ELECTROPHYSIOLOGICAL EFFECTS
OF ANGIOTENSIN II ON HYPOTHALAMIC
PARAVENTRICULAR NUCLEUS NEURONS OF THE RAT

by

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ABSTRACT

The role of the hypothalamic paraventricular nucleus (PVN) in cardiovascular and neuroendocrine regulation has been well documented. Much remains unknown however about the integration of synaptic signals within this nucleus and the neuronal subtypes and chemical messengers governing these processes. Angiotensin II (ANG) has been demonstrated to act as a neurotransmitter in PVN where it exerts considerable influence on neuronal excitability. The studies within this thesis were undertaken to delineate the actions of ANG on the membrane properties of PVN neurons and its effect on synaptic transmission within this nucleus.

We report that ANG activates a nitric oxide mediated negative feedback loop in the PVN. The magnitude of the depolarizing response to ANG appears to be dependant on this GABAergic inhibitory input demonstrating there exists within PVN an intrinsic negative feedback loop which modulates neuronal excitability in response to peptidergic excitation. We also demonstrate that the depolarizing response to ANG in magnocellular neurons is in part dependent upon increases in glutamatergic excitatory synaptic input. These data in combination highlight the multiple levels of synaptic integration controlling the output of magnocellular neurons in PVN.

PVN also contains significant populations of neurosecretory parvocellular neurons which exercise considerable influence over the adenohypophysis and therefore neuroendocrine regulation. ANG caused an AT_{1} receptor mediated
depolarization of these neurosecretory neurons. Voltage-clamp analysis revealed that ANG activated a non-selective cationic current and reduced a sustained potassium current characteristic of $I_K$. These studies identify multiple post-synaptic modulatory sites through which ANG can influence the excitability of neurosecretory parvocellular PVN neurons.

The findings in this thesis provide the framework for a cellular model of action of ANG within PVN to regulate the activity of this nucleus not only through direct cellular mediated ion channel interactions but also through modulation of synaptic input within the magnocellular system of PVN.
ACKNOWLEDGEMENTS

It took me ten years to finish my thesis. It was an unbelievable journey to finish it, and not without its ups and downs. Through all of this AI, you supported me unconditionally. Thank you for being my supervisor and my friend. I look forward to many great discussions on the golf course.

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This thesis got finished because my wife Tara pestered me about it relentlessly. Add this to the long list of reasons why she is the most amazing thing that has ever happened to me. Tara, thank you for saying yes even if you had to think about it for a moment. Thank you for introducing me to the Sacks and the Malcolms. Thank you for rescuing Sophia, twice. Thank you for making me the happiest guy I know. I love you and always will.
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    - Neuronal Subtypes: Magnocellular Neurons
    - Neuronal Subtypes: Parvocellular Neurons
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    - Information Integration in PVN: Intraneural Circuitry
    - Excitatory Neurotransmission in PVN
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  - Angiotensinergic Pathways in the CNS
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<td>AA</td>
<td>Arachidonic acid</td>
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<tr>
<td>ADM</td>
<td>Adrenomedullin</td>
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<tr>
<td>ANG</td>
<td>Angiotensin II</td>
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<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
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<td>AVP</td>
<td>Arginine vasopressin</td>
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<td>ACh</td>
<td>Acetylcholine</td>
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<td>AMPA</td>
<td>DL-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
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<tr>
<td>ARBs</td>
<td>Angiotensin II receptor blockers</td>
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<td>AP</td>
<td>Area Postrema</td>
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<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
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<tr>
<td>AIF</td>
<td>Angiotensin immunoreactive fibers</td>
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<tr>
<td>AT1,2</td>
<td>Angiotensin II receptors 1,2</td>
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<tr>
<td>BMI</td>
<td>Bicuculline methiodide</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>CHF</td>
<td>Congestive heart failure</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CVO</td>
<td>Circumventricular organ</td>
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<td>CRH</td>
<td>Corticotropic hormone</td>
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<tr>
<td>Dil</td>
<td>1,1-dioctadecyl-3,3',3',3'-methylindocarbocyanine perchlorate</td>
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<td>DMNV</td>
<td>Dorsal motor nucleus of the vagus</td>
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<td>EPSCs</td>
<td>Excitatory post-synaptic currents</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>EPSPs</td>
<td>Excitatory post-synaptic potentials</td>
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<tr>
<td>Erev</td>
<td>Reversal potential</td>
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<tr>
<td>GABA</td>
<td>(\gamma)-aminobutyric acid</td>
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<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic pituitary axis</td>
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<tr>
<td>(I_A)</td>
<td>Transient outward potassium current</td>
</tr>
<tr>
<td>(I_{Ca})</td>
<td>Calcium current</td>
</tr>
<tr>
<td>(I_K)</td>
<td>Delayed rectifier potassium current</td>
</tr>
<tr>
<td>(I_{KCa})</td>
<td>Calcium activated potassium current</td>
</tr>
<tr>
<td>(I_H)</td>
<td>Hyperpolarization activated cationic current</td>
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<tr>
<td>IPSCs</td>
<td>Inhibitory post-synaptic currents</td>
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<tr>
<td>IPSPs</td>
<td>Inhibitory post-synaptic potentials</td>
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<tr>
<td>ICV</td>
<td>Intracerebroventricular</td>
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<tr>
<td>KA</td>
<td>Kyurenic acid</td>
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<tr>
<td>(L)-NAME</td>
<td>(N^\omega)-nitro-L-arginine methylester</td>
</tr>
<tr>
<td>LTS</td>
<td>Low threshold spike</td>
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<tr>
<td>MPO</td>
<td>Medial preoptic area</td>
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<td>ME</td>
<td>Median eminence</td>
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<td>NSCC</td>
<td>Non selective cationic conductance</td>
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<tr>
<td>NA</td>
<td>Noradrenaline</td>
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<tr>
<td>NTS</td>
<td>Nucleus tractus solitarius</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate hydrogenase</td>
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<td>NMDA</td>
<td>(N)-methyl-D-aspartate</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
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<tr>
<td>OX-A</td>
<td>Orexin-A</td>
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<tr>
<td>OXY</td>
<td>Oxytocin</td>
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<tr>
<td>PVN</td>
<td>Paraventricular nucleus of the hypothalamus</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphotidyl inositol</td>
</tr>
<tr>
<td>RNSA</td>
<td>Renal sympathetic nerve activity</td>
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<tr>
<td>RMP</td>
<td>Resting membrane potential</td>
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<tr>
<td>RVLM</td>
<td>Rostroventrolateral medulla</td>
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<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneously hypertensive rat</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SON</td>
<td>Supraoptic nucleus</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>SFO</td>
<td>Subfornical organ</td>
</tr>
<tr>
<td>7-NI</td>
<td>7-Nitroindazole</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>TRH</td>
<td>Thyrotropin releasing hormone</td>
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Chapter 1: GENERAL INTRODUCTION
Nature constantly bombards us with a wide variety of conditions that each organism must successfully endure to insure its continued existence. The ability to maintain a constant internal milieu in the face of such unpredictable stimuli is critical to the survival of all organisms. Consequently, in order to meet the demands of a constantly changing environment, a myriad of biochemical, anatomical, and physiological systems have evolved to preserve homeostasis. The central nervous system (CNS) has traditionally been considered the most influential site in the regulation of homeostasis. This remarkable adaptive capacity of living organisms is governed largely by the hypothalamus which contains within its boundaries the majority of the important forebrain autonomic control centers (Swanson & Sawchenko, 1983; Swanson & Sawchenko, 1980). Paraventricular nucleus (PVN) in particular has been demonstrated to play important roles in regulation of the cardiovascular system, body-fluid homeostasis, immune function, and neuroendocrine control. The neuronal pathways and electrophysiological processes involved in PVN mediated control of autonomic function have been extensively studied (Swanson & Sawchenko, 1983; Swanson & Sawchenko, 1980). As a result, a number of neuromodulators, including peptidergic hormones, classical neurotransmitters, gases, and steroids have been found to exert a profound influence on PVN and accordingly its regulation of the periphery (Follwell & Ferguson, 2002a; Follwell & Ferguson, 2002b; Hermes et al., 2000; Daftary et al., 1998a; Li & Ferguson, 1996). Conventional wisdom suggests that a failure of peripheral homeostatic
mechanisms is responsible for many pathophysiological conditions including chronic hypertension, congestive heart failure, and endotoxemia (Coote, 2005; Patel & Zhang, 1996). Not surprisingly, more recent evidence has implicated breakdowns in communication between PVN neurons and neighboring nuclei in a number of these diseases (Coote, 2005).

PARAVENTRICULAR NUCLEUS

PVN is one of several critical nuclei involved in autonomic and neuroendocrine regulation (Swanson & Sawchenko, 1980). PVN is a heterogeneous nucleus comprised of magnocellular and parvocellular neurons. Magnocellular neurons are known to synthesize and secrete oxytocin (OXY) or vasopressin (AVP) into the circulation from terminals in the posterior pituitary and therefore exert considerable influence over blood pressure and reproductive functions (Swanson & Sawchenko, 1980). Parvocellular neurons on the other hand regulate anterior pituitary function via projections to the median eminence and also send projections to autonomic nuclei in the medulla, brainstem, and spinal cord (Swanson & Sawchenko, 1980). PVN’s heterogeneity and unique position within the hypothalamus allow this nucleus to act as an integration and modulation center exerting substantial power in the management of homeostasis.

AFFERENT PROJECTIONS

The primary role of afferent projections to PVN is to provide continuous monitoring of both the periphery and CNS in order to endow PVN with ‘insight’ as to the condition of the whole organism. Autoradiographic studies have documented projections to PVN from throughout the hypothalamus, including the
preoptic area, the ventromedial nucleus, the anterior and lateral hypothalamic area and the suprachiasmatic nucleus (SCN) (Swanson & Sawchenko, 1983; Swanson & Sawchenko, 1980). PVN also receives projections from the circumventricular organs (CVOs) which have been observed to influence PVN neurons projecting to the posterior pituitary and dorsal medulla (Ferguson et al., 1984a; Ferguson et al., 1984c). Afferent projections to PVN also include noradrenergic and adrenergic fibers from the brainstem. Indeed, PVN has been shown to contain one of the most dense catecholaminergic terminal fields in the brain. The A1 cell group of the ventral medulla, the A2 cell group of the nucleus tractus solitarius (NTS), and the A6 cell group of the locus coeruleus provide the majority of this catecholaminergic input to PVN (Swanson & Sawchenko, 1980). PVN also receives projections from the telencephalon (Swanson & Sawchenko, 1980), although the functions of these fibers are less well understood.

**EFFERENT PROJECTIONS**

Efferent projections from PVN have been known for some time largely as a result of anatomical studies completed in hypophysectomized animals. Four primary areas that receive efferent projections from PVN have been identified: (1) posterior pituitary (predominantly magnocellular neurons although a small population of parvocellular neurons project here), (2) median eminence (ME), and (3) autonomic nuclei in the medulla and, (4) interomediolateral cell column of the spinal cord (Swanson & Sawchenko, 1980; Saper et al., 1976).
NEURONAL SUBTYPES: MAGNOCELLULAR NEURONS

PVN consists of eight loosely demarcated subdivisions. AVP and OXY are synthesized as prohormones in the cell bodies of magnocellular neurons and are then processed during axonal transport. The cleavage product is a peptide known as neurophysin which is unique for AVP and OXY and can therefore be used as a marker to identify neurosecretory cells in PVN. Three of the eight anatomical subdivisions are known to consist of OXY and AVP magnocellular neurosecretory cells (Swanson & Sawchenko, 1983). These regions consist largely of bipolar and multipolar neurons with a few dendrites largely confined within the boundaries of the nucleus. Typically, these zones are divided anatomically and have been denoted the anterior, medial and posterior regions. It is the posterior subdivisions that most investigators refer to as the magnocellular region of PVN which contains neurosecretory cells that project to the neurohypophysis and it is this region where neurophysin-immunoreactive fibers are concentrated (Liposits, 1993; Hoffman et al., 1991).

NEURONAL SUBTYPES: PARVOCELLULAR NEURONS

The remaining five subdivisions of PVN are composed of smaller parvocellular neurons that morphologically resemble their magnocellular counterparts with the exception of their smaller size (Kiss et al., 1991; Swanson & Sawchenko, 1983). Interestingly, the observation that axon collaterals of some parvocellular neurons ramify locally and appear to contact dendrites of neighboring magnocellular and parvocellular neurons provided some of the
original inspiration for the hypothesis that integration may occur within the PVN (Renaud, 1976). Functionally, parvocellular PVN consists of two major output pathways. Those neurons which project either to the external lamina of the ME and regulate adenohypophyseal hormone release and those projecting caudally to the medulla (including NTS, dorsal motor nucleus of the vagus [DMNV], and rostroventrolateral medulla [RVLM]) and/or spinal cord which have implications in blood pressure and fluid-electrolyte regulation (Swanson & Sawchenko, 1983; Swanson & Sawchenko, 1980; Swanson & Kuypers, 1980).

NEURONAL SUBTYPES: INTERNEURONS

Historically a description of PVN typically consisted of detailed accounts of magnocellular and parvocellular neurons whose roles have been well documented (Swanson & Sawchenko, 1983; Swanson & Sawchenko, 1980). Scarce reference however is made to the plausibility that additional subtypes of neurons may exist within this nucleus. Recent evidence however suggests that within PVN there also exists a large population of interneurons which may play profound roles in the integration of information within this nucleus and thus exercise significant influence on its output (Daftary et al., 1998b; Elhawary et al., 1995).

Cajal documented morphological evidence for axon collaterals in PVN almost a century ago (Renaud & Bourque, 1991). This discovery laid the framework for the hypothesis that not only does the neurohypophysial pathway act as a predominant output for PVN but that this nucleus was potentially endowed with the capacity to modulate this output via recurrent
excitation/inhibition in order to fine-tune subsequent signals. The advent of intracellular recordings permitted the observation of short latency IPSPs following electrical stimulation of the pituitary stalk (Kandel, 1964). Similar observations were subsequently made in the parvocellular tuberoinfundibular system (Renaud, 1976). These illustrations lead to two distinct hypotheses: (1) that axon collaterals from neurosecretory neurons synapsed directly on the neuron of origin to produce a recurrent inhibitory feedback pathway (Renaud & Bourque, 1991; Renaud, 1976) or (2) that a similar pathway was activated indirectly through trans-synaptic activation of a local interneuron (Renaud, 1976). Recurrent facilitation was subsequently discovered in both the neurohypophysial (Koizumi et al., 1973) and tuberoinfundibular pathways (Sawaki & Yagi, 1976) and the notion that interneurons existed within PVN was firmly established.

Kiss et al. (Kiss et al., 1983a; Kiss et al., 1983b) described the total number and numerical ratio of extrinsic and intrinsic (local) innervations of magnocellular neurons in PVN and determined that over 40% of the synaptic connections within this area of the nucleus were intrinsic. This data suggests a high degree of local integrative control over neuronal activity exists within PVN. Ferguson et al. (Ferguson et al., 1984b) later observed that a large population of PVN neurons that were excited via subfornical organ (SFO) stimulation had no identifiable projections. The authors were unable to account for this observation but speculated the existence of interneurons within PVN as a potential explanation (Ferguson et al., 1984b). More recently, studies using radiolabeled aspartate as a neuronal tracer of glutamatergic interneurons provided convincing
data that: (1) glutamatergic interneurons exist within PVN and (2) glutamatergic fibers that project to PVN are scattered throughout other hypothalamic nuclei and the telencephalon (Csaki et al., 2000).

Interestingly, several groups (see sections on neurotransmission in PVN) have also documented/suggested the presence of interneurons in the perifornical area surrounding PVN, which are known to provide substantial excitatory and inhibitory input into this nucleus (Daftary et al., 1998b; Tasker & Dudek, 1993; Swanson & Sawchenko, 1980). The existence of interneurons within and around PVN was thus established; their role however in the integrative physiology which PVN governs is only now beginning to be addressed.

**PVN ELECTROPHYSIOLOGY**

The advent of the whole cell patch clamp ushered in an era of electrophysiological profiling. Immunohistochemical identification of neurons within PVN in combination with intensive classification of their respective electrical properties permitted neurophysiologists the potential to study separate subpopulations of neurons within this heterogeneous nucleus.

Tasker, Dudek, and Hoffman provided much of the early work classifying PVN neurons based on their electrical fingerprints and introduced the concept of the Type I and Type II neurons (Hoffman et al., 1991; Tasker & Dudek, 1991). Type I neurons (putative magnocellular) were shown to be relatively large cells that were immunoreactive for neurophysin and are situated near similarly stained neurophysin-positive neurons (Hoffman et al., 1991). These cells showed linear I-V relations, a prominent transient outward rectification generated by an A-type
potassium current (characteristically observed as a delayed return to baseline following a hyperpolarizing current pulse, which is required to de-inactivate the transient potassium current), and a delay to first spike during a depolarizing current pulse from a hyperpolarized potential (Tasker & Dudek, 1991). In addition these cells express little or no T-type Ca\(^{2+}\) currents characteristic of parvocellular neurons and generate spike trains with a delayed onset and little spike-frequency adaptation (Tasker & Dudek, 1991).

Type II neurons (putative parvocellular) are not neurophysin-positive and are located in the parvocellular regions of PVN (Hoffman et al., 1991). These cells demonstrate a variable but persistent low threshold depolarization (typically generating one or two action potentials) following a hyperpolarizing current pulse consistent with the activation of a low threshold calcium current. They do express a transient potassium current (\(I_K\)) although its magnitude is significantly smaller than a Type I neuron and typically show linear I-V relations until approximately -90mV with inward rectification appearing at more negative potentials. The identity of the conductance responsible for this phenomenon has not been identified although it is presumed to be a hyperpolarization activated cationic current (\(I_H\)) (Pape, 1996). Type II neurons fire repetitive spikes which show marked adaptation and no delayed onset during a prolonged depolarizing current pulse and bursting is rarely observed in Type II neurons (Tasker & Dudek, 1991).

While the characteristics used to describe magnocellular neurons have been updated with respect to their full complement of ion channels, subtle morphological characteristics, changes during lactation, hemorrhage and other
physiological events, their electrophysiological identification has remain virtually unchanged. Considerable debate however has emerged concerning the characteristics of the parvocellular subdivisions, specifically between those cells projecting to the median eminence (neurosecretory) and those projecting to pre-autonomic areas (non-neurosecretory). Stern characterized the cellular properties of pre-autonomic neurons in PVN by combining \textit{in vivo} retrograde tracing techniques with \textit{in vitro} current and voltage clamp recordings (Stern, 2001). Following 1,1-dioctadecyl-3,3,3',3'-methylinodocarbocyanine perchlorate (Dil) injection into either the NTS or DMNV, areas known to receive projections from PVN, retrogradely labeled neurons in PVN were found to express low threshold spikes and inwardly rectifying I-V consistent with traditional description of Type II neurons.

Consequently, Luther \textit{et al.} attempted to determine if electrophysiological differences existed between neurosecretory neurons and non-neurosecretory parvocellular neurons (Luther \textit{et al.}, 2002). Utilizing IV injections of the retrograde tracer flouro-gold they recorded from putative neurosecretory neurons, which were labeled, and also from unlabelled putative non-neurosecretory cells. Neurosecretory neurons were without prominent low threshold spikes (LTS) and T-type calcium current, while non-neurosecretory neurons were undistinguishable from traditional Type II parvocellular neurons in that they generated a prominent low threshold spike and robust T-type calcium current.
INFORMATION INTEGRATION IN PVN: INTRANUCLEAR CIRCUITRY

Morphological studies of PVN neurons have demonstrated that both magnocellular and parvocellular neurons are replete with synaptic connections. A substantial number of these connections are the result of axo-dendritic contacts between PVN and its numerous afferent projections. There remains evidence however, that many PVN dendrites remain confined within the nucleus raising the possibility that intranuclear connections may exist. The observation of interneurons within and around PVN has led to many studies directed toward examination of the role or roles of these intranuclear connections in controlling autonomic and neuroendocrine outputs controlled by PVN neurons.

EXCITATORY NEUROTRANSMISSION IN PVN

A discussion of PVN would not be complete without consideration of the messengers which modulate the activity of PVN neurons and as a consequence exert considerable control over autonomic and neuroendocrine regulation. The abundance of neurotransmitters and neuromodulators in the hypothalamus has proved to be fertile ground for exploration. Neurotransmitters identified in the hypothalamus include glutamate, γ-aminobutyric acid (GABA), glycine, noradrenaline (NA), opioids, serotonin, dopamine, and acetylcholine (ACh) (Swanson & Sawchenko, 1983). Excitatory neurotransmission in the hypothalamus has been characterized and glutamate has emerged as the predominant fast excitatory neurotransmitter. This observation has lead to an
intensive investigation into the functional role of glutamate in PVN in order to
decipher its role mechanistically in neuroendocrine regulation.

Anatomically, PVN has been demonstrated to contain some of the highest
concentrations of glutamate in the hypothalamus (Palkovits et al., 1986). Van den
Pol et al. (van den Pol et al., 1990) demonstrated large quantities of glutamate in
boutons making synaptic contact with PVN neurons. The source of this glutamate
is largely contained within glutamatergic inputs to this nucleus although the
magnocellular neurons themselves have been shown to contain a labile pool
(Meeker et al., 1989). Intranuclear connections represent a significant proportion
of the estimated afferent projections within PVN and more than 40% of the
synaptic terminals in PVN are intranuclear in origin (Kiss et al., 1983a;Kiss et al.,
1983b). Within PVN, radiolabeled neurons show a heterogeneous distribution
and are observed throughout the magnocellular and parvocellular subdivisions.
Extrinsic to PVN, glutamatergic inputs to this nucleus arise from throughout the
telencephalon and hypothalamus (Elhawary et al., 1995).

PVN is endowed with a high concentration of metabotropic and ionotropic
glutamate receptors. Ionotropic glutamate receptors, cation permeable ion
channels which are divided into N-methyl-D aspartate (NMDA), kainate, and DL-
$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) subgroups
based on their affinity for selective agonists have all been found in PVN. Herman
et al. (Herman et al., 2000) using in situ hybridization analysis of ionotropic
glutamate receptor subunit expression in PVN have suggested that
magnocellular neurons preferentially express NMDA subunits, pre-autonomic
parvocellular neurons express AMPA subunits, and neurosecretory parvocellular neurons express kainate subunits although the significance of this finding remains unknown. Metabotropic glutamate receptors which are coupled to G proteins and modulate a variety of intracellular second messengers have also been identified in PVN (Brann & Mahesh, 1997; van den Pol et al., 1994; Printz & Healy, 1983).

Wuarin and Dudek (Wuarin & Dudek, 1991) demonstrated electrophysiologically that glutamate is responsible for the majority of fast excitatory input to both magnocellular and parvocellular neurons in PVN and that AMPA, kainate, and NMDA play a shared role in mediating this excitation. In order to determine the functional effects of glutamate within PVN, early reports evaluated the effects of iontophoretic or bath applied glutamate agonists on magnocellular neurons and reported a rapid increase in excitability (Haller & Wakerley, 1980; Bioulac et al., 1978). Subsequent demonstrations included the observation that glutamate evoked depolarizations in magnocellular neurons that are rapid in onset and termination and are accompanied by a marked increase in membrane conductance (Bourque & Renaud, 1983). Tasker and Dudek (Tasker & Dudek, 1993) demonstrated that glutamate microapplication caused a tonic depolarization and increase in action potential frequency in PVN slices. Perhaps the most recent evidence comes from Bains and Ferguson (Bains & Ferguson, 1998; Bains & Ferguson, 1997b) who showed that NMDA agonists had potent excitatory actions on both parvocellular and magnocellular PVN neurons and that activation of NMDA receptors located on the dendrites of PVN neurons triggered
Ca\textsuperscript{2+} spikes. Although the precise function of these spikes is unclear the possibility that dendritically initiated spikes may serve as a local signal to trigger neuropeptide release in PVN is an intriguing theory. In accordance with this theory Hu and Bourque (Hu & Bourque, 1992; Hu & Bourque, 1991) showed that NMDA receptor activation mediates rhythmic bursting activity in rat supraoptic nucleus (SON) neurons \textit{in vitro}, a phenomena critical in the regulation of neuropeptide release.

It is clear from the previous section that a significant number of synaptic connections within PVN utilize glutamate as a neurotransmitter and furthermore that this neurotransmitter exerts a profound excitatory effect on neurons within PVN. As a consequence, the relationship between glutamate and those pathways that PVN exploits in order to influence neuroendocrine and autonomic systems have been well studied. For example, electrical stimulation of SFO results in excitatory responses in PVN neurons observed to be biphasic consisting of a transient, short latency response hypothesized to be mediated by glutamate coupled with a longer duration ANG mediated response (Bains & Ferguson, 1999; Bains & Ferguson, 1995). Electrical stimulation of SCN evoked excitatory post-synaptic potentials (EPSPs) in PVN that were sensitive to NMDA and non-NMDA receptor antagonists. In addition, chemical microstimulation within SCN was associated with transient increases in spontaneous EPSPs recorded from these PVN neurons suggesting that glutamate in part regulates circadian rhythmicity to PVN (Hermes \textit{et al.}, 1999).
Local excitatory inputs to PVN and SON neurons were studied with the use of electrical and chemical stimulation. Extracellular electrical stimulation of perinuclear (lateral) areas evoked multiple EPSPs and excitatory post-synaptic currents (EPSCs) in PVN and SON cells (Boudaba et al., 1997). Application of metabotropic glutamate receptor agonists caused an increase in the frequency of EPSPs/EPSCs that were blocked by TTX, suggesting that it was mediated by activation of local presynaptic excitatory neurons as these were subsequently blocked by ionotropic glutamate receptor antagonists.

What is the significance of these observations? Neuro- and adenohypophyseal regulation are likely the most documented pathways shown to be regulated in this manner. Stimulation of PVN with glutamate elicits AVP release and causes an increase in heart rate (Darlington et al., 1989). Injection of NMDA or AMPA into the PVN or SON in non-suckling lactating rats elicited a strong increase in OXY release which was abolished by treatment with antagonists of their respective receptors (Parker & Crowley, 1995). Osmotically stimulated AVP release from hypothalamo-neurohypophyseal explants is abolished by NMDA receptor antagonists (Swenson et al., 1998). There is also evidence that glutamate is involved in autonomic function as glutamate receptors in PVN have been shown to mediate the pressor response induced by carotid body chemoreceptor stimulation (Kubo et al., 1997). Daftary et al. (Daftary et al., 2000; Daftary et al., 1998b) demonstrated that noradrenergic excitation of magnocellular neurons was effectively abolished by glutamate antagonists and was TTX-sensitive suggesting this effect is mediated by an increase in
glutamatergic input. In addition, they observed that the NA mediated increase in EPSP frequency was maintained in a surgically isolated PVN preparation and also with NA microdrop application directly into PVN further implicating a glutamate interneuron in PVN. Follwell and Ferguson (Follwell & Ferguson, 2002b) examined the cellular mechanisms underlying the effects of orexin A (OX-A) on magnocellular neurons in PVN. Magnocellular neurons showed depolarizations in response to OX-A which were associated with a robust increase in EPSC frequency and amplitude. These effects were abolished by kynurenic acid illustrating the role of glutamate in mediating these responses.

As for the role of excitatory neurotransmission in regulation of anterior pituitary Herman et al. (Herman et al., 2002) have suggested that regulation of the hypothalamic-pituitary-adrenal (HPA) axis is a multisynaptic process involving the cortex, hypothalamus, and limbic system all of which are integrated within PVN via glutamate-GABA microcircuits. In particular limbic influences on adrenocorticotropic hormone (ACTH) secretion which are mediated by synaptic activation or inhibition of neurosecretory parvocellular neurons. Activation of the HPA axis is controlled by neurons localized within the medial parvocellular subdivision of PVN (Whitnall, 1993). Injection of glutamate into PVN stimulates ACTH release via increases in corticotropin releasing hormone (CRH) which is thought to be regulated through NMDA and metabotropic glutamate receptor activation (Joanny et al., 1997). The ramifications of these observations remain to completely understood; however, it is clear that damage to either of these
areas results in hypersecretion of corticosterone and therefore has important implications on HPA response to stressors.

INHIBITORY NEUROTRANSMISSION IN PVN

GABA is the predominant inhibitory neurotransmitter in the CNS and thus also in the hypothalamus. It has been estimated that almost 50% of the total synaptic contacts in PVN can be attributed to GABAergic nerve terminals (Oliver et al., 1998; Roland & Sawchenko, 1993). GABA therefore has considerable influence on neuroendocrine regulation and much effort has been devoted to characterizing its anatomical distribution and effects on pulsatile hormone release, stress integration, and modulating the actions of several well known peptide hormones including ANG, AVP, and adrenomedullin (ADM).

GABA is converted from glutamic acid, a reaction catalyzed by glutamic acid decarboxylase (GAD). Immunohistochemical studies of GABAergic anatomy within the CNS have largely relied on immunostaining with antisera against GAD or GABA itself. PVN appears to be uniformly invested with GABAergic varicosities. Decavel and van den Pol (Decavel & van den Pol, 1990) observed that GABA immunoreactive terminals account for almost 50% of the total synaptic input to PVN and are of both the axo-somatic and axo-dendritic variety. GABAergic input does however appear to be homogeneously distributed within PVN amongst the magnocellular and parvocellular areas (Oliver et al., 1998; Roland & Sawchenko, 1993).

GABA receptors, which belong to the ligand-gated ion channel superfamily, have been identified in PVN and mediate the majority of fast
inhibitory neurotransmission in this nucleus. Three different subtypes of GABA receptors have been identified based on their respective pharmacological and electrophysiological profiles. The GABA\textsubscript{A&C} receptors are Cl\textsuperscript{−} permeable ion channels while GABA\textsubscript{B} receptors are G protein coupled receptors. GABA\textsubscript{A} receptor mRNAs have been found in the magnocellular and parvocellular region of PVN where they appear to be localized within CRH neurons as well as in the peri-PVN area. In addition, GABA\textsubscript{B} receptors have been observed in parvocellular PVN although their role is less well defined (Cullinan, 2000; Wisden \textit{et al.}, 1992).

The origins of GABAergic projections to PVN are well documented. The periventricular area was found to contain a disproportionately high density of GABAergic neurons which in turn receives projections from throughout the hypothalamus, the prefrontal cortex, amygdala, SCN, and ascending brainstem pathways (Boudaba \textit{et al.}, 1996; Roland & Sawchenko, 1993). These regions are known to influence PVN yet have been shown to send relatively few projections into major subdivisions of PVN. The perinuclear zone thus acts as an important integratory synaptic relay center. Not surprisingly this area is thought to be an important modulator of inhibitory and excitatory projections into PVN, particularly in regard to stress integration, regulation of the anterior pituitary, and synchronization of AVP/OXY release from the neurohypophysis (Herman \textit{et al.}, 2002).

As introduced previously, evidence supporting the existence of inhibitory circuits within the neurosecretory system has been reported. Kandel (Kandel,
for example, demonstrated the presence of short latency IPSPs in goldfish neurosecretory neurons following stimulation of the pituitary stalk. More recently this recurrent inhibition in the neurohypophyseal pathway been postulated to modulate the discharge characteristics of PVN and SON neurons and therefore aid in the synchronization of neuropeptide release (Renaud & Bourque, 1991). Observations of this nature were sufficient to launch a detailed characterization of GABAergic neurotransmission within PVN. Tasker and Dudek (Tasker & Dudek, 1993) reported that local application of glutamate increases GABA\textsubscript{A} mediated inhibitory synaptic activity in PVN, an effect which they felt was probably due to the activation of local GABAergic inhibitory interneurons. The existence of such interneurons in the PVN was later verified (Boudaba et al., 1996) combining glutamate microstimulation techniques with \textit{in situ} hybridization of GABAergic neurons to physiologically map local inhibitory neurons to PVN (Boudaba et al., 1996). Very few of these GABAergic neurons however are thought to originate within the confines of PVN although immunohistochemical studies have confirmed the existence of GABA immunoreactive projections in PVN, which correlate both anatomically and chemically with medial parvocellular neurons (Boudaba \textit{et al.}, 1996;Roland & Sawchenko, 1993;Decavel & van den Pol, 1990;Meister \textit{et al.}, 1988). Interestingly, PVN projections have been found to co-express glutamate receptors and GAD mRNA suggesting that periventricular GABAergic neurons can integrate glutamatergic neurotransmission with consequent effect on integration within PVN (Herman \textit{et al.}, 2002).
The effects of GABA on PVN membrane properties have also been described. Extracellular recordings indicate GABA mediates inhibitory actions on the excitability of PVN and SON neurosecretory neurons (Renaud & Bourque, 1991). Similarly, intracellular recordings in SON neurons suggest that GABA reduces action potential firing frequency as a result of increasing a membrane conductance which is sensitive to external chloride concentration. These effects are abolished by the GABA\textsubscript{A} receptor antagonist bicuculline methiodide (BMI) and are reflective of an increase IPSP activity in these cells. An interesting paradox involving GABAergic transmission has also been observed in PVN. Bains and Ferguson (Bains & Ferguson, 1997b) observed that during NMDA induced excitation of magnocellular neurons in PVN, an increase in IPSPs was also observed in a significant population of these cells. The increase in IPSP frequency was found to be nitric oxide dependent, an intriguing observation in lieu of the demonstration of nitric oxide (NO) synthase (NOS) in magnocellular neurons. In combination with the aforementioned immunohistochemical studies identifying GABA immunoreactive projections in PVN associated with parvocellular neurons, the excitability of which is known to be influenced by nitric oxide (Bains & Ferguson, 1997a; Boudaba et al., 1996; Decavel & van den Pol, 1990; Decavel et al., 1989) these authors postulated that an ultra short loop inhibitory feedback circuit existed within the PVN, which could ultimately act to regulate the activity of the neuroendocrine cells.

Like glutamate, the relationship between GABA and PVN in modulating several well known neuroendocrine pathways has been studied. A circadian
pacemaker located in the SCN generates the diurnal activity of the HPA axis. The principal neurons participating in this axis controlling ACTH release are the CRH neurons located in the medial parvocellular PVN. Electrophysiological and immunohistochemical studies have identified neural connections between SCN and PVN which utilize GABA as a neurotransmitter and are thought to regulate the HPA axis (Wang et al., 2003; Buijs et al., 1994). While electrical stimulation of SCN results in an increase in excitability of PVN neurons identified as projecting to the median eminence an increase in IPSP frequency is also observed. Circulating levels of AVP and OXY exhibit circadian variations which is likely mediated by SCN and evoked IPSPs following SCN stimulation have also been observed in magnocellular neurons (Hermes et al., 1996). Autonomic variables such as blood pressure, heart rate, and body temperature are also known to display circadian rhythmicity and most recently spinally projecting neurons in PVN were found to be influenced by SCN stimulation strengthening the argument for GABAergic transmission within this pathway exerting significant influence on homeostatic function (Cui et al., 2001).

AVP acts at V1a receptors to excite GABAergic neurons that are presynaptic to a population of magnocellular PVN neurons. This has important consequences on magnitude of AVP release which is correlated with both the frequency and the pattern of action potentials generated at the cell soma, features that are dependent on intrinsic membrane properties and synaptic input (Renaud & Bourque, 1991). Functional roles for such inputs in the control of hormone secretion are supported by studies showing that intracerebroventricular
(ICV) injection of the GABA agonist muscimol reduces or abolishes AVP release consequent to hypovolemia, increases in plasma osmolarity, and ICV ANG injection. In addition, OXY also has been noted to alter cell excitability through a reduction of GABAergic inhibition (Brussaard et al., 1996) which likely facilitates bursting activity in OXY-synthesizing neurons during suckling.

Regulation of the anterior pituitary occurs primarily at the level of the hypothalamus through several well-established hypophysiotropic hormones which stimulate or inhibit the secretion of hormones from the adenohypophysis. In view of the importance of these adenohypophyseal hormones in maintaining mammalian homeostasis, the pathways underlying their regulation have been given considerable attention. PVN has been found to be a principal nucleus in the regulation of many of these hormonal systems including thyrotropin releasing hormone (TRH), ACTH, prolactin, gonadotropins, and growth hormone (Roland & Sawchenko, 1993). Subsequently, GABAergic innervation of this nucleus was found to influence TRH release (Fekete et al., 2002). Further, GABAergic innervation of CRH secreting parvocellular neurons has been observed (Miklos & Kovacs, 2002) and while application of BMI into PVN increased plasma corticosterone (Cole & Sawchenko, 2002). Interestingly, Cole and Sawchenko (Cole & Sawchenko, 2002) also report that application of glutamate into PVN fails to increase in plasma corticosterone but resulted in marked fos induction in identified GABAergic interneurons in the peri-PVN region suggesting that the activation of these neurons may underlie the lack of corticosterone release by this excitatory neurotransmitter.
ANGIOTENSIN II

Discovered in 1940 (Munoz et al., 1939) as the mysterious agent responsible for mediating the pressor effects of kidney extracts, the peptide hormone ANG has since dominated the literature. Traditionally, ANG is recognized for its peripheral endocrine roles in the regulation of vascular resistance and control of fluid electrolyte homeostasis. However, ANG has subsequently been shown to play important roles within the CNS to influence memory (Armstrong et al., 1996), reproduction (Phillips et al., 1995; Phillips et al., 1993c), and of course neuroendocrine regulation (Phillips & Sumners, 1998). The notion of ANG acting in the CNS as a neurotransmitter has garnered considerable interest and continues to inspire much debate. The requisite biochemical pathways for ANG production and ANG receptors have been found in many areas of the brain, particularly in the hypothalamus and specifically PVN. Pursuant to this observation an intense investigation of the actions of this hormone in modulating thirst, AVP, OXY, and ACTH secretion, enhancing sympathetic outflow, and modulating baro-reflex sensitivity have followed. Only recently though have the mechanisms underlying ANG’s ability to exert such a profound influence over such an enormous number of physiological pathways begun to be unraveled.

PHARMACOLOGY

ANG is an octapeptide whose structure has been extensively studied. Its amino acid sequence is known to be Asp-Arg-Val-Tyr-Ile-His-Pro-Phe and Printz et al. determined that the three dimensional structure of ANG consists of a
polypeptide backbone forming a gamma-turn stabilized by three hydrogen bonds (Printz et al., 1972). A description of ANG would not be complete without discussing the biochemical enzymatic cascade for the renin-angiotensin system (RAS). Renin is an aspartyl protease synthesized and stored largely in the juxtaglomerular apparatus of the kidney, which catalyzes the hydrolytic release of the decapeptide angiotensin I from angiotensinogen, an α-2 globulin synthesized by the liver. ANG which is found near the N terminus of angiotensinogen is formed following cleavage of angiotensin I via the carboxypeptidase angiotensin-converting enzyme (ACE). ANG has a relatively short half-life of 15-30s and is rapidly degraded by a series of aminopeptidases collectively referred to as angiotensinase (Regoli et al., 1974).

ANG has pharmacological actions on a wide range of tissues and exerts considerable influence in the maintenance of homeostasis. In the vasculature where ANG gained its initial notoriety as a circulating hormone, it is one of the most potent known vasoconstrictors acting directly on arterial smooth muscle resulting in contraction and a rapid and transient pressor effect. ANG has been implicated in modulating cellular growth and is known to cause significant hypertrophy and hyperplasia of vascular smooth muscle cells and cardiac myocytes (Matsusaka & Ichikawa, 1997). ANG exercises significant control over fluid electrolyte homeostasis via its actions on the kidney that includes inducing renal artery vasoconstriction, increasing proximal tubular sodium reabsorption, and inhibiting the secretion of renin (Ganong, 1984). Furthermore, ANG acts directly on the zona glomerulosa of the adrenal cortex to stimulate aldosterone
biosynthesis and release, a hormone which itself is of considerable interest in blood pressure regulation.

The documentation of an endogenous RAS in the CNS has resulted in an extensive description of this peptides action in the brain (Ferguson et al., 2001; Ferguson et al., 1999; Phillips & Sumners, 1998; Fitzsimons, 1998; Ganong, 1984; Mendelsohn et al., 1984a) One of the more intriguing observations is the ability of ANG to stimulate the sensation of thirst and the behavioral drive to drink, increase ACTH release and sodium appetite, and modulate cardiovascular regulation (Phillips & Sumners, 1998). ANG is particularly significant in the central control of blood pressure through at least three separate mechanisms including activation of the sympathetic nervous system (SNS) (via PVN projections to sympathetic nuclei in the brainstem), release of AVP, and inhibition of baroreflexes (via ANG induced inhibition of the vagal-NTS synaptic connection) (Phillips & Sumners, 1998).

ANGIOTENSINERGIC PATHWAYS IN THE CNS

The development of sophisticated neuroanatomical imaging techniques in conjunction with the advent of specific peptidergic and non-peptidergic ANG antagonists has lead to a comprehensive categorization of angiotensinergic pathways within the CNS. Perhaps the most intriguing of these descriptions was the initial observation that a broad distribution of ANG binding sites are found throughout the brain. As ANG is unable to cross the blood brain barrier (BBB) this information provided the first real suggestion for the existence of angiotensinergic neurons within the CNS. Subsequent identification of all the
components on the RAS within the CNS has permitted a much more intensive investigation of this peptide’s integrated physiological role.

Confirmation of the existence of an expansive angiotensinergic system in the CNS followed the demonstration of neuronal soma and fibers that bound immunocytochemical probes targeted against ANG (Lind et al., 1985a; Lind et al., 1985b; Lind et al., 1984; Healy & Printz, 1984; Changaris et al., 1978; Fuxe et al., 1976). Fittingly, these studies seemed to suggest an angiotensinergic network that largely matched the anatomical description of those pathways known to play significant roles in the control of fluid-electrolyte homeostasis (Lind et al., 1985b). Angiotensinergic neurons were consequently identified in the SFO, medial preoptic nucleus (MPO), SON, and PVN, nuclei all regarded for their participation in cardiovascular regulation. Intriguingly, SFO was observed to send ANG immunoreactive fibers (AIFs) to these same nuclei providing the first evidence suggesting this peptide acted as a neurotransmitter. Not surprisingly, neurosecretory cell bodies in SON and PVN were shown to be ANG immunoreactive and also sent AIFs to both the posterior pituitary and median eminence (Lind et al., 1985b).

The remainder of the fundamental RAS components within the CNS were identified shortly thereafter. Although studies have described the presence of renin or renin mRNA within the CNS (Yu & Di Nicolantonio, 1996; Lou et al., 1995; Phillips et al., 1993b) the distribution of this enzyme either in specific anatomical regions or in specific cell groups remains unknown. Angiotensinogen and ACE are widely distributed within the CNS and are predictably localized
within the regions described demonstrating ANG immunoreactivity (Rogerson et al., 1995; Mungall et al., 1995; Sernia, 1995; MacGregor et al., 1994; Lippoldt et al., 1993; Tsutsumi et al., 1993; Bunnenmann et al., 1993; Hong-Brown & Deschepper, 1993; Lynch et al., 1987; Correa et al., 1985; Kiss et al., 1983b). Despite the intensive categorization of ANG, ACE, angiotensinogen, and renin within the CNS, whether the full complement of the RAS apparatus exists in a single angiotensinergic neuron remains unknown. This mystery continues to inspire debate as to whether ANG is indeed a bona fide neurotransmitter (to be discussed in further in Effects on Neurotransmission).

ANGIOTENSIN RECEPTORS

A major advance in our understanding of ANG occurred with the synthesis of ANG analogs which exhibited clear antagonistic effects at the receptor level. Substitution of certain amino acids particularly sarcosine for phenylalanine in position 8 of ANG results in the formation of a potent ANG antagonist, the best known of which is saralasin (Regoli et al., 1974). Subsequent discoveries of new antagonists have in turn lead to the exploration of the role of this peptide in its natural physiological setting and also clinically in a number of pathophysiological conditions. Losartan, unlike its peptide counterparts, is a non-peptide ANG receptor antagonist derived from imidazole. Studies demonstrating varying levels of inhibition by ANG of adrenal cortical microsomes by saralasin and losartan provided the first evidence of divergent ANG receptors (Regoli et al., 1974). Consequently, two distinct subtypes of ANG receptors were identified on the basis of their differential affinity for non-peptide (PD 123177) and peptide (CGP
antagonists and termed AT₁ and AT₂ respectively. Most of the known actions of ANG are mediated by the AT₁ receptor which is a G protein coupled receptor distributed throughout the periphery and CNS. A number of studies have utilized both immunocytochemical and in situ hybridization techniques to describe the distribution of ANG receptors within the CNS. The autoradiographic distribution of labeled ANG as an indication of ANG binding sites within the CNS (Gehlert et al., 1991; Israel et al., 1985; Mendelsohn et al., 1984b) includes the SFO, ME, NTS, area postrema (AP), SON, and PVN. These hypothalamic regions are again similar to those described in the documentation of CNS RAS and are complementary to ANG’s role in the regulation of body fluids. In addition, data describing additional binding sites for ANG in the cerebellum, hippocampus, locus coeruleus, and superior colliculus suggest broader roles for this peptide than initially suspected (Gehlert et al., 1991; Mendelsohn et al., 1984b). More recent reports using in situ hybridization techniques have effectively confirmed such observations by describing the distribution of mRNA’s for AT₁A, AT₁B, and AT₂ receptors (Raizada et al., 1993; Kakar et al., 1992; Wooldridge, 1980).

**CELLULAR ACTIONS**

The distribution of the AT₁ and AT₂ receptor have been extensively studied in the CNS (See preceding section). While the description of the consequent electrophysiological effects of AT receptor activation continues to evolve it is clear that the receptor subtype infers significant downstream effects on target tissues.
The concept of AT receptor as a G protein coupled receptors is well documented (Phillips & Sumners, 1998; Sumners & Gelband, 1998). While the elucidation of the intracellular cascades activated following ANG-AT\textsubscript{1,2} receptor binding remains in its early stages, several interesting observations have been made. Studies reveal that AT\textsubscript{1} receptors are coupled to either activation of phospholipase C (PLC) and the subsequent stimulation of phosphatidyl inositol (PI) hydrolysis (Richards et al., 1999; Pueyo et al., 1996; Dudley et al., 1990) or to the inhibition of adenylyl cyclase (Dudley et al., 1990). PI effects are largely dependent upon mobilization of internal calcium and/or activation of protein kinase C (PKC). Stimulation of the AT\textsubscript{1} receptor has also been shown to increase transcription of several immediate early genes including c-FOS, FOS B, c-JUN, JUN B, and KROX-24 in the SFO, MPO, PVN, and SON (Richards et al., 1999; Culman et al., 1995) although the consequence of this activation is unknown. Most of the information on the effects of AT\textsubscript{2} receptor activation on neurons has been obtained from either tumor cell lines or from the newborn rat hypothalamus in culture. AT\textsubscript{2} receptors are also G protein coupled yet experiments in PC12 cells suggest their actions are largely dependent upon activation of phospholipase A2 and the subsequent production of arachidonic acid (AA) (Sumners & Gelband, 1998; Zhu et al., 1997; Sumners et al., 1994; Lokuta et al., 1994).
EFFECTS ON ION CHANNELS

Ion channels represent the cornerstone of neuronal communication and modulation of their function can exert pervasive changes on the function of a single cell through complex neuronal networks. ANG has been shown to act upon a variety of ionic conductances, including voltage dependent potassium and calcium currents as well as non-selective cationic currents (NSCC). The effects of ANG on these channels seem largely dependent upon the subtype of AT receptor, although the AT$_1$ receptor appears to be predominant in modulating neuronal excitability.

Potassium channels are ubiquitous amongst excitable cells. ANG has been shown to modulate the voltage dependent delayed rectifier potassium current ($I_K$), and $I_A$. The AT$_1$ receptor mediated effects on these channels have been explored extensively and have been implicated in facilitating neuronal excitation (through inhibition of this conductance) in several hypothalamic nuclei including SON and cultured brainstem/hypothalamic neurons (Sumners et al., 1996; Nagatomo et al., 1995). Li and Ferguson however show that ANG is without effect on $I_K$ in PVN magnocellular neurons and therefore does not likely mediate ANG induced excitatory actions in this nucleus (Li & Ferguson, 1996), although they did not examine the actions of ANG on $I_K$ of parvocellular neurons in this nucleus. Activation of the AT$_2$ receptor in cultured brainstem/hypothalamic neurons results in a potentiation of $I_K$ that can be mimicked by CGP42112A (AT$_2$ receptor agonist) and abolished by pertussis toxin suggesting the involvement of a G$_i$ or G$_o$ protein (Sumners & Gelband, 1998).
$I_A$ is known to play a important role in regulating spike frequency, a phenomenon critical in modulating neuropeptide/transmitter release (Connor & Stevens, 1971). This conductance therefore has important implications in PVN and SON magnocellular neurons which express a dominant $I_A$ (Luther & Tasker, 2000; Fisher & Bourque, 1998; Fisher et al., 1998). In cultured hypothalamic neurons, ANG has been shown to decrease $I_A$ in the presence of PD123319 ($AT_2$ receptor antagonist) verifying the $AT_1$ mediated nature of this response. This observation is consistent with the finding that ANG caused an $AT_1$ receptor mediated decrease in single channel open probability of $I_A$ (Sumners & Gelband, 1998; Wang et al., 1997). In addition, an ANG mediated reduction in $I_A$ has been observed in neurons in the SFO, SON, and PVN, effects maintained in synaptic isolation (TTX or $Ca^{2+}$ free/$Co^{2+}$ medium) and mediated by $AT_1$ (Li & Ferguson, 1996; Nagatomo et al., 1995). While ANG effects on $I_A$ are unlikely to initiate significant neuronal excitation in these nuclei, the regulation of neuropeptide release is a plausible target which may be finely synchronized by this interaction.

Calcium channels play important regulatory and electrical roles in all cells including neurons in the CNS. Increases in intracellular calcium release have been shown to have a spectrum of cellular actions including activation of $Ca^{2+}$-dependent enzymes and modulation of ion channels. Calcium channels can be differentiated based on their biophysical properties and sensitivity to pharmacological agents. ANG has been shown to act on several calcium channel subtypes (N-, T-, and L-Type) although these effects have not been documented in PVN (Sumners & Gelband, 1998; Guenther et al., 1996; Chorvatova et al.,
ANG has however been shown to modulate calcium channels thus influencing neuronal excitability (likely in combination with an increase in NSCC and a decrease in $I_K$) in cultured hypothalamic neurons. ANG increases total calcium current ($I_{Ca}$) in response to $AT_1$ receptor activation which is in part responsible for this neuronal excitation (Sumners & Gelband, 1998; Zhu et al., 1997; Sumners et al., 1996; Kang et al., 1992). The stimulation of $I_{Ca}$ largely appears to be similar in nature to the reduction of the $I_K$ as it involves $G_q$ protein cascade and PLC (Sumners & Gelband, 1998). $AT_2$ receptor stimulation does not appear to have an effect on $I_{Ca}$ in cultured neurons.

The NSCC, a voltage independent current, has recently been implicated in facilitating the depolarizing effects of a number of well-known hormones including ANG, ADM, and OX-A (Yang & Ferguson, 2002; Follwell & Ferguson, 2002b; Chakfe & Bourque, 2000; Bai & Renaud, 1998; Yang et al., 1992). The actions of ANG on NSCC are known to be $AT_1$ receptor mediated, the signal transduction pathways which trigger the activation of NSCC following $AT_1$ receptor stimulation however remain unknown (Bai & Renaud, 1998; Chorvatova et al., 1996). While a number of studies suggest the involvement of a NSCC based on the observed decrease in input resistance and reversal potential of the depolarizing current, few studies have investigated the relationship between the NSCC and ANG in well controlled voltage clamp experiments. Future studies will no doubt implicate NSCC in mediating neuropeptide actions, as focus on this conductance grows in view of its potential role in the integration of diverse signals regulating body fluid status.
EFFECTS ON NEUROTRANSMISSION

Important novel roles for ANG have recently been suggested in the modulation of somatic and dendritic ion channels, as well as the regulation of synaptic efficacy. It is through these complex interactions that ANG is able to modify the output of PVN. In addition to the traditional endocrine roles of circulating ANG, there is considerable evidence supporting additional functions as a neurotransmitter. This concept is derived from observations that seem to fulfill the established criteria for a neurotransmitter which are:

1. Substance must be contained in and synthesized by presynaptic terminals.
2. Specific high affinity receptors must be identified and appropriately distributed.
3. Exogenous application of the substance must have post-synaptic effects that are blocked by pharmacological antagonists.
4. Antagonists must be shown to block the effects of endogenous release.

In the case of ANG these traditional criteria are not met absolutely as the synthetic machinery necessary for its production have not been identified in nerve terminals within PVN. This situation is not unlike other neuropeptides which are primarily synthesized in the cell body of the peptidergic neuron and then transported to axon terminals for exocytotic release. In the case of ANGergic neurons, particularly those originating in SFO, studies have documented ANG immunoreactivity in somata which could presumably be transported anterogradely toward axon terminals for potential release following
action potential triggered calcium influx. There is however considerable evidence that each of the other neurotransmitter criterion are satisfied suggesting that ANG should indeed be considered as a neurotransmitter. AT₁ receptors have been documented throughout the hypothalamus and specifically in PVN. Perfusion experiments have demonstrated endogenous physiologically relevant release of ANG in the PVN (Wright et al., 1993). Exogenous administration of ANG and ANG-related peptides into this region mimics the effects of electrical or chemical stimulation of presumed ANGergic afferent connections (Li & Ferguson, 1993b). Finally, the physiological and electrophysiological consequences of activation of putative ANGergic input to PVN neurons have also been shown to be abolished by specific AT₁ receptor antagonists.

In addition to the anatomical and neurochemical data suggesting a potential for the use of ANG as a neurotransmitter, many electrophysiological studies have shown direct evidence in support of this hypothesis. PVN neurons projecting to the spinal cord and posterior pituitary receive excitatory ANGergic input from the SFO (Bains & Ferguson, 1995). In vitro studies have shown that ANG has excitatory effects on neurons within the PVN that are blocked by AT₁ receptor antagonists (Li & Ferguson, 1996; Li & Ferguson, 1993a). In addition to direct electrophysiological measurements, evidence suggests that many of the physiological responses governed by the SFO-PVN pathway are mediated by neurotransmitter actions of ANG. Microinjection of ANG into PVN causes elevations in blood pressure, drinking (Jensen et al., 1992), and AVP release (Lin et al., 1980). In addition, similar effects subsequent to stimulation of the SFO-
PVN pathway actions which are abolished following receptor blockade providing evidence for inhibition of the effects of endogenous ANG release (Bains et al., 1992).

**DENDRITIC ACTIONS**

Ion channels are known to play critical roles in modulating dendritic excitability and action potential propagation. The study of ANG’s effects on dendritic ion channels remains in its infancy. A number of studies have clearly demonstrated that magnocellular neurons of the SON and PVN express a profound $I_A$ (Li & Ferguson, 1996; Armstrong, 1995; Tasker & Dudek, 1991), a conductance which has been shown to be mediated by dendritically localized channels in hippocampus and cerebellum. Interestingly, following dissociation (and consequent loss of their dendritic trees) magnocellular neurons of the SON appear to lose their $I_A$ suggesting it is anatomically localized to the dendrite (Widmer et al., 1997). These data therefore suggest that ANGergic inhibition of $I_A$ in these SON and PVN neurons (through AT$_1$ receptors) (Li & Ferguson, 1996; Steardo et al., 1994) is likely the result of actions on this peptide on dendritic ion channels.

**SOMATIC ACTIONS**

The majority of studies characterizing the somatic actions of ANG on various neuronal systems report that ANG causes depolarizations and associated increases in action potential frequency (Bai & Renaud, 1998; Yang et al., 1992). These excitatory effects are the result of complex interactions between
ANG and a number of the previously mentioned ion channels and are largely AT₁ receptor dependent (Sumners & Gelband, 1998). In SON neurons for example, ANG receptor activation elicits a depolarization accompanied by a decrease in input resistance, which is maintained in synaptic isolation and is AT₁ receptor mediated (Yang et al., 1992). The mean reversal potential (E_{rev}) for these effects is approximately -25mV (suggesting activation of a NSCC).

ANG also has a powerful effect on synaptic pathways within the CNS. As described earlier, SFO projections to PVN neurons represent an important output pathway through which these cells influence neuroendocrine (Plotsky et al., 1987;Ferguson et al., 1984a) and autonomic (Bains & Ferguson, 1995;Ferguson et al., 1989;Ferguson et al., 1984a) systems. SFO stimulation results in excitation of neurosecretory cells identified as having projections to the posterior pituitary (Ferguson et al., 1984b), increases in plasma AVP concentration (Ferguson & Kasting, 1986), and an increase in blood pressure. Furthermore, these effects can be effectively mimicked by pressure injection of ANG directly into the PVN (Li & Ferguson, 1993a;Bains et al., 1992). Interestingly, electrical stimulation of the SFO resulted in a biphasic post-synaptic excitation, which, following treatment with a non-peptidergic AT₁ receptor antagonist, was partially abolished revealing an underlying rapid excitatory phase remaining (Bains et al., 1992). These data suggest that chemical neurotransmission at this SFO-PVN synapse is mediated by both a rapid (putative amino acid, likely glutamate) and a slow messenger which is known to be ANG. In accordance with such a hypothesis, glutamate has been shown to increase both blood pressure (Krukoff et al., 1994;Martin &
Haywood, 1992) and single neuron activity following administration into PVN, effects mediated, at least in part, by the NMDA receptor (Wuarin & Dudek, 1991; van den Pol et al., 1990).

**TERMINAL ACTIONS**

The modulation of synaptic transmission by ANG is not limited to the dendrites and soma. ANG has been observed to modulate neurotransmitter/peptide release and while this action is likely in part due to its effect on neuronal dendrites and somata, effects on synaptic terminals have also been implicated. In NTS, ANG differentially modulates inhibitory and excitatory synaptic activity via AT$_1$ receptors, effects thought to be critical in ANG’s modulation of both baro- and chemoreceptor reflexes (Kasparov & Paton, 1999; Casto & Phillips, 1986). Further support for interactions between GABA and ANG at the nerve terminal have been observed in central portions of SFO where numerous terminals contain both GABA and ANG immunoreactivity (Pickel & Chan, 1995). Recent data has also suggested that ANG excites spinally projecting PVN neurons by attenuation of GABAergic synaptic inputs through activation of presynaptic AT$_1$ receptors, which may underlie the pressor, tachycardic and renal sympathtoexcitatory responses to PVN stimulation (Li et al., 2003). Xiong and Marshall (Xiong & Marshall, 1990) suggest ANG may also modulate glutamate evoked PSP’s as iontophoretically applied ANG reduces the depolarization induced by glutamate in locus coeruleus neurons.
STATEMENT OF PROBLEM

This thesis comprises three manuscripts wherein the experiments conducted were designed to elucidate the electrophysiological actions of ANG in PVN. In particular ANG's role in the modulation of synaptic transmission within PVN and its effect on the membrane properties of neurosecretory neurons will be discussed. These studies were completed using the whole-cell configuration of the patch clamp technique. Current and voltage clamp recording were obtained from PVN neurons in coronal hypothalamic slices from male Sprague-Dawley rats.

1. Chapter 2 (manuscript one) examines the role of ANG in activating an inhibitory feedback loop within PVN and the chemical messengers upon which this system is dependent.

2. Chapter 3 (manuscript two) examines the role of ANG in activating an excitatory feedback loop in PVN and the role of glutamate interneurons within this system.

3. Chapter 4 (manuscript three) examines the cellular membrane properties which ANG modulates in neurosecretory PVN neurons to excite these cells.
Chapter 2: ANGIOTENSIN II ACTIVATES A NITRIC OXIDE DRIVEN INHIBITORY FEEDBACK IN THE RAT PARAVENTRICULAR NUCLEUS

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ABSTRACT

The hypothalamic paraventricular nucleus (PVN) has been shown to play major obligatory roles in autonomic and neuroendocrine regulation. Angiotensin II (ANG) acts as a neurotransmitter regulating the excitability of magnocellular neurons in this nucleus. We report here that ANG also activates a nitric oxide mediated negative feedback loop in the PVN that acts to regulate the functional output of magnocellular neurons. Thus, in addition to its depolarizing actions on magnocellular neurons, ANG application results in an increase in the frequency of inhibitory post-synaptic potentials in a population of these neurons without effect on the amplitude of these events. ANG was also without significant effect on the mean frequency or amplitude of mini synaptic currents analyzed in voltage clamp experiments. This increase in inhibitory input following ANG can be abolished by the nitric oxide synthase inhibitor N\textsubscript{ω}-Nitro-L-Arginine methylester (L-NAME), demonstrating a requisite role for nitric oxide in the activation of this pathway. The depolarization of magnocellular neurons that show increased IPSP frequency in response to ANG is significantly smaller than that observed in neurons in which IPSPs frequency was unaffected (3.2±1.1 vs. 8.0±0.5mV, p<0.05). Correspondingly, following nitric oxide synthase inhibition, the depolarizing effects of ANG on magnocellular neurons are augmented (2.7±0.4 vs. 6.5±0.5mV, p<0.05). The depolarization was also enhanced in the presence of the GABAergic antagonist bicuculline (1.9±1.2 vs. 11.9±2.3, p<0.001). These data demonstrate that there exists within the PVN an intrinsic negative feedback loop which modulates neuronal excitability in response to peptidergic excitation.
INTRODUCTION

The paraventricular nucleus (PVN) of the hypothalamus is one of the critical nuclei involved in neuroendocrine and autonomic regulation. These actions are mediated in part by the neurohypophysial hormones vasopressin (AVP) and oxytocin (OXY), which are synthesized in PVN and secreted from the pituitary (Swanson & Sawchenko, 1980). The magnocellular neurons of the PVN, which are responsible for AVP and OXY production and release, can be distinguished both morphologically and electrophysiologically from parvocellular neurons in the same nucleus (Hoffman et al., 1991; Tasker & Dudek, 1991). PVN neurons have been shown to receive afferents from other hypothalamic nuclei, the brainstem, and the subfornical organ (Swanson & Sawchenko, 1983; van den Pol, 1982). These cells integrate incoming signals from afferent fibers and send projections to the posterior pituitary, median eminence, brainstem, and spinal cord, and ultimately influence autonomic regulation through these connections (Swanson & Sawchenko, 1980). While the afferent signals to PVN provide the initial stimulus for excitation, it is thought that local synaptic regulation within the nucleus is responsible for preservation and propagation of the correct output. Interestingly, recent data suggests that a breakdown in these synaptic mechanisms may underlie the onset and/or maintenance of several well-described pathophysiological conditions (Patel & Zhang, 1996; Earle & Pittman, 1995; Ciriello et al., 1984).

The anatomical distribution and cellular actions of the predominant excitatory and inhibitory neurotransmitters of the CNS, glutamate and GABA in
the hypothalamus are well documented (van den Pol & Trombley, 1993; van den Pol et al., 1990; Decavel & van den Pol, 1990). Furthermore, the actions of glutamate and GABA on the magnocellular neurons of the supraoptic nucleus (SON) and PVN, and on parvocellular neurons in the PVN have been described (Bains & Ferguson, 1997b; Bains & Ferguson, 1997c; Renaud et al., 1992). Bains and Ferguson recently showed that a glutamate activated negative feedback loop within PVN, which may constitute a major regulatory mechanism modulating the strength of output signals (Bains & Ferguson, 1997b). They observed that following NMDA receptor activation, magnocellular neurons were depolarized concurrent with an increase in both action potential firing and membrane conductance. Paradoxically, a subset of these neurons were not only excited but demonstrated an increase in GABAergic inhibitory post-synaptic potentials (IPSPs), the occurrence of which were dependent on nitric oxide (NO) production (Bains & Ferguson, 1997b). Interestingly, vasopressin has also been observed to increase IPSPs in magnocellular neurons (Hermes et al., 2000). This increase in IPSPs could be abolished by tetrodotoxin (TTX) and bicuculline suggesting the involvement of a GABAergic interneuron (Bains & Ferguson, 1997b).

Angiotensin II (ANG) has been shown to influence a variety of neuroendocrine and autonomic functions (Ferguson et al., 1999; Ferguson & Washburn, 1998; Culman et al., 1995; Lenkei et al., 1994). ANG containing cell bodies, nerve terminals, and receptors have been localized in the PVN (Lenkei et al., 1998; Lenkei et al., 1994; Phillips et al., 1993a; Song et al., 1991; Lind et al., 1985a). Dawson and Krukoff recently demonstrated that systemic infusion of
ANG into PVN resulted not only in c-fos activation in neurons immunoreactive for AVP and OXY, but also neurons, which stained positive for AT₁ receptors and NADPH-d (Dawson et al., 1998). Magnocellular neurons of both the SON and PVN are known to contain nitric oxide synthase (NOS) (Miyagawa et al., 1994; Vincent & Hope, 1992; Nakamura et al., 1991) and our own recent studies have described a role for nitric oxide (NO) in synaptic transmission within PVN (Bains & Ferguson, 1997b). In addition, in vivo studies, indicate that microdialysis of NO into the PVN results in an increase in GABA release and a decrease in blood pressure (Horn et al., 1994).

We have utilized the whole cell patch clamp technique to characterize the actions of ANG on synaptic activity within the PVN. We report that application of ANG results in an increased frequency of IPSPs in magnocellular neurons as a consequence of the activation of a NO mediated GABAergic feedback loop in the PVN.

MATERIALS and METHODS

Slice Preparation

Experiments were performed using hypothalamic slices prepared as previously described (Li & Ferguson, 1996). Male, Sprague-Dawley rats (150-250g, Charles River, P.Q., Canada) were killed by decapitation; the brain was quickly removed from the skull and immersed in cold (1-4°C) artificial cerebrospinal fluid (aCSF). The brain was blocked and 400µm coronal sections were cut through the hypothalamus using a vibratome. Slices were hemisected,
trimmed into blocks containing PVN, and were incubated in oxygenated aCSF (95%O₂/5%CO₂) for at least 90 min. at room temperature. Prior to recording, the slice was transferred into an interface-type recording chamber and continuously perfused with aCSF at a rate of 1ml/min.

**Electrophysiology**

Electrophysiological experiments were performed using the whole-cell configuration of the patch-clamp technique to record from PVN neurons. Patch pipettes were pulled to a resistance of 5-7 MΩ and filled with the pipette solution described below. Signals were processed with an Axoclamp-2A amplifier. An Ag-AgCl electrode connected to the bath solution via a KCl-agar bridge served as reference. All signals were digitized using the CED 1401 plus interface and stored on computer for off-line analysis. Drugs were applied by switching the perfusion solution from aCSF to a solution containing the desired drug. IPSPs were quantified based on frequency and amplitude (>1mV) and shape (fast falling phase and slow decay) using Spike2 software (CED, Cambridge, UK). Each detected event was inspected visually to exclude obvious false IPSPs. Mean group values were compared with a Student’s paired “t” test. Dunnett’s multiple comparison test was utilized when multiple means were analyzed versus a control group following a one-way analysis of variance while a repeated measures ANOVA & Newman-Keuls multiple comparison test were employed to statistically compare multiple groups. Cumulative probability plots of IPSP amplitude and frequency were compared with the Kolmogorov-Smirnov test.
Solutions

The aCSF composition was (in mM): 124 NaCl, 2 KCl, 1.25 KH$_2$PO$_4$, 2 CaCl$_2$, 1.3 MgSO$_4$, 20 NaHCO$_3$, and 10 glucose. Osmolarity was maintained between 285 and 300 mOsm and pH between 7.3 and 7.4. The pipette solution contained (in mM): 140 Kgluconate, 0.1 CaCl$_2$, 2 MgCl$_2$, 1.1 EGTA, 10 HEPES, and 2 NaATP, and had a pH of 7.25 (adjusted with KOH if necessary), except in voltage clamp experiments where 140 mM KCl was substituted for Kgluconate. Intracellular calcium concentration (0.012 µM) calculated using WEBMAXC Standard (Maxchelator, Stanford University).

A stock solution of ANG (0.1 mM, Phoenix Pharmaceuticals) was prepared from which daily aliquots were made to the required concentration. Tetrodotoxin (TTX, Alamone Laboratories), was used to block voltage-activated Na$^+$ channels and was prepared daily from a stock solution (0.1 mM). L-NAME (Sigma) an inhibitor of nitric oxide synthase, kynurenic acid, a broad based glutamate receptor antagonist (Sigma), and bicuculline methiodide (BMI, Sigma) were prepared daily as required.

RESULTS

Whole-cell patch clamp recordings were obtained from a total of 35 electrophysiologically identified magnocellular PVN neurons. These cells all demonstrated a linear I/V relationship and a prominent outward rectification ($I_A$) when depolarized from hyperpolarized potentials (Fig. 2.1A). These neurons had a mean resting membrane potential (RMP) of $-59.7\pm1.3$mV, displayed action
potentials with a minimum spike amplitude of 60mV, and had a mean input resistance of 1200±53MΩ. Action potential and IPSP amplitude was measured throughout the duration of the recordings and no run-down was observed.

**ANG activates IPSPs in magnocellular neurons**

A total of 35 magnocellular neurons were tested for the effects of bath application of ANG using current clamp techniques. Following a control-recording period of at least 5 minutes, ANG was administered by bath perfusion in concentrations ranging from 0.01µM to 1µM for a period of 30 seconds. Neurons tested with ANG typically responded with a depolarization (6.2±0.7 mV; n=20, 66% of neurons tested), and peak effects were observed at a dose of 0.1µM (Fig. 2.1B, 0.1µM; 5.0±0.9 mV, n=16, p<0.001, unpaired t-test) which was therefore selected for the remainder of experiments. While changes in input resistance of cells influenced by ANG were variable (n=11; 5 showing increases, 3 decreases, and 3 no change) the group mean was not altered significantly by ANG application (-51±183 MΩ p>0.05, paired t-test).

In 75% (12/16) of these neurons the ANG induced depolarization was accompanied by an increase in IPSP frequency as illustrated in Figure 2.1B and 2.2A. This ANG induced increase in IPSPs showed a latency to onset ranging from 30s to 90s, and a duration of between 150-180 seconds in most neurons (Fig. 2.1B, 2.2B). The average increase in IPSPs for these 12 neurons (calculated in 30s bins, normalized to control period) following ANG application was 0-30s; 27.5±14.9%, 30-60s; 36.5±12.1%*, 60-90s; 31.4±14.1%*, 90-120s;
Figure 2.1 ANG increases IPSPs in magnocellular neurons. (A) The electrophysiological characteristics of a magnocellular neuron are illustrated here. Magnocellular neurons demonstrate a linear I/V relationship and a prominent outward rectification ($I_A$) when depolarized from hyperpolarized potentials. (B) The top whole cell current clamp recording depicts the response (-56mV resting membrane potential) of a magnocellular neuron to bath application of 0.1µM ANG (30s, application indicated by bar). 30pA hyperpolarizing current pulses are applied at 5 second intervals to evaluate the effects of ANG on input resistance in magnocellular neurons. ANG depolarizes these neurons concurrent with an increase in IPSPs as demonstrated in the lower traces, which have been expanded and labeled.
Figure 2.2 Frequency analysis of ANG induced increase of IPSPs in magnocellular neurons. (A) Current clamp recordings demonstrating the increase in frequency of IPSPs following application of 0.1µM ANG in a magnocellular neuron (B) Instantaneous rate frequency histogram demonstrating the increase of IPSP frequency of a single magnocellular neuron in response to application of 0.1µM ANG (application indicated by arrow). (C) Histogram depicting the increase in the IPSP frequency (as a %) following ANG versus control (each bin [30s, control and ANG] calculated as a percentage of the mean IPSP frequency [30s] of the control period) (ANG; 0-30s; 27.5±14.9%, 30-60s; 36.5±12.1%*, 60-90s; 31.4±14.1%*, 90-120s; 72.3±10.9%*, 120-150s; 42.9±12.8%*, * p<0.05, one way analysis of variance & Dunnett’s multiple comparison test) in the population of magnocellular neurons (n=12) which respond to 0.1µM ANG with an increase in IPSP frequency (application indicated by arrow).
72.3±10.9%*, 120-150s; 42.9±12.8%* (* p<0.05 compared to control, one way analysis of variance & Dunnett’s multiple comparison test) as illustrated in Figure 2.2C. The maximum increase in IPSPs (in a 30s bin, average of 12 neurons: 88.7±9.8% increase) occurred either 60-90s (n=3) or 90-120s (n=9) following ANG application, demonstrating a consistent time course of IPSP activation in these neurons. No significant receptor desensitization was observed as similar increases in IPSP frequency were observed in response to a second identical application of ANG (n=3).

While ANG increased the frequency of synaptic events the peptide was without effect on the amplitude of these synaptic events. As illustrated in the IPSP amplitude distributions shown in Figure 2.3, control magnitude of PSPs (light bars) was similar to that observed following 0.1µM ANG (dark bars - analyzed after ANG but prior to depolarization to avoid effects of increased driving force for Cl⁻). The amplitude distributions demonstrate that ANG is without effect on the amplitude of synaptic events observed. Similarly analysis of cumulative probability vs. amplitude plots also show ANG to be without effect on the amplitude of the observed IPSPs (n=12; p>0.05, Kolmogorov-Smirnov test). Cumulative probability/amplitude plots do however confirm that IPSPs measured during the depolarization induced by ANG are greater in amplitude (p<0.05, Kolmogorov-Smirnov test) than the IPSPs observed in the control periods presumably as a result of the increased driving force for Cl⁻ movement across the membrane (Data not shown). Application of BMI (10µM) abolished the increase
**Figure 2.3** ANG is without effect on the amplitude of the IPSPs. (A) Amplitude distributions of IPSPs before (light bars) and following (dark bars) application of 0.1µM ANG. The number of events falling into each amplitude bin in both distributions has been depicted as a percentage of the total number of events analyzed in the distribution (i.e. # of events of this magnitude/the total number of events recorded). The amplitude distributions demonstrate that ANG is without effect on the amplitude of synaptic events observed. (B) A cumulative probability vs. amplitude plot reveals ANG is without effect on the amplitude of the observed IPSPs (p<0.05, Kolmogorov-Smirnov test).
Figure 2.4  BMI abolishes the ANG induced IPSPs. (A) Current clamp trace demonstrating application of ANG (0.1µM) results in an increase in IPSP frequency in a magnocellular neuron. BMI (10µM) abolishes the IPSPs demonstrating their GABAergic nature. Subsequent application of ANG in the same neuron fails to elicit a similar increase in IPSP frequency in the presence of BMI.
in IPSPs in response to ANG (Fig. 2.4). Previous studies in our laboratory have also demonstrated the GABAergic nature of these IPSPs (inhibition in the presence of bicuculline) and the effect of membrane potential shifts on their amplitude (Bains & Ferguson, 1997b) supporting these observations.

**L-NAME abolishes ANG stimulated IPSPs in magnocellular neurons**

Previous studies showing that NMDA induced IPSPs in magnocellular neurons were NO dependent (Bains & Ferguson, 1997b) led us to investigate whether the ANG induced IPSPs were contingent on the production of NO. The increase in IPSP frequency in magnocellular neurons following application of ANG (145±8.8%, p<0.05 paired t-test vs. control) was abolished by prior administration of the non-selective NOS inhibitor L-NAME (L-NAME normalized to 100%; L-NAME+ANG; 82.3±9.7%, n=4) as illustrated in Fig. 2.5A,B. Interestingly, L-NAME also decreased the basal frequency of IPSPs suggesting a tonic role for such inputs in controlling magnocellular excitability but was without effect on the amplitude of the IPSPS. These observations suggest that NO plays a mandatory intermediary role causing the increased frequency of IPSPs in these neurons.

**IPSC amplitude and frequency are unchanged in TTX**

The synaptic mechanisms underlying the ANG mediated increase in IPSP frequency have not yet been identified. In order to establish whether the ANG/NO mediated increase in IPSP frequency was due to an action at the cell body or synaptic terminal of an inhibitory interneuron, voltage clamp recordings were obtained from an additional 6 magnocellular neurons of which 4 demonstrated clear statistically significant increases in IPSC frequency (in 10μM kynurenic acid
Figure 2.5  The ANG induced increase in IPSP frequency is dependent on the production of nitric oxide. (A) Current clamp recordings demonstrate that the increase in IPSPs in response to 0.1µM ANG can be abolished by pre-application of the nitric oxide synthase inhibitor, L-NAME (10µM). Note that the basal level of IPSPs is also decreased in L-NAME, suggesting nitric oxide plays a role in maintaining tonic inhibitory input in these neurons. (B) These bar graphs describe the effects of ANG on a population of magnocellular neurons (n=4) following nitric oxide synthase inhibition. The left graph demonstrates the increase and recovery of IPSP frequency following 0.1µM ANG application (145±8.8%, p<0.05 paired t-test, vs. control). The graph on the right demonstrates that following L-NAME application (10µM), ANG is without effect on IPSP frequency (L-NAME normalized to 100% [vs. recovery]; L-NAME+ANG; 82.3±9.7%, n=4) in the same population of neurons.
Figure 2.6 ANG is without effect on the frequency and amplitude of mini inhibitory synaptic currents. (A) Voltage clamp recordings showing that ANG (0.1µM) application results in an increase in the frequency of spontaneous IPSCs. Recordings are done at –60mV with a KCl (substituted for Kgluconate) electrode with 10µM kynurenic acid continuously bath applied to abolish EPSCs. Following application of TTX (0.1µM) however, miniature inhibitory synaptic activity was unaffected by ANG. (B,C) Bar charts summarizing effects of ANG on IPSC frequency (Fig. 6B) and amplitude (Fig. 6C). * p<0.01; repeated measures ANOVA & Newman-Keuls multiple comparison test. $ p>0.05; repeated measures ANOVA & Newman-Keuls multiple comparison test. # p>0.05; paired t-test).
- applied to block glutamate receptor mediated EPSCs) following application of 0.1μM ANG (n=4) as illustrated in Figure 2.6A,B (KA:1.0±0.1 vs. KA+Ang:1.8±0.3Hz; n=4, p<0.01 repeated measures ANOVA & Newman-Keuls multiple comparison test). In contrast, following application of TTX (0.1μM) to isolate mIPSCs, ANG was without effect on frequency (Fig. 2.6B KA,TTX 0.6±0.2Hz vs. KA,TTX+ANG 0.4±0.2Hz; n=4, p>0.05, repeated measures ANOVA & Newman-Keuls multiple comparison test), or amplitude (Fig. 2.6C TTX,KA 60.2±11.1pA vs. TTX,KA+ANG 44.8±7.8pA; n=4, p>0.05, paired t-test) of these remaining events, supporting the conclusion that NO evokes these effects at the cell body of an inhibitory interneuron.

**IPSPs modulate ANG responsiveness of magnocellular neurons**

It is important to note that not all of the magnocellular PVN neurons recorded in this study showed IPSPs. Those neurons that demonstrated spontaneous IPSPs did however have an attenuated response (Fig. 2.7D, 3.2±1.1mV, 0.1μM ANG, n=10) to ANG compared to those neurons that did not show tonic IPSPs (8.0±0.5mV, 0.1μM ANG, n=6, p<0.05 unpaired t-test). In addition, as illustrated in Figure 2.7, ANG induced depolarizations (0.1μM) in neurons in which this peptide caused increases in IPSP frequency were significantly enhanced in the presence of L-NAME (ANG - 2.0±0.7mV vs. L-NAME - 6.7±0.7mV, n=4, p<0.05; paired t-test). Consequently, magnocellular neurons were tested with ANG in the presence of BMI (10μM). Application of BMI not only abolished the IPSPs (Fig. 2.4) demonstrating the GABAergic nature of these events but also amplified the depolarization in response to ANG
Figure 2.7 The magnitude of the ANG induced change in membrane potential is dependent upon the presence of inhibitory synaptic input. (Ai) Current clamp recording depicting a magnocellular neuron (-55mV resting membrane potential) which showed a small response to bath application of ANG (0.1µM, application indicated by bar). Following application of L-NAME (10µM, application indicated by light gray extended bar), the response to ANG (0.1µM) in the same neuron is potentiated (-53mV resting membrane potential). Action potentials have been truncated. (Aii) The expanded traces demonstrate the high basal frequency of IPSPs observed in this neuron and the decrease in IPSP frequency in this neuron following L-NAME application. Note the lack of an increase in IPSP frequency before and following ANG application in this neuron in the presence of L-NAME. (B) Bar graph summarizing data demonstrating that neurons which respond to ANG with an increase in IPSPs show a reduced response to ANG (0.1µM) (3.2±1.1mV, 0.1µM ANG, n=10) compared to neurons with no IPSPs (8.0±0.5mV, 0.1µM ANG, n=6; p<0.05 t-test). (C) This graph illustrates that the change in membrane potential to ANG (2.0±0.7mV, 0.1µM ANG, n=4) is exacerbated in those neurons with a high degree of IPSPs following bath application of the NOS inhibitor L-NAME (6.7±0.7mV, 0.1µM ANG, n=4; p<0.05; t-test) and the subsequent removal of inhibitory input to these neurons. (D) ANG induced depolarization (1.9±1.2mV, 0.1µM ANG, n=5) is enhanced by application of BMI (10µM) which abolished GABAergic IPSPs (11.9±2.3mV, 0.1µM ANG, n=5; p<0.001; t-test).
(1.9±1.2mV vs. 11.9± 2.3mV, n=5, p<0.001; paired t-test, Fig. 2.7D). These data suggest a physiological correlate exists between these neurons, an intrinsic inhibitory feedback loop within the nucleus, and their level of excitation and subsequent output.

DISCUSSION

Despite the well-recognized importance of ANG in neuroendocrine regulation the mechanisms by which this peptide modulates the excitability of PVN neurons are not well understood. We show here that ANG, in addition to depolarizing magnocellular neurons, activates an inhibitory feedback system, which can reduce the magnitude of this excitation. The production of NO, presumably produced by depolarized NOS positive magnocellular neurons is essential to the activation of this inhibitory feedback loop (Miyagawa et al., 1994;Vincent & Hope, 1992;Nakamura et al., 1991). We suggest that NO acts on inhibitory interneurons to increase the frequency of inhibitory synaptic events in magnocellular neurons in a manner similar to that reported following NMDA or vasopressin receptor activation (Hermes et al., 2000;Bains & Ferguson, 1997b). These observations demonstrate that PVN possesses intrinsic circuitry that is capable of modulating the excitability of output neurons following multi-modally induced depolarization of magnocellular neurons.

Application of ANG results in the depolarization of magnocellular neurons without a significant change in input resistance. While the mechanisms which underlie the ANG induced depolarization remain unclear potential candidates include; an inhibition of $I_A$ (Wang et al., 1997;Li & Ferguson, 1996), activation of a
calcium current (Sumners et al., 1996) or non-selective cationic conductance (Yang et al., 1992), and the possibility of indirect contributions following activation of a glutamate interneuron in PVN (Daftary et al., 2000; Daftary et al., 1998b).

We have shown here that a sub-population of magnocellular neurons that were stimulated by ANG also demonstrated an increase in IPSP frequency. Intriguingly, we have previously reported that NO donors including SNAP and L-ARG increase the frequency of GABAergic PSPs in magnocellular neurons (Bains & Ferguson, 1997b), suggesting a potential role for NO as a mediator of ANG actions. Our observation that the ANG induced increase in IPSPs in magnocellular neurons can be abolished by administration of a NOS inhibitor supports this hypothesis. Evidence documenting NO’s role in facilitating acetylcholine, catecholamine, and neuroactive amino acid release (Kuriyama & Ohkuma, 1995) suggests a potential role in the presynaptic terminal, while data reporting direct effects of NO on putative GABA neurons within this nucleus supports the possibility that NO controls the excitability of these interneurons (Bains & Ferguson, 1997a). Our voltage clamp data suggest these effects are most likely the result of peptide induced effects on the firing frequency of GABAergic interneurons as while ANG increased the frequency of IPSCs it was without effect on the amplitude of spontaneous synaptic events. The location of these GABAergic interneurons is likely the perinuclear zone (Boudaba et al., 1996; Tasker & Dudek, 1993; Decavel & van den Pol, 1992; van den Pol, 1982). Their close proximity to PVN also makes them plausible targets for NO produced in the lateral magnocellular areas of PVN.
It is important to note that not all PVN magnocellular neurons recorded show IPSPs. Interestingly, those neurons showing IPSPs have an attenuated response to ANG (compared to cells not showing IPSPs). Furthermore, inhibition of inhibitory synaptic input with BMI or reduction of these events with L-NAME enhanced the depolarization observed in magnocellular neurons in response to ANG. In fact, in a number of magnocellular neurons application of BMI revealed an increase in EPSP frequency in response to ANG. These observations are in accordance with previous reports that NOS inhibition enhances the response of magnocellular neurons to NMDA receptor activation (Bains & Ferguson, 1997b). It remains unknown whether those neurons that demonstrate an increase in IPSP frequency will have an augmented response to ANG if the feedback loop is rendered non-functional (by GABA antagonists or NOS inhibitors) in vivo. This is of particular importance pathophysiologically where PVN has been implicated in the onset of both congestive heart failure and chronic hypertension (Patel & Zhang, 1996; Earle & Pittman, 1995; Eilam et al., 1994; Eilam et al., 1991; Ciriello et al., 1984).

PERSPECTIVES

The PVN is essential in the regulation of neuroendocrine and autonomic functions, including body fluid homeostasis and cardiovascular regulation. Neurons in the PVN must be able to monitor the body’s internal environment, integrate these messages, and send the appropriate signals to both other central nuclei and peripherally so that a constant environment is maintained. The negative feedback pathway we describe here represents an important circuit
whereby the regulation of neuronal excitability and thus the extent of neuropeptide release can be tightly controlled. Physiologically, this inhibitory system has been documented to moderate PVN output signals essential to the control of both cardiovascular and endocrine systems.

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Chapter 3: ANGIOTENSIN II INDUCED EXCITATION OF
PARAVENTRICULAR NUCLEUS MAGNOCELLULAR NEURONS: A ROLE
FOR GLUTAMATE INTERNEURONS

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ABSTRACT

The hypothalamic paraventricular nucleus (PVN) plays a critical role in cardiovascular and neuroendocrine regulation. Angiotensin II (ANG) acts throughout the periphery in the maintenance of fluid-electrolyte homeostasis and has also been demonstrated to act as a neurotransmitter in PVN exerting considerable influence on neuronal excitability in this nucleus. The mechanisms underlying the ANG mediated excitation of PVN magnocellular neurons have yet to be determined. We have used whole cell patch clamp techniques in hypothalamic slices to examine the effects of ANG on magnocellular neurons. Application of ANG resulted in a depolarization of magnocellular neurons, a response which was abolished in tetrodotoxin (TTX) suggesting an indirect mechanism of action. Interestingly, ANG also increased the frequency of EPSP/Cs in magnocellular neurons an effect which was abolished following application of the glutamate antagonist kynurenic acid. ANG was without effect on the amplitude of EPSCs suggesting a pre-synaptic action on an excitatory interneuron within PVN. The ANG induced depolarization was shown to be sensitive to kynurenic acid revealing the requisite role of glutamate in mediating the ANG induced excitation of magnocellular neurons. These observations indicate that the ANGergic excitation of magnocellular PVN neurons are dependent upon an increase in glutamatergic input and thus highlight the importance of a glutamate interneuron in mediating the effects of this neurotransmitter.
INTRODUCTION

The hypothalamic paraventricular nucleus (PVN) plays major obligatory roles in autonomic and neuroendocrine regulation. Functional studies have demonstrated the involvement of PVN in the control of fluid electrolyte homeostasis, feeding behavior, cardiovascular regulation, stress adaptation, and the milk ejection reflex (Ferguson & Washburn, 1998; Swanson & Sawchenko, 1980). PVN is a heterogeneous nucleus whose neurons can be differentiated based upon both morphological and electrophysiological criteria. Morphologically, PVN can be separated into the magnocellular neurons known to synthesize and secrete oxytocin (OXY) or vasopressin (AVP) into the circulation from terminals in the posterior pituitary, and the parvocellular neurons, which contain among others somatostatin, thyrotropin releasing hormone (TRH), \( \gamma \)-amino-butyric-acid (GABA), neurotensin, and corticotropin releasing hormone (CRH) (Swanson & Sawchenko, 1983; Sawchenko & Swanson, 1982; Swanson & Sawchenko, 1980). Electrophysiologically, PVN consists of Type I (magnocellular – OXY/AVP) and Type II (parvocellular) neurons, each of which has a unique electrical fingerprint (Luther & Tasker, 2000; Hoffman et al., 1991; Tasker & Dudek, 1991). Interestingly, recent reports by Daftary suggest the presence of a third previously unclassified glutamate interneuron in PVN thought to mediate noradrenergic input to PVN from the brainstem (Daftary et al., 2000; Daftary et al., 1998b) although this group of neurons has yet to be characterized.

The actions of numerous peptides and hormones in PVN have been well documented. Recently, more attention has been paid to the synaptic regulation of
magnocellular neurons along with the traditional membrane delimited mechanisms modulated by transmitters and other messengers. Angiotensin II (ANG) has been shown to influence a variety of neuroendocrine and autonomic functions (Ferguson et al., 1999; Ferguson & Washburn, 1998; Culman et al., 1995; Lenkei et al., 1994). ANG containing cell bodies, nerve terminals, and receptors have been well documented in the PVN (Lenkei et al., 1998; Lenkei et al., 1994; Phillips et al., 1993a; Song et al., 1991; Lind et al., 1985a). Early autoradiographic reports by Mendelsohn and Gehlert demonstrated significant displaceable ANG binding in PVN, which following the development of antagonists specific for AT₁ and AT₂ receptors was shown to be AT₁ specific (Gehlert et al., 1991; Gehlert et al., 1986; Mendelsohn et al., 1984b). Functional studies then demonstrated that direct administration of ANG into PVN resulted in, the excitation of neurosecretory cells identified as having projections to the posterior pituitary, increases in plasma AVP concentration, and an increase in blood pressure (Ferguson & Wall, 1992; Shoji et al., 1989). Furthermore, blockade of the AT₁ receptor, by a specific antagonist (losartan) was shown to abolish these physiological actions of locally administered ANG (Kirby et al., 1992), and of postsynaptic effect of SFO stimulation induced ANG release (Li & Ferguson, 1993b).

In vitro extracellular single unit recordings showed that bath application of ANG dose dependently increased the firing rate of neurons in PVN (Li & Ferguson, 1993a). This effect was maintained in synaptic isolation (low Ca²⁺/high Mg²⁺) and blocked by losartan indicating the response was AT₁ receptor.
mediated. Using whole cell voltage clamp recordings, Li and Ferguson later demonstrated that ANG inhibited the transient outward potassium current \( I_{\text{A}} \), an effect which was also shown to be \( \text{AT}_1 \) receptor mediated (Li & Ferguson, 1996). However, there is to date no direct evidence explaining the specific mechanisms underlying the depolarizing effects of ANG on magnocellular neurons.

Recent biochemical reports demonstrating the apparent lack of \( \text{AT}_1 \) receptors in the magnocellular region of PVN have provided an interesting and important caveat to these suggestions (Lenkei et al., 1998; Lenkei et al., 1994). ANG receptors have been observed however in the parvocellular regions of PVN and Oldfield et al. demonstrate that the \( \text{AT}_1 \) receptor coexists strongly with neurons in the anterior parvocellular area of the nucleus which direct axons to the median eminence (Stern et al., 2003). Intriguingly, it has also been shown that while ANG activates PVN magnocellular neurons, there is also a population of unidentified neurons, which are influenced by activation of angiotensinergic pathways originating in SFO as well as by ANG itself (Li & Ferguson, 1993b). It is possible that these unidentified ANG-responsive neurons in PVN may be a subset of parvocellular interneurons within PVN, a suggestion supported by recent evidence suggesting the presence of a glutamatergic interneuron within PVN (Daftary et al., 2000; Csaki et al., 2000; Daftary et al., 1998b; Boudaba et al., 1997).

We have utilized the whole cell patch clamp technique to characterize the actions of ANG on magnocellular neurons in PVN slices. We report here that bath application of ANG results in the excitation of magnocellular neurons by
both direct and synaptic mechanisms, the latter occurring as a consequence of enhanced glutamatergic input to these neurons.

METHODS and MATERIALS

Slice Preparation

Experiments were performed using hypothalamic slices prepared as previously described (Li & Ferguson, 1996). Briefly, Male Sprague-Dawley rats (150-250g, Charles River, P.Q., Canada) were killed by decapitation, and the brain was quickly removed from the skull and immersed in cold (1-4°C) artificial cerebrospinal fluid (aCSF). The brain was blocked and 400 μM coronal sections were cut through the hypothalamus using a Vibratome. Sections were hemisected, trimmed into blocks containing PVN, and were incubated in oxygenated aCSF (95%O₂/5%CO₂) for at least 90 min. at room temperature. Prior to recording, the slice was transferred into an interface-type recording chamber and continuously perfused with aCSF at a rate of 1ml/min. All procedures were carried out in accordance with the guidelines of the CCAC and were approved by the Queen’s University Animal Care Committee.

Electrophysiology

Electrophysiological experiments were performed using the whole-cell configuration of the patch-clamp technique to record from PVN neurons. Patch pipettes were pulled to a resistance of 5-7 MΩ and filled with the pipette solution described below. Seal resistance was at least 1GΩ and as large as 10GΩ. Signals were processed with an Axoclamp-2A amplifier. An Ag-AgCl electrode connected to the bath solution via a KCl-agar bridge served as reference. All
signals were digitized (5000Hz) using the C.E.D. 1401 plus interface and stored on computer for off-line analysis. Drugs were applied by switching the perfusion solution from aCSF to a solution containing the desired drug. EPSPs were quantified based on frequency and amplitude (>1mV) and shape (fast rising phase and slow decay) using Spike2 software (CED, Cambridge, UK). Similarly, EPSCs were quantified based on frequency and amplitude (>5pA) and shape (fast falling phase and slow decay). Each detected event was inspected visually to exclude obvious false EPSP/Cs. Mean group values were compared with a paired Student’s “t” test. Dunnett’s multiple comparison test was utilized when multiple means were analyzed versus a control group following a one-way analysis of variance while a repeated measures ANOVA & Newman-Keuls multiple comparison test were employed to statistically compare multiple groups. Cumulative probability plots of EPSC amplitude were compared with the Kolmogorov-Smirnov test.

**Solutions**

The aCSF composition was (in mM): 124 NaCl, 2 KCl, 1.25 KPO₄, 2 CaCl₂, 1.3 MgSO₄, 20 NaHCO₃, and 10 glucose. Osmolarity was maintained between 285 and 300 mOsm and pH between 7.3 and 7.4. The pipette solution contained (in mM): 140 Kgluconate, 0.1 CaCl₂, 2 MgCl₂, 1.1 EGTA, 10 HEPES, and 2 NaATP, and had a pH of 7.25 (adjusted with KOH if necessary).

A stock solution of ANG (Phoenix Pharmaceuticals, California) was prepared from which daily aliquots were made to the required dilution. Tetrodotoxin (TTX, Alamone Laboratories, Israel) and kynurenic acid (KA, Tocris-
Cookson, UK) were prepared daily from stock solutions for those experiments where it was necessary to block voltage-activated Na\(^+\) channels or antagonize NMDA (N-methyl-D-aspartate) and non-NMDA receptors respectively.

**RESULTS**

Whole-cell patch clamp recordings were obtained from a total of 60 PVN neurons which displayed a prominent ‘A current’ following hyperpolarizing current pulses, had a linear I/V relationship, and therefore were classified as magnocellular neurons (Fig. 3.1A). These neurons had a mean resting membrane potential (RMP) of -57.7±1.5mV (mean±SEM), displayed action potentials with a minimum spike amplitude of 60mV, and had a mean input resistance of 1100±80M\(\Omega\) (mean±SEM). Action potential and EPSP amplitude were measured throughout the duration of the recordings and no run-down was observed.

**ANG Effects on Magnocellular Neurons**

**Membrane Potential**

A total of 47 magnocellular neurons were tested for the effects of bath application of ANG in the current clamp configuration. Following a control recording period of at least 5 minutes, ANG was administered by bath perfusion in concentrations ranging from 0.01\(\mu\)M to 10\(\mu\)M for a period of 30 seconds. The majority of the neurons tested (n=35, 74%), responded to ANG with a depolarization concurrent with an increase in action potential firing frequency as illustrated in Figure 3.1B,C. The remaining neurons tested with ANG either
Figure 3.1  ANG depolarizes magnocellular neurons. (A) The electrophysiological characteristics of a magnocellular neuron are illustrated here. Magnocellular neurons demonstrate a linear I/V relationship and a prominent outward rectification ($I_h$) when depolarized from hyperpolarized potentials. (B) Bar graph summarizing the responses of magnocellular neurons to ANG application. 74% of magnocellular neurons responded to ANG with a depolarization, while 5% hyperpolarized and 21% were unaffected. (Ci-iii) Whole cell current clamp recordings illustrating the response of three magnocellular neurons to bath application of $10\mu$M, $0.1\mu$M, $0.01\mu$M ANG respectively (30s, application indicated by bar). 20pA hyperpolarizing current pulses are applied at 5 second intervals to evaluate the effects of ANG on input resistance. Inset shows that the depolarization of these neurons mediated by ANG are dose dependent.
hyperpolarized (5%, n=2) or were unaffected (21%, n=10) showing no clear change in membrane potential (Fig. 3.1B). The depolarizing response of these magnocellular neurons to ANG was found to be dose dependent (10.0µM; 11.8±4.2mV n=5, 1.0µM; 9.1±0.9mV n=10, 0.1µM; 6.9±0.6mV n=16, 0.01µM; 1.4±0.8mV n=4) and demonstrated a sigmoidal dose response relationship with an estimated EC$_{50}$ of 5.6x10$^{-8}$. In some cases following recovery of the neuron to resting RMP, ANG application was repeated (n=4) and a second depolarization was observed indicating that magnocellular neurons do not show significant desensitization to ANG. The depolarization observed in the magnocellular neurons was also accompanied by a change in input resistance, which was measured as the maximum voltage change to a hyperpolarizing current pulse. To eliminate the possibility that the depolarization of the neuron and subsequent activation of voltage sensitive channels rather than ANG was responsible for the observed change in input resistance current injection was used to hyperpolarize the membrane potential to RMP following the ANG induced depolarization and the input resistance was measured at this potential. Although relatively large changes in input resistance were observed these effects were not consistent with 5 of 8 cells tested showing a decreased input resistance (-410±81MΩ) in response to ANG while in 3 there was an increase (547±80MΩ). The mean change in input resistance for all of the neurons was not however significantly altered by ANG application (-51.3±183.5, p<0.05, paired t-test).
Figure 3.2  ANG mediated depolarization of magnocellular neurons is significantly reduced by TTX. (Ai) Current clamp recording illustrating that the ANG (0.1µM, 30s, application indicated by bar) induced depolarization of a magnocellular neuron is significantly reduced by pretreatment with 1.0µM TTX (application indicated by light gray extended bar). (Aii) Bar graph summarizes the effects of ANG on magnocellular neurons following application of TTX. ANG also inhibits $I_A$ in magnocellular neurons which is maintained in TTX. (Bi) These current clamp traces illustrate that $I_A$ (elicited by applying a hyperpolarizing current pulse sufficient to deinactivate this conductance) which is observed as a delay in the return to baseline membrane potential following a hyperpolarizing current pulse is inhibited by 0.1µM ANG an effect which was maintained during bath perfusion with TTX and summarized in the bar graphs presented.
Depolarizing Effects of ANG on Magnocellular Neurons are Significantly Reduced in TTX

ANG effects on magnocellular neurons were also examined during sodium channel block with TTX (1.0µM) in order to determine whether these were direct effects. Figure 3.2Ai-ii. illustrates that the depolarizing effect of ANG (0.1µM) was significantly reduced following bath application of TTX (ANG 0.1µM; 7.0±0.5mV, TTX/ANG; 2.1±0.6mV, n=6, p<0.05, paired t-test). These data suggest that the action of ANG on magnocellular PVN neurons results from actions at a second subpopulation of PVN neurons, a surprising observation in view of previous voltage clamp studies reporting that actions of ANG on voltage gated ion channels (I_A) in magnocellular PVN neurons are maintained in synaptic isolation (Li & Ferguson, 1996). This observation led us to reexamine whether the effects of ANG on I_A in magnocellular neurons were observed in TTX. Indirect assessment of I_A was carried out using current clamp techniques to measure the delay in return to baseline membrane potential following a standard hyperpolarizing current pulse (Fig. 3.2Bi-ii). ANG inhibited I_A as measured by the return to baseline (Control 1.1±0.1s vs. ANG 0.5±0.1, n=11, p<0.001, paired t-test) an effect which was maintained during bath perfusion with TTX (Control 1.3±0.1s vs. ANG/TTX 0.3±0.1s, n=6, p<0.001, paired t-test). I_A was further assessed by measuring the delay to first spike during a depolarizing current pulse from a hyperpolarized membrane potential. Such measurements also indicated ANG induced reduction in I_A with a decrease in the time to first spike being observed as reported previously from our laboratory (Bains et al., 2001).
Figure 3.3  ANG mediates an increase in EPSPs in magnocellular neurons. (A) Current clamp trace demonstrating that ANG (0.1µM, application indicated by bar, 30s) application results in a depolarization of a magnocellular neuron which is accompanied by an increase in EPSP frequency which is illustrated in the expanded traces below. (B) Histogram demonstrating the increase in EPSP frequency of a single magnocellular neuron in response to application of 0.1µM ANG (application indicated by bar, 30s). (C) The mean change in EPSP frequency in the population of magnocellular neurons tested is summarized in this bar graph.
These data are in agreement with previous voltage clamp experiments showing ANG effects on $I_A$ in magnocellular neurons in PVN (Li & Ferguson, 1996) and SON (Nagatomo et al., 1995).

**ANG Increases EPSP/Cs in Magnocellular Neurons**

Anatomical studies have confirmed the presence of glutamatergic interneurons within the PVN (Csaki et al., 2000), while electrophysiological recordings have identified a physiological role for such intranuclear connections in mediating noradrenergic input to PVN from the brainstem (Daftary et al., 2000; Daftary et al., 1998b). Our unexpected observation that depolarizing effects of ANG were abolished in TTX led us to focus on the potential role of other synaptic inputs to magnocellular neurons in mediating these effects. We first carried out a retrospective analysis of excitatory postsynaptic potentials (EPSPs) in magnocellular neurons which we had previously tested with ANG in these studies. Of these neurons, 63% (n=22) were found to have spontaneous EPSPs. ANG application (0.1µM) resulted in a significant increase in EPSP frequency in a population of these magnocellular neurons (n=10) as demonstrated in Figure 3.3A,B. The mean change in EPSP frequency is summarized in Figure 3.3C (Control; 2.2±0.5 vs. ANG; 3.3±0.6Hz n=10, p<0.005, paired t-test). In addition, when the EPSP frequency control data is normalized to 100% and compared to ANG we observe a 62.4±22.0% (n=10, p<0.05, t-test) increase in EPSP frequency. In the remaining magnocellular neurons (n=12) we were unable to quantify an increase in EPSP frequency as a result of the commensurate increase in action potential frequency following depolarization. In order to
eliminate the effects of action potential frequency or the change in membrane potential which would modify the amplitude of the observed events, these synaptic events were next analyzed in a separate population of magnocellular neurons using voltage clamp techniques (Fig. 3.4). ANG was seen to cause significant increases in the frequency of EPSCs (Control; 2.3±0.5 vs. ANG; 3.7±0.9Hz n=6, p<0.005, paired t-test). As with the EPSP data when the EPSC frequency control data is normalized to 100% and compared to ANG we observe a 60.5±13.0% (n=6, p<0.05, t-test) increase in EPSC frequency. The amplitude of the EPSCs as assessed by both cumulative amplitude distributions (n=6; p>0.05, Kolmogorov-Smirnov test) or assessment of mean amplitude (Control; 44.0±2.41pA vs. ANG; 43.8±0.5pA, p>0.05, t-test, Fig. 3.4D) was unaffected (Fig. 3.4D inset) in response to ANG application.

**ANG Induced Increase in Synaptic Activity is mediated by Glutamate**

In order to determine the neurotransmitter responsible for mediating the increase in synaptic events observed following ANG application on magnocellular neurons in PVN, additional experiments were performed utilizing the non-specific glutamate antagonist kynurenic acid (KA). Figure 3.5 clearly shows that pretreatment with KA (10µM) completely abolished both spontaneous EPSCs (n=5) and the increase in EPSC frequency in magnocellular neurons following ANG (0.1µM, n=3) administration. As predicted from previous studies (Daftary *et al.*, 1998b; Marrion, 1997) such observations demonstrate that the EPSCs are
Figure 3.4 ANG also influences EPSCs. (A) Voltage clamp traces demonstrating that ANG increases the frequency of EPSCs without augmenting the amplitude of these events (cells held at -60mV). (B) Histogram demonstrating the increase of EPSC frequency of a single magnocellular neuron in response to application of 0.1µM ANG (application indicated by bar, 30s). (C) The mean change in EPSC frequency in the population of magnocellular neurons tested is summarized in this bar graph. (D) The amplitude of these events assessed by both cumulative amplitude distributions (n=6; p>0.05, Kolmogorov-Smirnov test) or assessment of mean amplitude (INSET) is found to be unaffected.
Figure 3.5  Kynurenic acid abolishes the ANG induced increase in EPSC frequency. (A) Voltage clamp trace demonstrating that application of ANG (0.1μM) results in an increase in EPSC frequency in a magnocellular neuron (cells held at -60mV). Treatment of this neuron with KA (10μM) abolishes spontaneous EPSCs demonstrating their glutamatergic nature (n=5). Subsequent application of ANG in the same neuron fails to elicit a similar increase in EPSC frequency in the presence of KA (n=3).
glutamatergic and in addition provide supplementary support for the existence of a glutamate interneuron mediating the excitatory effects of ANG in PVN.

**Depolarizing Effects of ANG on Magnocellular Neurons are Abolished by Kynurenic Acid.**

The observations above raised the intriguing possibility that the ANG induced depolarization of magnocellular neurons is mediated by glutamate and therefore should be abolished by glutamate antagonists. This hypothesis was tested in experiments where KA (10 µM) was bath applied prior to the application of ANG (0.1 µM) to examine the contribution of the increase in EPSPs to the observed depolarization (Fig. 3.6). Such pretreatment of slices abolished the increase in excitatory events and also significantly reduced the depolarization induced by ANG (ANG; 8.7±1.3 mV vs. KA; 0.80±0.4 mV, n=6, p<0.001, paired t-test, Fig. 3.6B). These observations support the requisite role of glutamatergic input to magnocellular neurons in their response to ANG.

**DISCUSSION**

Despite the numerous studies identifying physiological roles for ANG in PVN (For review see: (Ferguson et al., 2001; Ferguson et al., 1999; Ferguson & Washburn, 1998; Phillips & Sumners, 1998), the cellular mechanisms through which this peptide depolarizes magnocellular neurons are still poorly understood. In the present study, using whole-cell patch clamp recording techniques, we show that while the majority of magnocellular neurons do respond to ANG with dose-dependent depolarizations, these effects are abolished in TTX suggesting
Figure 3.6  ANG mediated depolarization of magnocellular neurons is abolished by KA. (A) Current clamp recording illustrating a magnocellular neurons which depolarizes in response to bath application of ANG (application indicated by bar, 30s). Following KA (10µM, indicated by extended light bar) treatment subsequent applications of ANG fail to depolarize the neuron. (Aii). Expanded traces of this recording demonstrate the increase in EPSP frequency following ANG application. KA application abolishes spontaneous EPSPs and the increase in EPSP frequency to ANG application. (B) Bar graph summarizing the effects of KA on the ANG mediated depolarization of these neurons which shows KA significantly reduced the depolarization mediated by ANG.
they result from direct actions of ANG on a separate subpopulation of neurons in our slice preparation. Furthermore, we have demonstrated that such depolarizing drive to PVN magnocellular neurons appears to be the result of increased excitatory glutamatergic input which presumably results from ANG actions increasing the activity of glutamate interneurons within the PVN.

Previous studies have reported that ANG inhibits the transient outward potassium current, I_A, of anatomically identified magnocellular neurons using whole cell voltage clamp techniques to record from these neurons in hypothalamic brain slice preparations (Li & Ferguson, 1996). Li and Ferguson demonstrated that the effect of ANG on I_A was maintained in low Ca^{2+} indicating that inhibition of this current was a direct effect of ANG on the magnocellular neurons rather than being synaptically mediated (Li & Ferguson, 1996). Losartan, an AT_1 receptor antagonist, abolished the ANG inhibition of I_A verifying the receptor-mediated nature of the response. Our current clamp data support this conclusion by demonstrating that I_A is inhibited in magnocellular neurons by bath administration of ANG, an effect maintained in the presence of TTX. However, while this inhibition of I_A (which is known to play an important regulatory role in neuronal burst firing (Connor & Stevens, 1971)) may underlie the increases in spike frequency observed in magnocellular neurons following AT_1 receptor activation, it is unlikely to be responsible for the significant depolarizations observed in these neurons, which can occur even in quiescent cells.

The dissociation of these two effects is further emphasized by our unexpected observation that bath application of TTX, in contrast to its effects on
I_A, significantly reduced the depolarizing response of the magnocellular neurons to ANG. The elimination of the ANG mediated depolarization in magnocellular neurons would suggest that this action of ANG results from an indirect component which requires synaptic input. These data on the magnocellular neurons suggest that the ANG induced depolarization of the magnocellular neurons is the result of this peptide’s actions on a secondary group of neurons either within or in the area immediately surrounding PVN (i.e. included in our slice preparation).

PVN is characteristically thought of as a heterogeneous nucleus composed of magnocellular and parvocellular neurosecretory neurons, and preautonomic neurons (Swanson & Sawchenko, 1983; Swanson & Sawchenko, 1980). Historically, the existence of excitatory interneurons had been postulated but only recently has this hypothesis been substantiated. The presence of short latency EPSPs was discovered in the neurohypophysial system as early as 1973 (Raffaele et al., 2000). Ferguson et al. later observed that a large population of PVN neurons (almost 40% of those recorded) exists which does not project to posterior pituitary, median eminence, or dorsomedial medulla, again providing circumstantial evidence for interneurons within the PVN (Ferguson et al., 1984b). Intriguingly, many of these cells were also excited by activation of SFO efferents, a synaptic effect which in some cases was abolished by losartan (Li & Ferguson, 1993b). Most recently, using radiolabeled aspartate as a neuronal marker for glutamatergic interneurons Csaki et al. provided histochemical data demonstrating that glutamatergic interneurons exist within PVN and
glutamatergic fibers that project to PVN are scattered throughout other hypothalamic nuclei and the telencephalon (Csaki et al., 2000).

Boudaba et al. provided the first evidence of the existence of excitatory synaptic circuits in PVN and SON demonstrating that electrical or chemical stimulation of the dorsomedial hypothalamus or perifornical region resulted in evoked EPSPs in these nuclei (Boudaba et al., 1997). Daftary et al. later demonstrated that noradrenergic (NA) excitation of magnocellular neurons was effectively abolished by glutamate antagonists and TTX sensitive suggesting this effect is mediated by an increase in glutamatergic input (Daftary et al., 2000; Daftary et al., 1998b). In addition, they observed that the NA mediated increase in EPSP frequency was maintained in a surgically isolated PVN preparation and also with NA microdrop application directly into PVN indicating that the source of the EPSPs was a glutamatergic interneuron located within PVN. This suggestion gained recent support from histochemical studies demonstrating glutamatergic interneurons within PVN (Csaki et al., 2000), although to date there are no reports in which chemical phenotyping of these cells have been combined with patch clamp techniques to allow recordings to be obtained from this specific subset of PVN neurons.

We demonstrate here that these glutamatergic interneurons are also apparently responsible for the depolarization of the magnocellular neurons following ANG application. The observation that the ANG induced depolarization in magnocellular neurons is TTX sensitive and therefore synaptically mediated suggested an alternate explanation for this peptide’s depolarizing effect on PVN
magnocellular neurons. Interestingly, the majority of the magnocellular neurons which responded to ANG also displayed a significant increase in the frequency of EPSPs suggesting glutamatergic input played an important role in the response of these neurons to ANG. However, we were unable to quantify an increase in EPSP frequency in several of the magnocellular neurons depolarized by ANG as the large increase in action potential frequency concurrent with depolarization made it impossible to accurately count EPSPs which in many cases likely drive these action potentials. We thus also evaluated the ANG effects using voltage clamp techniques which avoid this problem and observed ANG induced increases in EPSC frequency in the majority of magnocellular neurons tested. In addition, ANG was without effect on the amplitude of these EPSCs suggesting that the effect of ANG is not the result of peptidergic effects on the nerve terminal rather that this effect of ANG is likely the result of actions at the cell body of these glutamate interneurons. Subsequent experiments where the increase in EPSC frequency was abolished following application of the glutamate antagonist kynurenic acid demonstrate the glutamatergic nature of these synaptic events. Finally, we tested our conclusion that glutamate inputs play an essential role in ANG signaling in PVN by examining the ability of glutamate antagonists to influence the depolarizing effects of ANG in magnocellular neurons. As predicted the depolarization of magnocellular neurons in response to ANG was found to be inhibited by application of kynurenic acid adding further support to the conclusion that glutamate interneurons play an essential prerequisite role in mediating ANG induced depolarization of PVN magnocellular neurons.
Interestingly, Li *et al.* demonstrated that ANG stimulates non-neurosecreatory spinally projecting parvocellular PVN neurons by attenuating GABAergic synaptic inputs through activation and presynaptic AT₁ receptors (Stern *et al.*, 2002). Although the presynaptic disinhibition of parvocellular neurons by ANG is an intriguing observation it does not seem a likely candidate to play a major role in modulating the excitation of magnocellular neurons via ANG. In fact, we have previously shown that ANG causes a nitric oxide dependent increase in the frequency of inhibitory synaptic activity in magnocellular neurons which appears to influence the excitability of these neurons (Stern, 2001).

**CONCLUSIONS**

The emergent notion of ANG’s action as a neurotransmitter in the CNS has led to a variety of studies aimed at characterizing its actions electrophysiologically. We provide evidence here that ANG not only excites magnocellular neurons in PVN, a nucleus regarded for its critical role in neuroendocrine regulation but does so via an increase in excitatory input to these neurons. These observations would also explain the excitation of magnocellular neurons by ANG in extracellular recordings given the apparent lack of AT₁ receptors in mRNA *in situ* hybridization studies. Future studies will attempt to describe the ionic conductances, which underlie the observed depolarization and a more detailed electrophysiological characterization of the glutamate interneuron and its response to ANG.
ACKNOWLEDGEMENTS

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Chapter 4: ANGIOTENSIN DEPOLARIZES PARVOCELLULAR NEURONS IN PARAVENTRICULAR NUCLEUS THROUGH MODULATION OF PUTATIVE NONSELECTIVE CATIONIC AND POTASSIUM CONDUCTANCES

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ABSTRACT

Neurosecretory parvocellular neurons in the hypothalamic paraventricular nucleus (PVN) exercise considerable influence over the adenohypophysis and thus play a critical role in neuroendocrine regulation. Angiotensin II (ANG) has been demonstrated to act as a neurotransmitter in PVN exerting significant impact on neuronal excitability and also influencing CRH secretion from the median eminence and therefore release of ACTH from the pituitary. We have used whole cell patch clamp techniques in hypothalamic slices to examine the effects of ANG on the excitability of neurosecretory parvocellular neurons. ANG application resulted in a dose-dependent depolarization of neurosecretory neurons, a response which was maintained in tetrodotoxin (TTX) suggesting a direct mechanism of action. The depolarizing actions of this peptide were abolished by losartan demonstrating these effects to be AT₁ receptor mediated. Voltage-clamp analysis using slow voltage ramps revealed that ANG activates a voltage independent conductance with a reversal potential of -37.8±3.8mV suggesting effects on a non-selective cationic current. Further, a sustained potassium current characteristic of I_K was significantly reduced (29.1±4.7%) by ANG. These studies identify multiple post-synaptic modulatory sites through which ANG can influence the excitability of neurosecretory parvocellular PVN neurons and, as a consequence of such actions, control hormonal secretion from the anterior pituitary.
INTRODUCTION

The paraventricular nucleus of the hypothalamus (PVN) plays essential roles in neuroendocrine and autonomic regulation (Swanson & Sawchenko, 1980). Extensive anatomical analysis has resulted in a thorough classification of this nucleus’ architecture resulting in the traditional view of PVN as a nucleus consisting predominantly of magnocellular and parvocellular neurons. Magnocellular neuroendocrine cells are recognized for their role in the production, storage, and secretion of the peptide hormones vasopressin and oxytocin (Swanson & Sawchenko, 1980). Parvocellular neurons on the other hand consist of two discrete subdivisions, pre-autonomic cells (non-neurosecretory) which play integral roles in the control of autonomic output through descending connections to the medulla and spinal cord, and neuroendocrine (neurosecretory) cells which are involved in the regulation of the anterior pituitary via projections to the median eminence (Swanson & Sawchenko, 1980).

For over a decade, the electrophysiological properties of these neurons have been utilized as an experimental tool to permit differential analysis of these subpopulations of neurons (Hoffman et al., 1991; Tasker & Dudek, 1991). While the description of magnocellular neurons has remained relatively unchanged, considerable debate has emerged concerning the electrical profiles of the neurosecretory and non-neurosecretory parvocellular subdivisions. Luther et al. demonstrate that the electrical criterion traditionally cited as being characteristic with parvocellular neurons is consistent with pre-autonomic parvocellular neurons.
projecting to the spinal cord (Luther et al., 2002). Specifically, non-neurosecretory neurons demonstrate a low threshold depolarization (typically generating one or two action potentials) following a hyperpolarizing current pulse consistent with the generation of a prominent low threshold spike and a robust T-type calcium current. In contrast, neurons which project to the median eminence have a separate electrophysiological identity characterized by lack of prominent LTS, and a relatively small T-type calcium current. In accordance with this view, Stern observed that neurons in PVN retrogradely labeled from pre-autonomic nuclei were found to express low threshold spikes and an inwardly rectifying I-V relationship as described above (Stern, 2001). Additionally, Cui et al. demonstrate that paraventricular neurons containing a retrograde label transported from the thoracic (T(1)-T(4)) intermediolateral column displayed a hyperpolarization-activated inward rectification and a LTS (Cui et al., 2001) consistent with the pre-autonomic neurons characterized by Stern. As a consequence of these observations the traditional models of PVN input/output and the regulation of its primary cell types by both classical neurotransmitters and neuropeptides have been modified in order to reflect the increasingly recognized heterogeneity of this nucleus.

Angiotensin II (ANG), a hormone traditionally recognized for its peripheral endocrine roles in the regulation of vascular resistance and control of fluid electrolyte homeostasis has been suggested to act as a neurotransmitter regulating the excitability of PVN neurons. Angiotensinergic fibers, cell bodies, and receptors have been reported in PVN (Lenkei et al., 1998;Lenkei et al.,
1994; Phillips et al., 1993a; Song et al., 1991; Lind et al., 1985a), and ANG has been shown to influence a variety of neuroendocrine and autonomic functions (Ferguson et al., 1999; Ferguson & Washburn, 1998; Culman et al., 1995; Lenkei et al., 1994). Early reports have focused primarily on ANG actions on magnocellular neurons (Li & Ferguson, 1996; Li & Ferguson, 1993a; Ferguson & Wall, 1992; Shoji et al., 1989). Interestingly, Lenkei et al. observed a high level of AT₁ receptor mRNA expression within the parvocellular areas of PVN and the surrounding periventricular area, but no expression in the magnocellular PVN (Lenkei et al., 1998; Lenkei et al., 1997; Lenkei et al., 1994).

Intriguingly, Oldfield et al. demonstrate that the distribution of AT₁ receptor correlates strongly with neurons in the anterior parvocellular division of PVN which direct axons to the median eminence (Oldfield et al., 2001). In addition, ICV injection of ANG stimulates ACTH release (Ganong & Murakami, 1987; Spinedi & Rodriguez, 1986) not a surprising discovery in view of the observation that AT₁ receptor mRNA is localized in CRH containing neurons (Aguilera et al., 1995). Indeed, activation of known ANG positive SFO efferents to PVN results in an elevation of hypophysial-portal plasma irCRF levels and increased circulating ACTH (Plotsky et al., 1988). The ionic mechanisms by which ANG mediates its effects upon parvocellular neurosecretory neurons however remain unresolved.

We have utilized the whole cell patch clamp technique to characterize the actions of ANG on neurosecretory parvocellular neurons. We report that application of ANG results in an excitation of these neurons which is mediated
directly by AT₁ receptors. Moreover, the excitation is likely the result of the activation of a non-selective cationic conductance and/or the inhibition of the delayed rectifier potassium current.

**MATERIALS and METHODS**

**Slice Preparation**

Experiments were performed using hypothalamic slices prepared as previously described (Li & Ferguson, 1996). Briefly, Male Sprague-Dawley rats (150-250g, Charles River, P.Q., Canada) were killed by decapitation, and the brain was quickly removed from the skull and immersed in cold (1-4°C) artificial cerebrospinal fluid (aCSF). The brain was blocked and 400µM coronal sections were cut through the hypothalamus using a Vibratome. Sections were hemisected, trimmed into blocks containing PVN, and were incubated in oxygenated aCSF (95%O₂/5%CO₂) for at least 90 min. at room temperature. Prior to recording, the slice was transferred into an interface-type recording chamber and continuously perfused with aCSF at a rate of 1ml/min at room temperature.

**Electrophysiology**

Electrophysiological experiments were performed using the whole-cell configuration of the patch-clamp technique to record from PVN neurons. Patch pipettes were pulled to a resistance of 5-7 MΩ and filled with the pipette solution described below. Seal resistance was at least 1GΩ and as large as 10GΩ. Signals were processed with an Axoclamp-2A amplifier. An Ag-AgCl electrode connected to the bath solution via a KCl-agar bridge served as reference. All
signals were digitized using the C.E.D. 1401 plus interface and stored on computer for off-line analysis. Drugs were applied by switching the perfusion solution from aCSF to a solution containing the desired drug. Mean group values were compared with a Student’s paired “t” test for those neurons where comparisons of two values were obtained in the same cell. Mean group values were compared with an unpaired “t” test when comparing values obtained from two separate populations of neurons.

**Solutions**

The aCSF composition was (in mM): 124 NaCl, 2 KCl, 1.25 KPO₄, 2 CaCl₂, 1.3 MgSO₄, 20 NaHCO₃, and 10 glucose. Osmolarity was maintained between 285 and 300 mOsm and pH between 7.3 and 7.4. The pipette solution contained (in mM): 140 Kgluconate, 0.1 CaCL₂, 1.1 EGTA, 10 HEPES, and 2 NaATP, and had a pH of 7.25 (adjusted with KOH if necessary).

A stock solution of ANG (Phoenix Pharmaceuticals, California) was prepared from which daily aliquots were made to the required dilution. Tetrodotoxin (TTX, Alamone Laboratories, Israel) was prepared daily from stock solutions for those experiments where it was necessary to block voltage-activated Na⁺ channels. Losartan (generously provided from Dupont Pharmaceuticals, Delaware) was made daily to the required concentration.

**RESULTS**

Whole-cell patch clamp recordings were obtained from a total of 80 putative neurosecretory parvocellular PVN neurons. These neurons had a mean resting membrane potential (RMP) of -58.5±1.9mV (mean±SEM), displayed
action potentials with a minimum spike amplitude of 60mV, and had a mean input resistance of 1155±75MΩ. Neurosecretory parvocellular neurons expressed neither a prominent I_A nor a low threshold spike as reported by Luther et al. (Luther et al., 2002). Neurons which displayed a prominent ‘A current’ following hyperpolarizing current pulses and had a linear I/V relationship were classified as magnocellular neurons while those neurons which featured a low threshold calcium conductance and whose I/V demonstrated an inward rectification at hyperpolarized potentials were classified as non-neurosecretory pre-autonomic parvocellular (Luther et al., 2002; Stern, 2001). Neither of these groups of neurons were considered further in the present study.

Angiotensin II Depolarizes Neurosecretory PVN Neurons

A total of 57 (RMP; -57.0±1.5mV) neurosecretory parvocellular neurons were tested for the effects of bath application of ANG using current clamp techniques. Following a control-recording period of at least 5 minutes, ANG was administered by bath perfusion in concentrations ranging from 0.01µM to 10µM for a period of 30 seconds. Of the 57 neurosecretory neurons tested with ANG, 38 (67%) depolarized, 3 (5%) hyperpolarized, and 16 (28%) were unaffected by peptide administration as illustrated in Figure 4.1. These depolarizing responses were dose dependent (10.0µM; 10.0±1.5mV n=6, 1.0µM; 8.8±0.5mV n=7, 0.1µM; 6.7±0.5mV n=21, 0.01µM; 0.8±0.8mV n=4) and followed a sigmoidal dose response relationship with an estimated EC_{50} of 5.5x10^{-8}. In some cases following recovery of the neuron to resting RMP, ANG application was repeated
Figure 4.1  Angiotensin II depolarizes neurosecretory parvocellular neurons. (A) Whole cell current clamp recordings illustrating the response of three neurosecretory parvocellular neurons to bath application of 0.01µM, 0.1µM, and 1.0µM ANG (30s, application indicated by bar). Action potentials have been truncated. (B) Bar graph summarizing the responses of neurosecretory parvocellular neurons to ANG application. 67% of neurosecretory parvocellular neurons responded to ANG with a depolarization, while 5% hyperpolarized and 28% were unaffected (C) Sigmoidal dose response curve shows that the depolarization of these neurons mediated by ANG are dose dependent.
and a second depolarization (ANG 0.1μM 6.9±1.0mV vs. 6.2±0.9mV n=4, p>0.5, paired t-test) was observed indicating that these neurons do not show significant desensitization to ANG.

The effect of ANG on the input resistance of neurosecretory neurons was also determined in 10 cells which responded to ANG with a depolarization. In the majority of neurons (n=7) ANG (0.1μM) decreased input resistance (-283±34MΩ: Control;1030±120MΩ, ANG;747±88MΩ) while the remaining 3 cells were unaffected. The reversal potential ($E_{\text{rev}}$) of the underlying conductance responsible for the actions of ANG on these neurons was extrapolated from an I/V relationship. In those neurons which demonstrated a depolarizing response concomitant with a decrease in input resistance following ANG application the $E_{\text{rev}}$ was found to be -45.5±3.5mV (n=7) suggesting potential effects on a non-selective cationic conductance ($I_{\text{NSCC}}$).

**Angiotensin II Effects of Neurosecretory Neurons are maintained in TTX**

Several recent reports have documented that the effects of a number of well known neuropeptides on PVN neurons, including ANG, adrenomedullin, AVP, and orexin-A as well as noradrenaline (NA) are dependent upon a modulation of synaptic input to these neurons. The effects of ANG before and after bath application of tetrodotoxin (TTX) on neurosecretory neurons were therefore evaluated to determine whether the ANG response in these neurons was maintained in synaptic isolation. The results of these experiments are shown in Figure 4.2A,B. The excitatory effects of ANG on neurosecretory neurons were maintained in TTX (0.1μM ANG: 6.6±0.6mV vs. 1.0μM TTX /ANG 6.2±0.3mV,
Figure 4.2 Angiotensin II mediated depolarization of neurosecretory parvocellular neurons is maintained in TTX and abolished by losartan. (A) Current clamp recording illustrating that the ANG (0.1µM, 30s, application indicated by bar) induced depolarization of a neurosecretory parvocellular neuron is maintained by pretreatment with 1.0µM TTX (application indicated by light gray extended bar). Action potentials have been truncated. (B) Bar graph summarizes the effects of ANG on neurosecretory parvocellular neurons following application of TTX. (C) Current clamp recording illustrating that the 0.1µM ANG (30s, application indicated by bar) induced depolarization of a neurosecretory parvocellular neuron is significantly reduced by pretreatment with 1.0µM losartan (application indicated by white extended bar). (D) Bar graph summarizes the effects of ANG on neurosecretory parvocellular neurons following application of losartan.
n=6, p>0.5, paired t-test) suggesting a direct interaction of ANG on the membranes of these cells that is independent of a change in synaptic input.

**Angiotensin II Effects on Neurosecretory Neurons are Losartan Sensitive**

Neuroanatomical studies indicate a high degree of AT₁ receptor mRNA localization in the parvocellular region of the PVN (Lenkei et al., 1998; Lenkei et al., 1997; Lenkei et al., 1994) particularly neurosecretory CRH neurons. The actions of ANG were therefore studied in the presence of the non-peptidergic AT₁ receptor specific antagonist losartan to determine if this receptor was indeed responsible for the observed depolarizations (Figure 4.2C,D). The effects of ANG on this subset of parvocellular neurons are abolished following bath application of 1.0µM losartan (0.1µM ANG: 7.2±0.4mV vs. Losartan/ANG 1.4±0.7mV, n=5, p<0.001, paired t-test) demonstrating the AT₁ receptor mediated nature of these depolarizations.

**Angiotensin II Activates a Putative NSCC in Neurosecretory Neurons**

Voltage clamp experiments were conducted in order to determine the identity of the current responsible for the effects of ANG on neurosecretory PVN neurons. Our input resistance data suggested the likely conductance was a non-selective cationic current (I_{NSCC}) based on the observed $E_{rev}$ extrapolated from I/V relationships taken during the depolarizations. This hypothesis is in accordance with previous studies documenting that the depolarizing effects of ANG and other peptides on PVN neurons are mediated by the activation of I_{NSCC} (Follwell & Ferguson, 2002a; Follwell & Ferguson, 2002b; Yang et al., 1992). We thus examined the effects of ANG on this current in voltage clamp following
Figure 4.3  Voltage-clamp recordings displaying activation of NSCC following angiotensin II application. (A) Voltage-clamp recordings showing the currents produced by a 10 mV s\(^{-1}\) depolarizing ramp. Shown here is the control current, the current elicited by 0.1\(\mu\)M ANG and the recovery current following ACSF wash. The difference current obtained by subtracting control currents from currents measured during ANG administration are displayed in the inset. (B) Average linear difference current obtained from the 7 cells (out of 12) that responded to 0.1\(\mu\)M ANG during this slow ramp protocol. The reversal potential of this current was found to be -37.8±3.8mV.
application of TTX (1.0µM) to block voltage gated sodium channels. Bath application of 0.1µM ANG during slow (10mV s⁻¹) depolarizing voltage ramps (-80 to +20mV) revealed an increase in conductance over this voltage range in 7 of 12 (58%) neurosecretory neurons tested (Figure 4.3). The proportion of ANG effects on voltage ramps was found to be similar to the observed depolarizing effects (Chi-square, P>0.5) of ANG in current clamp. The subtracted current was linear over the voltage range -80 to -20mV (Figure 4.3A inset) suggesting activation of a voltage independent current with a mean reversal potential of -37.8±3.8mV (Figure 4.3B), consistent with activation of $I_{\text{NSCC}}$.

**Angiotensin II Inhibits $I_K$ in Neurosecretory Neurons**

Interestingly, several of the ramps revealed that the difference current between control and ANG application was non-linear in the 0 to +20 mV voltage range suggesting the influence of an inwardly rectifying current. Our input resistance data estimated a reversal potential of -45.5±3.5mV which is lower than the reversal potential elicited by the $I_{\text{NSCC}}$ (-37.8±3.8mV). These observations in combination with the demonstration that ANG has effects on the delayed rectified potassium current ($I_K$) (Zhu et al., 1999;Gelband et al., 1999;Bains & Ferguson, 1997b) lead us to suspect this current was modulated by ANG. This hypothesis was tested using a voltage step protocol (250 ms steps from -60 to +20mV were employed) in the presence of TTX. Bath application of 0.1µM ANG caused a significant decrease in $I_K$ (29.1±4.7%, Figure 4.4) in 4 of 7 (P<0.005, paired t-test) neurosecretory neurons as tested at the +20 mV step. The proportion of ANG effects on $I_K$ was found to be similar to the observed depolarizing effects
Figure 4.4 Voltage-clamp recordings displaying a decrease in $I_K$ following angiotensin II application. (A) Voltage-clamp step protocols (250 ms steps from -60 to +20 mV in the presence of TTX) revealed 0.1µM ANG application results in a decrease in whole cell potassium currents. (B) $I$-$V$ relationship of the 4 $I_K$ cells that responded to 0.1µM ANG (out of a total of 7 tested), illustrating that ANG caused a significant decrease in this current in neurosecretory parvocellular PVN neurons in the -10 to +20 mV range (*$P < 0.05$).
(Chi-square, P>0.5) of ANG in current clamp. Although no recovery was seen from this decrease in current, this effect was unlikely to be the result of current run-down as application of 1nM ANG (a dose which does not result in a depolarization) had no significant effect on $I_K$ (n=4, data not shown).

DISCUSSION

The complexity of PVN when combined with the difficulty in selectively studying one population of cells has made it difficult to thoroughly characterize each of its cellular subtypes. Therefore while the electrophysiological properties of PVN neurons have been studied in detail, only two broad categories are traditionally cited: magnocellular (Type I) and parvocellular (Type II) neurons. Whole cell patch clamp techniques in combination with sophisticated immunohistochemical identification of neurons have permitted a rigorous characterization of PVN neurons.

Recently, Stern characterized the cellular properties of pre-autonomic non-neurosecretory neurons in PVN by combining in vivo retrograde tracing techniques with in vitro patch clamp recordings (Stern, 2001). Subsequently, Luther et al. documented utilizing IV injections of the retrograde tracer fluorogold that neurosecretory neurons whose projections extended to the median eminence had unique electrophysiological fingerprints of their own (Luther et al., 2002). These studies in combination demonstrated that neurosecretory neurons were without a prominent LTS and a small T-type calcium current while non-neurosecretory neurons were undistinguishable from traditional Type II parvocellular neuron in that they generated a prominent low threshold spike and
a robust T-type calcium current. We have used these observations to characterize the effects of ANG on neurosecretory parvocellular neurons.

We report here that ANG excites neurosecretory neurons in a dose-dependent fashion that does not rely on synaptic input as the depolarizations are maintained in synaptic isolation. In addition, the excitation is abolished by the AT₁ antagonist losartan demonstrating that the ANG effects on this subset of PVN neurons are mediated by the AT₁ receptor. These observations are particularly intriguing given that the majority of studies thus far have concentrated on the actions of ANG on magnocellular neurons (Li & Ferguson, 1996; Li & Ferguson, 1993a; Ferguson & Wall, 1992; Shoji et al., 1989). These reports have themselves garnered considerable debate with respect to AT₁ receptor localization with PVN. The autoradiographic distribution of labeled ANG as an indication of ANG binding sites within the CNS (Gehlert et al., 1991; Israel et al., 1985; Mendelsohn et al., 1984b) includes the SFO, ME, SON, and PVN complementary sites to this peptides actions in cardiovascular regulation. Interestingly, high levels of AT₁ receptor mRNA expression within the parvocellular areas of PVN and the surrounding periventricular area have been documented but the magnocellular region of PVN is devoid of such mRNA providing an interesting caveat to those observations (Lenkei et al., 1998; Lenkei et al., 1997; Lenkei et al., 1994) detailing the role of ANG in PVN. Indeed, recent immunohistochemical studies have observed that AT₁ receptors are found within specific regions of parvocellular PVN, namely neurosecretory neurons (Oldfield et al., 2001).
The observation that the ANG mediated depolarization of neurosecretory neurons is maintained in TTX indicates that ANG exerts direct effects on these cells. The actions of ANG on a variety of other neuronal populations have been described and $I_{NSCC}$ and $I_K$ are frequently cited as the conductances responsible for the observed excitation of these neurons (Ferguson et al., 2001; Sumners & Gelband, 1998; Bai & Renaud, 1998; Sumners et al., 1994; Yang et al., 1992). The isolated difference current in the voltage ramps, the ANG induced current is voltage independent between –80 and –20 mV and its reversal potential is similar to previously characterized NSCCs (Bai & Renaud, 1998). The actions of ANG on NSCC which we report here are AT$_1$ receptor mediated as they are abolished in losartan, however the signal transduction pathways which trigger the activation of NSCC following AT$_1$ receptor stimulation remain unknown (Bai & Renaud, 1998; Chorvatova et al., 1996).

The effects of ANG on the voltage dependent delayed rectifier, $I_K$, a current critical in neuronal repolarization have been documented with conflicting observations. Sumners et al. demonstrate that in newborn hypothalamic/brain stem cultures, ANG reduces $I_K$, an effect that is mediated by the AT$_1$ receptor (Sumners et al., 1996). This reduction involves a Ca$^{2+}$/calmodulin/CaM KiI signaling pathway as well as PKC (Sumners & Gelband, 1998). In addition, Nagatomo et al. found that in a small population of SON neurons $I_K$ was weakly sensitive to ANG (Nagatomo et al., 1995). Contrary to findings in hypothalamic cultures and SON, Li and Ferguson show that $I_K$ is not reduced by ANG in PVN.
neurons although this study focused on magnocellular neurons exclusively (Li & Ferguson, 1996).

Interestingly, a population of non-neurosecretory neurons express a prominent hyperpolarization-activated current ($I_h$). $I_h$, a cationic current, is renowned for its roles in regulating neuronal burst firing and rhythmicity, cardiac pacemaking, and phototransduction. Egli et al. demonstrate ANG provokes a shift of the $I_h$ activation curve to more depolarized values an effect mediated by the AT₁ receptor (Egli et al., 2002). These authors suggest that the excitatory effect of ANG in this subset of PVN neurons is at least partially due to an increased population of active $I_h$ channels. We did not however observe a substantial $I_h$ in neurosecretory parvocellular neurons and therefore this mechanism would appear to be unlikely to contribute substantially in this population of neurons.

Li et al. demonstrated that ANG stimulates non-neurosecretory spinally projecting parvocellular PVN neurons by attenuating GABAergic synaptic inputs through activation of presynaptic AT₁ receptors (Li et al., 2003). This raises the intriguing possibility that ANG acting at presynaptic terminals to facilitate/inhibit the release of a neurotransmitter could contribute to our observations. Although we do not observe the presence of miniature synaptic events in the presence of TTX, this mechanism remains a possibility.

In summary we have shown that ANG excites neurosecretory parvocellular neurons likely through a combination of activation of a putative NSCC and inhibition of $I_K$. These observations are consistent with previous
observations of the mechanism of action of ANG on hypothalamic neurons and may in part explain the cellular mechanisms by which ANG helps regulate secretion in the adenohypophysis.

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Chapter 5: GENERAL DISCUSSION
September 1997 marked the transition from undergraduate study to the world of research for me, and specifically an introduction to PVN. Undeniably, as of 1997 PVN was already the subject of an enormous research effort and the fundamental structure and function of this nucleus were well known. The major functional units within PVN, the magnocellular and parvocellular neurons were known to have distinct electrophysiological characteristics which permitted real time classification of neurons while performing current and voltage clamp experiments. Experimental paradigms could therefore be tailored to specific PVN cellular subtypes and were thus received with much enthusiasm. Combining patch clamp techniques with our understanding of the architecture of PVN, we explored the functional/physiological design of this nucleus with a particular focus on synaptic communication and its modulation by ANG. What did we learn?

**PVN CIRCA 1997**

PVN was regarded as a heterogeneous nucleus composed of magnocellular and parvocellular neurons. The advent of powerful immunochemical labeling techniques in combination with increasingly sophisticated patch clamp techniques had resulted in a broad characterization of these cells into Type I (putative magnocellular) and Type II (putative parvocellular) neurons based upon their electrophysiological profiles (Tasker & Dudek, 1991). At the time, little or no recognized evidence of interneurons in this nucleus existed, although speculation of their existence was growing.

The afferent and efferent connections of PVN had been extensively mapped and were consistent with its functional role in cardiovascular,
gastrointestinal, and neuroendocrine regulation (Swanson & Sawchenko, 1983; Swanson & Sawchenko, 1980). Although there was literature describing extensive afferent inputs to PVN from other regions of the hypothalamus, there was minimal, if any, clear data in support of any extensive integration occurring within the nucleus as a consequence of intranuclear circuitry, and thus PVN was considered largely a routing center. In the traditional model of PVN very little integration occurred within the nucleus due to a lack of interneurons which could effectively integrate and modulate incoming signals. Synaptic input was therefore thought to be channeled in a rather linear fashion from nuclei of origin via PVN which acted as the autonomic and neuroendocrine relay center to various outputs centers in order to effect change. The importance of PVN therefore lay in the maintenance of the fidelity of information and directing signals appropriately.

In view of PVN's central role in autonomic and neuroendocrine regulation and its extensive connections throughout the hypothalamus and brainstem, delineating synaptic pathways and neurotransmission within this nucleus were becoming increasingly popular. PVN neurons had been known for some time to be sensitive to both glutamate and GABA and numerous studies had examined the functional roles of these transmitters within PVN (van den Pol & Trombley, 1993; Decavel & van den Pol, 1990). Interestingly work from our lab was the first to show that glutamate activated a negative feedback system with PVN providing the first evidence for integration of an electrical system within PVN, which was dependent upon glutamate, GABA, and NO (Bains & Ferguson, 1997b).
Figure 5.1  Summary of ANG actions in PVN Circa 1997. ANG inhibits I_A in PVN magnocellular neurons (Li & Ferguson, 1996). Glutamate activates an NO mediated inhibitory feedback loop in PVN magnocellular neurons (Bains & Ferguson, 1997b).
ANG, a peptide hormone well known for its role in fluid-electrolyte balance, also known to exist in large numbers of afferent fibers to PVN (predominantly from SFO) seemed a logical candidate to modulate neuronal excitability within PVN. The cellular actions of ANG within PVN were not however clearly understood. While PVN neurons were known to contain a full complement of ion channels including $I_A$, $I_K$, $I_{Na}$, and $I_{KCa}$ (Bains & Ferguson, 1998; Li & Ferguson, 1996; Komatsu et al., 1991) only preliminary work had been undertaken to study the ability of ANG to modulate specific ion channels. Aside from studies from our own lab showing that ANG inhibited $I_A$ in magnocellular neurons and was without significant effect on $I_K$ in this population of neurons (Li & Ferguson, 1996), few studies had addressed the mechanisms by which ANG excited neurons in PVN. Studies using hypothalamic cultures had investigated the effects of ANG on $I_K$, $I_A$, $I_{Ca}$, (Wang et al., 1997; Sumners et al., 1996; Kang et al., 1993; Kang et al., 1992) while studies in SON had shown the peptide acted upon $I_A$ and NSCC (Nagatomo et al., 1995; Yang et al., 1992).

**PVN CIRCA 2007**

I have come to call the interval years: A decade of functional integration within PVN. Work described in this thesis (Latchford & Ferguson, 2005; Latchford & Ferguson, 2004; Latchford & Ferguson, 2003) and elsewhere (Hermes et al., 2000; Daftary et al., 1998b; Boudaba et al., 1997; Bains & Ferguson, 1997b; Boudaba et al., 1996) has identified important roles for both GABA and glutamate interneurons in PVN in both regulating inputs to and outputs from this essential autonomic control nucleus of the hypothalamus. During this time we
show that ANG activates local inhibitory (NO driven GABA) and excitatory (ANG driven glutamate) synaptic loops within PVN as well as having direct effects on membrane bound ion channels in parvocellular neurosecretory neurons. We now know that ANG’s actions in controlling autonomic and neuroendocrine axes involve multiple action sites with multiple levels of complex integration, a very significant change in perspectives from the simplistic perspective of excitatory actions on certain neurons of 10 years ago.

As discussed earlier, the actions of ANG have indeed been studied in hypothalamic cultures (Wang et al., 1997;Sumners et al., 1996;Kang et al., 1993;Kang et al., 1992) and on magnocellular neurons (Li & Ferguson, 1996;Nagatomo et al., 1995;Yang et al., 1992) but only recently has ANG been studied extensively in parvocellular neurons. This is a somewhat surprising fact in view of the abundance of AT₁ receptors in the parvocellular division of PVN (Lenkei et al., 1998;Lenkei et al., 1997;Lenkei et al., 1994), and the established role of ANG in CRH release and excitation of the sympathetic nervous system (Hogarty et al., 1992;Plotsky et al., 1988;Swanson & Sawchenko, 1983). It was not until the discovery that neurosecretory and non-neurosecretory neurons had distinct electrical fingerprints (Luther et al., 2002;Stern, 2001) that specific studies were designed to determine how ANG acted within these subsections of PVN. We were able to show that ANG activates a NSCC and has inhibitory effects on I_K. ANG has been shown to modulate both of these channels within other neuronal populations, effects similar to those observed on neurosecretory PVN cells.
Figure 5.2  Summary of ANG actions in PVN Circa 2007. ANG excites PVN non-neurosecretory neurons through presynaptic disinhibition (Li et al., 2003). Glutamate activates an NO mediated inhibitory feedback loop in PVN magnocellular neurons (Bains & Ferguson, 1997b). ANG activates a NO driven inhibitory feedback loop in PVN (Latchford & Ferguson, 2003). ANG excites PVN magnocellular neurons via augmentation of glutamatergic synaptic input (Latchford & Ferguson, 2004). Angiotensin depolarizes parvocellular PVN neurons through modulation of $I_{NSCC}$ and inhibition of $I_K$ (Latchford & Ferguson, 2005).
(Sumners & Gelband, 1998; Hu & Bourque, 1992). It has been clearly established that hormone secretion from magnocellular neurons is directly correlated with both the frequency and pattern of action potentials generated at the magnocellular cell soma (Poulain & Wakerley, 1982). Thus we would also expect that ANG’s action on parvocellular neuron to increase action potential frequency would also result in increased CRH and consequently ACTH secretion.

**PATHOPHYSIOLOCAL CONSEQUENCES of PVN DYSFUNCTION**

Future research will need to more clearly delineate the role of ANG in PVN in modulating the hypothalamic-pituitary-adrenal (HPA) stress axis. Limbic influences on adrenocortical hormone secretion are mediated by hypophysiotropic CRH neurons in PVN (Herman et al., 2002). The role of ANG in modulating the HPA stress axis however remains poorly understood. Future studies examining how limbic input is modulated by ANG in order to regulate the excitability of CRH neurons may shed important new light on understanding the regulation of stress in the human population. For example, PVN AT$_1$ receptor expression is increased during repeated restraint stress and after 24 hours of isolation stress (Saavedra & Benicky, 2007; Armando et al., 2007). Stimulation of these receptors is essential for HPA axis activation and in fact can be blocked by ANG antagonists but the ionic mechanisms which mediate these phenomena are not known. This evidence suggests that AT$_1$ receptor blockade may have a place in the prevention and treatment of stress-related disorders.

Conventional teaching of the pathophysiology of most cardiovascular diseases has until recently focused on pathological changes within the heart
and/or vasculature itself to explain the onset and maintenance of these complex disorders. Progressive new theories however are emerging which hypothesize that the CNS likely plays a critical role in congestive heart failure (CHF) and hypertension. Intriguingly, recent work has suggested that dysfunctional hypothalamic feedback loops likely (the short loop NO feedback we describe in this thesis) play a critical role in the development of CHF and hypertension (Coote, 2005; Zhang et al., 1998; Zhang & Patel, 1998; Patel & Zhang, 1996).

Neurohumoral responses in CHF include increased thirst and sodium appetite, increased AVP release, increased ACTH and cortisol release, and increased sympathetic renal nerve activity (Felder et al., 2003). Unfortunately, the mechanisms underlying autonomic/CNS dysfunction in CHF are not well established but clearly involve the hypothalamus and in particular PVN. Perhaps the first study demonstrating that PVN was clearly involved in the pathogenesis of CHF was performed by Patel et al. (Patel et al., 1993) who showed that metabolic activity with the parvocellular and magnocellular regions of PVN was increased in ischemia induced CHF models. Interestingly, a number of neuroactive peptides have been shown to be elevated in CHF including ANG, aldosterone and pro-inflammatory cytokines all of which have documented actions within the PVN (Francis, 1998). ANG levels known to be elevated in CHF act within PVN to increase renal sympathetic nerve activity (RNSA), increased renin release, and ultimately increased peripheral ANG release. Normally, PVN is under potent inhibitory influence of GABA and NO but these feedback loops are thought to be dysfunctional in CHF and therefore have exaggerated responses to
ANG. A vicious feed forward cycle is established. For example, electrical stimulation of PVN usually results in a mild pressor response but this is dramatically increased with injection of BMI or NOS inhibitors within PVN (Zhang & Patel, 1998; Zhang et al., 1997).

Interestingly aldosterone has been shown to increase the binding of ANG to AT1 receptors in SFO and PVN, to promote ACE activity, and to increase AVP mRNA and AVP release within PVN (De Nicola et al., 1994). Increased AVP levels in CHF may contribute to the profound increase in vascular resistance in patients with CHF (Hirsch et al., 1987). The decreased expression of nNOS in ischemia induced models of CHF suggest that NO may also contribute to the increased sympathoexcitation and vasopressin levels associated with CHF (Patel & Zhang, 1996). We show that inhibition of NOS results in a decrease in inhibitory feedback in magnocellular neurons concomitant with an augmented excitatory response to ANG. Whether or not a disruption of this pathway exists in CHF remains to be investigated, but is a plausible explanation for the increase in AVP seen in these models. The observation of increased fos-like immunoreactivity (fos-LI) in the magnocellular PVN of CHF than in sham operated rats further suggests enhanced activation of these neurosecretory cells (Vahid-Ansari & Leenan, 1998).

Li et al. demonstrate that AT1 receptors within PVN mediate an excitatory effect on renal sympathetic nerve discharge, arterial blood pressure, and heart rate (Li et al., 2003). Increases in RSNA increase circulating levels of renin, ANG, and aldosterone, and are each partly responsible for the increase in intravascular
volume observed in CHF. The expansion of blood volume seen in CHF has been shown to increase heart rate reflexively via volume receptors at the venous-atrial junctions of the heart (Coote, 2005). This reflex response to an increase in plasma volume consists of a distinctive unique pattern of sympathetic activity to maintain fluid balance in which PVN is intimately involved. Neurons in PVN show early gene activation on stimulation of atrial stretch receptors (Coote, 2005). A similar pattern of cardiac sympathetic excitation and renal inhibition can be evoked by electrical stimulation of PVN neurons (Coote, 2005). In CHF the atrial reflex is blunted and evidence suggests that this is a result of downregulation of NOS and NO production and reduced GABA activity in the PVN (Zhang & Patel, 1998). Unlike their magnocellular counterparts we did not observe significant increases in GABAergic input to neurosecretory cells within PVN.

PVN is also known to play an important role in the regulation of blood pressure. Not only do PVN neurons synthesize, store, and secrete AVP from the posterior pituitary, but they also send axonal projections to the brainstem (NTS, AP) and spinal cord (IML), which play significant roles in controlling autonomic output (Swanson & Sawchenko, 1983; Swanson & Sawchenko, 1980). Indeed, certain forms of hypertension (eg. 1 kidney 1 clip) are abolished in those animals, which undergo electrolytic lesion of PVN (Earle & Pittman, 1995). ANG also plays a central role (Steckelings et al., 1992) in cardiovascular disease. Elevated central concentrations of ANG and the AT$_1$ receptor have been found in spontaneously hypertensive rats (SHR) (Meyer et al., 1990; Phillips & Kimura, 1988). ICV injection of ANG significantly increases the expression of c-FOS and
c-JUN in SHR compared with normotensive controls (Zhu et al., 1999; Lebrun et al., 1996). Microinjection studies demonstrate that ANG administered into PVN results in an increase in sympathetic nerve activity (Zhang et al., 1997). Recent data from Li et al. (Li et al., 2003) indicate that ANG stimulates pre-autonomic non-neurosecretory parvocellular neurons through pre-synaptic disinhibition. An interesting observation in view of Zhang et al. who previously demonstrated that ANG and glutamate excite renal sympathetic nerve activity (Zhang et al., 1997).

NO in PVN has been shown to have an inhibitory effect on RSNA (Li et al., 2002; Zhang et al., 1997). Microinjection injection of a NOS inhibitor into PVN increases RNSA and elevates blood pressure dramatically in response to ANG or glutamate. The sympathoinhibitory effect of NO is eliminated by bicuculline demonstrating the involvement of GABA as the inhibitory modulator (Zhang & Patel, 1998). NO therefore potentiates GABAergic synaptic inputs to spinally projecting PVN neurons which is mediated by a cGMP-protein kinase G pathway (Li et al., 2004). Interestingly, GABA receptor binding sites measured using quantitative autoradiography and GAD levels were significantly lower in SHR PVN when compared with normotensive controls (Horn et al., 1998; Kunkler & Hwang, 1995). The basal firing rate of presympathetic PVN neurons were significantly decreased in normotensive versus SHR and both the frequency and amplitude of GABAergic spontaneously inhibitory postsynaptic currents were reduced in SHR (Li & Pan, 2006). These data suggest that the elevated levels of ANG associated with hypertension may stimulate PVN either through
exaggerated presynaptic disinhibition or a dysfunctional GABAergic feedback system.

Intriguingly, Hosoya et al. (Hosoya et al., 1995) demonstrated that there exists oxytocinergic innervation to the upper thoracic sympathetic preganglionic neurons in the rat. Immunohistochemical studies indicate that these neurons originate in the parvocellular divisions of PVN (Hosoya & Matsushita, 1979). Stimulation of PVN has been shown to evoke increases in heart rate which can be abolished following intrathecal administration of an oxytocin antagonist (Yang et al., 2004). An increase in heart rate is a common component in many cardiovascular diseases. In hypertension tachycardia increases blood pressure by augmenting cardiac output while in CHF it decreases cardiac output by decreasing diastolic filling time. In our experimental paradigms we putatively identify the neuron based upon its electrical fingerprint and not its location within PVN. Presumably oxytocinergic neurons within the parvocellular divisions of PVN retain these electrophysiological characteristics. We show that ANG results in a glutamate mediated excitation of magnocellular neurons. Future research possibilities include determining if the elevated ANG levels observed in CHF act upon an exaggerated glutamatergic pathway within PVN to increase oxytocinergic output to the thoracic sympathetic spine.

Over the last decade we have witnessed an explosion of research in PVN focused on functional integration within this nucleus. How this data impacts on our perception of cardiovascular disease remains to be seen. A common theme has certainly emerged throughout this discussion. Elevated levels of ANG are
apparent in both CHF and hypertension and PVN seem to be central in the pathogenesis of these disorders. Whether dysfunctional feedback or inappropriate feed-forward loops are responsible for these phenomena remains a mystery. What is clear however are that these observations are of particular importance in the clinical setting where ACE inhibitors (e.g. enalapril), angiotensin receptor blockers (e.g. losartan), and aldosterone antagonists (e.g. spironolactone) have become first line therapy in managing heart failure (Flather et al., 2000; Pitt et al., 2000; Pitt et al., 1999). Additionally they are used commonly in the treatment of hypertension and also in the setting of post myocardial infarction. Traditionally these drugs have been viewed as afterload and intravascular volume reducers with concomitant changes in ventricular and arterial remodeling. No studies however have addressed potential roles of these medications in treating CHF and HTN by augmenting autonomic outflow and neuroendocrine secretion from PVN and therefore they remain intriguing possibilities for future research.
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