVP had no effect on the other three cells, which had experienced abnormal or damaging recording phenomena such as resealing and re-rupturing. In control ACSF (Fig 1A), 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 1B). 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. 1 D). The summary of the effect of AVP on all neurons that responded by decreased accommodation is shown in Figure 1E. 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).

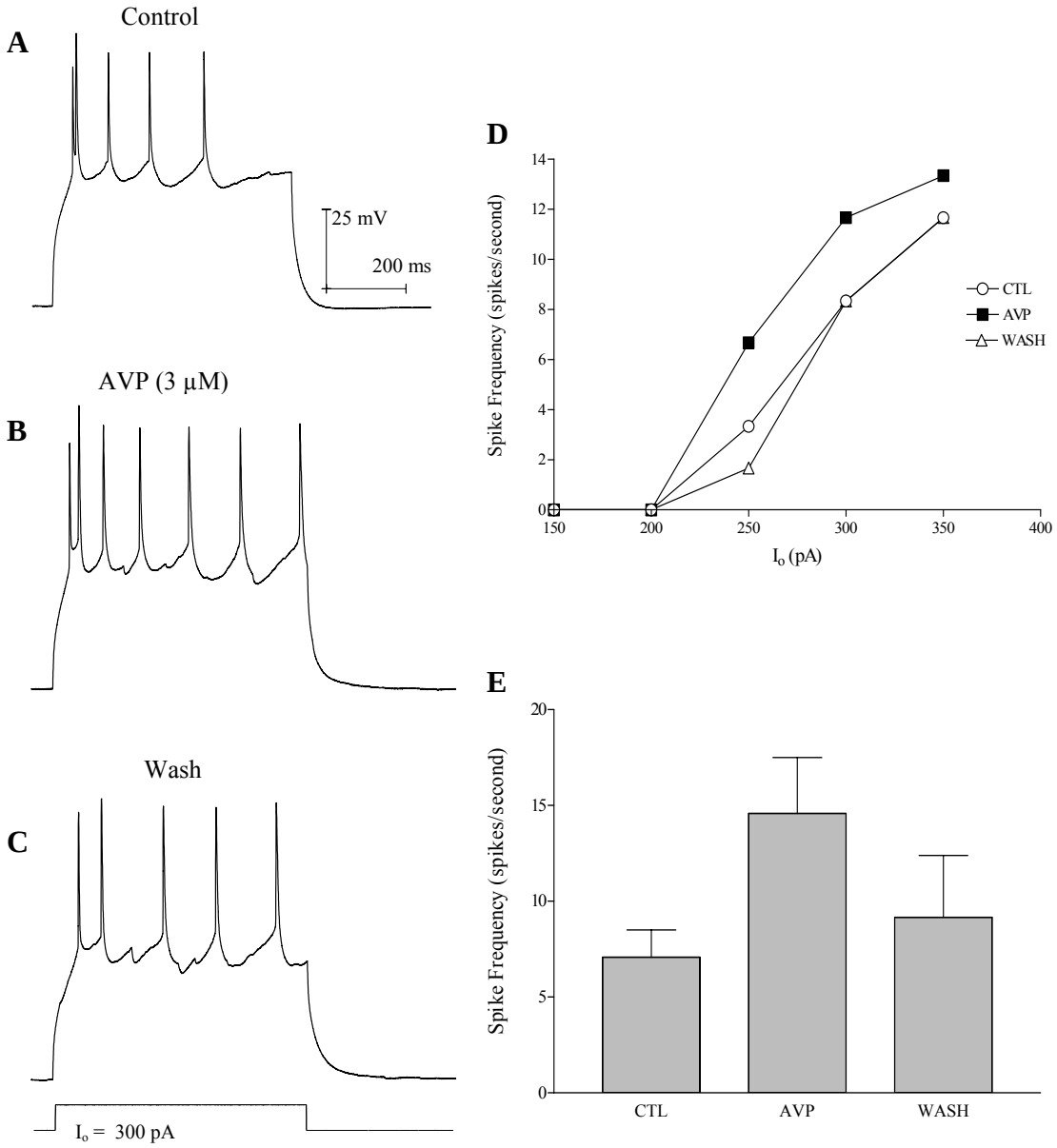

Figure 1. Spike accommodation decreases in AVP. A-C show current clamp recordings of membrane potential in a representative LA neuron in response to a 300 pA depolarizing current step. A. In control, the depolarizing current step reveals an accommodating neuron with spike frequency adaptation.B. In arginine-vasopressin (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, AVP (1 to 3µM), and wash (n=4). Scale in A is the same for B and C. All recordings performed at V_m= -60mV.

Hyperpolarization-Activated Current is Decreased in AVP

Previous studies 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 in the presence of Ba^{2+} (1mM) and tetrodotoxin (TTX, 1μM) to isolate I_h. The holding potential was -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 2A). 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. The hyperpolarization-activated current, I_h , was calculated as the difference between the instantaneous current and the steady state current ($I_h = I_i - I_{ss}$). 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 2B).

Current-voltage (I/V) relationships were constructed to examine the change in membrane conductance mediated by AVP. Figure 2C 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 2D) shows I_h for the same cell, AVP inhibits I_h amplitude. I_h amplitude upon hyperpolarization to -130 mV was reduced from -57.1 ± 17.5 pA in control to 49.5 ± 13.7 pA in AVP.

The voltage dependence of activation of I_h was analyzed by constructing activation curves for I_h in control and in the presence of AVP (n=4). The data was fit

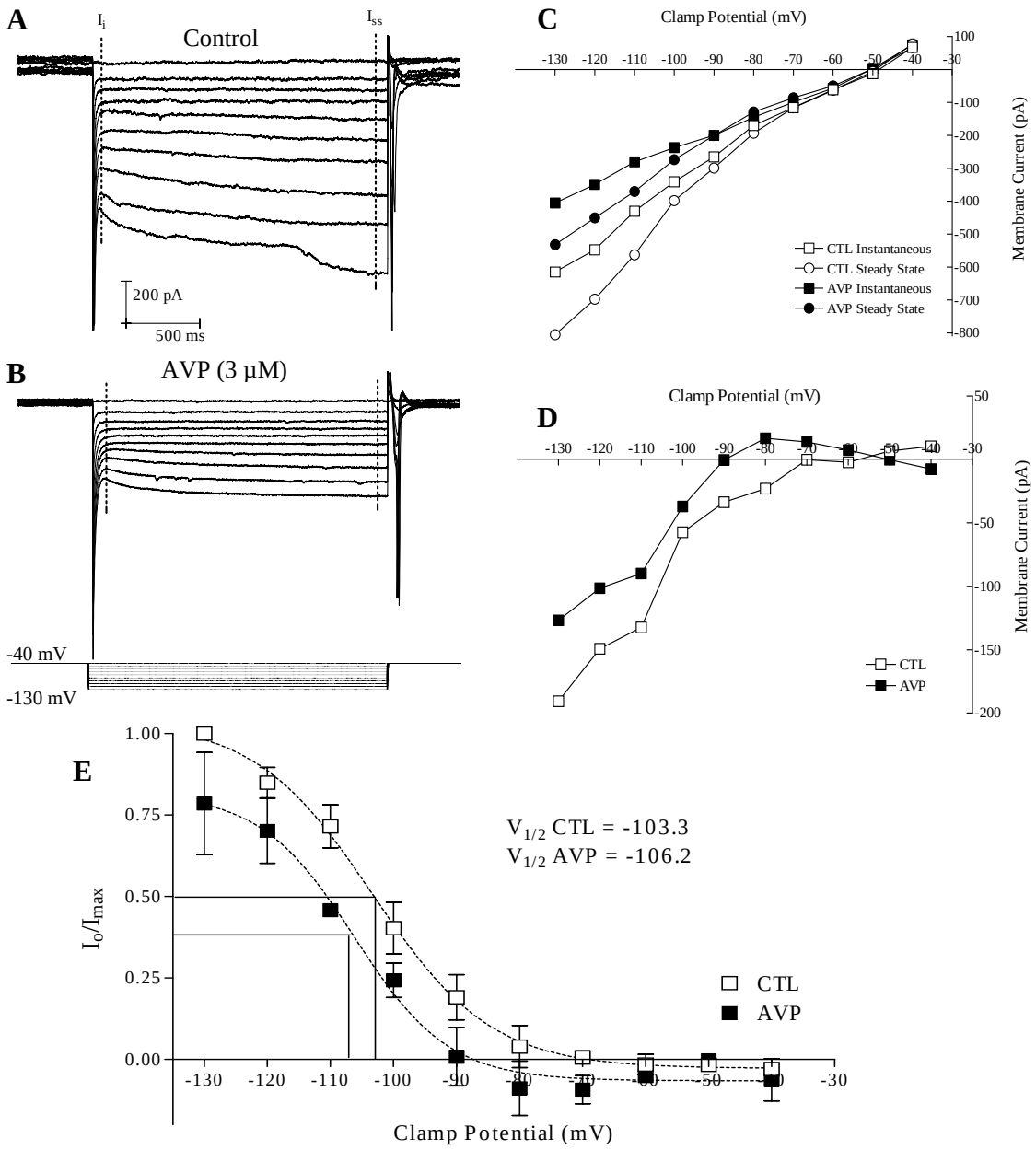

Figure 2. Arginine-vasopressin (AVP) inhibits the hyperpolarization-activated current (I_h) in LA neurons. Representative voltage clamp recordings (V_h=-40 mV in TTX, 1 µM and Ba^{++}, 1 mM) are shown in A (control) and B (AVP, 1 µM). Hyperpolarizing voltage steps (2s duration) elicit an instantaneous (ionic) current, I_i, followed by a slow inward rectification that reaches steady state (I_{ss}) by the end of the step. C. Current-voltage relationships of I_i (squares) and I_{ss} (circles) in control (CTL, open symbols) and AVP (3µM, filled symbols) from the neuron shown in A and B. The I/V relationship shows that AVP reduces both the instantaneous current and the inward current, I_h. D. The subtraction current/voltage relationship (I_i-I_{ss}) shows specific inhibition of I_h. E. Voltage-dependent activation curves for I_h in CTL and AVP (1 and 3 µM) (n=4). Data is fit with a Boltzmann curve, and $V_{1/2}$ values were obtained from the fitted data.

with a Boltzmann curve to determine the voltage of half-maximal activation ($V_{1/2}$) and slope. In control, $V_{1/2}$=-103.3 ± 2.0 mV, (slope = -9.4 ± 1.7). In AVP (1 or 3μM), $V_{1/2}$ was shifted in the hyperpolarizing direction to $V_{1/2}$=-106.2 ± mV (slope = -7.5 ±). This suggests AVP may act to decrease I_h by shifting the activation curve to hyperpolarized potentials.

Effects of Oxytocin

Accommodation is Increased in Oxytocin

The effect of oxytocin (OT) on action potential accommodation was examined using current clamp protocols identical to those described for AVP. In 4 of 8 neurons, OT increased accommodation; OT had no effect on the other four. Figure 3 shows a representative LA neuron where OT increased accommodation. In control ACSF (Fig 3A), 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) added to the ACSF, the 200 pA stimulus elicited no action potentials. Further depolarization by a 400 pA stimulus elicited only a single action potential (Fig 3B). The effect of OT on spike accommodation occurred over a range of input current intensities (Fig. 3C). The summary of the effect of OT on neurons that responded by increased accommodation is shown in Figure 3D. The firing frequency of LA neurons was decreased from 9 ± 5 spikes/s in control (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, the effect of OT on I_h amplitude was also investigated. Cells in the LA were

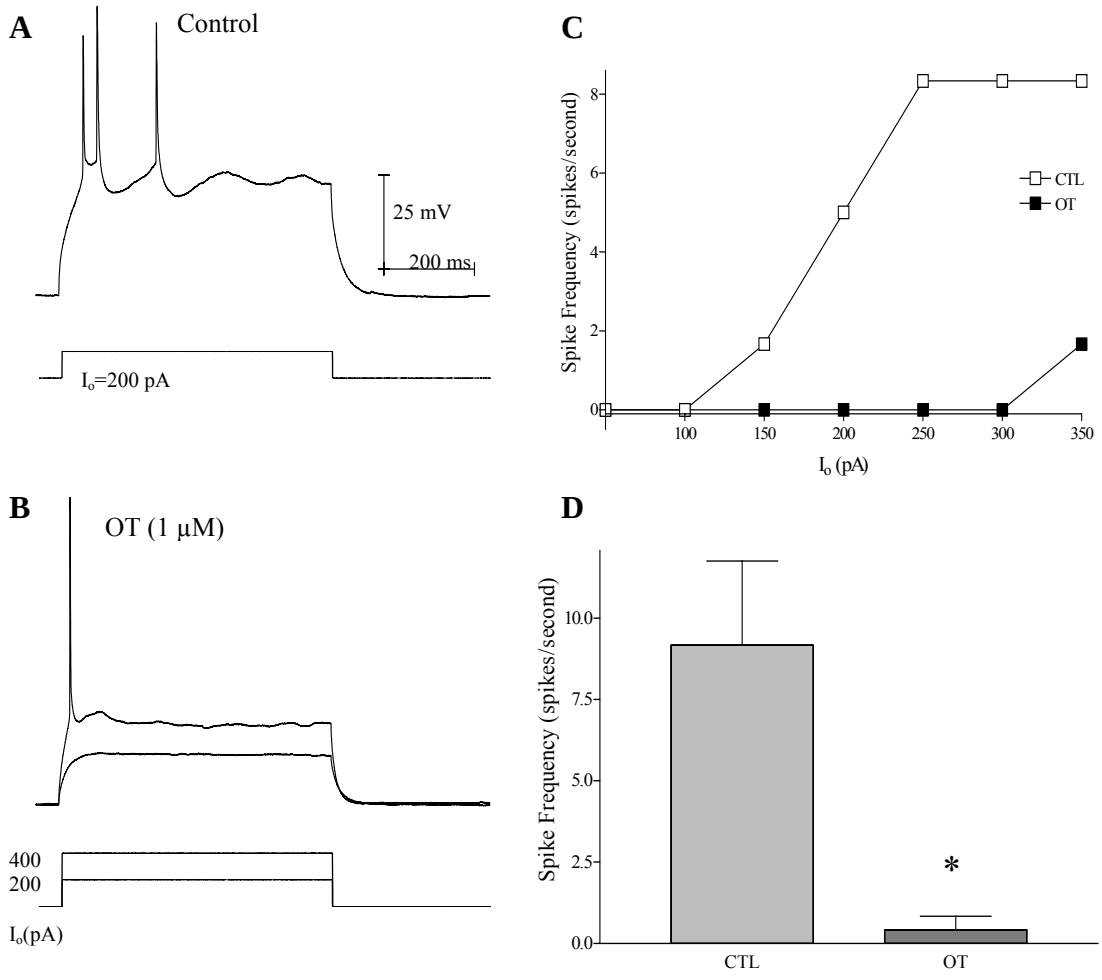

Figure 3. Spike accommodation increases in OT. A and B show current clamp recordings of membrane potential in a representative LA neuron in response to depolarizing current steps. A. In control, a 200 pA depolarizing current step elicits repetitive firing in a strongly accommodating neuron. B. In oxytocin, (OT, 1 μM) the same 200 pA depolarizing current step does not evoke action potentials, and at 400 pA evokes only a single action potential. 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 in CTL and OT (1 μM) (n=4). *, $p<0.05$.

voltage-clamped to a holding potential of -60 mV (V_h=-60 mV) and given 350 ms voltage steps from -40 to -115 mV. Hyperpolarization beyond -70 mV revealed a slowly inactivating inward sag that resembled hyperpolarization-activated current (I_h) (Fig. 4A). Following superfusion of OT (1 μM), I_h amplitude was tested again to determine effects of OT on I_h amplitude (Fig 4B).

Figure 4C 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 (I_i-I_{ss}) current (Fig 4D) shows I_h for the same cell, OT increases I_h amplitude. I_h was increased from -145.1 ± 89.6 pA to -225.8 ± 93.0 pA in OT (1 μM).

The voltage dependence of activation of I_h was analyzed by constructing activation curves for I_h in control and again in the presence of OT (n=4). The data was fit with a Boltzmann curve to determine the voltage of half-maximal activation ($V_{1/2}$) and slope. In control, $V_{1/2}$=-99.60 ± 2.4 mV, and slope = -6.868 ± 1.9. In OT (1 μM), $V_{1/2}$= -102.5 ± 12.3 mV, and slope = -8.525 ± 7.8.

LA Cells Unresponsive to OT

In some LA cells (n=4/9) OT (1 μM) had no effect on either accommodation properties or I_h activation. 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. Figure 5 shows a recording from a representative LA neuron that did not respond to the superfusion of OT. In control ACSF (Fig 5A), delivering a 250 pA depolarizing stimulus elicits 9 action potentials. In the same cell in the presence of OT (1μM) added to the CSF, 250 pA again elicits 9 action potentials with

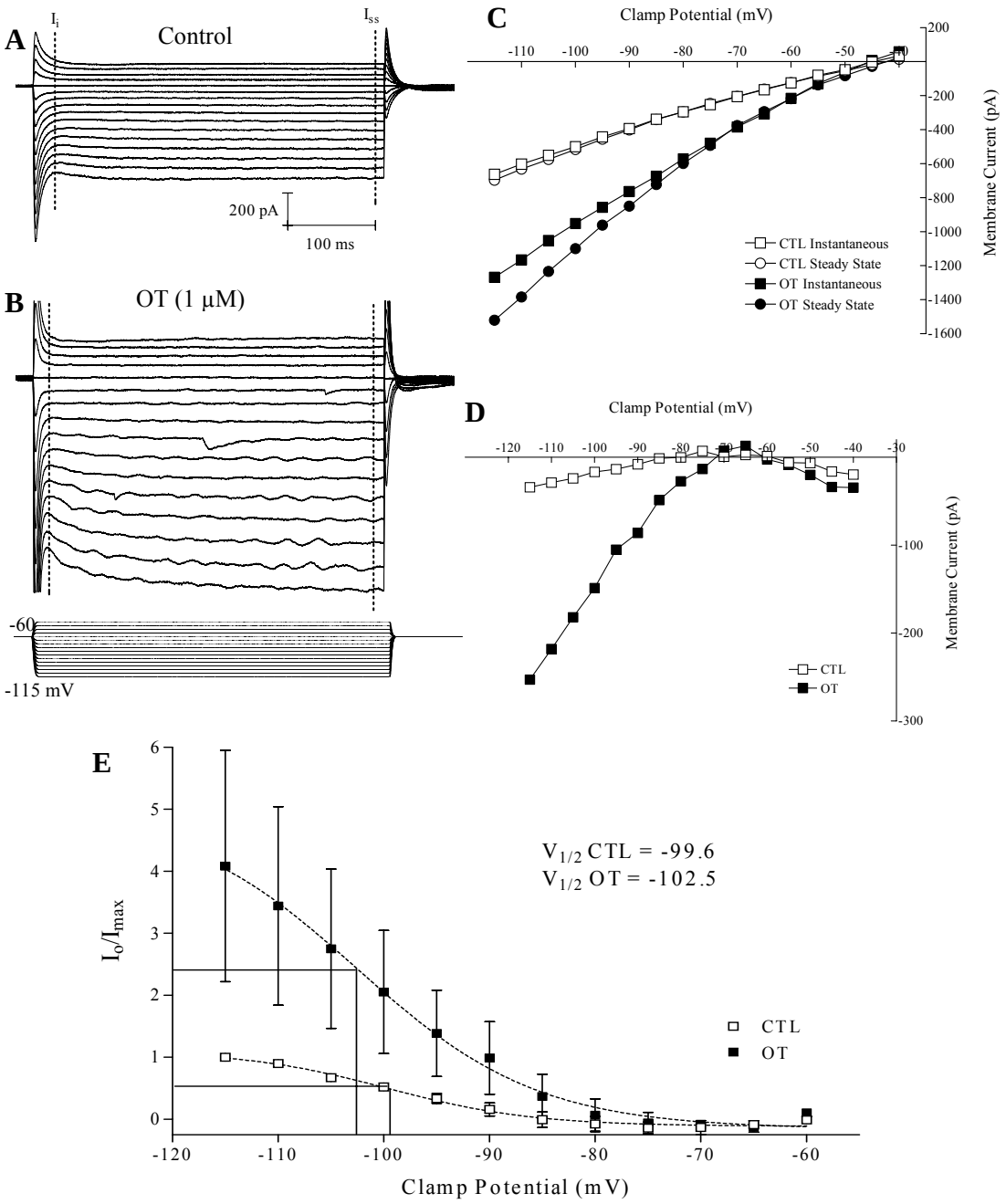

Figure 4. Oxytocin (OT) increases the hyperpolarization-activated current (I_h) in LA neurons. Representative voltage clamp recordings (V_h=-60 mV) are shown in A (control) and B (OT, 1µM). Voltage steps (350 ms duration) elicit an instantaneous (ionic) current, I_i, followed by a slow inward rectification (I_{ss}). C.Current-voltage relationships of I_i (squares) and I_{ss} (circles) in control (CTL, open symbols) and OT (1µM, filled symbols) from the neuron shown in A and B. The I/V relationship shows that OT increases both the instantaneous current and the inward current, I_h. D. The subtraction (I_i-I_{ss})/V relationship shows specific increase of I_h. E. I_h activation curve in control (CTL, open symbols) and OT (1 µM, filled symbols) (n=4). Data is fit with a Boltzmann curve, $V_{1/2}$ values were obtained from the fitted data.

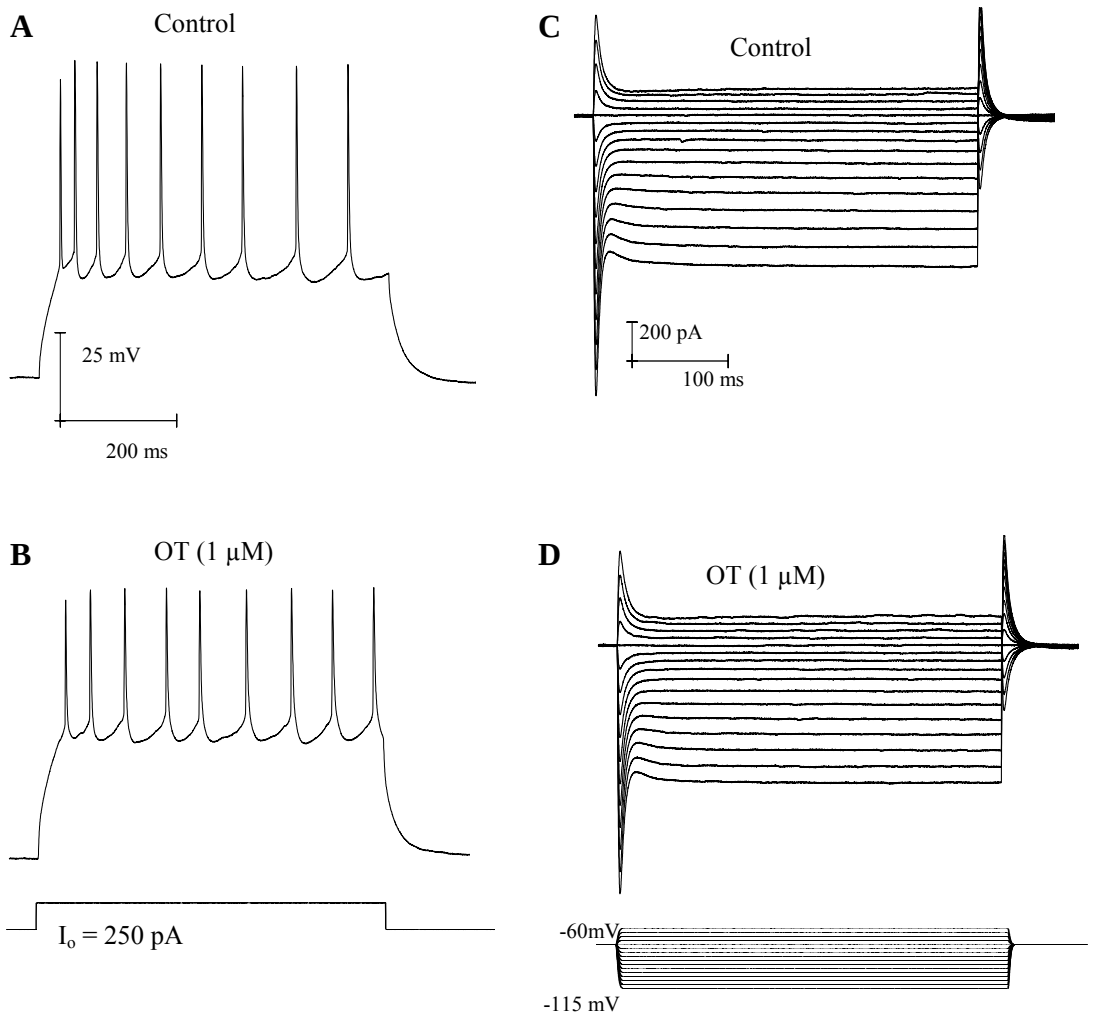

Figure 5. In 4 of 8 LA neurons, oxytocin (OT) had no effect on repetitive firing or I_h amplitude . A and B show current clamp recordings of membrane potential in a representative LA neuron in response to a 250 pA depolarizing current step. In control (A) and oxytocin,1 μM (B) the 250 pA depolarizing current step elicits similar repetitive firing patterns. Representative voltage clamp recordings (V_h=-60 mV) are shown in C (control) and D (OT, 1 μM). Voltage steps (350 ms duration) elicit an instantaneous (ionic) current, I_i, followed by a slow inward rectification that reaches steady state (I_{ss}) by the end of the step. Scale in A is the same in B. Scale in C is the same in D.

a similar repetitive firing pattern (Fig 5B). Similarly, in voltage clamp (V_h = -60mV), hyperpolarizing steps to -115 mV to evoke I_h were unchanged in the presence of OT (1 uM).

CHAPTER FOUR

Discussion

Vasopressin and oxytocin were both shown to modulate excitability in the lateral amygdala (LA). AVP (1 or 3 μM) decreased action potential accommodation in LA cells (n=4 of 7) and decreased hyperpolarization-activated current (I_h) as well as inducing a slight hyperpolarization of the activation curve for I_h, all of which contribute to increased excitability. OT (1μM) increased action potential accommodation in LA cells (n=4 of 9) and increased I_h amplitude, resulting in decreased LA excitability. These results indicate complementary functional roles for AVP and OT in the modulation of LA excitability.

Vasopressin has been shown to increase excitability in many areas of the CNS including the spinal cord, facial motoneurons, and hypoglossal motoneurons (Raggenbass et al., 1991; Raggenbass, 2000). 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, 1993). 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, et al., 1994). The results of the current study are similar in that AVP induces an increase in excitability, but the mechanism through which it appears to work—the modulation of I_h—has not been observed elsewhere. 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, 2006). 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 hyperpolarizing shift in the voltage-dependence of activation. A hyperpolarized voltage dependence reflects that more hyperpolarization is needed to induce I_h. In conditions of high AVP concentration, excitability is increased. It follows that the AVP-induced decrease in accommodation seen in the LA would be associated with decreased I_h. Together, these data suggest that AVP increases the excitability of LA neurons by decreasing current amplitude via shifting the voltage-dependence of I_h to more hyperpolarized potentials.

Oxytocin 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. In the CeA, there are two reported classes of cells, one of which is inhibited by OT, the other is excited by OT. Its overall effect is inhibitory, as the cells it excites are GABAergic and inhibit other cells in the CeA. Despite often increasing cellular excitability, as in the CeL, OT often works in opposition to AVP, inhibiting cells that are excited by AVP through excitation of different groups of cells. The results of this project are similar to previous studies 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. AVP increased excitability in LA cells through decreasing action potential accommodation and decreasing I_h, and OT decreased excitability through increasing action potential accommodation (decreasing spike frequency) and increasing I_h amplitude.

The decreased excitability in the presence of OT may also be the result of the effects of OT on I_h in the LA. An increase in I_h would be expected to increase cell accommodation, resulting in decreased excitability, congruent with results in the current study. These opposing roles for AVP and OT in the LA suggest that they may be important in modulating excitability.

It was also found that some cells in the LA were insensitive to the presence of OT. In 4 of 8 cells recorded in OT, there was no change in accommodation or I_h amplitude. 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). The cells that exhibited increased firing rates after the application of OT in the CeL were shown to be GABAergic and project to the medial division of the CeA (CeM), thus causing inhibition of cells in the CeM that are excited by AVP. Again, this demonstrates the complementary effects of AVP and OT. As in the CeA, the effects of AVP and OT in the LA are also complementary to each other, although the effects in the LA are likely

dependent on direct modulation of cellular excitability rather than through the mediation of inhibitory circuitry.

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, 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. Therefore, controlling excitability 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 may be important in ASD.

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