Cell type specific retrograde signaling determines synaptic gain.

by

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Abstract

Maintenance of internal body homeostasis requires the integration of information about the ongoing state of the body's internal systems. In order for distinct cell types to produce a correct physiological response to a global signal, neural mechanisms must exist which allow neurons to selectively "listen" to a common relevant input. Using a physiologically defined synaptic connection in the hypothalamus, we have outlined a mechanism by which synaptic gain may be modulated in a cell type specific fashion to produce an appropriate outcome in response to a state dependent signal.

Osmotic homeostasis (water and salt balance) is maintained in part by the regulation of vasopressin (VP) and oxytocin (OT) secretion from the supraoptic nucleus (SON) to regulate antidiuresis and natriuresis, respectively. The organum vasculosum of the lamina terminalis (OVLT) is a central osmosensory nucleus and produces an excitatory glutamatergic synapse on the SON to increase VP and OT secretion in response to hyperosmolality under euvolemic conditions. Under hypovolemic conditions, antidiuresis is further enhanced but natriuresis is depressed. We therefore hypothesize that Angiotensin II (Ang II), a signaling molecule secreted under hypovolemic conditions, may act differentially on the OVLT evoked responsiveness of VP versus OT neurons.

We demonstrate that application of Ang II facilitates osmotic-evoked spiking in the VP cells, but depresses osmotic-evoked spiking in OT cells. We found that Ang II modulates excitatory transmission at the OVLT-SON pathway in a presynaptic fashion. Ang II modulation is triggered by a postsynaptic mechanism

and requires the retrograde signaling molecules of nitric oxide and endocannabinoids to facilitate or depress, respectively, OVLT to SON neurotransmission. Thus, under hypovolemic conditions where VP secretion is favorable while OT secretion is not, Ang II can initiate a postsynaptic signaling event to modulate incoming synaptic activity based on cell type. This modulation of synaptic gain allows the generation of a physiological response appropriate for restoring euvolemia.

Résumé

Le maintien de l'homéostasie corporelle interne exige l'intégration d'informations concernent l'état des systèmes internes de l'organisme. Afin que différents types de cellules produisent une réponse appropriée à un signal physiologique, des mécanismes neuronaux doivent exister afin de permettre aux neurones d'être attentifs aux stimuli pertinents. En utilisant une connexion synaptique physiologiquement définie dans l'hypothalamus, nous avons défini un mécanisme par lequel un gain synaptique peut être modulée sur des cellules spécifiques pour produire un résultat approprié à un signal interne.

L'homéostasie osmotique (équilibre de l'eau et du sel) est maintenue en partie par la régulation de la sécrétion de vasopressine (VP) et d'oxytocine (OT) du noyau supraoptique (NSO) pour réguler l'antidiurèse et la natriurèse respectivement. L'organe vascularisé de la lame terminale (OVLT) est un noyau central osmosensible qui détient une connexion synaptique glutamatergique qui excite les neurones du NSO afin d'augmenter la sécrétion de VP et OT en réponse à l'hyperosmolarité dans des conditions euvolemiques. Dans des conditions hypovolémiques, l'antidiurèse est d'avantage augmentée, alors que la natriurèse est diminuée. Nous lançons donc l'hypothèse que l'angiotensine II (Ang II), une molécule de signalisation sécrétée dans des conditions hypovolémiques, pourrait agir différemment sur les neurones VP par rapport aux neurones OT, lorsque libéré par l'OVLT.

Nous avons démontré que l'application d'Ang II facilite le déchargement de potentiels d'action évoqués osmotiquement dans les cellules VP, mais abaisse le

déchargement de potentiels d'action évoqués osmotiquement dans les cellules OT. Nous avons trouvé que l'Ang II module la transmission excitatrice dans la connection OVLT-NSO d'une manière présynaptique. La modulation par Ang II est déclenchée par un mécanisme post-synaptique qui nécessite la signalisation rétrograde des molécules d'oxyde nitrique et d'endocannabinoides afin de faciliter ou de diminuer respectivement, la neurotransmission entre l'OVLT et le SON. Ainsi, dans des conditions hypovolémiques où la sécrétion de VP est favorable alors que la sécrétion d'OT ne l'est pas, l'Ang II peut initier un événement post-synaptique de signalisation afin de moduler l'activité synaptique entrant en fonction du type cellulaire. Cette modulation du gain synaptique permet la génération d'une réponse physiologique appropriée pour la restauration euvolémique.

Originality and Overview

The results presented in this thesis have been reported at scientific conferences and meetings locally and internationally. Portions of this work have been published (Stachniak et al., 2011) Overviews of each chapter and of the original contributions to general knowledge are summarized below.

Chapter 1 Introduction

This chapter provides an overview and background of osmotic and volemic homeostasis, as well as highlighting a role for angiotensin II (Ang II) in these processes. A discussion of mechanisms that regulate activity of magnocellular neurosecretory cells (MNCs) is included. An original contribution in this chapter is the formulation of a hypothesis regarding the mechanisms behind the bidirectional regulation of vasopressin and oxytocin neurons under hypovolemic conditions.

Chapter 2 Methods

This chapter describes the materials and methods used in Chapters 3 to 5, as well as outlining the experimental design for those chapters.

Chapter 3 Modulation of osmotic gain

This chapter examines the effects of the hypovolemic signaling peptide angtiotensin on the OVLT to SON synapse. Original contributions in this chapter include the observation that the excitation induced in MNCs by osmotic stimulation of the OVLT can be either facilitated or inhibited by Ang II, depending on MNC cell type. Further, the observation that Ang II differentially

regulates OVLT to SON synaptic transmission in vasopressin and oxytocin neurons provides a novel description of cell type specific synaptic regulation.

Chapter 4 Synaptic mechanisms of osmotic modulation

This chapter elucidates the mechanisms that underlie the differential regulation of synaptic transmission. Experiments in this chapter utilize several classical electrophysiological assays to determine the site of modulation of OVLT to SON synaptic transmission by Ang II, as well as pharmacological manipulations to probe signaling mechanisms. Original contributions in this chapter include the observation that presynaptic release probability of the OVLT synapse in MNCs is modulated in a bidirectional fashion by Ang II. The identification of nitric oxide and endocannabinoids as retrograde signaling molecules in the MNCs was guided by previous findings, but our identification of a physiologically relevant context in which to interpret such a retrograde signaling mechanism provides new insights into how synaptic integration may give rise to physiological consequences.

Chapter 5 Synaptic modulation alters MNC activity

This chapter first identifies and records a modulation of spontaneous synaptic transmission by Ang II, and then uses this recording to shape activity in naïve MNCs. This is accomplished through the generation of an artificial synaptic template from spontaneous synaptic recordings. This template is then "played back" to MNCs as a current injection waveform to alter spontaneous firing behavior. Original contributions in this chapter include the finding that spontaneous currents in MNCs undergo Ang II induced modulations in both frequency and amplitude in a cell type specific manner, which may partially

account for changes in vasopressin and oxytocin section under hypovolemic conditions. As well, we provide the novel finding that synaptic modulation by Ang II, in the absence of any other modulatory effects of the neuropeptide, is sufficient to elicit a change in activity of MNCs.

Chapter 6 General discussion

This chapter summarizes the findings of the previous chapters and expands the discussion of the findings therein. A discussion of how the mechanisms outlined in the previous chapters might generate cell type specific synaptic modulation is included. Original contributions in this chapter include the hypothesis that vasopressin and oxytocin are regulated in a biphasic manner by hypovolemia, such that under mild hypovolemic conditions, the bidirectional regulation of synaptic transmission could blunt or facilitate oxytocin or vasopressin secretion, respectively. In severe hypovolemia, they would not be differentially regulated. The vasopressin component of this theory has previously been proposed and supported in the literature, as reviewed in chapter 1.

Publications

Stachniak TJ, Sudbury JR, Trudel E, Choe KY, Ciura S and Bourque CW (2011). Osmoregulatory Circuits in Slices and En-Bloc Preparations of Rodent Hypothalamus. In: Isolated brain circuits. Ballanyi K.(Ed.). Humana-Springer. (In press).

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Stachniak TJ, Bourque CW. Visually guided whole cell patch clamp of mouse supraoptic nucleus neurons in cultured and acute conditions.Am J Physiol Regul Integr Comp Physiol. 2006 Jul;291(1):R68-76.

Presentations

Electrophysiological recordings from organotypic cultures of the murine supraoptic nucleus. Tevye Stachniak and Charles Bourque. (Abstract) The Physiologist. 2005 Jun; 48(3). (Poster) 2005 APS Conference. Neurohypophyseal Hormones: From Genomics and Physiology to Disease, July 16-20, 2005, Steamboat Springs, CO

Electrophysiological recordings from organotypic cultures of the murine supraoptic nucleus. Tevye Stachniak and Charles Bourque. (Abstract) Society for Neuroscience Abstract viewer 2005. (Poster) 2005 SfN 35th annual meeting, November 12-16, 2005, Washington, DC.

Angiotensin II enhances excitatory synaptic transmission from OVLT to SON. (Abstract, Presentation) The Canadian Physiological Society, CPS Winter Meeting 2007, January 31 to February 3, 2007, Beaupre, QC.

Presynaptic enhancement excitatory transmission from OVLT to SON by angiotensin II. (Abstract, Poster) The 7th world congress on neurohypophysial hormones, Sept. 18 to 22, 2007, Regensburg, Germany.

Presynaptic enhancement excitatory transmission from organum vasculosum of the lamina terminalis to supraoptic nucleus by angiotensin II. (Abstract) Society for Neuroscience Abstract viewer 2007. (Poster) 2007 SfN 37th annual meeting, November 3-7, 2007, San Diego, CA.

Angiotensin II modulation of OVLT signaling to the supraoptic nucleus. (Invited Presentation) Canadian Workshop on the neurophysiology of Homeostasis. 2nd annual Canadian Neuroscience Meeting, May 25-28, 2008, Montreal, QC.

I think I need a drink: Neural control of osmoregulation. (Invited Presentation) Department of Physiology & Biophysics Seminar, Friday Oct 24th, 2008, 2:30-3:30pm, Dalhousie University, Room 3-H1, Sir Charles Tupper Medical Bldg, Halifax, NS.

Angiotensin II enhances osmotic signalling by presynaptic facilitation. (Poster) 2009 neuroscience retreat, June 12-13, Mont Gabriele, Ste-Adele, QC.

Fate vs. Free Will: Suggestions from a Neuroendocrinologist. (Invited Presentation) Philopolis, March 20-21, 2010. Montreal, QC.

Tell me what I want to hear: Cell type specific synaptic modulation. (Invited Presentation) Center for Research in Neuroscience Graduate Student Association Seminar, Thursday Mar 25th, 2010,12-1pm, McGill University, Room L7-140, Montreal General Hospital, Montreal, QC.

Angiotensin II facilitates osmosensitivity: An assessment by synaptic playback. (Abstract, Poster) 2010 Canadian neuroscience meeting, May 15-18, Ottawa, ON.

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List of abbreviations

aCSF Artificial cerebrospinal fluid

AHP After hyperpolization

AM-251 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide, CB1 antagnoist

AMPA 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid

Ang II Angiotensin II

ANP Atrial natriuretic peptide

AP Area postrema

AT1R angiotensin type 1 receptor

AT1aR angiotensin type 1 receptor, isoform a

AT1bR angiotensin type 1 receptor, isoform b

AT2R angiotensin type 2 receptor

BK Large potassium conductance

c- fos FBJ murine osteosarcoma viral oncogene homolog

cAMP cyclic adenosine monophosphate

CB1 Cannabinoid receptor 1

cGMP cyclic guanosine monophosphate

CNS Central nervous system

DAG Diacylglycerol

DAP Depolarizing after potential

DNQX 6,7-dinitroquinoxaline-2,3-dione, AMPA receptor antagonist

eCB Endogenous cannabinoid

ECF Extracellular fluid

eGFP Enhanced green fluorescent protein

EGTA ethylene glycol tetraacetic acid

EPSC Excitatory postsynaptic current

EPSP Excitatory postsynaptic potential

GABA gamma-aminobutyric acid

ICV Intracerebroventricular

IEI Inter-event interval

IPSC Inhibitory postsynaptic current

L-NAME N^G-nitro-L-arginine methyl ester hydrochloride

LTD Long term depression

LTP Long term potentiation

mGluR metabotropic glutamate receptor

MNC Magnocellular neurosecretory cell

MnPO Median preoptic nucleus

Mosm/kg Milliosmoles per kilogram

NA Noradrenaline

NADPH Nicotinamide adenine dinucleotide phosphate

NMDA N-Methyl-D-aspartic acid

NO Nitric oxide

NOS Nitric oxide synthase

NSO Noyau supraoptique

NTS Nucleus of the solitary tract

OT Oxytocin

OVLT Organum vasculosum of the lamina terminalis

PBS Phosphate-buffered saline

PEG Polyethylene glycol

PKG protein kinase G

PLC Phospholipase C

PPR Paired pulse rate

PVN Paraventricular nucleus

SCN suprachiasmatic nucleus

SFO Subfornical organ

SICC Stretch inhibited cation channel

SNAP S-Nitroso-N-acetylpenicillamine

SNP Sodium nitroprusside

SON Supraoptic nucleus

TRPV1 transient receptor potential cation channel, vanilloid, member 1

TTX Tetrodotoxin

VLM Ventrolateral medulla

VP Vasopressin

VPeGFP Vasopressin enhanced green fluorescent protein

Δ Change in

Table of contents

Chapter 1 Introduction

The maintenance of internal homeostasis in the face of a changing external environment is the critical challenge all organisms must face in order to sustain life (Cannon, 1929). The complex interplay of a body's regulatory systems presents a unique challenge to the nervous system: how can we interpret and integrate ongoing perturbations to a given system in a way that permits an appropriate physiological response? If the primary function of the nervous system is to collect and transmit information, its primary challenge is to organize that information. In the case of homeostasis, that organization is provided by the hypothalamus. In the following work, we outline a mechanism by which the mysterious hypothalamus organizes and interprets information relating to fluid homeostasis.

1.1 Fluid homeostasis

The regulation of fluid homeostasis within an organism is, at its most basic level, an issue of salt and water maintenance. Osmolality, the concentration of salt (or other osmolyte) per unit weight of solution, is maintained at constant levels within a physiological system, in order to avoid the generation of an osmotic imbalance between extracellular and intracellular fluid compartments: an osmotic pressure. As such, many organisms, including all mammals, have developed mechanisms to regulate their extracellular fluid (ECF) osmolality. Homeostatic feedback loops designed to regulate osmolality consist of internal detectors of osmotic perturbations of the ECF (osmosensors) which relay information to

effector systems in order to correct osmotic imbalance. This study will focus on the activity of an osmosensory relay in the central nervous system that regulates secretion of osmotic regulatory hormones from the neurohypophysis.

1.1.1 Osmotic homeostasis

Maintenance of osmotic homeostasis is crucial for avoiding an imbalance between intracellular and extracellular compartments, leading to physical stress on an organism (Bourque, 2008). Osmotic pressure results from the tendency of water to follow salt in physiologic systems, which results in cell shrinkage in response to extracellular hypertonicity, or cell swelling in response to extracellular hypotonicity as water exits or enters the cell, respectively (Zhang & Bourque, 2003). Salt will also move down a concentration gradient to join a hypotonic compartment if possible, but the semi-permeable nature of biological membranes restricts such movement. Accordingly, the body establishes specialized polarized epithelia containing tight junctions to effectively control the movement of water and solute (Andreoli & Schafer, 1976).

The detection of osmotic deficits results in the production of both behavioral and physiological effector responses (Figure 1.1). Behavioral responses include the sensation of thirst, which is necessary to induce drinking required to replace internal water deficits, and salt appetite to restore sodium deficits. Increasing tonicity will increase thirst (Stricker, 1969; Hatton & Bennett, 1970) while inhibiting salt appetite (Weisinger, Denton, & McKinley, 1983), whereas decreasing tonicity will invoke the opposite effects (Stricker & Wolf,

1969; Stricker, 1969), in both cases acting to restore osmotic pressure to a set point to about 300 mosm/kg in mammals (Bourque, 2008). Physiological responses include both neural and endocrine activity directed at correcting fluid volume and tonicity. Neural modulation of kidney function can be engendered through a change in vascular tone in both afferent and efferent glomerular arterioles, which will modify the glomerular filtration rate (DiBona & Kopp, 1997). Reductions in glomerular filtration result in decreases in both sodium excretion (natriuresis) and water excretion (diuresis) (Thompson & Pitts, 1952). Increased renal sympathetic nerve activity reduces natriuresis (Gill, Jr. & Casper, 1969) and diuresis (DiBona & Kopp, 1997). Neuroendocrine regulation of osmolality includes the regulation of magnocellular neurosecretory cell (MNC) activity in the supraoptic nucleus (SON) and hypothalamic paraventricular nucleus (PVN) which produce and transport vasopressin (VP, the antidiuretic hormone) and oxytocin (OT) into the neurohypophysis, where they are subsequently secreted into the bloodstream via the posterior pituitary (Antunes-Rodrigues, de Castro, Elias, Valenca, & McCann, 2004).

1.1.2 Hypothalamic networks for osmotic homeostasis

The central regulation of fluid balance is mediated by a number of discrete nuclei within the brain (Figure 1.2). These include the sensory circumventricular organs such as the hypothalamic organum vasculosum of the lamina terminalis (OVLT). The OVLT, along with the subfornical organ (SFO) and area postrema (AP), respond to neural, endocrine and osmotic signals by modulating their

activity (Ferguson, Donevan, Papas, & Smith, 1990), which is then communicated to the various hypothalamic integration centers such as the median preoptic nucleus (MnPO), and output nuclei, which include the magnocellular components of the PVN and SON. These neurons send their axons through the median eminence into the posterior pituitary, where they release the neuropeptides VP and OT directly into the bloodstream in direct proportion to neural activity (Poulain & Wakerley, 1982).

Early lesion studies identified the anteroventral third ventricular region as the major site of osmosensation in the brain, as lesions in this area render animals adipsic (McKinley et al., 2004a). Conversely, electrical (Andersson, Larsson, & Persson, 1960) or osmotic (Buggy, Hoffman, Phillips, Fisher, & Johnson, 1979; Andersson, Jobin, & Olsson, 1967; Eriksson, Simon-Oppermann, Simon, & Gray, 1987) stimulation of this region elicits drinking, suggesting a role as a primary osmosensor. Within this region, the OVLT has been singled out as an especially important area in detecting osmotic changes (McKinley et al., 2004b; Thrasher & Keil, 1987). Thus with the OVLT acting as a primary sensory nucleus, and the SON effecting a homeostatic response by release of neurohypophysial hormones, we may consider these two nuclei to be a simplified osmosensory loop in which to study the organization and interpretation of signals in osmoregulatory networks (see section 1.1.3b).

1.1.3 Hypertonicity and neurohypophysial hormone release

We have known for decades now that fluid osmolality regulates VP release, with Verney's proposed "osmoreceptor" (Verney, 1947) responding to changes in blood osmolality and altering VP secretion accordingly. VP secretion represents one of the most important physiological responses to osmotic perturbation (Verbalis, 2003). The antidiuretic effect of VP on the kidneys is mediated by an insertion of aquoporin-2 into the apical membrane of collecting duct principle cells, which increases water reabsorption from the glomerular filtrate (Nielsen et al., 1999). Conversely, the secretion of OT has been demonstrated to elicit natriuresis in rodents (Verbalis, Mangione, & Stricker, 1991), in addition to its more widely recognized roles in parturition and lactation (Bealer, Armstrong, & Crowley, 2010; Russell, Leng, & Douglas, 2003). Natriuresis is triggered by the direct actions of oxytocin on the kidney (Antunes-Rodrigues et al., 2004) and indirect actions through the release of atrial natriuretic peptide (Haanwinckel et al., 1995) to inhibit sodium reabsorption. The direct actions may be mediated by the activation of cyclic guanosine monophosphate (cGMP) through nitric oxide production (Soares et al., 1999) and the closure of sodium channels in the apical membrane of the cortical collecting duct (Stoos, Carretero, & Garvin, 1994). The closure of these channels renders sodium unable to move across the collecting duct membranes, and thus sodium reabsorption is prevented which increases natriuresis (De Wardener, 1978). Functionally, this means that an osmotic stimulation of either VP or OT cells will elicit a reduction in osmotic pressure by increasing antidiuresis or natriuresis respectively.

1.1.3a Osmosensitivity mediated by stretch inhibited cation channels

Until quite recently it was unclear exactly how increases in osmolality were transduced into increases in VP and OT secretion. The factors involved in the acute regulation of MNC firing include both intrinsic and extrinsic mechanisms (Bourque, Oliet, Kirkpatrick, Richard, & Fisher, 1993). The detection and transduction of osmotic perturbations in the ECF is accomplished by the integration of a number of concerted mechanisms in the SON and OVLT. These include an intrinsic osmosensitivity (i.e. a change in activity evoked by a change in osmolality) mediated by the stretch inhibited cation channel (SICC) (Oliet & Bourque, 1993a) recently identified as a splice variant of the transient receptor potential cation channel, vanilloid, member 1 (TRPV1) channel present in the SON (Sharif-Naeini, Witty, Seguela, & Bourque, 2006). Changes in ECF tonicity alter osmotic pressure to induce inversely proportional and sustained changes in MNC size (Zhang & Bourque, 2003). Decreases in cell size produces a reduction in mechanical pressure, as demonstrated by the ability of increased pipette pressure to counteract hyperosmotic-induced reductions in cell size in whole cell recordings (Zhang, Kindrat, Sharif-Naeini, & Bourque, 2007). This mechanical pressure is transduced through the actin cytoskeleton to reduce inhibition of SICCs (Zhang et al., 2007). Although it is not yet clear exactly how the cytoskeleton interacts with SICCs to modulate their probability of opening, increased cortical actin density is associated with increased mechanosensitive activity of SICCs (Zhang et al., 2007).

Activation of these channels during cell shrinkage increases cation current influx, which subsequently depolarizes the neuron (Ciura & Bourque, 2007). Notably, the activity of these SICCs under isotonic conditions means that a hypotonic stimulus can increase basal stretch and close channels to produce a hyperpolarization and inhibition of the osmosensitive neuron (Oliet & Bourque, 1993b).

1.1.3b Osmosensitivity mediated by OVLT to SON synapses

The SICC identified as the source of osmosensitivity in MNCs (Oliet & Bourque, 1993a) has also been found in the osmosensitive OVLT (Ciura & Bourque, 2006). The presence of the SICC in the OVLT produces the osmosensitivity of the glutamatergic projections from the OVLT to the SON (Richard & Bourque, 1995). A subset of OVLT neurons respond to changes in osmolality (Ciura & Bourque, 2006; Nissen, Bourque, & Renaud, 1993) and give rise to monosynaptic connections to the SON, as indicated by the ability of electrical stimulation of the SON to evoke antidromic action potentials of constant latency (Nissen et al., 1993). Further, Lucifer yellow stained OVLT neurons project to the SON (Nissen et al., 1993), and both anterograde (Armstrong, Tian, & Wong, 1996; Camacho & Phillips, 1981) and retrograde (Wilkin, Mitchell, Ganten, & Johnson, 1989) labeling studies have identified OVLT-SON connectivity. At the electron microscope level, these projections are seen to give rise to synaptic terminals throughout the SON (Armstrong et al., 1996).

The osmotic information transmitted from the OVLT provides important regulatory control of VP and OT secretion from the SON (Negoro, Higuchi, Tadokoro, & Honda, 1988; Brimble, Dyball, & Forsling, 1978; Leng, Mason, & Dyer, 1982; Voisin & Bourque, 2002). The absence of OVLT-mediated activity blunts the osmotic control of VP secretion (Thrasher, Keil, & Ramsay, 1982; Thrasher & Keil, 1987; Leng, Blackburn, Dyball, & Russell, 1989), and MNCs may require excitation induced by glutamate secretion from OVLT to express osmosensitivity (Leng et al., 1989). OVLT synaptic activity is therefore integrated with the intrinsic osmosensitivity of the SON to optimize the osmotic evoked release of VP (Voisin & Bourque, 2002). Since VP is secreted in proportion to MNC firing rate (Poulain & Wakerley, 1982), the excitatory OVLT to SON projections combined with the osmosensitivity conveyed by SICCs in both the OVLT and SON make these nuclei components of Verney's osmosensor.

1.1.3c Osmosensitivity mediated by glial release of taurine

In addition to intrinsic osmosensitivity and synaptic control of MNC activity, taurine release from astrocytes has been suggested to regulate MNC activity in an osmosensitive fashion (Hussy, 2002). Taurine immunoreactivity has been reported in astrocytic processes within the SON, intercalating between MNC cell bodies or in close apposition to axonal boutons (Decavel & Hatton, 1995) and closely associated with immunostaining for extrasynaptic glycine receptors on MNCs (Deleuze, Alonso, Lefevre, Duvoid-Guillou, & Hussy, 2005). Taurine acts as an agonist at MNC glycine receptors to inhibit cell firing (Hussy,

Deleuze, Pantaloni, Desarmenien, & Moos, 1997). Secretion of taurine is increased by hypoosmotic stimulation, and decreased by hyperosmotic stimulation (Deleuze, Duvoid, & Hussy, 1998), suggesting that the basal amount of taurine release evident in the SON (Hussy et al., 1997) provides for a bidirectional regulation of MNC activity. This dynamic modulation by astrocytes adds an important component to the osmotic regulation of MNC activity.

The intrinsic, synaptic, and glial osmotic modulations of activity produce a correlation between osmotic pressure and magnocellular activity, and hence mediate a relationship between osmolality and neurohypophysial hormone secretion. Thus we can observe a linear relationship between serum osmolality and serum VP concentration which reflects the osmosensory gain (i.e. the change in MNC activity corresponding to a given change in ECF osmolality). Modulations in this gain help to maintain both osmotic and volemic homeostasis (see section 1.2.1 and Figure 1.3).

1.2 Volume Homeostasis

Like the regulation of osmotic balance, volume homeostasis is an issue of salt and water balance. Moving through extracellular fluid compartments, water follows salt down osmotic pressure gradients. As such, the regulation of salt will greatly impact extracellular fluid volume. In order to maintain blood volume and pressure, it is therefore critical to regulate the intake and output of both salt and water. To this end, the nervous, renal, and endocrine systems interact to maintain euvolemia.

Reductions in fluid volume trigger thirst, salt appetite, antidiuresis, and antinatriuresis (Antunes-Rodrigues et al., 2004). Retention and recovery of both water and sodium is critical to the restoration of fluid volume (Stricker & Jalowiec, 1970). As such, hypovolemia-induced salt appetite is potentiated by low sodium (Stricker & Jalowiec, 1970). In contrast to hyperosmotic evoked thirst, hypovolemia may evoke water intake even in the presence of hyponatremia (Andersson, 1978).

Detection of hypovolemia is accomplished in a similar (but not identical, see (Buller, Smith, & Day, 1999; Schreihofer, Stricker, & Sved, 1994) fashion to detection of blood pressure. Baroreceptors in the aortic arch and carotid sinus respond to changes in blood pressure by modulating activity through the aortic depressor nerve and carotid sinus nerve to autonomic nuclei in the brainstem, including the nucleus of the solitary tract (NTS) and ventrolateral medulla (VLM) (Calaresu, Ciriello, Caverson, Cechetto, & Krukoff, 1984). Additionally, stretch receptors in the heart atria (Koizumi & Yamashita, 1978; Grindstaff, Grindstaff, & Cunningham, 2000) and ventricles (Wang, Flora-Ginter, Leadley, Jr., & Goetz, 1988) detect volume changes and send afferents to the central nervous system (CNS) to alter homeostatic regulation (Cunningham et al., 2002; Wang, Sundet, Hakumaki, & Goetz, 1983). Drops in blood pressure trigger sympathetic outflow, inducing vasoconstriction of resistance arteries to increase blood return to the heart and elevating blood pressure (Kirchheim, 1976). Sympathetic activation also reduces blood flow to the kidneys, reducing glomerular filtration rate. Increases in renal sympathetic nerve activity also occur (see section 1.1.1), elevating blood

volume and pressure over longer time periods by triggering antidiuresis and reducing natriuresis (Antunes-Rodrigues et al., 2004).

Reductions in blood volume elicited by hemorrhage reduce urine volume and sodium excretion (Edwards, Zimmerman, Schwab, Heublein, & Burnett, Jr., 1988). In order to avoid difficulties associated with the experimental use of hemorrhage, a number of protocols to experimentally induce reductions in fluid volume have been developed. A commonly used experimental model for hypovolemia is administration of subcutaneous polyethylene glycol (PEG), which sequesters fluids in the extravascular space, thereby reducing blood volume (Stricker & MacArthur, 1974). Stricker describes a method for converting PEG induced changes in hematocrit or plasma protein levels to approximate measures of volume depletion (Stricker, 1968), which allows changes in physiological parameters to be compared at multiple levels of volume depletion. PEG treatment also reliably induces water and sodium retention (Stricker & Jalowiec, 1970; Bennett & Gardiner, 1986). Alternatively, inflating a balloon in the inferior vena cava can block the return of venous blood to the heart to simulate hypovolemia, resulting in reductions in both urine output and sodium excretion (Fitzsimons & Moore-Gillon, 1980).

1.2.1 Hypovolemia and VP release

Volume depletion of about 5-10% by either hemorrhage (Chen, Morris, Key, & Chen, 2004) or PEG induced hypovolemia (Dunn, Brennan, Nelson, & Robertson, 1973) is sufficient to induce VP release. Hypovolemia induces VP

release, due in part to activation of atrial and ventricular stretch receptors (Wang et al., 1983; Grindstaff et al., 2000; Quail, Woods, & Korner, 1987; Menninger, 1979). Blood pressure signaling through baroreceptor pathways in the A1 region of the VLM also contributes to VP neuron activation (Buller et al., 1999). However, even after performance of sinoaortic denervation to remove baroreceptor input, a residual hypotensive activation of VP neurons is apparent, possibly due to circulating angiotensin II (Ang II) (Dampney, Polson, Potts, Hirooka, & Horiuchi, 2003), which can activate SON neurons by signaling through the SFO (see section 1.3). Larger depletions of volume dramatically increase release of VP (Weitzman, Glatz, & Fisher, 1978).

The dual nomenclature assigned to VP (vasopressin or antidiuretic hormone) reflects two discrete activities of this neuropeptide in the body, namely vascular pressor and renal antidiuretic effects. These activities arise at two different concentrations of plasma VP. Under normal conditions, urine osmolality increases as a linear function of plasma VP concentrations. Within the range of 1-5 pg/mL, urine will continue to increase in osmolality in relation to VP levels, but for serum VP levels greater than 10 pg/mL, there is no additional antidiuresis (Robinson, 1985). Elevations in blood pressure resulting from the vasopressor actions of VP begin at around 10-20 pg/mL, reaching a statistically significant difference at 30 pg/mL (Malayan, Ramsay, Keil, & Reid, 1980). Thus VP activity can be seen as biphasic; antidiuretic at low concentrations and vasopressive at high concentrations. It has also been suggested that the secretion of VP also occurs in two phases in response to hypovolemia (Verbalis, 2003). Plots of

hypovolemic reductions against VP secretion generate exponential relationships, with VP secretion increasing slowly between 0 and 10% hypovolemia (plasmaVP < 10 pg/mL), and rapidly for volume reductions exceeding 15% (plasma VP > 15 pg/mL) (Dunn et al., 1973). Similar results have been reported by Stricker et al., who find that volume reductions exceeding 22% rapidly increase VP secretion, while reductions below this level only increase VP levels gradually (plasma VP < 12 pg/mL) (Stricker, Schreihofer, & Verbalis, 1994). It has been suggested by Verbalis that for mild reductions in volume (below 10%), the primary effect of hypovolemia is to modulate the slope of the VP-osmolality relationship (Verbalis, 2003).

Thus we see that the linear relationship between VP and osmolality, generated as described in 1.1.3, can be modified under certain pathophysiological conditions (Figure 1.3) (Verbalis, 2003). The osmosensory gain regulating VP secretion is increased by hypovolemia or hypotension and decreased by hypervolemia or hypertension (Verbalis, 2003). As a result, water balance is modulated by the current state of blood pressure or volume in a manner consistent with the restoration of homeostasis.

1.2.2 Hypovolemia and OT release

The oxytocin response to hypovolemia is more complex. Oxytocin is natriuretic (Figure 1.1) at physiological hormone levels (Verbalis et al., 1991) and stimulates atrial natriuretic peptide (ANP) secretion (although not in humans (Rasmussen, Simonsen, Sandgaard, Hoilund-Carlsen, & Bie, 2004)), and as such

is counterproductive in situations requiring salt retention to restore blood volume. Some studies using hemorrhagic stimuli sufficient to elicit VP release show no change in OT levels (De Wardener et al., 1968; Ginsburg & Smith, 1959; Beleslin, Bisset, Haldar, & Polak, 1967; Clark & Silva, Jr., 1967; Poulain, Wakerley, & Dyball, 1977), while others do see increases in OT in response to hemorrhage (Lipinska, Zebrowska-Badalla, & Lipinska, 2006; Rosella-Dampman, Hartman, & Summy-Long, 1987; Kasting, 1988; Fabian, Forsling, Jones, & Lee, 1968; Kadekaro et al., 1998). A closer assessment of mild hypovolemic stimuli suggests that VP and OT differ in their responsiveness to hypovolemia. After measuring hormone secretion in response to polyethylene glycol (PEG) induced hypovolemia, Koehler et al. produced an experimentally-determined regression model which suggests that oxytocin has a much higher secretion threshold (i.e. OT is secreted at blood volume depletions greater than 25%) compared to vasopressin (secreted at depletions greater than 0%) (Koehler, McLemore, Martel, & Summy-Long, 1994). Similarly, Stricker et al. found that OT displayed an estimated release threshold of 22-28% blood volume depletion in response to PEG (Stricker et al., 1994). Together, these studies suggest that mild hypovolemic stimuli are not sufficient to elicit OT secretion.

In response to graded hemorrhage, activation of OT cells required greater volume decreases to induce activity as assessed by c-fos (FBJ murine osteosarcoma viral oncogene homolog) co-expression (Roberts et al., 1993; Buller et al., 1999). Recording action potentials from putative VP and OT cells in rats subjected to inferior vena cava occlusion as a model of hypovolemia, Khanna et

al. noted an increased activity in both VP and OT cells in response to severe caval occlusion, but only VP cells increased their activity in response to moderate occlusion (Khanna, Sibbald, Smith, & Day, 1994). In summary, although VP and OT are regulated in a similar fashion by osmolality, studies of hypovolemia induced secretion may suggest blunted OT release compared to VP release in response to mild hypovolemia.

1.2.3 Ang II facilitates volemic homeostasis

In response to reductions in blood volume due to hemorrhage, PEG administration, or simulated hypovolemia with caval ligation, renin is released from the kidney, resulting in an elevation Ang II plasma concentrations (Antunes-Rodrigues et al., 2004; Leenen & Stricker, 1974; Abdelaal, Mercer, & Mogenson, 1976; Brown, Davies, Lever, Robertson, & Verniory, 1966; Scornik & Paladini, 1964). Ang II increases blood pressure by acting on the vasculature to increase vascular tone and pressure, and on the kidney to increase blood volume by reducing water excretion (Hall, Guyton, & Mizelle, 1990) and reducing sodium excretion by triggering the release of aldosterone, an antinatriuretic hormone (Mulrow & Ganong, 1962; Tomaschitz, Pilz, Ritz, Obermayer-Pietsch, & Pieber, 2010).

In addition to its peripheral effects, Ang II acts at the CNS to influence fluid homeostasis (Antunes-Rodrigues et al., 2004; Fitzsimons, 1998). Ang II in the brain stimulates an immediate thirst followed by a delayed salt appetite, where the time-course of each matches the pattern seen with acute experimental

hypovolemia (Fitzsimons, 1998), suggesting that Ang II may underlie hypovolemic induced thirst. This theory is supported by the observation that mice lacking Ang II display no thirst responses to hypovolemia, while osmotic thirst is unimpaired (McKinley et al., 2008). Ang II facilitates sympathetic outflow, which elevates mean arterial pressure, as well as increasing renal sympathetic nerve activity to increase blood volume by increasing sodium retention (Chen & Toney, 2001; Chen & Toney, 2003). The majority of these effects are mediated by the angiotensin type 1 receptor (AT1R), in particular the AT1aR isoform (Davisson, Oliverio, Coffman, & Sigmund, 2000; Lazartigues et al., 2002; Lazartigues et al., 2008; Ito et al., 1995; Chen, Bassi, Walther, Thomas, & Allen, 2010), although both AT1bR and angiotensin type 2 receptor (AT2R) knockout mice also show drinking deficits (Davisson et al., 2000; Hein, Barsh, Pratt, Dzau, & Kobilka, 1995). Taken together, these results indicate that Ang II is an important mediator of volemic homeostasis, acting to correct fluid deficits by promoting positive water and sodium balances under hypovolemic conditions.

1.3 Ang II activates MNCs in the SON

It has been suggested that the transition of peripheral neurohormone signals into the central nervous system is mediated by sensory circumventricular organs (Fry & Ferguson, 2007; Ferguson et al., 1990). As such, the transition of Ang II signaling from the periphery to the CNS is thought to occur via a stimulation of the subfornical organ (SFO) by circulating Ang II (Rowland, Goldstein, & Robertson). Ang II in the circulation increases activity in SON-

projecting SFO neurons, which in turn modulate SON activity (Ferguson & Renaud, 1986). Activation of SON neurons by electrical stimulation of SFO can be blocked by inhibiting Ang II, indicating that SFO-SON projections are angiotensinergic (Jhamandas, Lind, & Renaud, 1989; Wilkin et al., 1989). SFO neurons are activated by hypovolemia and hypotension (Potts, Ludbrook, Gillman-Gaspari, Horiuchi, & Dampney, 2000; Nicolaidis, Ishibashi, Gueguen, Thornton, & de Beaurepaire, 1983) which suggests that Ang II levels will be elevated in the SON under hypovolemic conditions (see Figure 1.2). Central Ang II has been shown to increase VP secretion (Antunes-Rodrigues et al., 2004; McKinley et al., 2001) and OT secretion (Ferguson & Kasting, 1988; Lang et al., 1981) from the hypothalamus.

Neurohypophysial hormone secretion in response to Ang II is likely mediated by the AT1R, as demonstrated by the ability of the specific AT1R antagonist losartan to block MNC excitation in response to electrical (Li & Ferguson, 1993) or Ang II stimulation (Xu & Xinghong, 1999) of the SFO. Supraoptic MNCs express AT1Rs, as indicated by immunohistochemical labeling with AT1R specific antibodies (Phillips, Shen, Richards, & Raizada, 1993; Pfister et al., 1997). Although AT2Rs have also been identified in the SON (Reagan et al., 1994), and on the dendrites of VP neurons in the PVN (Coleman, Anrather, Iadecola, & Pickel, 2009), the majority of Ang II effects observed in MNCs are mediated by AT1Rs (Li & Ferguson, 1996; Yang, Phillips, & Renaud, 1992; Ozaki, Soya, Nakamura, Matsumoto, & Ueta, 2004). The canonical signaling pathway for the AT1R is G-protein (G_q) mediated activation of phospholipase C

(PLC) leading to phosphoinositol cleavage and calcium signaling (de Gasparo, Catt, Inagami, Wright, & Unger, 2000).

Thus we consider the transition of Ang II signaling into the CNS as a means by which volemic state can be communicated to the central nervous system. Within the CNS, Ang II mediates a variety of functions, some of which are outlined in section 1.2.3. As outlined in the following sections, Ang II has been shown to influence MNC activity through a modulation of intrinsic conductances, cell excitability, and circuit function. We have therefore selected Ang II as a candidate molecule to examine how hypovolemic signaling might influence osmosensory regulation of VP and OT secretion.

1.3.1 Ang II modulates MNC conductances

A number of studies provide us information about how Ang II may alter MNC activity. Ang II reduces a transient potassium current I_A in MNCs (Nagatomo, Inenaga, & Yamashita, 1995; Li & Ferguson, 1996) which will increase excitability (Fisher, Voisin, & Bourque, 1998). Ang II inhibits the fast afterhyperpolarization (AHP) in SON neurons (Nagatomo et al., 1995). The fast AHP is mediated by the large potassium conductance (BK) and is activated following an action potential (Scott & Brown, 2010). Inhibition of the fast AHP may promote the induction of phasic firing (Bourque, Kirkpatrick, & Jarvis, 1998). Ang II also increases resting membrane potential in MNCs (Latchford & Ferguson, 2004; Chakfe & Bourque, 2000; Yang et al., 1992). Ang II mediated

modulation of these intrinsic conductances in MNCs will impact MNC excitability and firing rates. However, such global changes to MNC activity will not be specific to the osmoregulatory function of MNCs.

1.3.2 Ang II modulates the SICC

Previous work in our lab has demonstrated that Ang II can excite MNCs by activating the SICC that produces intrinsic osmosensitivity in these cells (Zhang & Bourque, 2008; Chakfe & Bourque, 2000). Ang II acts to increase the probability of opening of SICCs (Chakfe & Bourque, 2000). This occurs in conjunction with an increased osmotic and mechanical sensitivity of MNCs, mediated by Ang II activation of phospholipase C (PLC) and calcium release (Zhang & Bourque, 2008). The increase in osmosensitive current is produced by an Ang II evoked increase in cortical actin density (see section 1.1.3a). Ang II therefore results in a depolarization and an increase in the intrinsic osmosensitivity of MNCs in the SON. This increased MNC osmosensitivity induced by Ang II suggests a mechanism by which Ang II might modulate the VP-osmolality relationship (see Figure 1.3).

However, both OT and VP neurons express intrinsic osmosensitivity (Sharif-Naeini et al., 2006; Blackburn & Leng, 1990; Chakfe & Bourque, 2000), and are regulated in a similar fashion by osmotic pressure (Negoro et al., 1988). Studies reporting Ang II modulation of SICC channel activity or excitability have not made a distinction between OT and VP neurons (Chakfe & Bourque, 2000; Zhang & Bourque, 2008). If SICC channel activity is regulated the same way in

both OT and VP neurons, then increased osmosensitivity of MNCs would not be sufficient to explain the blunted OT release observed under mild hypovolemic conditions (section 1.2.2). It therefore remains to be tested whether Ang II differentially activate the SICC current in VP and OT cells.

1.3.3 Ang II modulates synaptic transmission

In previous studies, Ang II has been shown to influence the frequency of synaptic events. Ang II modulates frequency of spontaneous excitatory post-synaptic potentials (EPSPs) in MNCs of the SON and PVN (Latchford & Ferguson, 2004; Ozaki et al., 2004). Increases in osmotically induced EPSP frequency are correlated with increased spike output in MNCs (Richard & Bourque, 1995), suggesting that modulation of synaptic transmission is another mechanism by which Ang II may facilitate MNC osmosensitivity. As mentioned in section 1.1.3b, Ang II-induced secretion of VP is abolished in OVLT lesioned animals (Thrasher & Keil, 1987), indicating that OVLT-SON transmission is necessary for Ang II-induced activation of VP cells. This finding suggests that Ang II may also mediate its effects on MNCs through a modulation of OVLT-SON transmission. A previous report of neuropeptide modulation in this pathway indicated that presynaptic inhibition of OVLT transmission prevents MNC action potential generation in response to osmotic stimulation of OVLT (Richard & Bourque, 1996). A recent study indicates that the neuropeptide endothelin can differentially modulate synaptic transmission in VP and OT neurons (Zampronio, Kuzmiski, Florence, Mulligan, & Pittman, 2010). Ang II induced modulation of

synaptic transmission therefore presents a possible mechanism by which osmosensitivity may be differentially affected in VP and OT neurons. As such, we have chosen to focus our study on the synaptic modulation of MNC osmosensitivity.

Hypothesis 1: Ang II differentially modulates OVLT to SON neurotransmission to increase osmotic evoked responsiveness in VP neurons and blunt osmotic evoked responsiveness in OT neurons.

1.4 Modulation of MNC activity

Maintenance of fluid homeostasis requires the concerted efforts of multiple systemic reflexes. Neuropeptides such as Ang II represent a common link for nervous, endocrine, cardiovascular, and renal systems, and often utilize multiple signaling mechanisms to exert their effects. An examination of neuropeptide signaling mechanisms in MNCs may therefore identify potential modulatory mechanisms for Ang II. Neuropeptides have been demonstrated to modulate MNC activity through modification of the activity of MNC ion channels (Kozoriz, Kuzmiski, Hirasawa, & Pittman, 2006; Slugg, Ronnekleiv, Grandy, & Kelly, 1999). In addition to these intrinsic mechanisms, a number of neuropeptides exert effects through the modulation of synaptic activity, as noted for endothelin (Zampronio et al., 2010). Likewise, prokinecitin-2, and ghrelin modulate the frequency of excitatory synaptic events in MNCs (Yuill, Hoyda, Ferri, Zhou, & Ferguson, 2007; Yokoyama et al., 2009), while neuropeptide FF

increases the frequency of inhibitory synaptic events (Jhamandas, MacTavish, & Harris, 2006). Leng et al. suggest that in MNCs inhibitory transmission increases the linearity of response of action potential frequency to increasing excitatory drive, as well as expanding the dynamic range over which MNCs respond to excitatory input (Leng et al., 2001). Thus modulation of synaptic input may have a profound effect on MNC activity.

In MNCs, Ang II has previously been shown to act through many of the mechanisms outlined above, which makes predicting a mechanism for neuromodulation difficult. As mentioned in section 1.3.1, Ang II inhibits the voltage activated current I_A (Li & Ferguson, 1996). Current density is higher in VP neurons than OT neurons, resulting in a reduced responsiveness to current injection in VP neurons (Fisher et al., 1998). I_A inhibition may therefore preferentially enhance VP neuron responsiveness to synaptic input. Ang II also influences synaptic transmission in MNCs (Latchford & Ferguson, 2004; Ozaki et al., 2004) as well as activating the SICC and enhancing responsiveness to osmotic stimulation (Zhang & Bourque, 2008). In fact, it is reasonable to assume that Ang II may act through multiple mechanisms simultaneously to influence activity. This issue will be addressed more fully in section 1.5. Proceeding with our hypothesis that Ang II mediates a differential regulation of osmosensory neurotransmission, we will attempt to define a synaptic mechanism by which transmission may be altered. Synaptic modulation is typically divided into the categories of postsynaptic and presynaptic modulation.

1.4.1 Postsynaptic alterations in MNC neurotransmission

Like many synapses in the nervous system, SON neurons display activity dependant modulations in the form of long term potentiation (LTP) and long term depression (LTD) (Panatier, Gentles, Bourque, & Oliet, 2006). Expression of LTP/LTD is mediated by postsynaptic increases or decreases in AMPA (2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid) receptor current, and requires the activity of NMDA (N-Methyl-D-aspartic acid) receptors (Panatier et al., 2006). A novel mechanism for NMDA receptor mediated plasticity was identified in the SON, with the observation that D-serine is the endogenous co-agonist of NMDA receptors at MNC synapses (Panatier et al., 2006). NMDA receptor activity and LTP/LTD can therefore be regulated by gliotransmission, in that calcium dependant secretion of D-serine from astrocytes is necessary for expression of LTP (Henneberger, Papouin, Oliet, & Rusakov, 2010).

Glial regulated modulation of synaptic plasticity has also been shown to occur in response to noradrenergic stimulation of astrocytes, which subsequently release ATP to mediate AMPA receptor insertion in MNCs via P2X7 receptors, thus facilitating transmission (Gordon et al., 2005). Glial derived ATP also induces a form of distributed plasticity, where increased AMPA receptor insertion occurs at multiple synapses (Gordon et al., 2009). This form of distributed plasticity requires metabotropic glutamate receptor (mGluR) activation of astrocytic calcium, and has been suggested to permit adaptations to global changes in afferent activity (Gordon et al., 2009). Although the observed distributed plasticity is rapid, induced within minutes of onset of excitatory

stimulation, it persists for up to 45 minutes (Gordon et al., 2009). Such a persistent modulation of activity is a useful mechanism for mediating long term changes in homeostatic circuits (Gordon & Bains, 2006; Wamsteeker & Bains, 2010).

1.4.2 Presynaptic alterations in MNC neurotransmission

As with postsynaptic modulations, persistent changes in activity of MNC synaptic inputs can be generated by presynaptic mechanisms. Exposure to noradrenaline (NA) results in a "priming" of glutamatergic terminals that persists for at least 15 minutes (Gordon & Bains, 2003). This priming arises from a downregulation of mGluRs on the presynaptic terminal, which reduces presynaptic inhibition. Subsequent re-application of NA results in a substantially facilitated release of glutamate. A pharmacological survey of presynaptic mGluR receptors present at presynaptic terminals in the SON reveals the presence of group III mGluRs, and mGluR8 in particular (Panatier, Poulain, & Oliet, 2004). Group III mGluRs subserve the function of regulating synaptic transmission by inhibiting transmitter release in response to glutamate spillover (Kuzmiski & Bains, 2010). A compelling new finding suggests that the down regulation of mGluRs in MNCs induces a metaplastic state (Kuzmiski, Pittman, & Bains, 2009). Down regulation of mGluRs facilitates the subsequent induction of LTP, indicating glutamatergic synapses are more plastic.

Although long term alterations in synaptic transmission allow for the ability to adapt to persistent changes in physiological state, such persistent

changes may lack sufficient adaptability to respond to ongoing alterations in internal state. Alternatively, rapid, reversible modulation of presynaptic transmission has been demonstrated in MNCs.

1.4.2a Dormant presynaptic terminals

Delaney et al. describe a rapid and reversible presynaptic inhibition induced by noradrenaline that results from a decrease in the number of active release sites (Delaney, Crane, & Sah, 2007). The inhibition is not dependant on calcium influx, nor does it change release probability. Conversely, the presence of dormant presynaptic synapses under basal conditions (Moulder et al., 2008) means that the rapid activation of these synapses (Chang, Jiang, Moulder, & Mennerick, 2010) could facilitate neurotransmission. In the SON, Trudel and Bourque demonstrate that presynaptic inhibition of OVLT transmission resulting from stimulation of the SCN is both rapid and reversible and propose that this inhibition is mediated by the silencing of presynaptic terminals (Trudel & Bourque, 2010). As recruitment of dormant presynaptic terminals can be triggered by diacylglycerol (DAG) analogs (Chang et al., 2010), and Ang II can signal through PLC in MNCs (Zhang & Bourque, 2008), we might propose that Ang II can enhance transmission at OVLT-SON synapses by PLC mediated activation of DAG independent of release probability.

1.4.2b Presynaptic release probability

A number of signaling intermediates have been shown to alter presynaptic probability of release of terminals made onto magnocellular neurons (Oliet, Baimoukhametova, Piet, & Bains, 2007; Kombian, Hirasawa, Mouginot, & Pittman, 2002). For example, dopamine (Price & Pittman, 2001) or galanin (Kozoriz et al., 2006) reduce EPSC amplitude while increasing paired pulse ratio, indicating a reduction in release probability (Zucker & Regehr, 2002) (see also Figure 2.6).

Somatodendritic release of OT from OT neurons induces a reduction in glutamatergic presynaptic release probability (Hirasawa, Kombian, & Pittman, 2001). OT triggers release of endogenous cannabinoids (eCB), which act on presynaptic N-type calcium channels to inhibit neurotransmitter release (Hirasawa et al., 2001). The ability of eCBs to modulate calcium dynamics makes them appealing candidates for short term regulation of synaptic transmission. Additionally, retrograde release of eCBs allows synaptic modulation in a target selective fashion (Oliet et al., 2007), which suggests a possible mechanism for differential regulation of activity in VP and OT cells.

1.4.3 Retrograde messengers in the SON

As mentioned above, retrograde signaling has been implicated in the modulation of presynaptic function in MNCs. Retrograde transmission is mediated by retrograde messengers such as members of the eCB family

(Chevaleyre, Takahashi, & Castillo, 2006) or nitric oxide (NO) (Ozaki et al., 2000).

1.4.3a Endocannabinoids in the SON

Supraoptic neurons express fatty acid amide hydrolase, a hydrolysis enzyme for eCBs, and show immunoreactivity for the cannabinoid receptor type 1 (CB1) in presynaptic processes (Hirasawa et al., 2004). In the SON, eCB activity can rapidly suppress spontaneous excitatory postsynaptic current (sEPSC, synaptic currents which occur in response to presynaptic action potentials) frequency, an effect which washes out within a few minutes (Zampronio et al., 2010). Although blockade of eCB signaling did not alter basal miniature EPSC (mEPSC, random synaptic currents which occur in the absence of presynaptic action potentials) frequency in coronal slices (Di et al., 2005), it seems likely that a basal amount of synthesis and release of eCB in the SON may occur in vivo. Di et al. were able to detect both anandamide and 2-arachidonoyl glycerol in the SON under control conditions, and the relative amounts of these compounds displayed an activity-dependent increase (Di et al., 2005). An interesting finding by Sabatier and Leng suggests that eCBs can selectively inhibit OVLT to SON transmission in OT cells in response to application of α-MSH (Sabatier & Leng, 2006).

1.4.3b Nitric oxide in the SON

Many neurons in the SON express the synthesis enzyme for NO, nitric oxide synthase (NOS) (Stern, 2004). Intracerebroventricular (ICV) administration Ang II stimulates NADPH-diaphorase (nicotinamide adenine dinucleotide phosphate) containing neurons in SON MNCs (Zhu & Herbert, 1997), suggesting Ang II may activate NO production. NO can act as a retrograde signal in MNCs to facilitate inhibitory postsynaptic current (IPSC) frequency (Bains & Ferguson, 1997; Latchford & Ferguson, 2003; Ozaki et al., 2000). NO mediated facilitation of glutamate release has been suggested to produce the somatodendritic release of VP in the SON (Gillard et al., 2007). NO production has been demonstrated to potentiate glutamatergic transmission through the activation of cGMP/PKG (protein kinase G) signaling to enhance presynaptic N-type Ca^{2+} channel activity (Huang, Chan, & Hsu, 2003). Application of a cGMP analogue to mimic downstream effects of NO altered frequency and amplitude of IPSCs and EPSCs in the SON (Ozaki et al., 2000).

Thus we find that retrograde messengers have the potential to rapidly and reversibly alter the probability of release of MNC synaptic inputs through calcium dependant alterations in presynaptic probability of release. The ability of eCBs to depress release probability and NO to facilitate of release probability makes these molecules attractive potential candidates for differential synaptic modulation. Indeed, the observation by Di et al. that MNCs can facilitate release of GABA (gamma-aminobutyric acid) through NO production and also depress release of glutamate through eCB production demonstrates the ability of MNCs to

differentially regulate synaptic transmission via retrograde signaling (Di, Maxson, Franco, & Tasker, 2009).

Hypothesis 2: The differential signal modulation by Ang II on MNCs is accomplished by an increased presynaptic probability of release in VP cells, and a decreased presynaptic probability of release in OT cells, mediated by NO and eCB production, respectively.

1.5 What impact does synaptic modulation have on MNC activity?

As described in section 1.3, Ang II has substantial and diverse effects on MNC activity. A number of these modulations would be expected to alter MNC activity or osmosensitivity. As previously noted, Ang II reduces the transient potassium current I_A in MNCs (Li & Ferguson, 1996; Nagatomo et al., 1995). The presence of I_A in MNCs would tend to reduce spike output in response to synaptic input, as I_A activates in response to rapid depolarizations (Fisher & Bourque, 1998). The suggestion that I_A may specifically localize to dendrites in VP neurons (Widmer, Boissin-Agasse, Richard, & Desarmenien, 1997) is in accordance with a potential role for I_A in reducing synaptic responsiveness. Ang II produces an increase in resting membrane potential in MNCs (Chakfe & Bourque, 2000; Latchford & Ferguson, 2004) by activation of the mechanosensitive SICC (Zhang & Bourque, 2008). This would increase spike output, and also enhance intrinsic osmosensitivity (Zhang & Bourque, 2008).

Overlaid on the modulation of intrinsic activity is the increase in excitatory synaptic currents evoked by Ang II in the SON (Ozaki et al., 2004). Although it has long been recognized that both membrane potential and synaptic activity contribute to the coordinated output of activity in magnocellular neurons, the relative contributions of each to osmosensory physiology are not so clearly defined. Leng and colleagues (Leng et al., 1982) have suggested that the intrinsic osmosensitivity of MNCs serves to render the cell sensitive to osmotic input by moving the membrane potential closer to spike threshold. A computational model comparing frequency of excitatory potentials to action potential frequency suggests that membrane depolarization increases the intrinsic osmosensitivity of MNCs would therefore play a permissive but not instructive role in maintaining the VP/osmolality relationship. This notion is supported by the observation that lesions of the lamina terminalis render VP secretion insensitive to osmotic perturbations, despite the fact that MNCs remain intact and are still able to secrete VP (McKinley et al., 2004b). In MNCs, spontaneous EPSP frequency is correlated with SON spike output (Richard & Bourque, 1995), and in the absence of osmosensory afferents from the OVLT, MNC osmosensitivity is dramatically reduced (Leng et al., 1989). Based on these observations, we may predict a prominent role for synaptic transmission in regulating MNC activity.

However, the observation that OVLT specific lesions produce a blunted, but not absent modulation of VP secretion in response to osmolality (Thrasher & Keil, 1987) indicates that intrinsic osmosensitivity does play a role in determining MNC activity. Moreover, the demonstration that osmotic perturbations of >2%

are able to evoke or facilitate firing in isolated MNCs (Bourque, 1989; Oliet & Bourque, 1992) supports a prominent role for intrinsic osmosensitivity, especially with large deviations in osmotic pressure (see also (Bourque, 1998)). A novel computational model of MNC firing activity suggests that for low (~1-5 Hz) firing rates, synaptic activity dominates the control of MNC firing, but above 5 Hz, MNC firing rates are insensitive to synaptic activity in the absence of depolarizing current (Nadeau & Mouginot, 2011).

Excitatory inputs play a critical role in the maintenance of osmosensory activity in the SON (Richard & Bourque, 1995). However, the sum total of synaptic modulation, depolarization, and removal of the suppressive effects of I_A necessitates a complex interpretation of any Ang II induced activity modulation in MNCs. To what extent then, can we attribute a change in MNC activity to a change in synaptic modulation? To answer this question, we attempt to isolate the synaptic component of the Ang II modulation, in order to assess the contribution of synaptic modulation to spike output.

Hypothesis 3: Modulation of synaptic transmission is sufficient to produce a functionally relevant impact on MNC firing rate.

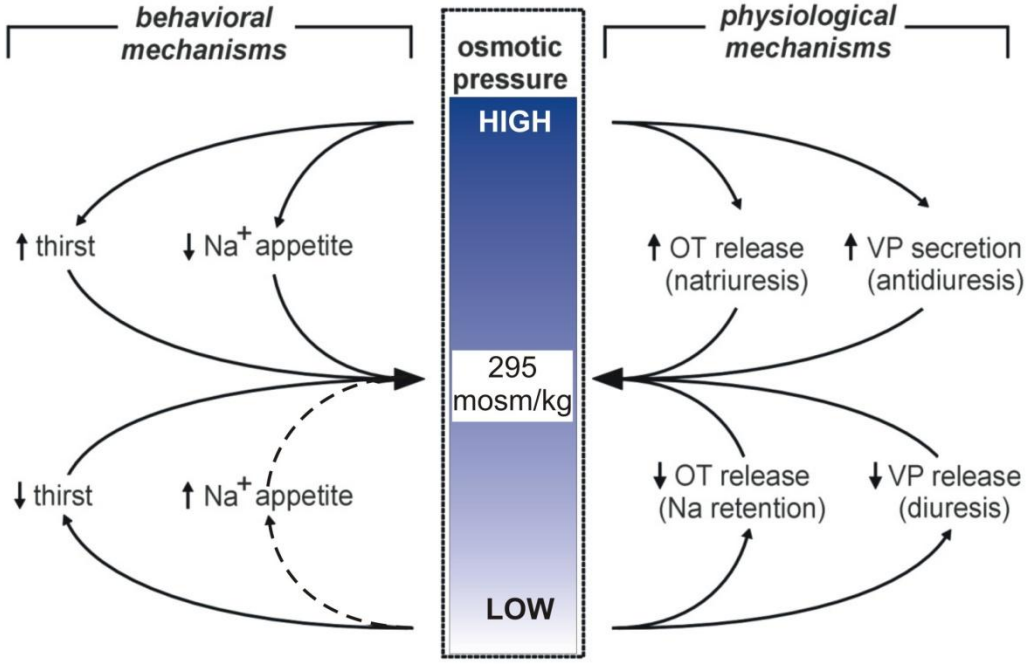

Figure 1.1 Homeostatic mechanisms of osmotic regulation

Osmotic pressure varies around a set point of 295 mosm/kg in rats. The set point is defended by behavioral and physiological mechanisms that will alter salt and water intake and excretion. Increases in osmotic pressure will result in increased water intake through elevated thirst and increased water retention through VP induced antidiuresis. The opposite is true for sodium, in that increased osmotic pressure suppresses salt appetite and increases natriuresis by increasing OT secretion. Inverse responses are triggered by decreases in osmotic pressure. These physiological and behavioral modifications will act to restore osmotic pressure to the set point.

Adapted from (Voisin & Bourque, 2002).

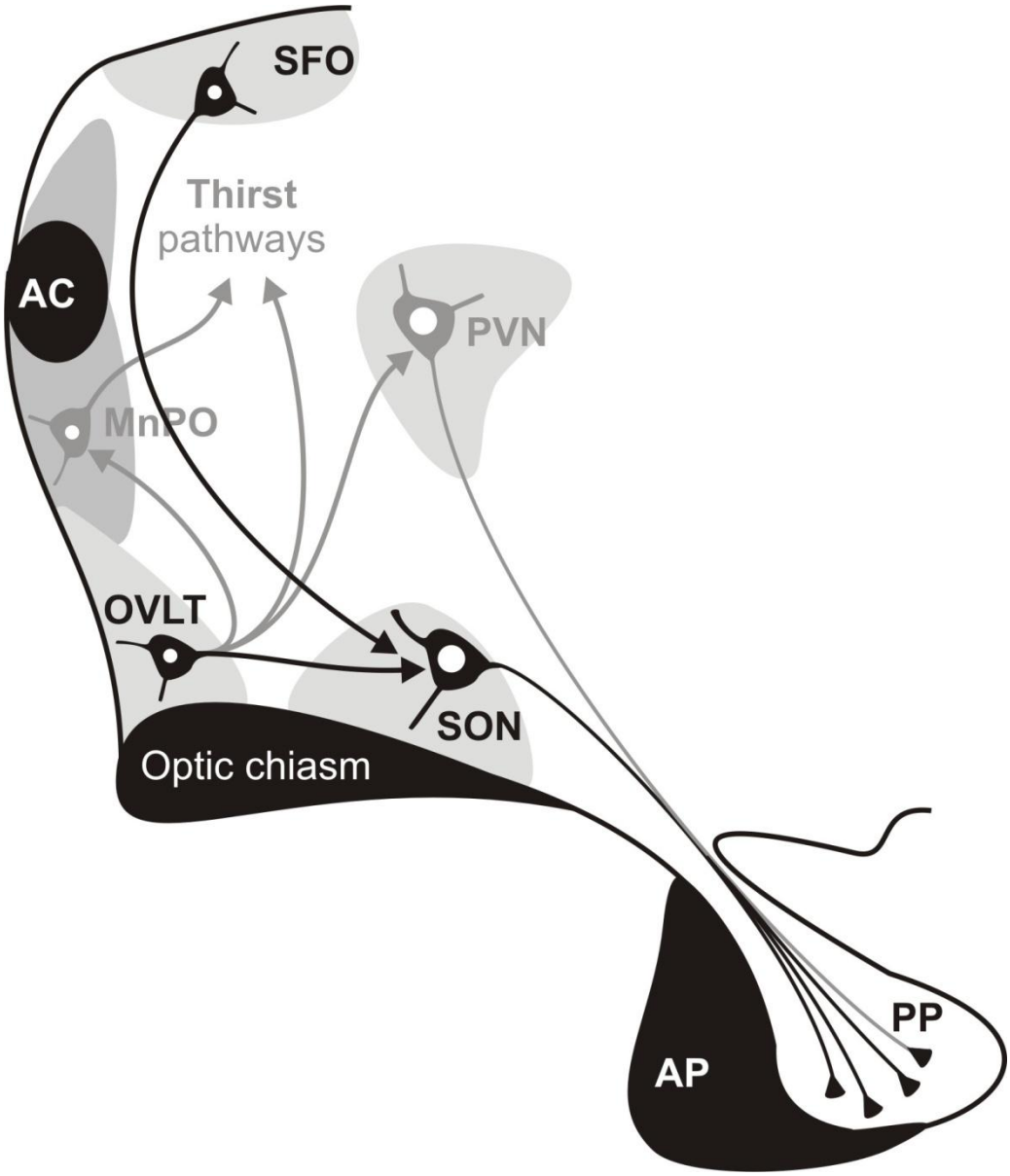

Figure 1.2 Hypothalamic networks for osmotic homeostasis

The central regulation of fluid balance is mediated by a number of interconnected nuclei. A simplified schematic illustrates some of the connections involved in osmotic homeostasis. Sensory circumventricular organs, including the OVLT (organum vasculosum lamina terminalis) and SFO (subfornical organ) detect changes in osmotic pressure or plasma angiotensin. Information regarding osmotic balance detected by the OVLT is relayed to integrative centers like the

MnPO, to higher brain areas regulating thirst, or to magnocellular neurons in the SON (supraoptic nucleus) or PVN (paraventricular nucleus). Activation of SFO neurons by circulating plasma Ang II results in enhanced neural transmission to the SON, mediated by angiotensinergic fibers. The SFO is therefore a source of Ang II for the SON (details in section 1.3).

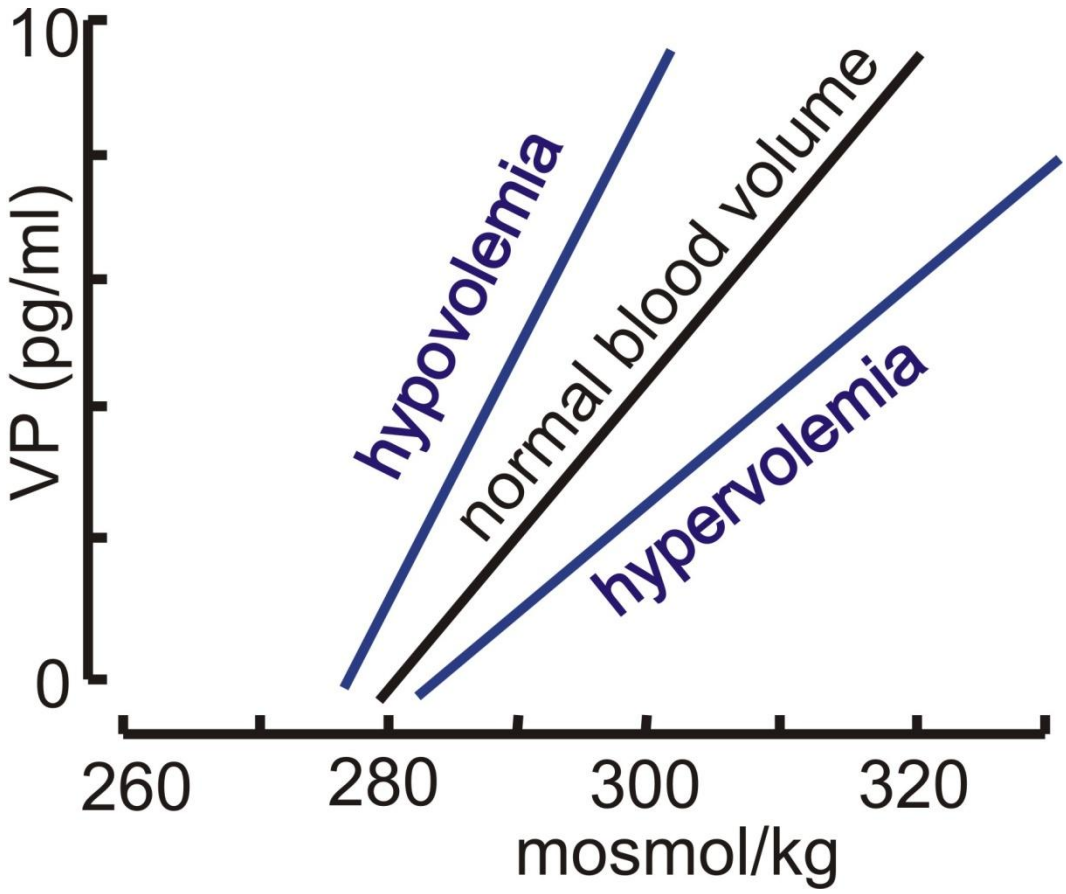

Figure 1.3 Modulation of the vasopressin/osmolality relationship

The osmotic regulation of MNC activity generates a linear relationship between plasma VP and osmolality. Under certain pathophysiological conditions the slope of this relationship is altered, reflecting a change in osmosensory gain. Hypovolemia increased the slope of the VP-osmolality relationship to increase water retention and thus restore blood volume. Hypervolemia decreases the slope of the relationship, which will favor diuresis.

Adapted from (Verbalis, 2003).

Chapter 2 Methods

2.1 Rationale and Experimental Design

We propose that Ang II enhances excitatory transmission from the OVLT to the SON in a presynaptic manner. The evidence outlined above indicates that Ang II increases osmosensitivity of central fluid homeostatic mechanisms, and potentiates VP release in a manner that is physiologically relevant. We will therefore investigate the extent to which OVLT-SON excitatory transmission is altered in the presence of Ang II, and probe the mechanisms by which this alteration occurs. By using an established horizontal slice preparation containing both the OVLT and SON (Trudel & Bourque, 2003), we will track changes in OVLT-evoked activity in the SON by whole cell patch clamp of MNCs. Richard and Bourque have shown that although the OVLT does give rise to inhibitory currents, these currents are not osmosensitive (Richard & Bourque, 1995). We therefore chose to perform all recordings in the presence of picrotoxin to block all GABA-mediated events.

VP and OT neurons are indistinguishable by visual inspection. However, as roughly 70% of MNCs in the rat SON are VP cells, a random sampling of MNCs would tend to reflect the properties of VP rather than OT cells. The distinctive (but partially overlapping) distributions of VP and OT cells in the angled hypothalamic slice permit an even higher proportion of VP cell recordings by selectively patching cells based on relative location (Stachniak et al., 2011) (Figure 2.1). In order to readily identify cells as either vasopressinergic or oxytocinergic, it is possible to take advantage of a transgenic rat line expressing

enhanced green fluorescent protein (eGFP) under the vasopressin promoter (VPeGFP) (Ueta et al., 2005). Visualization of the fluorescent label in VP cells (Figure 2.2) is accomplished using a mercury lamp (Olympus, Model No. BH2-RFL-T3) through a green filter (Olympus, U-MNB2). Cells were categorized as putative vasopressin based on recording position or presence of eGFP, or putative oxytocin based on absence of eGFP combined with recording position (Figure 2.3). Definitive characterization was accomplished by post-hoc immunohistochemisty for VP or OT in recorded cells labeled with neurobiotin (Figure 2.4).

Having established an experimental model and methodology for cell identification, we will undertake the experiments outlined below to examine our hypotheses.

Hypothesis 1: Ang II differentially modulates OVLT to SON neurotransmission to increase osmotic evoked responsiveness in VP neurons and blunt osmotic evoked responsiveness in OT neurons. [Chapter 3]

The native stimulus for activation of osmosensitive neurons in the OVLT is a change in osmotic pressure. By application of a hyperosmotic solution to the OVLT, we may observe evoked changes in spike rate of MNCs under current clamp conditions. We will apply hyperosmotic solutions specifically to the OVLT by means of a pressure pulse through a glass micropipette (Figure 2.5). For better control of OVLT activity, we will also evoke synaptic responses from OVLT by

electrical stimulation. For both cases, we will then assess the effects of Ang II in VP and OT neurons.

Hypothesis 2: The differential signal modulation by Ang II on MNCs is accomplished by an increased presynaptic probability of release in VP cells, and a decreased presynaptic probability of release in OT cells, mediated by NO and eCB production, respectively. [Chapter 4]

Using evoked EPSC amplitude as our readout, we will assess the mechanisms of action of Ang II. First, we will identify the site of action using various electrophysiological technical manipulations designed to differentiate presynaptic from postsynaptic modulations as well as identify calcium dependant modulation of release probability. Paired pulse facilitation (Figure 2.6) appears at a variety of synapses and is generally accepted to represent an effect of residual presynaptic calcium (Thomson, 2000; Zucker & Regehr, 2002). Likewise, asynchronous release has been noted as a calcium dependant mechanism at hippocampal (Cummings, Wilcox, & Dichter, 1996) and supraoptic (Iremonger & Bains, 2007) synapses. We will then apply pharmacological manipulations to identify the mechanisms of neuromodulation of Ang II.

Hypothesis 3: Modulation of synaptic transmission is sufficient to produce a functionally relevant impact on MNC firing rate. [Chapter 5]

The multifaceted actions of Ang II present a problem for the interpretation of any mechanistic findings in terms of information processing. We will attempt to isolate the effects of a specific component of Ang II neuromodulation, namely the change in synaptic inputs, on MNC spike output. We will first assess the change in spontaneous synaptic activity in response to Ang II. We will then adopt a synaptic "playback" paradigm, whereby MNCs will respond to current injection of an artificial waveform generated from recordings of spontaneous synaptic activity to mimic the effects of synaptic transmission. Assessing spike output under these conditions will indicate whether synaptic neuromodulation is sufficient to regulate MNC activity.

2.2 Tissue Preparation

All protocols are performed in accordance with the McGill University Animal Care Committee guidelines. Animals include wild type male Long-Evans rats (50-80g, Charles River) and transgenic VPeGFP Wistar rats (Ueta et al., 2005) provided by Dave Andrew (Queens University, Kingston, ON). Due to limited availability of transgenic rats, we use both male and female rats (50-250g). Rats are anaesthetized with halothane (0.5mL/100g into a bell jar), sacrificed by decapitation and the brains quickly removed. Brain tissue is trimmed, angle mounted, and cut at a thickness of 400 μm for angled horizontal slice preparations as previously described, or alternatively flat mounted for cutting coronal sections (Trudel & Bourque, 2003).

Upon extraction the brain is immersed in ice-cold (0-4°C) oxygenated (5%/95% CO2/O2 mixture) artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl, 120; KCl, 3; NaH_2PO_4, 1.23; $MgCl_2$, 1.48; $CaCl_2$, 1; $NaHCO_3$, 25.95; and D-glucose, 10. The ventral brain region is trimmed with a razor blade into a block suitable for sectioning by making coronal cuts 3 mm rostral to the optic chiasma, and just rostral to the pons, and by making sagittal cuts bisecting the temporal lobes. The resulting block of tissue is glued by the dorsal surface to an angled (35°) mounting block so that the rostral pole slants upward. The assembly is then placed on the stage of a vibratome (Leica VT1200) with the ventral surface facing the blade and submerged in ice-cold oxygenated aCSF. The first cut (speed setting of 0.14, vibration amplitude 1.5mm on the Leica VT-1200) is made just caudal to the optic chiasma such that portions of the optic tracts visible in the discarded tissue form a small V shape. A single 400 μm slice is then cut and transferred to a recording chamber. The slice is submerged dorsal side up, immobilized with a C-shaped platinum wire fitted with nylon strings, and continuously perfused with 32°C oxygenated aCSF containing 100 μM picrotoxin, at a rate of 1-3 ml/min. The slice is generally left alone for 45 minutes prior to recording, to allow any cellular debris to wash clear of the recording field. Although each brain yields only one slice that maintains intact nuclei, with training the gross architecture of the slice readily indicates the suitability of a slice for recording even to the naked eye. A further microscopic evaluation of the orientation of the relevant nuclei is generally sufficient to suggest connectivity, as

good slices tend to have a stereotypic appearance. Magnocellular neurons are identifiable based on cell size (Oliet & Bourque, 1992).

For VP/OT immunohistochemical staining, 2 rats were deeply anaesthetized with Urethane (Sigma, 4g/mL, 0.7mL/100g animal i.p.) and perfused transcardially with 200 ml phosphate-buffered saline (PBS), followed by ice cold 4% paraformaldehyde (Fisher) in PBS. Brains were removed and postfixed in 4% paraformaldehyde overnight. Angled horizontal brain sections were cut on a Vibratome Series 1000 (Technical Products International, St. Louis, MO) at a thickness of 200 μm.

2.3 Recording

The slice is immobilized with a C-shaped platinum wire fitted with nylon strings. Slices are continuously perfused with 32°C oxygenated (95% O2; 5% CO2) aCSF. Picrotoxin (100 μM) is added to all solutions to remove inhibitory currents. Perfusion is accomplished by gravity flow. For mEPSC analysis, a switch to aCSF plus tetrodotoxin (TTX, 500 nM) was performed before the start of each recording, and the abolition of the sodium current was verified before recordings began.

Stimulation of the OVLT is accomplished by passing current through a bipolar electrode embedded adjacent to the OVLT (Trudel & Bourque, 2003). Bipolar stimulating electrodes were made from a pair of teflon-coated platinum-iridium wires (50 μm o.d.; A-M Systems Inc., Everett, WA). Coating was removed for the final 250μm of each wire. For stimulation, both wire tips were

inserted into the OVLT. Stimuli (10 to 70μA; 0.1 to 0.5ms) were delivered via an isolated stimulator device (DS2; Digitimer Ltd., Hertfordshire, England) triggered via the acquisition system (see below). Osmotic OVLT stimulation is accomplished by applying a hyperosmotic puff (+20 or +60 mosmol mannitol). Both osmotic and Ang II puffing were delivered using a picrospritzer (General Valve Corporation) through 3-6 MΩ glass pipettes aimed towards the nucleus, positioned about 0.5mm away from the center of the OVLT or edge of the SON. A 6 psi puff was delivered in a 0.5s/0.5s on/off fashion, which permitted visualization of the resulting tissue movement at the tip of the pipette to verify puff delivery.

Whole cell voltage clamp recording of MNCs is performed as described previously (Trudel & Bourque, 2003; Stachniak & Bourque, 2006). Glass capillary patch pipettes (1.2 mm o.d., 0.68 mm i.d., A-M Systems Inc.) were pulled on a micropipette puller (P-87, Sutter Instrument Co.), and filled with an internal solution containing (in mM): Potassium gluconate, 110; $MgCl_2$, 1; KCl, 10; HEPES 10 and adjusted to pH 7.4 with KOH, 282 milliosmolar with mannitol. In some cases (current clamp), the internal solution contained KMeSO4 instead of K gluconate and the addition of 4 mM ATP, 1mM GTP. Internal solutions with low dose (Rees, Palmer, Schulz, Hodson, & Moncada, 1990) N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME, 1 μM) or GDP-β-S (a non-hydrolyzable form of guanosine diphosphate, 1 μM) were used where indicated. Pipette resistance was 2-4 MΩ. In some cases, neurobiotin (Vector Labs 0.2% w/v) is

added to the pipette for post-fixation identification of cell phenotype, and osmolality of the internal solution was adjusted accordingly.

Cells are visualized using an Olympus BX51WI upright microscope coupled to a Photometrics (Tucson, AZ) Coolsnap cf2 digital camera run on RSImage software (Roper Scientific). Electrodes are visually guided to each cell using a motorized micromanipulator (SD Instruments Inc., Grants Pass, OR). Positive pipette pressure is maintained while manipulating the electrode towards the cell in order to minimize debris, and sweeping the pressurized flow over the intended cell can help insure that a clean giga-ohm seal is made prior to whole cell recordings. Under these conditions, pipette series resistance is 5-15 MΩ. Membrane potentials were not corrected for a liquid junction error of +2 mV. Unless otherwise stated, holding potential is -70 mV. Cell conductance and series resistance is monitored by applying a 50 mV hyperpolarizing pulse at the end of each sweep. Whole cell current and voltage is recorded using an Axopatch-1D amplifier (Axon Instruments Inc.), displayed on an oscilloscope and digitized using pCLAMP 8.0 software. For synaptic playback experiments, whole cell voltage was recorded on a Multiclamp 700B amplifier attached to a Digidata 1440A interface controlled with pCLAMP 10 software. Signals are analyzed offline using Clampfit 8 and Clampfit 10 software.

For neurobiotin labeled cells, each recording was followed by a 4 minute current injection protocol in voltage clamp, consisting of an oscillating current pulse to from -70 mV to +70 mV for 500 msec at 1Hz.

2.4 Drugs

Angiotensin II was purchased from Phoenix Pharmaceuticals (Burlingame, CA) in 20 mg lyophilized aliquots, stored at -80 degrees, diluted in aCSF and used immediately. We found that Ang II left in solution in a -20 degree freezer was of questionable stability. Although Ang II at 10^{-7} M was effective in initial bath applications, the issue of peptide stability engendered an increase in Ang II dose to supersaturating concentrations (10^{-5} M) for puffing applications. These precautions produced reliable responses to Ang II application. Ang II (10^{-5} M) was applied by pressure puff for all experiments except the following: miniature experiments (section 4.2.5), calcium experiments (section 4.2.2), osmotic puff experiments (section 3.2.1). DNQX (6,7-dinitroquinoxaline-2,3-dione, Tocris, Ellisville MO, final concentration 20 μM) and AM-251 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide, Tocris, final concentration 0.5 μM) were dissolved 1:10000 in DMSO. TTX (10^{-5} M) was purchase from Alamone Labs (Jerusalem, Israel). All other drugs were purchased from Sigma.

2.5 Analysis

2.5.1 Osmotic puff experiments

For osmotic puff experiments, cells in the OT group include identified neurobiotin labeled OT cells and eGFP negative OT cells. Cells in the VP group include neurobiotin labeled cells, eGFP positive cells and presumptive VP cells located in posterior, medial SON. Basal cell firing rates were adjusted to around 3

Hz by modulating current injected. Recorded firing frequencies ranged from about 0.5-6 Hz. A stable baseline of 30 sec was established and then a hypertonic pressure injection (puff, +20 or +60 mosm mannitol) was applied to the OVLT for 30-40 seconds. Angiotensin II was bath applied or puffed on the SON. In response to Ang II application, cells were classified as excited, inhibited, or non-responsive based on a 10% change in firing rate. Firing rate was readjusted by injection of current (0.1-5 pA) as necessary to compensate for any changes induced by Ang II. No significant differences were found between treatment conditions, so the groups were combined into one data set. Also, no significant differences were found between basal firing rates in any conditions and so values were normalized to basal firing frequency. Changes in firing frequency (Δ Hz) were determine by counting the number of spikes in 10 second intervals and averaging over 3 intervals for the 30 second period before puff onset (control condition), then comparing this to the 10 to 40 second period after puff onset for (hypertonic condition). Cells were removed from analysis if they showed no response to osmotic puff, if basal firing shifted dramatically before puffing, or if they had spike rates that fell dramatically over the course of the experiment.

2.5.2 Evoked current synaptic experiments

Sweeps containing OVLT-evoked EPSCs were averaged, and the average evoked response was used to position cursors either side of the peak average response, such that the mean peak amplitude from each individual evoked EPSC was recorded. This value was subtracted from the mean baseline value for each

sweep to give the evoked EPSC amplitude. The average EPSC amplitude derived from a stable region before, during, and after drug application from various trials was subjected to the student's paired T-test in Microsoft Excel.

Trials were excluded if the series resistance varied by more than 6 MΩ over the course of the trial, or if the holding current was greater than 200 pA at any point. Paired pulse ratio was determined by dividing the second EPSC amplitude (paired stimulus delay +60 msec) by the first EPSC amplitude. Paired pulse ratio values of less than 0.1 or greater than 10 were excluded. If the trials contained a relatively large percentage of failures, that trial was subjected to failure analysis instead of analyzing the change in amplitude. Any evoked EPSC less than 5 pA was considered a failure, and percent failure (# of failures/# of sweeps) was calculated before, during, and after Ang II treatment and then compared using the student's paired T-test. Example traces were lowpass filtered at 400 Hz for illustration purposes. Conductance was calculated based on average current change from -70 to -120 mV, and cells showing conductances greater than 3 nS were excluded from analysis. Putative OT cells included immunohistochemically identified neurobiotin labeled OT cells (n= 6) and VPeGFP negative cells (n=9). Putative VP cells included immunohistochemically identified neurobiotin labeled VP cells (n=5), VPeGFP positive cells (n=4), and cells presumed VP based on recording position (n=6).

To help discriminate between pre- and post-synaptic modulation, asynchronous release events were analyzed in cells displaying synchronous EPSC facilitation (VP) or depression (OT). Evoked synchronous EPSCs generally

decayed to sufficiently discriminate asynchronous events within 15 msec of onset. Events were therefore examined in a period from 15 msec after evoked synchronous EPSC onset to just before the stimulus artifact for the paired pulse protocol (+60 msec from the first stimulus, for an analysis time of ~40 msec per sweep). Twelve sweeps (2 minutes elapsed time) from the control period were compared to twelve sweeps from the Ang II treatment period. Event frequency was determined for each cell by counting the number of events and dividing by elapsed time. Median event amplitude for each cell was also determined and these averages were compared by student's T-test. Event amplitudes and inter-event intervals for all events were grouped across cells then compared by Kolmogorov-Smirnov test and expressed as cumulative probability plots.

2.5.3 Spontaneous synaptic experiments

Spontaneous synaptic currents were recorded at 10 kHz in VP cells, and at 2 kHz in OT cells and then detected using the template match function of Clampfit 10. Detected synaptic event amplitudes and inter-event intervals (IEI, time in ms between each event) were subjected to a Kolmogorov-Smirnov statistical analysis. Total event amplitudes or IEIs from stable regions (about 3 minutes) directly before or during Ang II treatment were compared for each trace. Graphs representing the cumulative probabilities of event amplitudes and IEIs were constructed from these sections of the continuous recordings, and the median amplitude and IEI were determined. The median values from all recordings were compared using the student's paired T-test in Microsoft Excel.

The analysis of synaptic events was restricted to recordings where series resistance was less than 20 MΩ, and did not vary by more than 3 MΩ. In an attempt to increase the portion of synaptic events that were osmotically evoked, we recorded under hypertonic conditions (+30 mosm mannitol) in 5/9 of the VP cells tested. This did not result in a statistically significant change in response.

2.5.4 Synaptic playback experiments

Based on the median frequencies and amplitudes for the group spontaneous EPSC (sEPSC) data, we selected representative traces showing comparable changes in frequency and amplitude to playback as a stimulus waveform. Four to six 30 second continuous intervals were selected from control and Ang II conditions, highpass filtered at 1 Hz, then intercalated with alternating conditions every 30 seconds, to produce a waveform oscillating in frequency and amplitude (see Figure 2.7). In 2/4 input files, all noise appearing in the inter-event interval (IEI) period was reduced to zero, while 2/4 input files included noise. This did not have a noticeable effect on the resulting outputs. This stimulus was played back to the cell as an input waveform in current clamp. Native spontaneous glutamate and GABA-mediated potentials were blocked with DNQX (20μM) and picrotoxin (100 μM). Output traces were not included in the analysis if resistance was less than 200 MΩ or firing rate rose or fell dramatically over the course of the experiment. To match basal firing to osmotic puff experiments, output traces were not included if basal firing was greater than 4 Hz.

Firing rates for each time interval (30 sec for sample output traces, 10 seconds for combined output traces) were normalized to the rate in the preceding time interval. For determining normalized spike output, the charge integral (area under the curve, or total charge transferred) was determined for sEPSCs detected in clampfit using the threshold function for event detection. Charge integral was summed for each time interval, and then normalized to the preceding time interval to plot change in normalized spike output against change in normalized charge integral. The use of charge transfer rather than current in this experiment provides a combined estimate of how frequency and amplitude are changing within a given time frame. Charge integral outliers in the normalized plot were removed according to Chauvenet's criterion (Chauvenet, 1960).

2.6 Statistics

All values are reported as either mean +/- standard error or median +/- standard error in the case of IEI and amplitude. A p-value of <0.05 is considered statistically significant. Statistical tests include Student's paired T-test within each experiment, Student's T-test assuming unequal variance across experiments. The chi-squared test was used to determine whether the number of cells that were excited, inhibited, or non-responsive to Ang II application differed between OT and VP cells. For synaptic playback experiments, the z-test was used for determining whether spike rates changed significantly. The above tests were performed in Microsoft Excel 2003, while the Kolmogorov-Smirnov test was done in Clampfit 10.

2.7 Immunohistochemistry.

For neurobiotin labeled cells, 1 cell per slice was recorded and its relative position in the nucleus noted. At the conclusion of the recording, tissue sections were immediately fixed in phosphate buffered saline (PBS) containing 4% paraformaldehyde, left 2 days and then washed in PBS and blocked at room temperature (PBS, 1% normal goat serum and 0.3% Triton X-100, 1 h). Sections were incubated for 1 to 4 days at 4°C with the primary antibodies, PS-41 mouse monoclonal anti-vasopressin (1:25) and VA-10 rabbit polyclonal anti-oxytocin (1:250), kindly provided by Dr. Hal Gainer (Altstein, Whitnall, House, Key, & Gainer, 1988; Ben Barak, Russell, Whitnall, Ozato, & Gainer, 1985). Sections were then washed in PBS and incubated in labeled goat anti-mouse (Alexa Fluor 488, 1:200, Invitrogen A11001), anti-rabbit secondary (Alexa Fluor 568, 1:200, Invitrogen A11011), and AMCA avidin (1:500, Vector A-2008) for 2 h, and washed in PBS. Neurobiotin/immunolabeled cells were visualized using RSImage software (Roper Scientific) connected to an Olympus IX71 microscope attached to a Lambda DG-4 fluorescence unit (Sutter). Some images were acquired and processed using MetaMorph software (Molecular Devices, Palo Alto, CA) with a spinning disk confocal system (PerkinElmer, Wellesley, MA) connected to a Leica (Nussloch, Germany) DMS upright microscope.

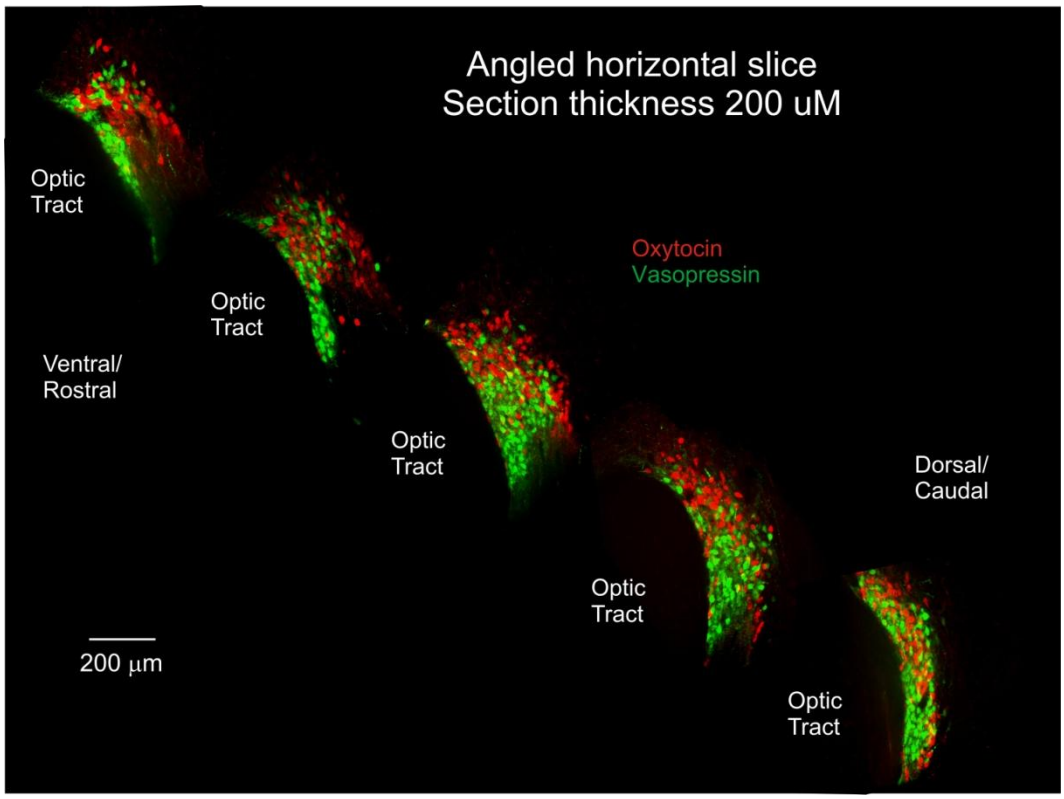

Figure 2.1 VP and OT distribution in the SON in an angled horizontal slice. Serial sections were taken through the SON in an angled horizontal plane and display different distributions for VP (green) and OT (red) neurons. VP cells tend to be more medial and caudal, while OT cells tend to be more rostral. From left to right, the illustrated sections proceed from more ventral and rostral sections to more dorsal and caudal sections. Slices used in recording experiments most closely resembled the middle section. Note the high density of VP cells in the caudal portion of this section.

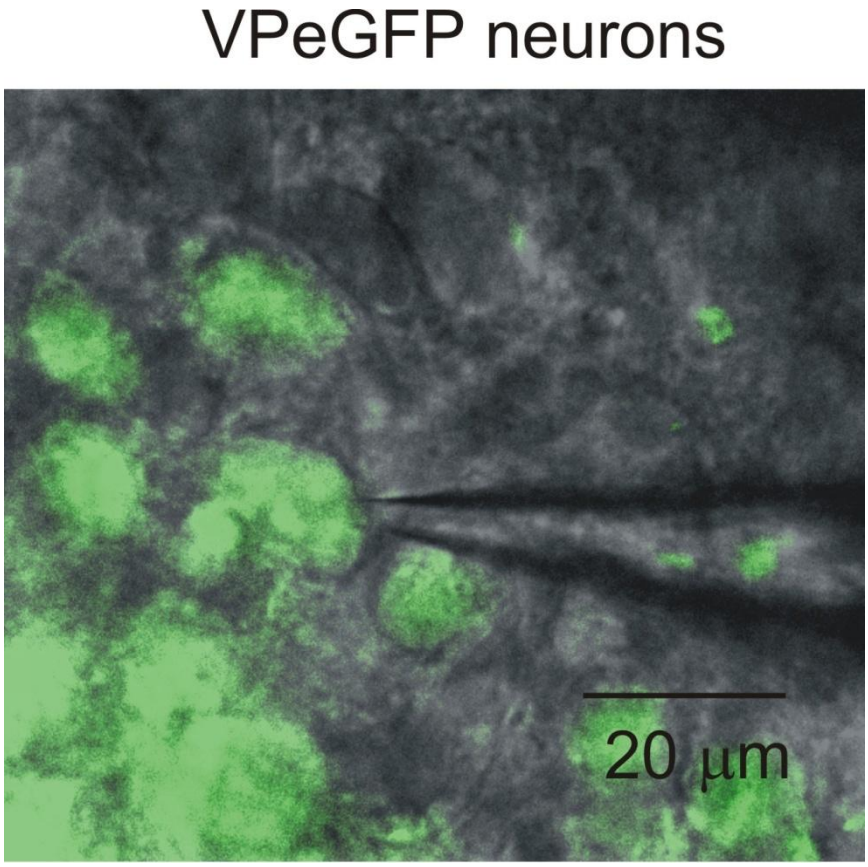

Figure 2.2 Visualization of the fluorescent label in VPeGFP cells

Transgenic animals expressing eGFP under the VP promoter were used to identify VP cells (fluorescent green cells) and putative OT cells (unlabeled cells) during recording.

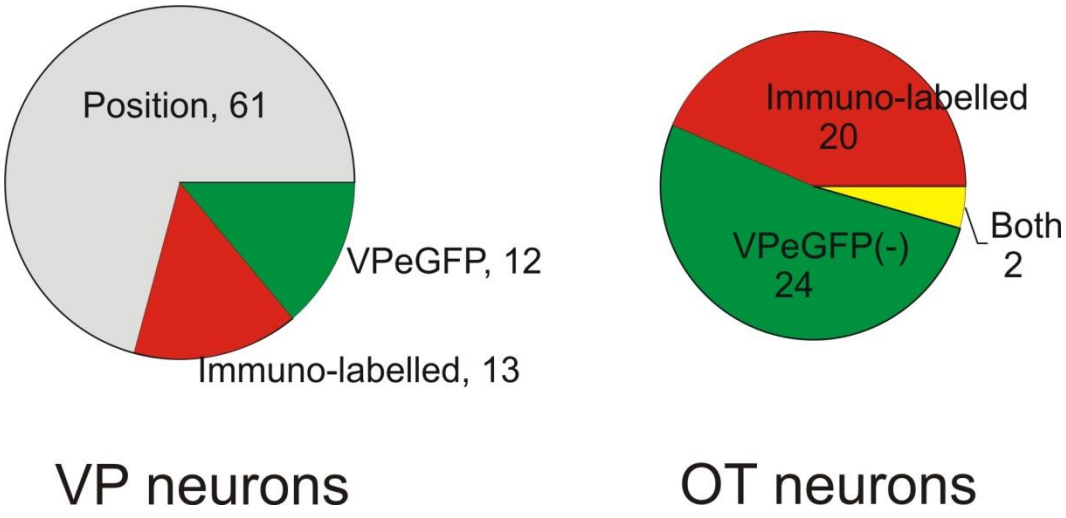

Figure 2.3 Cell type identification

Cells were categorized as putative vasopressin based on recording position or presence of eGFP, or putative oxytocin based on absence of eGFP combined with recording position. Definitive identification was made by labeling with neurobiotin and staining with VP and OT antibodies. The majority of putative VP cells were distinguished based on recording position alone, while putative OT cells were always identified with either immune-labeling or the absence of eGFP, or both. The total number of cells identified by each method in this study is indicated for VP and OT cells.

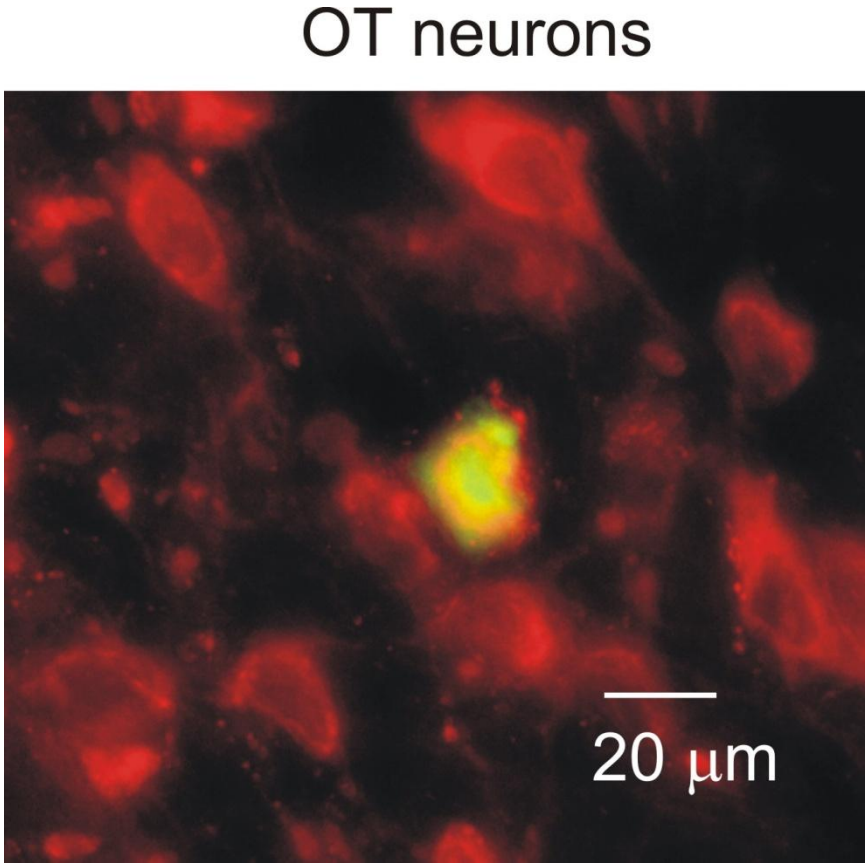

Figure 2.4 Immunohistochemical labeling for recorded OT cells

A subset of recorded VP and OT cells were labeled with neurobiotin (green, overlay appears yellow) and then stained for VP (not shown) or OT (shown here in red).

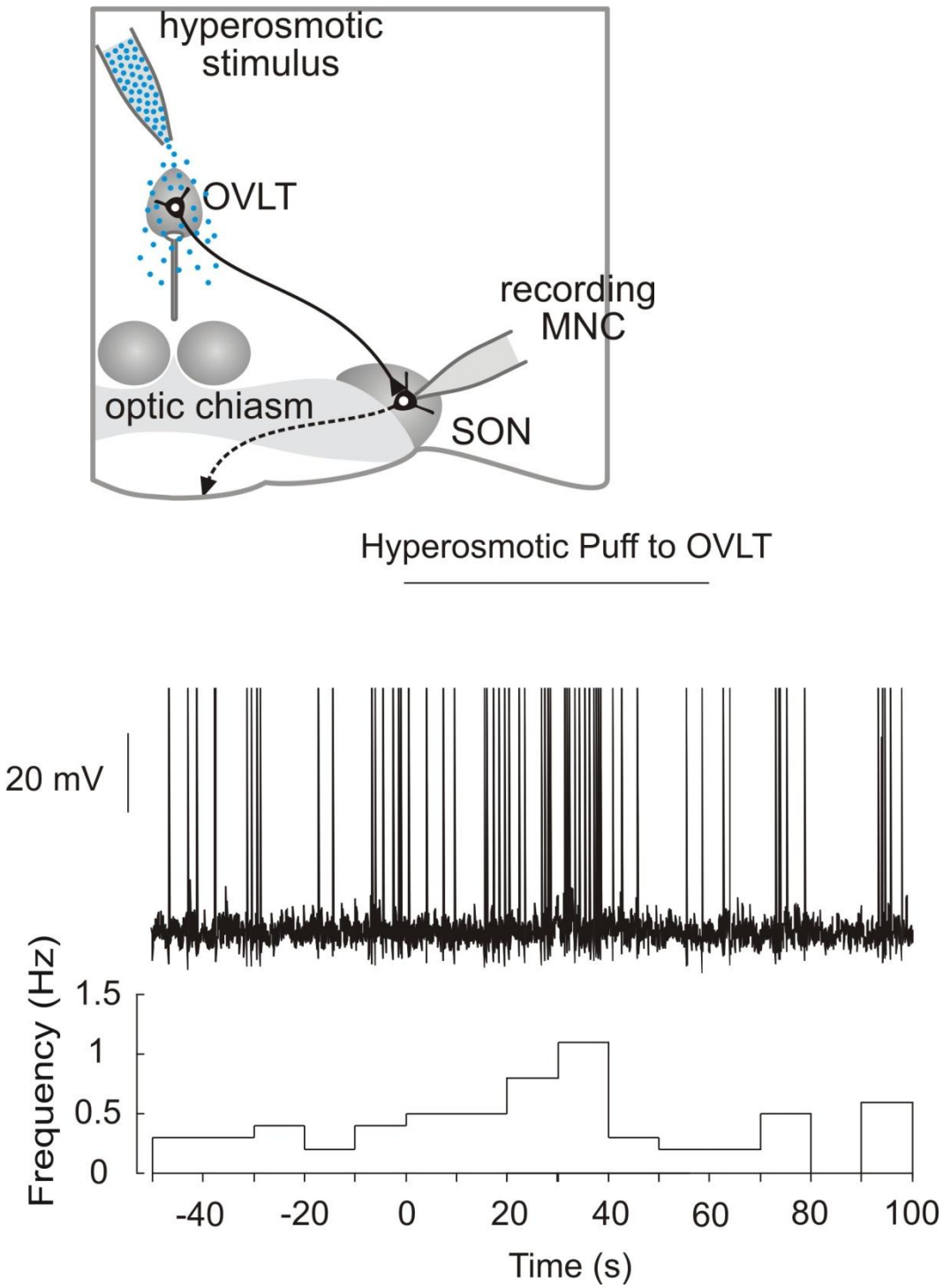

Figure 2.5 Osmotic stimulation of OVLT evokes MNC firing

As illustrated in the schematic diagram, a hyperosmotic stimulus was applied to OVLT while recording from a MNC in the SON. Osmotic puffing resulted in a

reversible increase in action potential frequency of MNCs. Firing frequency is indicated below, divided into 10 second bins.

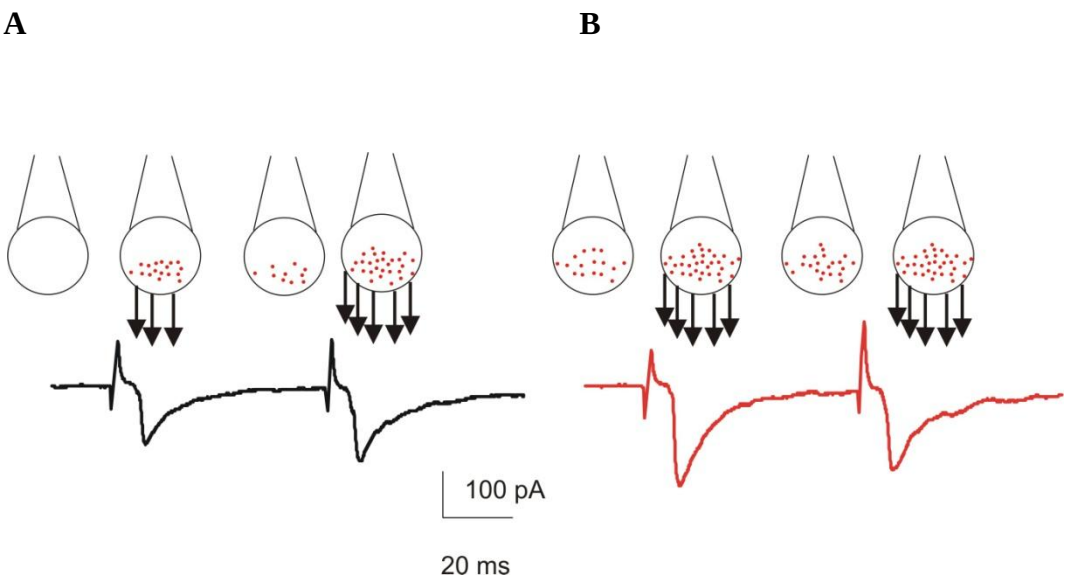

Figure 2.6 Paired pulse ratio

(A) Eliciting two evoked currents in rapid succession results in a facilitation of the second current due to residual calcium (red dots) in the presynaptic terminal. Any manipulation which increases presynaptic calcium (B) results in facilitation of the first current, but has less effect on the second current as release saturates. The paired pulse ratio is the size of the second current divided by the size of the first. Any modulation which results in a reduction in paired pulse ratio ($2^{nd}/1^{st}$ in B compared to $2^{nd}/1^{st}$ in A) therefore indicates a calcium dependent modulation of release probability.

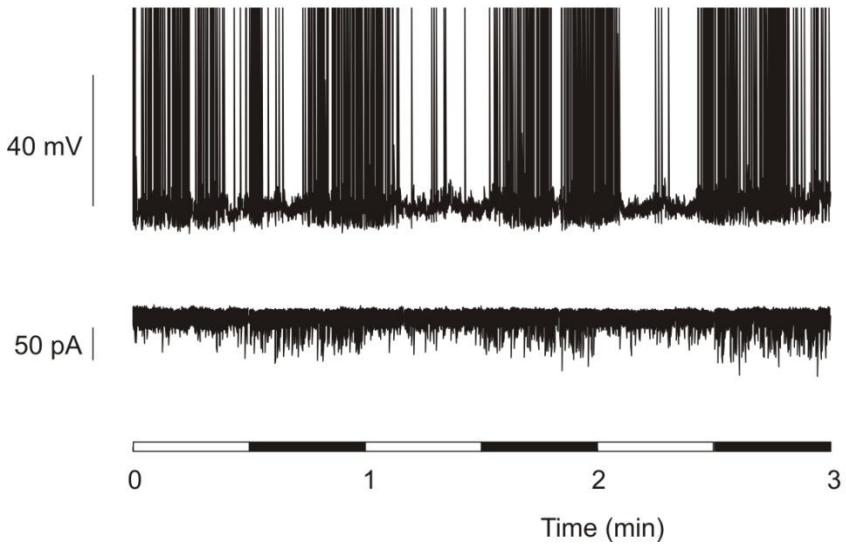

Figure 2.7 Artificial synaptic template and resulting MNC activity

Synaptic activity recorded under control (above the white bars) and Ang II (above the black bars) conditions are intercalated in an alternating fashion every 30 seconds. The resulting artificial tempate (middle trace) oscillates in frequency and amplitude. This synaptic waveform was played back to MNCs as a dynamic current clamp command. Native spontaneous glutamate and GABA-mediated potentials were blocked with DNQX (20μM) and picrotoxin (100 μM), leaving only the artificial synaptic input. MNCs generate action potentials in response (top trace), and action potential frequency for each time period is normalized to preceding period. Action potential amplitude has been truncated.

Chapter 3 Modulation of Osmotic Gain

3.1 Introduction

The ongoing regulation of VP secretion by osmolality provides a continual correction of fluid tonicity towards a homeostatic set point. Under hypovolemic conditions, osmotically evoked VP secretion is enhanced, resulting in a greater antidiuresis in reponse to increasing tonicity (Verbalis, 2003). The secretion of Ang II under hypovolemic conditions (Abdelaal et al., 1976), combined with the observation that Ang II increases the osmosensitivity of MNCs (Zhang & Bourque, 2008), along with other lines of evidence outlined in section 1.3, suggest that Ang II may mediate the increase in osmotic gain (used herein to mean the increase in MNC activity evoked by a given increase in osmolality, section 1.1.3) underlying enhanced VP secretion.

In contrast to VP, the regulation of OT in hypovolemia is less well characterized. However, based on the observation that in rats OT elicits natriuresis by triggering ANP secretion from the heart (Haanwinckel et al., 1995), and that under hypovolemic conditions, natriuresis is suppressed (Bennett & Gardiner, 1986; Stricker & Jalowiec, 1970), we might predict that OT release is not facilitated by hypovolemia (see section 1.2.2). This differential regulation of OT and VP neurons under mild hypovolemic conditions leads us to hypothesize that Ang II differentially modulates OVLT to SON neurotransmission to increase osmotic gain in VP neurons and blunt osmotic gain in OT neurons.

Using an angled horizontal rat brain slice which maintains both the OVLT and SON, and connectivity between the two (Trudel & Bourque, 2003), we have

examined the osmotic gain of OT and VP neurons in response to Ang II. We applied a hyperosmotic stimulus to the OVLT, which excites MNCs in the SON (Richard & Bourque, 1995). The resulting increases in action potential frequency were recorded in identified SON MNCs, and the osmotic gain was compared in the presence and absence of Ang II. In doing so, we may test the idea that Ang II differentially modulates the synaptically evoked osmotic excitation of VP and OT neurons.

3.2 Results

3.2.1 Bidirectional modulation of osmotic gain

Before testing the effects of Ang II, we first established synaptic connectivity of the OVLT and SON in our slice by verifying that a hypertonic stimulus applied to the OVLT increased MNC firing rate. Current clamp recordings from VP and OT (identified as outlined in section 2.1) revealed spontaneous activity in MNCs that generally ranged from 0.5 to 7 Hz, as well as some cells that were not spontaneously active. Positive or negative current (+2 to -5 pA) was injected to bring action potential (spike) frequency to a stable firing rate of about 3 Hz for at least 30 seconds. A 40 second application of hypertonic aCSF to the OVLT resulted in a significant increase in spiking in both vasopressin (3.32 +/- 0.39 Hz compared to 3.00 +/- 0.38 Hz at baseline, $p<0.0005$, $n=12$) and oxytocin cells (3.22 +/- 0.35 Hz compared to 2.49 +/- 0.27 Hz at baseline, $p<0.005$, $n=19$). The evoked increases in spike rate were significantly greater in OT cells than in VP cells (+0.74 +/- 0.16 Hz in OT compared to +0.32 +/- 0.10 Hz

in VP cells, $p<0.05$ by Student's T-test assuming unequal variances, n=12 and 19). Within a given recording, multiple applications of hypertonic stimulation to the OVLT evoked similar increases in firing (data not shown). Following termination of the hypertonic saline application, spike rates recovered within 60 to 90 seconds.

Application of Ang II (10^{-5} M) to the SON resulted in increased firing (10% or greater increase in frequency) in 42% of VP cells and 42% of OT cells, and resulted in decreased firing (10% or greater reduction in frequency) in 21% of VP cells and 33% of OT cells. The remainder of the cells (37% of VP cells and 25% of OT cells) showed changes in firing rates of less than 10% and were defined as non-responsive to Ang II application. The relative proportions of VP and OT neurons that increased, decreased, or were unaffected by Ang II did not differ between cell type (N.S. by chi-squared test). Holding current was sometimes adjusted after Ang II application in an attempt to compensate for any changes in spike rate. Once a stable firing rate had again been attained, the osmotic OVLT stimulus was repeated.

In the presence of Ang II, vasopressin neurons showed an increase in spiking to osmotic activation of the OVLT (3.32 +/- 0.36 compared to 2.78 +/- 0.33 Hz at baseline, $p<0.0001$, n=19). The increase in firing rate evoked by osmotic OVLT stimulation was significantly greater in the presence of Ang II (+0.71 +/- 0.12 Hz compared to +0.32 +/- 0.10 Hz in the absence of Ang II, $p<0.005$). Conversely, in the presence of Ang II, OT neurons no longer showed a significant increase in spiking in response to osmotic activation (2.87 +/- 0.40 Hz

with hypertonic OVLT stimulation compared to 2.67 +/- 0.40 Hz at baseline, p= 0.08, n=12). This represented a significant reduction in the amount of excitation induced by osmotic stimulation of OVLT, which produced an increase of 0.19 +/- 0.13 Hz in the presence of Ang II compared to an increase of 0.74 +/- 0.16 Hz in the absence of Ang II ($p<0.05$, n=19). Osmotic induced spiking in the presence of Ang II is expressed as a percentage of the osmotic induced spiking in control conditions for each cell type in Figure 3.1 to highlight the alterations in osmotic gain. Ang II application resulted in a significant change in the amount of excitation elicited by OVLT stimulation, facilitating osmotic excitation of VP cells, and inhibiting osmotic excitation of OT cells.

3.2.2 Bidirectional synaptic modulation

To determine if the modulation in osmotic excitation induced by Ang II resulted from a change in synaptic transmission between OVLT and SON, we applied an electrical stimulation to the OVLT and recorded the evoked synaptic currents. A pair of pulses separated by 60 milliseconds was applied once every 10 seconds. Electrical current pulse duration and amplitude (section 2.3) were adjusted to evoke an EPSC with an absolute amplitude of about 50 pA.

Electrical stimulation of the OVLT reliably evoked excitatory synaptic currents in SON MNCs. Bath application of Ang II (10^{-7} M) resulted in a significant increase in absolute EPSC amplitude (48.7 +/- 8.3 pA vs 40.4 +/- 6.9 pA in control conditions, $p<0.005$, n=8) in putative VP cells. In order to clearly observe the timecourse of the onset and offset of Ang II effects, we next applied a

concentrated (10^{-5}M) puff of Ang II dissolved in aCSF directly to the SON. This produced no significant alteration of the observed facilitation evoked by Ang II. As this more rapid application method increased the temporal resolution of the effects of Ang II on synaptic transmission, and restricted the Ang II application to the region of the SON, we subsequently used Ang II puffing for all our experiments.

In VP cells, puff application of Ang II resulted in an increase in evoked EPSC amplitude (73.6 +/- 13.9 pA compared to 58.1 +/- 9.2 pA in control conditions, $p<0.05$, n=12). OT cells showed a reduction in peak amplitude in response to Ang II (39.1 +/- 5.5 pA compared to 43.9 +/- 5.9 pA in control, $p<0.001$, n=19). These effects are summarized in Figure 3.2. The onset of the Ang II effects were rapid (within 30 seconds of puff onset), and reversible, as amplitudes generally returned to baseline within 90 seconds of puff cessation. Ang II effects on evoked EPSC amplitude were reasonably stable for the duration of application (3 minutes), and in test trials with longer applications were stable for the duration of the recording (10+ minutes, data not shown).

3.2.3 Membrane input conductance modulation

As outlined in section 1.3.2 Ang II has previously been shown to activate a SICC in MNCs, which increases conductance to excite the cell (Chakfe & Bourque, 2000). To test whether Ang II produces differential effects on this excitatory conductance in VP and OT cells, we monitored conductance levels in MNCs by delivering a 50 mV hyperpolarizing pulse once every 10 seconds.

Application of Ang II produced an increase in cell conductance (Figure 3.3). Both cell types showed comparable increases in conductance, with VP neurons increasing from 2.17 +/- 0.14 nS to 2.28 +/- 0.14 nS ($p<0.05$, n=15), while OT neurons showed a conductance increase from 2.11 +/- 0.23 to 2.21 +/- 0.25 ($p<0.05$, n=9). No significant differences were found between cell types either in terms of basal conductance or in change in conductance ($p= 0.41$, $p= 0.48$ by Student's T-test assuming unequal variance, n=15 and 9).

3.3 Discussion

The osmotic stimulation experiments outlined above demonstrate that Ang II differentially regulates osmotic gain of VP versus OT neurons. VP neurons show increased responsiveness to an osmotic stimulation of OVLT, while OT neurons show a blunted responsiveness. This divergent modulation of osmotic gain is consistent with previous observations regarding enhanced VP secretion under hypovolemic conditions (Verbalis, 2003), and with previous observations indicating OT secretion is blunted under mild hypovolemic conditions (section 1.2.2). This experiment provides the novel observation that Ang II induces a state dependent, cell type specific modulation of osmotic gain.

3.3.1 Possible physiological impact of Ang II modulation of osmotic gain

Increasing firing rates of VP neurons results in increased VP secretion (Poulain & Wakerley, 1982) and subsequent increases in antidiuresis (Robinson, 1985). The dynamic range of VP concentrations for VP induced antidiuresis is 0-6

pg/mL in humans (Robinson, 1985), which corresponds to a range in plasma osmolality of about 12 milliosmoles/kg (Verbalis, 2003). Plasma osmolality in rats corresponding to an equivalent dynamic range (corrected for differences in basal plasma osmolality) generate VP neuron firing rates in the range of 0 to 5 Hz (Wakerley, Poulain, & Brown, 1978). Thus we can state that the total dynamic range of VP firing rates for VP-induced antidiuresis is about 5 Hz. The observed increase in firing rate in VP cells in response to osmotic activation of the OVLT (+0.32 Hz) therefore represents a change in activity corresponding to about 6% of the total dynamic range of firing rates for VP induced antidiuresis *in vivo*. In the presence of Ang II, OVLT stimulation evoked an increase in firing rate that corresponds to about 15% of the total dynamic range for VP induced antidiuresis.

The linear dynamic range of OT concentrations for eliciting natriuresis is 0-15 pg/mL in rats (Verbalis et al., 1991). This corresponds to a plasma osmolality range of about 14 mosm/kg in rats (Balment, Brimble, & Forsling, 1980). OT firing rates for this range of osmolality vary from 0 to 4 Hz (Wakerley et al., 1978). The initial osmotic stimulation of OT firing rates therefore represents 18% of the linear dynamic range for OT induced natriuresis. Ang II reduced OT firing rates in response to OVLT stimulation such that only 5% of the linear dynamic range of OT firing was elicited, and this change was not significantly different from basal firing rates.

Speaking solely in terms of osmotic regulation then, the Ang II induced modulation of VP and OT cell firing rates represents a sizeable portion of the respective dynamic ranges of these neurons. Thus we expect, given that MNC

firing promotes OT and VP release (Poulain & Wakerley, 1982), that Ang II-mediated modulation of osmotic induced firing will have a substantial impact on the regulation of osmotic balance by neurohypophysial hormones. Under mild hypovolemic conditions elevations in plasma Ang II, signaling through the SFO, will therefore facilitate antidiuresis and depress natriuresis to restore blood volume.

3.3.2 Integration of synaptic and intrinsic signals

Previous studies have demonstrated that Ang II activates a depolarizing conductance in magnocellular neurons (Chakfe & Bourque, 2001; Latchford & Ferguson, 2004; Yang et al., 1992) (see sections 1.3.1 and 1.3.2). Here we confirm that MNC conductance is increased in the presence of Ang II. However, as we saw no differences in the activation of the conductance between VP and OT cells, this cannot fully account for the differential modulation OT and VP secretion under mild hypovolemic conditions.

The divergent effects of Ang II on evoked EPSC amplitude (Figure 3.2) are consistent with the observed divergence in osmotic gain illustrated in Figure 3.1. The bidirectional modulation of evoked synaptic currents by Ang II is consistent with the bidirectional modulation of osmotic gain in response to osmotic stimulation of the OVLT. These results therefore suggest a prominent role of synaptic modulation in the control of MNC firing rates. This is in accordance with observations that Ang II no longer elicits VP secretion in animals with OVLT specific lesions(Thrasher & Keil, 1987).

However, it should be noted that since Ang II can increase the intrinsic osmosensitivity of MNCs by modulating the SICC (Zhang & Bourque, 2008), as well as impacting MNC activity through other mechanisms (see section 1.3), we expect that synaptic modulation will comprise only a component of the total activity of Ang II in this system. In fact, since we modulated holding current after the application of Ang II, we were able to compensate for any changes in steady state currents in our osmotic stimulation experiments, but not voltage gated currents such as I_A, which likely still played a role in determining osmotic gain. It is difficult to make a rigorous assessment of the relative contributions of synaptic or intrinsic modulations by Ang II due to the multiple overlapping and often synergistic effects evoked by Ang II (Li & Ferguson, 1996; Latchford & Ferguson, 2004; Zhang & Bourque, 2008; Antunes-Rodrigues et al., 2004) (section 1.5). We will therefore attempt to address this issue more fully in Chapter 5 by separating the synaptic component of Ang II from its other effects.

3.3.3 Cell type specific gain modulation

VP cells show a substantial (>2 fold) facilitation of osmotic induced spiking in the presence of Ang II. In OT cells, the presence of Ang II reduces the osmotic OVLT- induced spiking to such an extent that spike rate is no longer significantly different from baseline. The repeatability of osmotic OVLT-induced spiking in control conditions, combined with observed recovery of osmotic induced spike rates in a subset of cells after Ang II washout preclude the notion of an experimental artifact, but rather suggest a genuine modulation of osmotic gain

at the OVLT-SON synapse. This surprising finding suggests that MNCs are able to selectively listen to or ignore an incoming synaptic signal. The facilitation or depression of evoked EPSC amplitude in VP or OT cells provides a mechanism by which such a selection process may arise, whereby inputs (osmotic) relevant to an ongoing signal (hypovolemic) are selectively enhanced or depressed. In addition to the implications for restoration of homeostatic balance (sections 1.2 and 3.3.1), this mechanism may have implications for cell-type specific signal processing.

The observed modulation of osmotic induced firing in MNCs provides a novel example of how a cell type specific modulation of synaptic transmission functions within a defined physiological context. The phenomenon of cell-type specific input selectivity has been previously described in MNCs (Oliet et al., 2007; Zampronio et al., 2010) and in the cortex (Reyes et al., 1998). In the visual cortex, Jia and colleagues demonstrate that orientation tuned neurons in the visual cortex receive synaptic inputs coding for a wide range of stimulus orientations (Jia, Rochefort, Chen, & Konnerth, 2010). The authors are unable to conclude how the neuron derives its tuning curve from such disparate inputs, and indeed even provide evidence that classical explanations such as dendritic filtering and spatial summation cannot easily explain the data, as the inputs for various orientation tunings are spatially and dendritically segregated. Our observations, although not immediately translatable into the field of visual orientation, suggest that synaptic modulation may hold the key to information processing questions such as this. An investigation of the mechanisms behind the modulatory effects of

Ang II may provide us with clues as to how cell-type specific input selectivity arises. A more detailed description of the mechanism of Ang II modulation will be outlined in chapter 4.

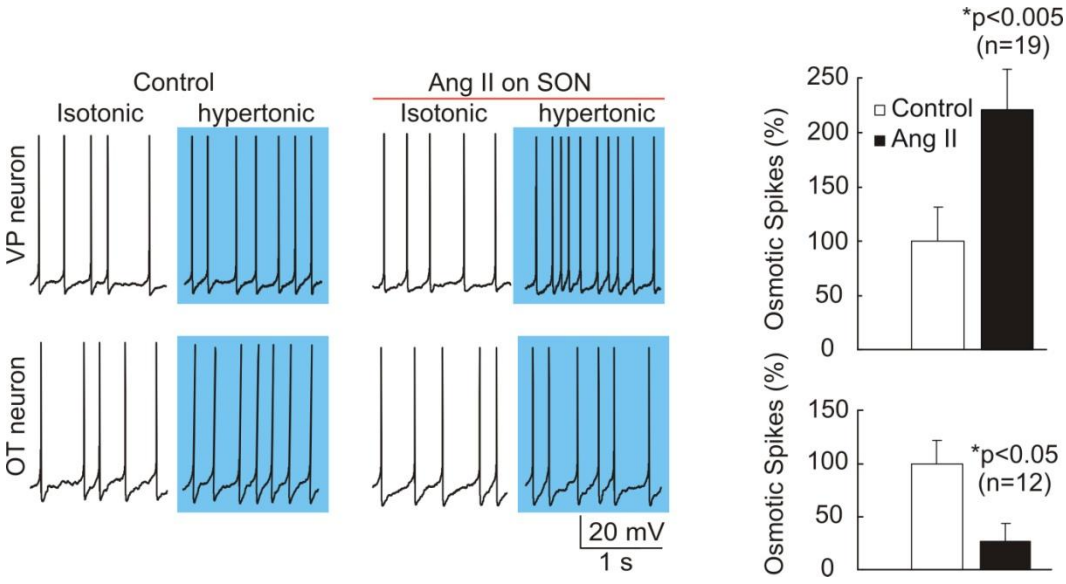

Figure 3.1 Ang II modulates osmotic induced action potential firing rate.

In control conditions, hypertonic stimulation of the OVLT results in increased spiking in both VP and OT neurons (left panels). In the presence of Ang II, hypertonic OVLT stimulation results in a significantly larger increase in spike rate in VP cells (middle panel). In OT cells, hypertonic OVLT stimulation produces a significantly smaller increase in action potential firing rate in the presence of Ang II (middle panel). By normalizing increase in action potential frequency to the increase in frequency observed in control conditions, we see that Ang II significantly enhances osmotic induced firing in VP cells and significantly depresses osmotic induced firing in OT cells (right panel). Action potential amplitude has been truncated.

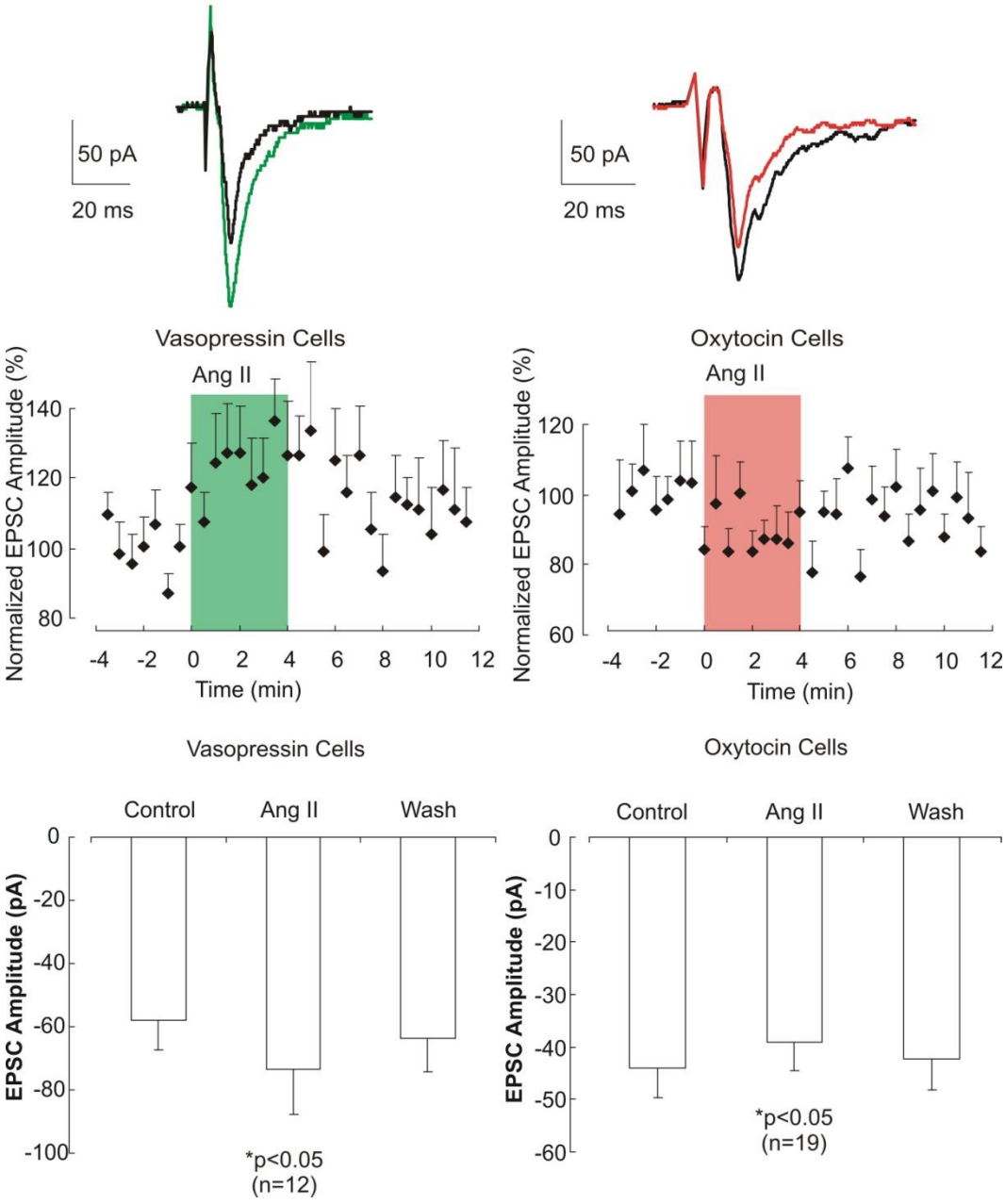

Figure 3.2 Evoked EPSC amplitude is modulated by Ang II

Electrical stimulation of the OVLT generates an evoked current in MNCs (top, black traces, examples are an average of 12 EPSCs). In VP cells, application of Ang II increases evoked EPSC amplitude (top left, green trace). OT cells showed a reduction in peak amplitude in response to Ang II (top right, red trace). The timecourse of Ang II effects is illustrated in the middle panels. Each point

represents an average of 3 evoked EPSCs set 10 seconds apart within each trial, normalized to the average baseline value. These values are then averaged across trials (n=12 for VP, n=19 for OT) to give mean +/- standard error. Onset of Ang II modulation is rapid, and recovers within a few minutes. Average evoked EPSC amplitudes are quantified in the lower panels for VP and OT. Ang II significantly increases EPSC amplitude in VP cells, and decreases EPSC amplitude in OT cells, an effect that washes out with removal of Ang II.

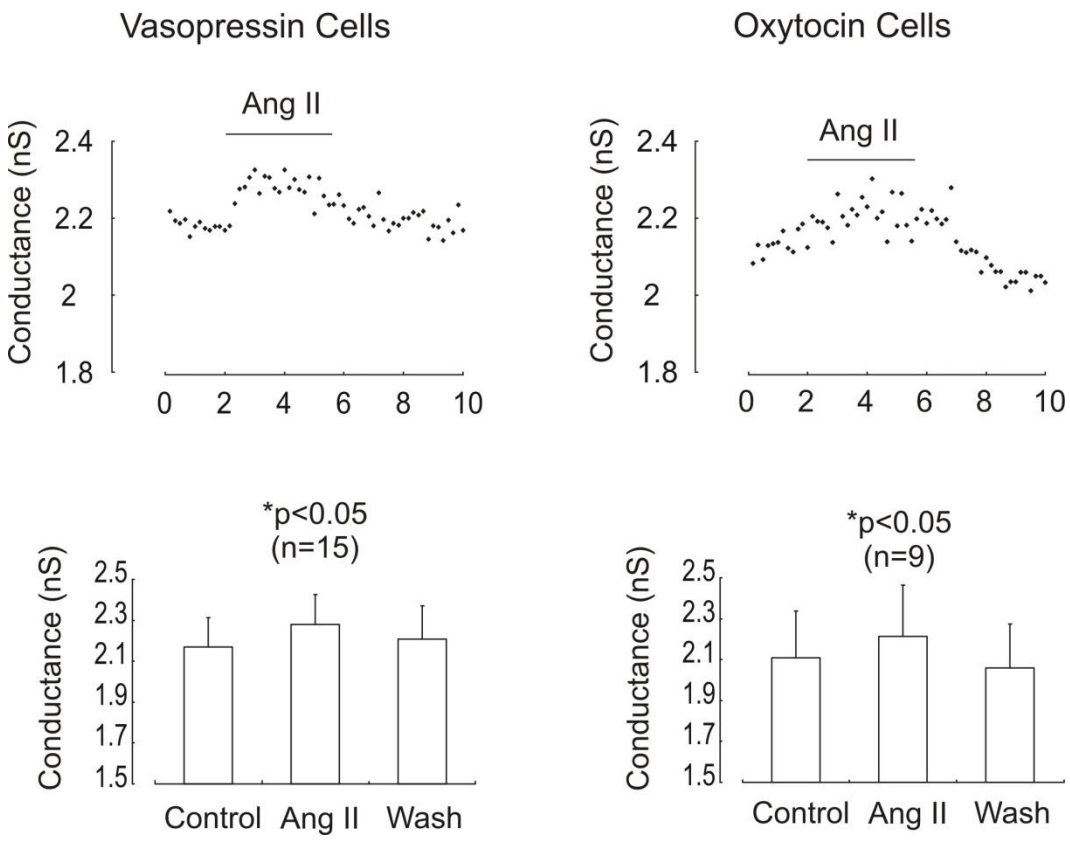

Figure 3.3 Modulation of membrane input conductance

Application of Ang II produced a reversible increase in cell conductance in both VP (left) and OT cells (right). The average timecourse of conductance modulation is shown in the top panels. Both cell types showed significant increases in input conductance, which recovers after Ang II washes out.

Chapter 4 Synaptic mechanisms of osmotic modulation

4.1 Introduction

Chapter 3 identifies a synaptic modulation induced by Ang II that coincides with changes in synaptically induced osmotic stimulation. In order to examine the mechanism of Ang II induced changes in OVLT-SON synaptic transmission, it is first necessary to determine where these changes in synaptic strength occur. Changes in synaptic strength are thought to underlie the formation of memories in the nervous system (Pastalkova et al., 2006), and the idea that long term potentiation (LTP) of synapses is the cellular basis for memory focused a great deal of attention on the process of synaptic modulation. As LTP can be mediated by modulations of either the presynaptic or postsynaptic terminal (Glanzman, 2010), the study of LTP has yielded the discovery of a number interesting synaptic modulatory mechanisms, as well as electrophysiological tools for assessing changes in pre- or postsynaptic function.

At glutamatergic synapses, postsynaptic facilitation or depression is mediated by changes in AMPA receptor number (Bredt & Nicoll, 2003). Activity dependant increases or decreases in postsynaptic receptor number result in facilitation or depression of synaptic transmission (section 1.4.1). Identification of an increase in AMPA receptors can be done by examining the amplitude of quantal events, such as the currents that arise asynchronously following activation of a synapse (Goda & Stevens, 1994). An increase or decrease in the amplitude of quantal events indicates an insertion or removal of postsynaptic AMPA receptors.

Neurotransmitter release is triggered when an action potential invades presynaptic terminals and evokes voltage-gated influx of calcium ions, which then bind to and activate the secretory apparatus to trigger exocytosis (Suudhof, 2008). Modulation of presynaptic release may therefore be accomplished by changing the number of presynaptic terminals activated by action potentials (Doussau, Humeau, Benfenati, & Poulain, 2010), as is the case for SCN-mediated inhibition of OVLT to SON transmission (Trudel & Bourque, 2010) (section 1.4.2a). Modulation may also occur by altering calcium levels or by calcium-independent mechanisms involving modulation of the secretory machinery to alter probability of release (Capogna, 1998).

Discriminating between pre- and postsynaptic modulations in synaptic transmission establishes a foundation on which to further explore the mechanisms of synaptic modulation. We therefore first attempt to determine whether the Ang II modulation of synaptic transmission observed in Chapter 3 is mediated by a presynaptic or postsynaptic mechanism.

4.2 Results

4.2.1 Bidirectional modulation of paired pulse ratio

Electrically evoked EPSCs consistently showed paired pulse facilitation at the OVLT-SON synapse. In parallel to the modulation of evoked EPSC amplitude, Ang II reliably produced a modulation of paired pulse ratio (PPR, Figure 4.1). In VP cells, PPR fell from 2.63 +/- 0.17 in control conditions to 1.98 +/- 0.14 in the presence of Ang II ($p<0.0005$, $n=12$). In OT cells, PPR rose from

2.04 +/- 0.15 to 2.49 +/- 0.24 in the presence of Ang II ($p<0.005$, n=19). Note that baseline PPR differed between the two groups ($p<0.01$), indicating a higher basal probability of release in OT cells. Paired pulse facilitation represents a calcium dependant increase in release probability (Zucker & Regehr, 2002; Katz & Miledi, 1968). Ang II modulation of PPR suggests that the observed changes in EPSC amplitude are a calcium dependant modulation of release probability.

4.2.2 Calcium dependant facilitation

Consistent with the calcium dependant interpretation of the PPR findings, we find that Ang II evoked facilitation of putative VP neurons was greater under conditions of reduced extracellular calcium (Ang evoked facilitation was 114 +/- 4% of control in 2 mM external Ca^{2+}, $p<0.001$, n=13; 120 +/- 11% of control in 1mM external Ca^{2+}, $p<0.05$, n=10; 207+/- 59% of control in 0.5 mM external Ca^{2+}, $p<0.05$, n=7). Figure 4.2 illustrates an experiment in which the effects of Ang II on synaptic transmission were assessed in the presence of 1 mM external calcium. Extracellular calcium was then increased to 2 mM, and Ang II was reapplied. Ang II induced facilitation was reduced in higher external calcium, consistent with the idea that Ang II facilitation is calcium dependent.

4.2.3 Bidirectional modulation of failure rate

Electrically triggering presynaptic release by increasing stimulation intensities from sub to near threshold values (a minimal stimulation protocol) evokes synaptic currents that may fail with a given frequency (Raastad, Storm, &

Andersen, 1992). With increase stimulus intensities, the probability of observing an evoked synaptic event transitions abruptly from zero to a sustainable non-zero value, and this value can be used as a description of release probability of the activated synapse (Dobrunz & Stevens, 1997). As with the paired pulse ratio, the rate of failures at a given synapse is calcium dependent (Jiang, Sun, Nedergaard, & Kang, 2000).

Minimal stimulation of the OVLT produced traces in which evoked EPSCs failed with a stable frequency. The addition of Ang II resulted in a change in failure rate Figure 4.3. VP cells showed a reduction in failure rate (31.5 +/- 4.4% compared to 38.5 +/- 5.0% in control conditions, $p<0.05$, n=15) consistent with an increased probability of release. OT cells showed an increase in failure rate (43.1 +/- 5.8% compared to 35.1 +/- 5.7% in control conditions, $p<0.01$, n=15) consistent with a decrease in probability of release.

4.2.4 Electrical stimulation of OVLT evokes asynchronous release in MNCs

Consistent with previous observations (Iremonger & Bains, 2007), the synchronous evoked EPSC was followed by the occurrence of high frequency, small amplitude asynchronous currents (Figure 4.4). Asynchronous currents are quantal release events that arise in response to presynaptic action potentials, but with a delayed timecourse as compared to synchronous release events (Goda & Stevens, 1994). A number of experimental manipulations by Iremonger and Bains indicate that asynchronous release in MNCs is not a function of polysynaptic release (Iremonger & Bains, 2007). A minimal stimulation protocol demonstrates

that both synchronous and asynchronous release have identical activation thresholds in MNCs, suggesting they arise from stimulation of the same presynaptic axon. Further, low concentrations of the AMPA receptor antagonist DNQX failed to preferentially inhibit asynchronous release, as would be expected if asynchronous release relied on polysynaptic activation (Iremonger & Bains, 2007). Finally, the slow calcium buffer EGTA (ethylene glycol tetraacetic acid, 25 μM) preferentially inhibits asynchronous release, but has no effect on synchronous release, ruling out the possibility that asynchronous events arise from synchronous release of a polysynaptic pathway (Iremonger & Bains, 2007). Thus asynchronous currents are a calcium dependant form of presynaptic release arising from the same terminals as the synchronously evoked currents. Although the mechanisms underlying asynchronous events are distinct from synchronous release (Geppert et al., 1994), their quantal nature and calcium dependence may inform us, respectively, about changes in postsynaptic AMPA receptors or changes in presynaptic calcium dependant release probability.

4.2.5 Bidirectional modulation of asynchronous release frequency

Application of Ang II resulted in an increase in the frequency of asynchronous currents in VP cells, and a decrease in asynchronous current frequency in OT cells. Median asynchronous frequency was significantly altered in both OT and VP cells, while median amplitude was not affected (Figure 4.5). VP cells show an increase in asynchronous event frequency (105.4 +/- 6.8 Hz compared to 79.9 +/- 5.4 Hz in control conditions, $p<0.01$, $n=7$) with no change in

amplitude (19.1 +/- 3.4 pA compared to 19.4 +/- 5.1 pA in control conditions, N.S. n=7). OT cells show a reduction in asynchronous event frequency (80.8 +/- 12.0 Hz compared to 103.2 +/- 13.7 Hz in control conditions, $p<0.01$, n=7) with no change in amplitude (24.6 +/- 4.5 pA compared to 23.5 +/- 4.3 pA in control conditions, N.S. n=7).

To add statistical rigor to our analysis of asynchronous event frequency and amplitude, we further applied the Kolmogorov-Smirnov test to cumulative probability plots of asynchronous events for both VP and OT cells. Due to the relatively small number of asynchronous events available for analysis in a given time window, it was necessary to pool event amplitude and inter-event interval across multiple cells for the control and Ang II treatment conditions. A Kolmogorov-Smirnov analysis applied to these pooled data sets revealed that in both VP and OT cells, asynchronous frequency was altered ($p<0.05$ for VP, $p<0.05$ for OT) while asynchronous amplitude was unchanged. Expressed as cumulative probability distributions, it is apparent that inter-event interval is decreased (population data set is left-shifted) in VP cells, and increased (population data set is right-shifted) in OT cells (Figure 4.6), confirming the differences observed in the median values. These observations are consistent with the idea that Ang II increases probability of release in VP cells while reducing probability of release in OT cells, without affecting quantal size.

4.2.6 Modulation of miniature EPSC frequency

In the presence of tetrodotoxin (500 nM), we found that Ang II increases miniature frequency but not amplitude (Figure 4.7). MNCs show an increase in miniature event frequency (6.96 +/- 1.35 Hz compared to 3.20 +/- 0.57 Hz in control conditions, $p<0.05$, n=5) with no change in amplitude (12.2 +/- 2.0 pA compared to 12.8 +/- 1.6 pA in control conditions, N.S. n=5). In determining the effects of Ang II on miniature events, we constructed cumulative probability plots of approximately 200 data points from control and Ang II treated time-points. We then applied the Kolmogorov-Smirnov statistical test to determine whether the two data sets came from the same population. The inter-event interval was significantly altered in 5/5 cells tested, with all cells showing a reduction in inter-event interval. Event amplitude was altered (decreased) in only 1/5 cells tested and in the one altered recording median amplitude was not affected. Together with the asynchronous data, these findings confirm that the Ang II induced synaptic modulation is not mediated by an increase in post-synaptic receptors.

In 2 cells identified as OT by neurobiotin staining and immunolabeling, Ang II also increased mini frequency (data not shown). As this data diverges from our main findings regarding depression of probability of release in OT cells, it suggests that modulation of miniature event frequency occurs by a different mechanism than modulation of evoked events (see section 6.6). As such, we did not further pursue the mechanism by which miniature event frequency is altered by Ang II.

4.2.7 Retrograde signaling mediates Ang II modulation of release probability

Di et al. demonstrate that both the glucocorticoid-induced facilitation of GABAergic and depression of glutamatergic inputs to MNCs can be blocked by including the G-protein blocker GDP-β-S in their patch pipette (Di, Malcher-Lopes, Marcheselli, Bazan, & Tasker, 2005). As this treatment inhibits G-protein signaling strictly in the postsynaptic cell, blockade of presynaptic release implicates retrograde transmitters as signaling intermediates of the synaptic modulations (Di et al., 2005). We therefore included GDP-β-S (10^{-6} M) in our patch pipettes to see if the Ang II induced modulations in synaptic transmission would be affected. Postsynaptic G protein blockade prevented Ang II induced changes in evoked EPSC amplitude (Figure 4.8). Mean amplitude was 74.3 +/- 13.3 pA in the presence of Ang II and pipette GDP-β-S compared to 70.4 +/- 11.4 pA with GDP-β-S alone (N.S. n=8). This finding implicated retrograde signaling in the mediation of Ang II evoked changes in presynaptic release probability.

In OT neurons we observe a presynaptic suppression of evoked EPSCs in response to Ang II. Previous studies of presynaptic depression in MNCs indicate that depression may be mediated by endocannabinoids (Zampronio et al., 2010; Hirasawa et al., 2004). We therefore tested whether endocannabinoids mediate the Ang II induced suppression of transmission in OT cells. In OT cells application of 0.5 μM AM-251, a CB1 antagonist, resulted in the reversible blockade of Ang II induced suppression of transmission (Figure 4.9). Evoked EPSC amplitude was 39.2 +/- 5.0 pA compared to 38.7 +/- 5.1 pA in control conditions (N.S. n=11). After washout of AM-251, Ang II produced a significant reduction in evoked

amplitude in OT cells (29.7 +/- 4.4 pA compared to 34.0 +/- 4.3 pA in control conditions, $p<0.01$, n=10).

VP neurons show an enhancement of release probability in the presence of Ang II. A number of studies have demonstrated a role for nitric oxide (NO) in the facilitation of release in MNCs for both excitatory (Ozaki et al., 2000) and inhibitory transmission (Di et al., 2009; Bains & Ferguson, 1997). NO has even been implicated as a mediator of the effects of Ang II on synaptic facilitation of inhibitory current in MNCs the PVN (Latchford & Ferguson, 2004). We therefore endeavored to determine whether the enhancement of excitatory release probability evoked by Ang II in VP neurons was mediated by NO. First we demonstrated that NO mimics the effects of Ang II on evoked EPSCs (Figure 4.10). Sodium nitroprusside (SNP, 10^{-7} M) elicited an increase in evoked EPSC amplitude (57.5 +/- 7.9 pA compared to 50.9 +/- 7.2 pA in control conditions, $p<0.005$, n=8), a modulation which was associated with a reduction in paired pulse ratio (1.60 +/- 0.15 compared to 1.82 +/- 0.15 in control conditions, $p<0.001$, n=8). Further, a blockade of NO production by bath application of the NO synthase inhibitor L-NAME prevented the Ang II evoked increase in EPSC amplitude (Figure 4.11). Application of L-NAME (10^{-5} M) did not produce a significant change in EPSC amplitude (40.3 +/- 8.8 pA compared to 42.1 +/- 8.8 pA in control conditions, N.S., n=6). Addition of Ang II in the presence of L-NAME did not facilitate EPSC amplitude (29.9 +/- 6.9 pA compared to 40.3 +/- 8.8 pA in the L-NAME condition, N.S. n=6). In 2 test cases, bath application of

L-NAME subsequent to Ang II application was able to reverse the induced facilitation of release (Figure 4.11).

Ang II induced facilitation of transmission depends on NO production in the postsynaptic neuron (Figure 4.12). When we applied a low dose (1 μM) of L-NAME directly to the postsynaptic cell by putting it into our patch pipette, Ang II evoked facilitation was blocked (63.0 +/- 19.6 pA compared to 62.6 +/- 19.6 pA in L-NAME internal alone, N.S. n=9). Together with the GDP-β-S findings, these results demonstrate that a cell autonomous inhibition of Ang II signaling intermediates is sufficient to prevent Ang II induced facilitation of transmission.

4.3 Discussion

Our results demonstrate that Ang II induces a modulation of presynaptic transmission in the OVLT-SON synapse. We describe the Ang II induced bidirectional modulation of calcium dependant release probability, which occurs in a cell type specific manner. In contrast to previous studies that have identified an effect of Ang II on presynaptic release at excitatory synapses in MNCs (Ozaki et al., 2004; Latchford & Ferguson, 2004), our study examines modulation of a discrete synaptic connection with a defined physiological function (Trudel & Bourque, 2003). Finally, our study demonstrates that cell type specific bidirectional regulation of synaptic function is mediated by two retrograde messengers, NO and eCB.

4.3.1 Ang II modulates presynaptic release probability via the postsynaptic cell

Using a combination of three independent measures of presynaptic release probability, we have determined that Ang II differentially modulates probability of release in VP and OT cells. Paired pulse ratio varied inversely with the effects of Ang II on evoked EPSC amplitude, as would be expected for calcium dependent alterations in release probability (see Figure 2.6). The frequency of asynchronous currents was also bidirectionally regulated by Ang II in VP and OT cells. As asynchronous release is dependent on presynaptic calcium levels (Chang & Mennerick, 2010), the modulation of asynchronous frequency induced by Ang II indicates an alteration in presynaptic calcium. Failure rate was reduced in VP cells and increased in OT cells by Ang II, consistent with an increased release probability in VP cells and a decreased release probability in OT cells. The observation that paired pulse ratio (Katz & Miledi, 1968), asynchronous release (Chang & Mennerick, 2010), and failure rate (Jiang et al., 2000) are all modulated by changes in presynaptic calcium is consistent with the idea that Ang II modulates probability of release at OVLT-SON synapses by altering presynaptic calcium.

Conversely, asynchronous current amplitude was not affected by Ang II, nor was the amplitude of miniature EPSCs. These data show that the observed modulation of release probability occurs in the absence of changes in the number of postsynaptic receptors at the OVLT-SON synapse.

Interestingly, we were able to disrupt the modulatory effects of Ang II on presynaptic transmission by interfering with G protein signaling or NO production

specifically in the postsynaptic cell. This finding suggests that cell autonomous regulation of synaptic transmission occurs through retrograde signaling. Further, the ability of postsynaptic G protein inhibition to block Ang II induced effects indicates that Ang II acts at receptors on the postsynaptic cell. However, this does not rule out the possibility that Ang II also acts at presynaptic receptors as well. Indeed, there is some evidence to support this possibility (see section 6.6).

Ang II modulation of evoked EPSC amplitude is mediated through the AT1 receptor, as indicated by the reversible blockade of the modulatory effects of Ang II by the AT1 receptor antagonist losartan (10^{-5} M, bath application) in both VP cells (n=1, data not shown) and OT cells (n=1, data not shown).

4.3.2 Retrograde signaling in synaptic integration

Mounting evidence proclaims a role for eCBs as retrograde inhibitors of presynaptic release (Oliet et al., 2007; Di et al., 2009; Di & Tasker, 2008; Chevaleyre et al., 2006), and for NO as a retrograde facilitator of release (Ozaki et al., 2000; Taqatqeh et al., 2009; Latchford & Ferguson, 2003; Bains & Ferguson, 1997; Di et al., 2009; Li, Wang, Mayhan, & Patel, 2006). In addition to providing further evidence to support the idea of retrograde modulation of neurotransmission, our study demonstrates that the locus of origin for the modulatory signal, the postsynaptic cell, plays a critical role in determining the type of neuromodulation. Such a postsynaptic decision implies that in addition to regulating postsynaptic excitability (Oliet et al., 2007), retrograde signaling may be necessary to allow the differential regulation of synapses to match the

physiological function of the postsynaptic cell. The existence of two opposing retrograde signals provides a mechanism by which signal integration may be regulated in a cell type specific fashion. We therefore propose that presynaptic modulations by NO and eCB represent a signaling pathway by which a cell may regulate or integrate synaptic input. This certainly appears to be the case for glucocorticoid signaling (Di et al., 2009), oxytocin signaling (Oliet et al., 2007), angiotensin signaling (Latchford & Ferguson, 2003) and endothelin signaling (Zampronio et al., 2010). Thus MNCs differentially regulate incoming synaptic signals through the use of retrograde signaling. The cellular mechanisms which may permit this differential regulation will be discussed in detail in section 6.2.

4.3.3 Can retrograde signaling balance excitatory and inhibitory inputs?

Our study demonstrates that Ang II facilitates excitatory transmission at the OVLT SON synapse through the release of NO. In contrast, a number of studies show that NO secretion facilitates inhibitory transmission in MNCs (Ozaki et al., 2000; Bains & Ferguson, 1997). Latchford and Ferguson show that Ang II can facilitate action potential dependent release of IPSCs (Latchford & Ferguson, 2003), while Reis et al. show that exogenous NO application can block ICV Ang II evoked increases in antidiuresis and natriuresis (Reis et al., 2007). Further, a microdialysis study of glutamate and GABA from the SON indicates that both neurotransmitters are dramatically increased by application of NO (Engelmann, Wolf, & Horn, 2002). In attempting to evaluate how the summation of the Ang II increases in both excitatory and inhibitory transmission may impact

neurohypophysial hormone release, we note that Leng and colleagues predict that balanced increases in both types of transmission is necessary to generate a linear relationship between osmolality and MNC firing rate (Leng et al., 2001). Although the computational model used in that study reliably describes the behavior of MNCs, no intrinsically osmosensitive inhibitory synaptic inputs to the SON have yet been described, leaving the source of balanced inhibitory input unclear (Leng et al., 2001). Ang II-induced facilitation of both excitatory and inhibitory inputs through the production of NO could represent a mechanism by which a balance in synaptic transmission is reached. As inhibitory currents were continuously inhibited in our study by application of picrotoxin, we cannot speculate as to whether a simultaneous and balanced facilitation of excitatory and inhibitory transmission may occur in MNCs.

4.3.4 Basal release probability in OT and VP cells

Our observation that VP and OT cells express significantly different paired pulse ratios under baseline conditions has not been previously reported. However there is some precedence in the literature for the presence of differential release probabilities in synaptic inputs to MNCs. Oliet et al. found that evoked IPSCs had differential release probabilities in VP vs OT neurons (Oliet et al., 2007). Although they found no differences in EPSC PPR, the stimulation paradigm employed in their study was not specific to the OVLT-SON pathway. In fact, connectivity in this pathway is absent in coronal slices, which lack spontaneous action potential mediated synaptic events in the SON (Trudel &

Bourque, 2003). Nonetheless, the observed differences in presynaptic release probabilities for both IPSCs and OVLT evoked EPSCs indicates that distinct synaptic connections exist in the SON, and that these connections are specific to the type of cell being innervated. Such target cell dependant differences in release probability have also been described in the cortex (Reyes et al., 1998; Markram, Wang, & Tsodyks, 1998).

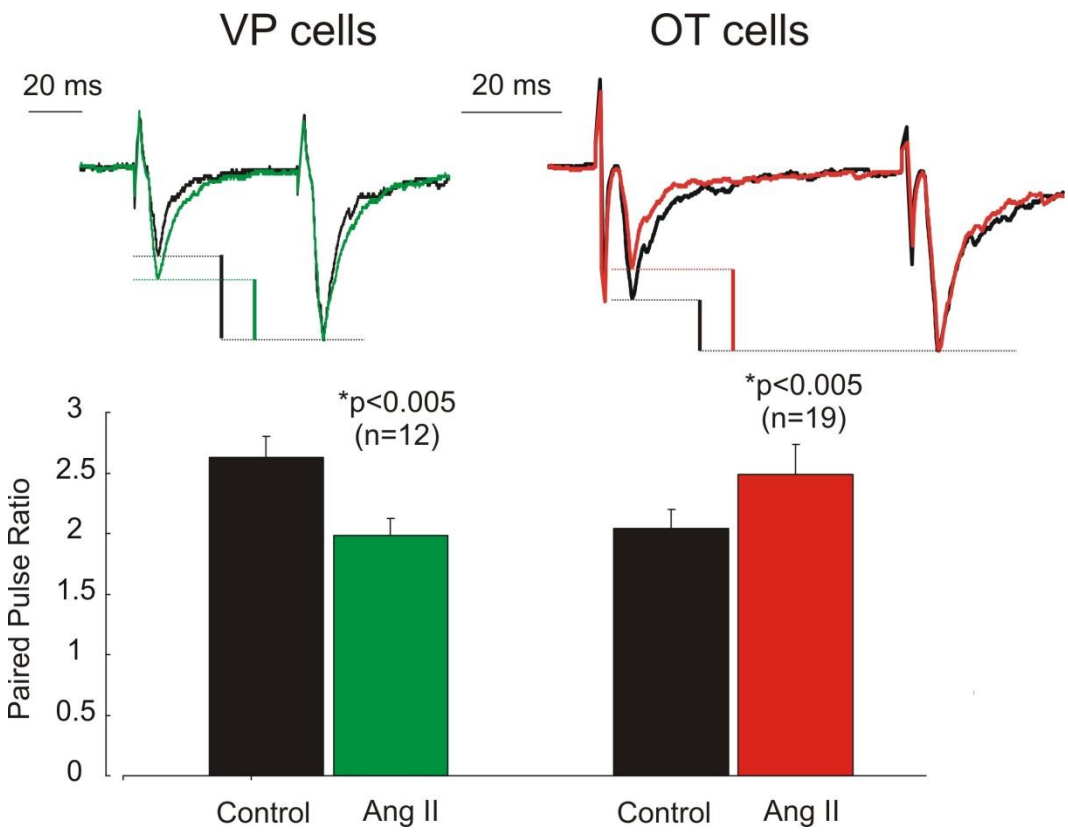

Figure 4.1 Modulation of paired pulse ratio

Electrically evoked EPSCs consistently showed paired pulse facilitation at the OVLT-SON synapse (top, black traces, examples are an average of 12 EPSCs). In VP cells, application of Ang II increases 1st EPSC amplitude, which decreases paired pulse ratio (top left, green trace, scaled to 2nd current amplitude in control). OT cells show decreases in 1st EPSC amplitude in Ang II, which increases paired pulse ratio (top left, red trace, scaled to 2nd current amplitude in control). The change in paired pulse ratio is quantified in the bottom panel for VP (left) and OT (right) cells. Stimulus artifact has been truncated.

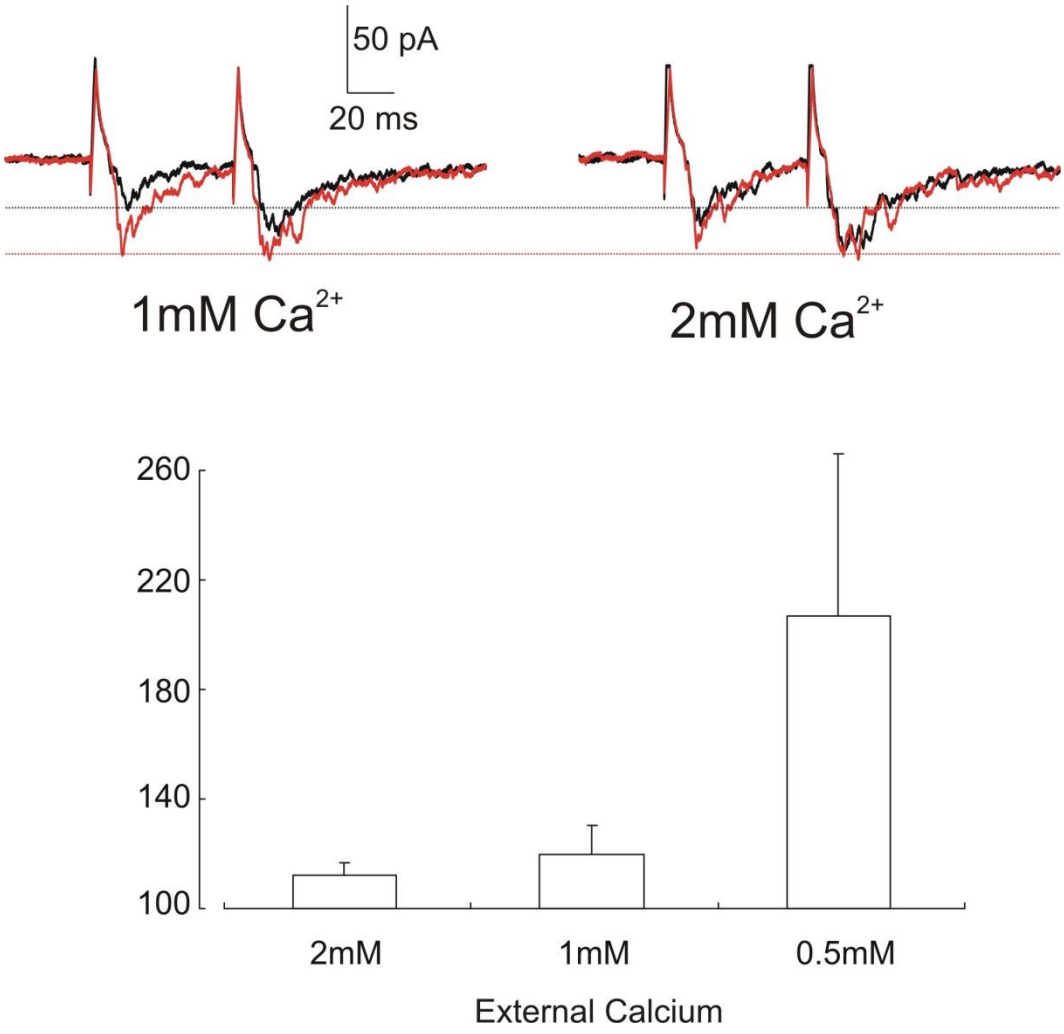

Figure 4.2 Ang II modulation is calcium dependant

Reduced extracellular calcium levels result in greater facilitation of evoked EPSC amplitude by Ang II. In the presence of 1 mM extracellular calcium, Ang II (top left, red trace) increases EPSC amplitude compared to control (top left, black trace). Switching to aCSF containing 2 mM calcium increases control EPSC amplitude (top right, black trace) but reduces the extent of facilitation induced by Ang II (top right, red trace). In separate experiments, the extent of Ang II facilitation of EPSC amplitude, expressed as a percent of control amplitude, is increased with decreasing extracellular calcium concentration (bottom panel,

$p<0.001$, $n=13$ for 2 mM calcium; $p<0.05$, $n=10$ for 1 mM calcium; $p<0.05$, $n=7$ for 0.5 mM calcium). Stimulus artifacts have been truncated.

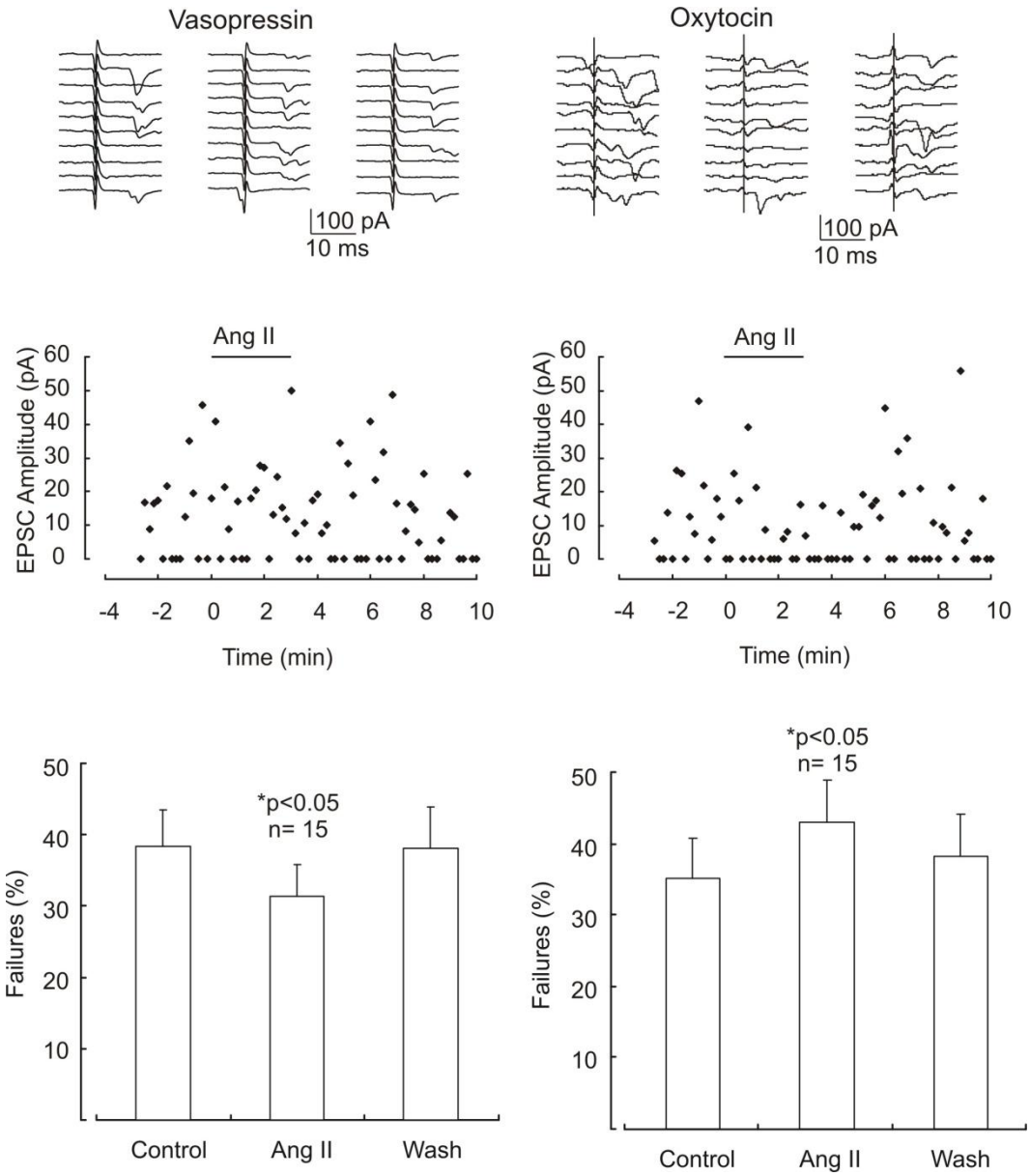

Figure 4.3 Ang II modulates failure rate

EPSCs evoked with minimal stimulation result in occasional failures (top panels, 10 consecutive sweeps). In the presence of Ang II, the frequency of failures is reduced in VP cells (left) and increased in OT cells (right). The timecourse of Ang II effects on failure rate is illustrated in the middle panels. Evoked EPSCs of less than 5 pA are considered failures and set to 0. Quantification of the percentage of failures arising in the presence of Ang II indicates a significant reduction in

failures in VP cells (bottom left), and a significant increase in failures in OT cells (bottom right). Stimulus artifacts have been truncated.

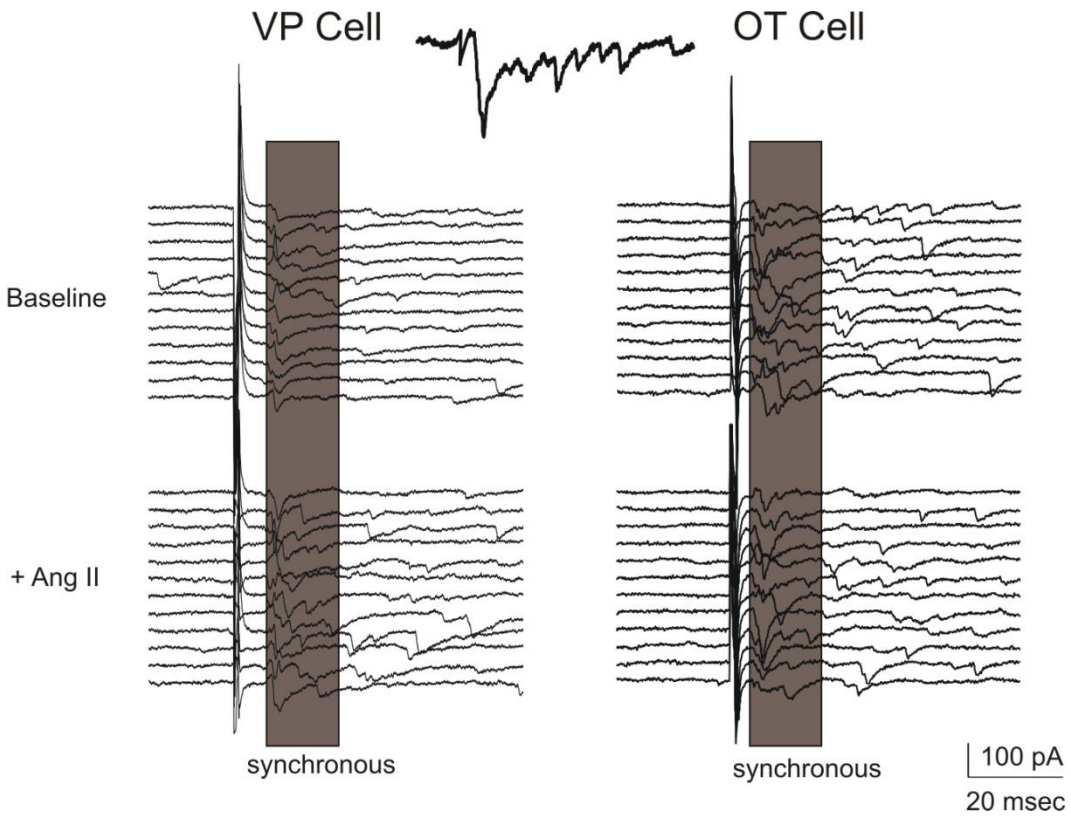

Figure 4.4 Ang II modulates asynchronous release frequency

EPSCs evoked by electrical stimulation of the OVLT resulted in both synchronous and asynchronous release events. An example trace is shown in the center. During the first 15 milliseconds following onset of the synchronous component (grey bars) asynchronous events could not be easily distinguished. In the subsequent 40 milliseconds, asynchronous event occurred at high frequency. In response to Ang II application (bottom), VP cells (left) showed an increase in asynchronous frequency, while OT cells (right) showed a decrease in asynchronous frequency compared to control conditions (top). Synchronous events have been electronically subtracted. Stimulus artifacts have been truncated.

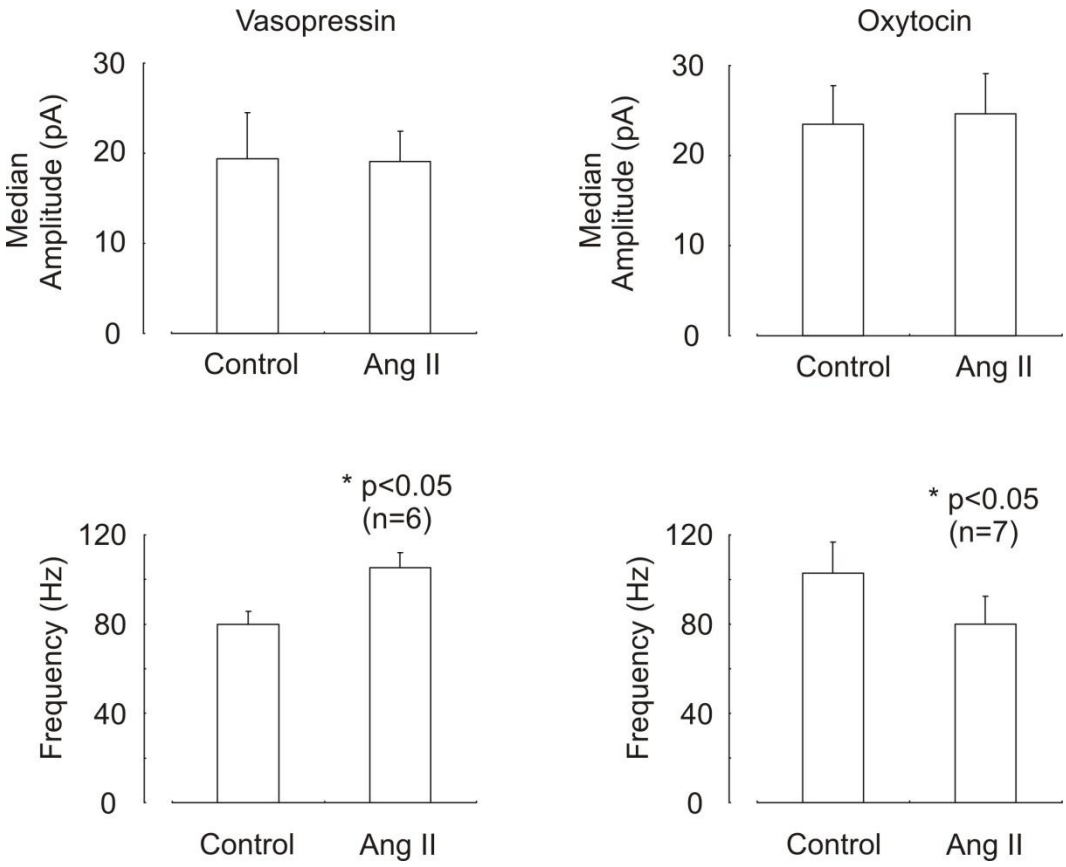

Figure 4.5 Median frequency and amplitude of asynchronous events

In the presence of Ang II, VP cells (left) showed an increase in median asynchronous frequency (bottom), while OT cells (right) showed a decrease in median asynchronous frequency (bottom) compared to control conditions. Neither cell type showed a change in asynchronous event amplitude (top).

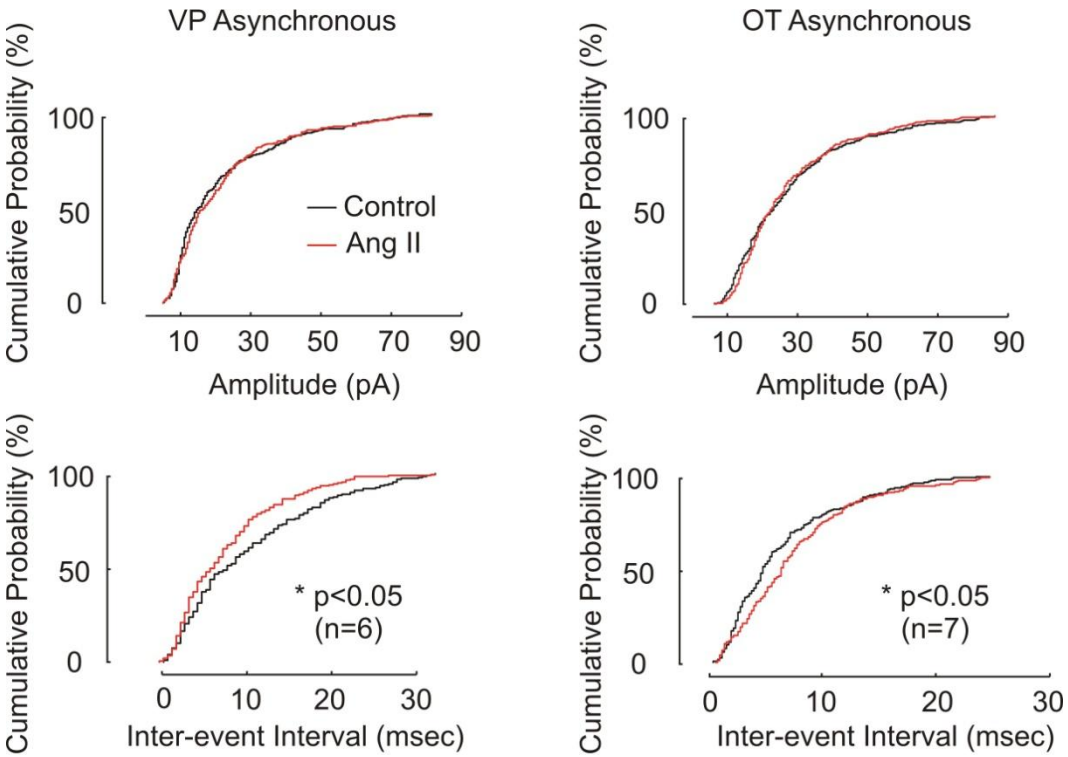

Figure 4.6 Cumulative probability plots of asynchronous amplitude and IEI

In the presence of Ang II (red), VP cell (left) asynchronous events show a leftward shift in cumulative probability of inter-event interval (IEI, bottom) compared to control conditions (black) indicating a in increase in event frequency. OT cell (right) asynchronous events show a rightward shift in IEI (bottom), indicating a decrease in asynchronous frequency. Again, asynchronous event amplitude is not affected in either cell type (top). Plots are pooled events from 6 VP and 7 OT cells.

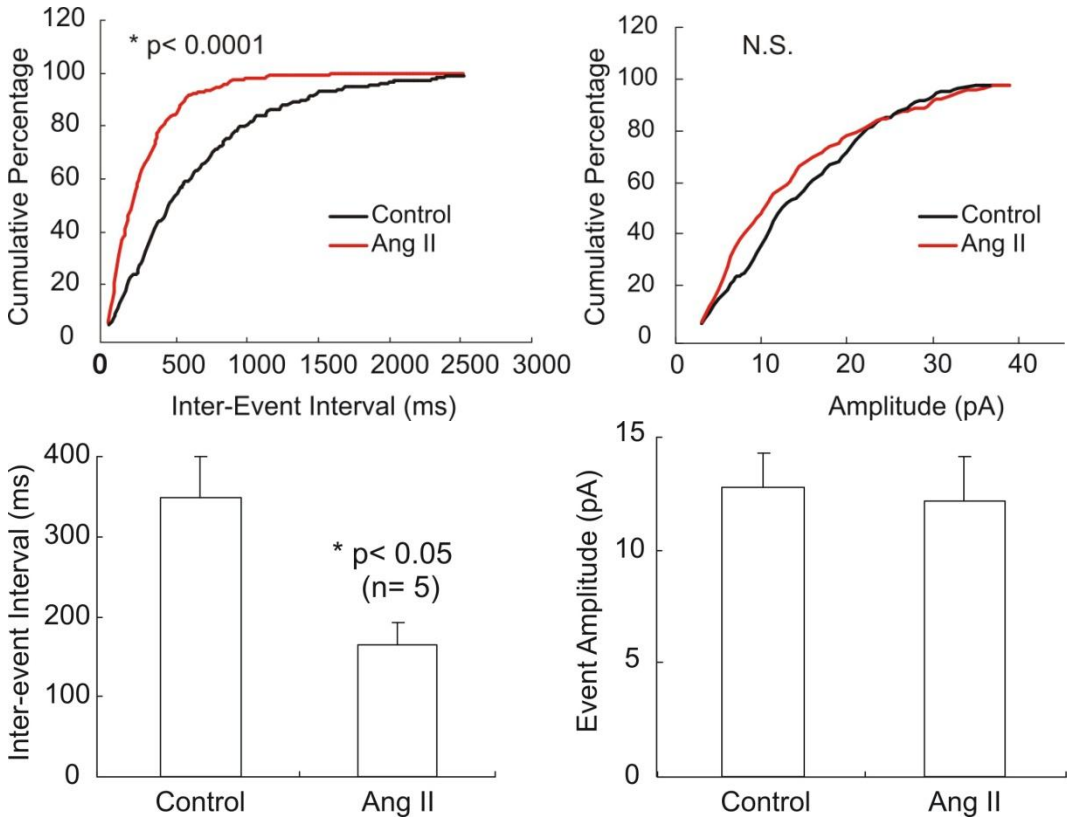

Figure 4.7 Ang II modulates miniature EPSC frequency

In the presence of tetrodotoxin (500 nM), Ang II reduces inter-event interval of miniature currents but does not affect amplitude. (Top) Cumulative probability plots of IEI (left) and amplitude (right) of miniature events from a sample cell show a leftward shift in IEI, indicating an increase in frequency. (Bottom) Median IEI and amplitude for 5 cells show a significant reduction in IEI. Amplitude is unchanged.

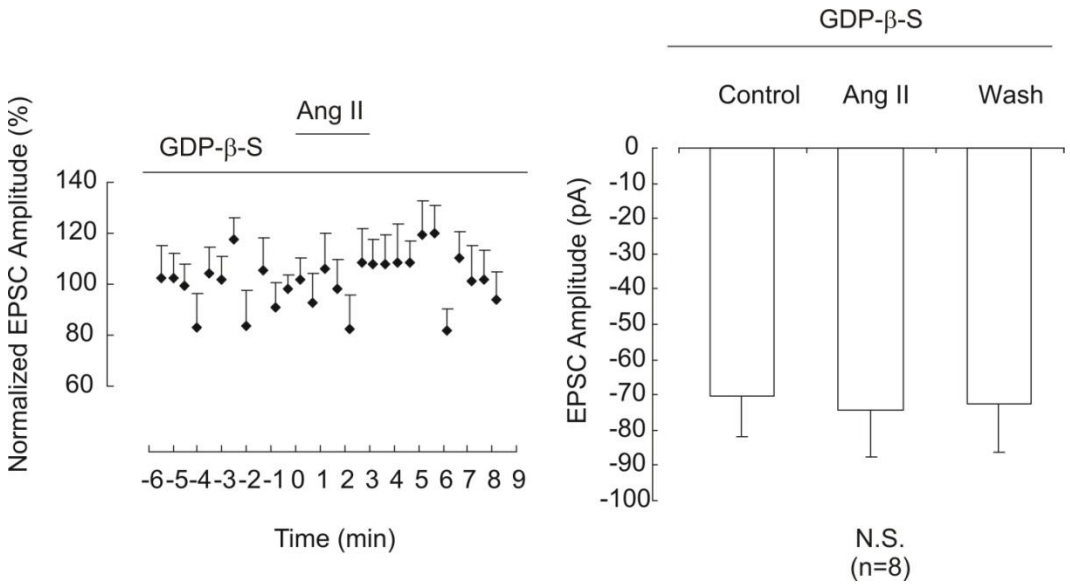

Figure 4.8 Postsynaptic G protein blockade prevents Ang II modulation

Including the G protein blocker GDP-β-S (10^{-6} M) in the patch pipette prevents Ang II induced modulations of evoked EPSC amplitude. (Left) A timecourse of evoked events shows no change in normalized event amplitude in response to Ang II. (Right) Mean data from 8 cells show no significant change in evoked EPSC amplitude in the presence of GDP-β-S. Data are plotted as in Figure 3.2.

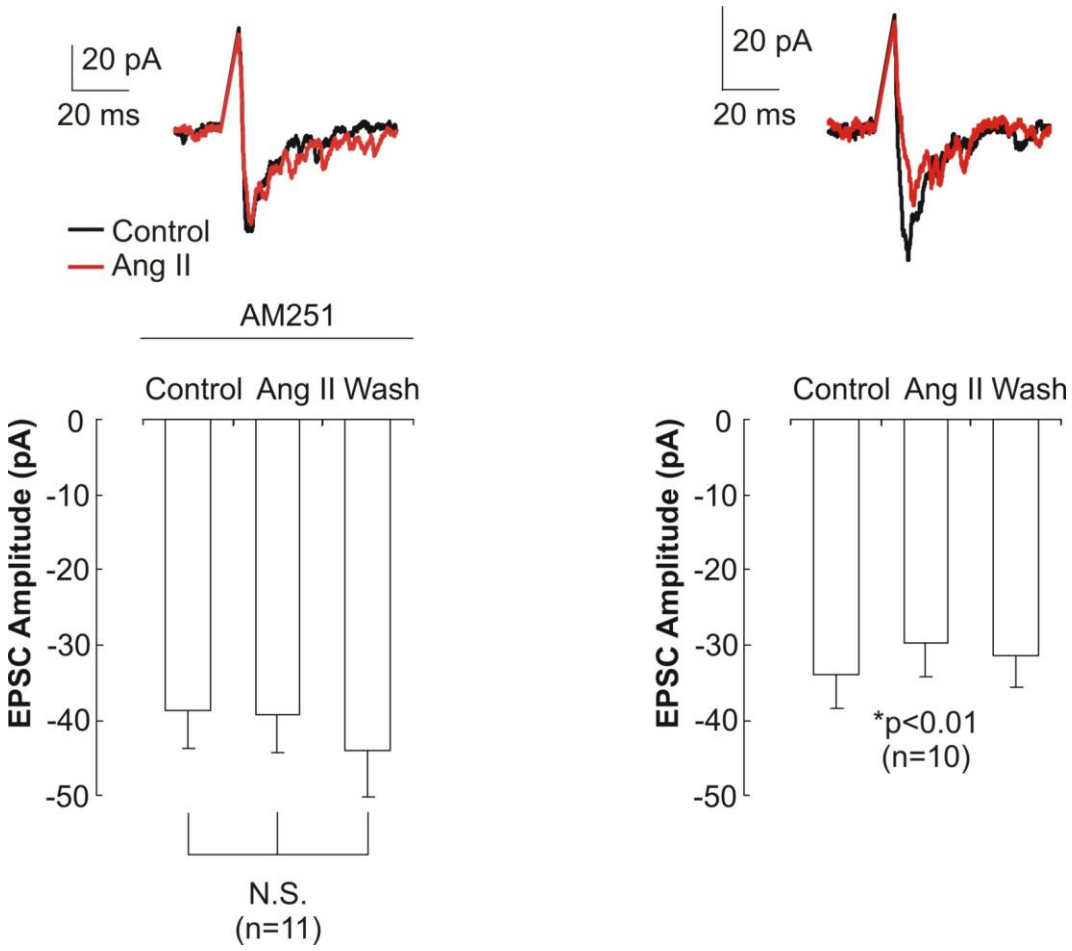

Figure 4.9 Ang II EPSC inhibition in OT cells is mediated by endocannabinoids In the presence of the CB1 antagonist AM-251 (left), Ang II (red) fails to depress EPSC amplitude in OT cells. Following washout of AM-251 (right), subsequent reapplication of Ang II (red) now produces a significant reduction in amplitude compared to control conditions (black). (Bottom) Quantification of evoked EPSC amplitude indicates that blockade of endocannabinoid receptors prevents Ang II inhibition of EPSC amplitude in OT cells.

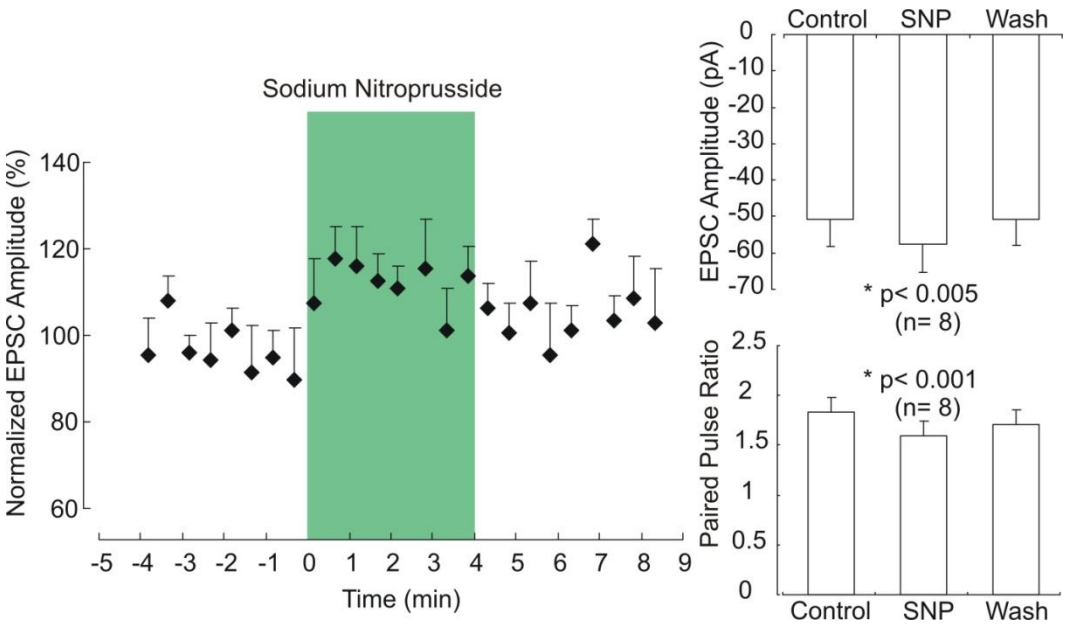

Figure 4.10 Nitric oxide mimics effects of Ang II on VP cells

Application of the nitric oxide donor sodium nitroprusside (SNP, 10^{-7} M) produces a facilitation of evoked EPSC amplitude very similar to that evoked by Ang II. (Left) A timecourse of the effects of SNP on evoked EPSCs shows facilitation of EPSC amplitude. (Right) EPSC amplitude facilitation occurs in parallel with a reduction in paired pulse ratio. Data are plotted as in Figure 3.2.

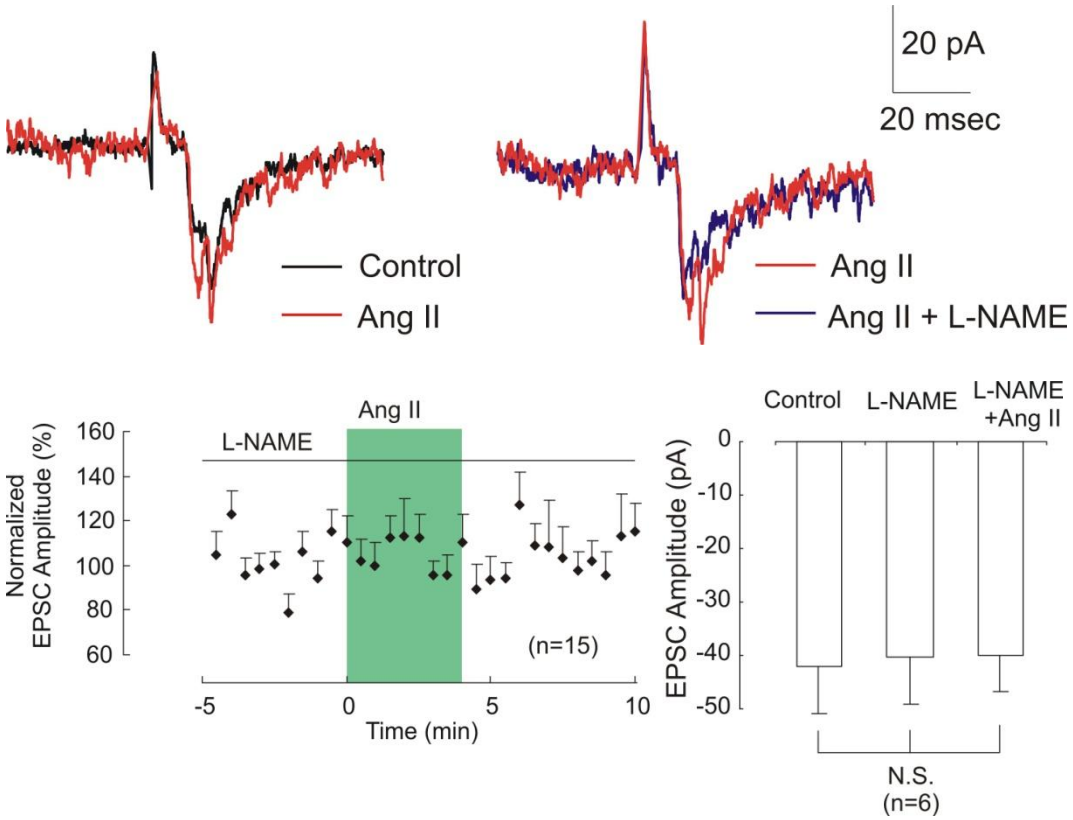

Figure 4.11 Blockade of NO production prevents Ang II facilitation

Bath application of L-NAME (10^{-5} M) inhibits Ang II induced facilitation in VP cells. (Top, left) Sample traces illustrate that application of Ang II (red) enhances evoked EPSC amplitude compared to control (black). (Top, right) Subsequent application of L-NAME (blue) reduces EPSC amplitude compared to in Ang II alone (red). (Bottom) In the inverse experiment, bath application of L-NAME prior to Ang II prevents Ang II induced facilitation of EPSC amplitude in VP cells. Data are plotted as in Figure 3.2.

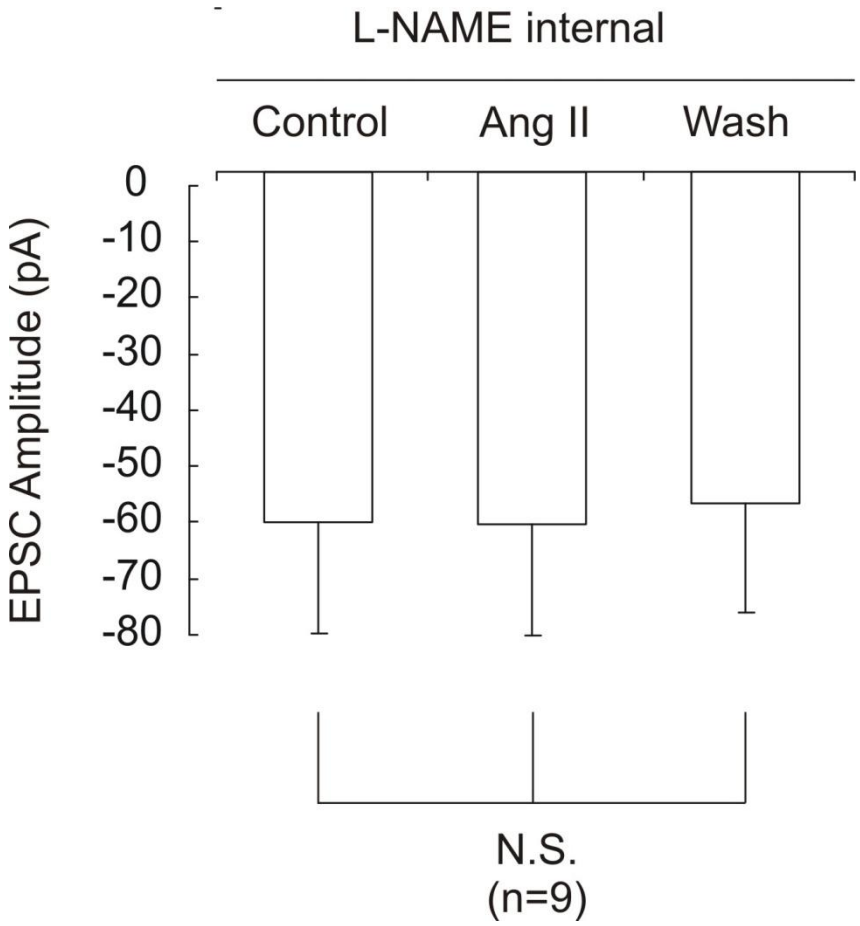

Figure 4.12 Postsynaptic blockade of NO production prevents Ang II facilitation Whole cell recordings that included L-NAME (10^{-6} M) in the internal pipette solution prevent Ang II induced facilitation in VP cells, indicating that NO production in the postsynaptic cell was necessary for Ang II to induce changes in evoked EPSC amplitude.

Chapter 5 Synaptic modulation alters MNC activity

5.1 Introduction

The preceding chapters outline a synaptic mechanism by which Ang II differentially modulates osmotic input from the OVLT in VP and OT cells. Chapter 3 identifies a differential effect of Ang II on OVLT evoked osmotic excitation in VP and OT cells. Chapter 4 outlines a synaptic mechanism by which such a differential effect may arise. Although the findings are compelling in their potential to explain how osmotic regulation of VP and OT is altered under mild hypovolemic conditions, our interpretations regarding the role of synaptic transmission in this process may be limited by the multiple modulatory effects evoked by Ang II in MNCs (see section 1.5). Alterations in intrinsic activity (Chakfe & Bourque, 2001), excitability (Nagatomo et al., 1995), and osmosensitivity (Zhang & Bourque, 2008) will also contribute to the overall effects of Ang II modulation.

We therefore make an effort to address the issue of whether changes in MNC activity by Ang II can be attributed to synaptic modulation. We first quantify the effects of Ang II on spontaneous excitatory synaptic activity in VP and OT cells. These currents are the result of spontaneous action potentials occurring in presynaptic cells, and represent the native signal attended to by the MNCs. As such, they provide a more physiologically relevant index of synaptic function than evoked currents. By first identifying modulations in these currents, and then asking the MNCs to "interpret" them for us by injecting these currents into MNCs and allowing them to fire action potentials in response, we seek to

verify that synaptic modulation by Ang II has a physiologically relevant impact on spike output. In doing so, we also hope to be able to estimate how much of the spiking activity in MNCs is attributable solely to synaptic input.

5.2 Results

5.2.1 Bidirectional modulation of spontaneous synaptic currents

Spontaneously occurring excitatory postsynaptic currents (sEPSC) were recorded at -70 mV in the absence (3 minutes baseline recording) and presence of Ang II (10^{-5} M, 3 minute application). In VP cells, application of Ang II resulted in an increase in both the median frequency 4.86 +/- 0.80 Hz compared to 3.32 +/- 0.60 Hz in control conditions, $p<0.005$, $n=9$) and median amplitude (26.8 +/- 2.4 pA compared to 24.8 +/- 2.1 pA in control conditions, $p<0.005$, $n=9$) of sEPSCs (Figure 5.1). A comparison of spontaneous events from baseline and Ang II treated conditions using cumulative probability plots confirmed that IEI was reduced and spontaneous event amplitude was increased in VP cells. Statistical analysis of each cell with the Kolmogorov-Smirnov test revealed that 9 out of 9 cells showed a significant change in inter-event interval (IEI), while 7 out of 9 cells showed a significant change in event amplitude. Sample cumulative plots from one cell are illustrated in Figure 5.2.

In OT cells, application of Ang II resulted in a decrease in both the median frequency (7.27 +/- 1.00 Hz compared to 8.33 +/- 1.31 Hz in control conditions, $p<0.01$, $n=8$) and median amplitude (33.6 +/- 3.6 pA compared to 34.7 +/- 3.5 pA in control conditions, $p<0.05$, $n=8$) of sEPSCs (Figure 5.1). A comparison of

spontaneous events from baseline and Ang II treated conditions using cumulative probability plots confirmed that IEI was increased and amplitude was decreased in OT cells. Application of the Kolmogorov-Smirnov test revealed that, 5 out of 8 cells showed a significant change in IEI, while 4 out of 8 cells showed a significant change in event amplitude. All cells showed a statistically significant change in at least one parameter; one cell showed a significant change in both. Sample cumulative plots from one cell are illustrated in Figure 5.2.

5.2.1a Modulation of sEPSC amplitude

In contrast to miniature events (section 4.2.6) and asynchronous events (section 4.2.4), which represent quantal release, spontaneous events include action potential derived events. As these events represent the concerted evoked release of one or more vesicles (multi-vesicular release) from a terminal in response to an action potential, changes in EPSC amplitude no longer imply changes in post-synaptic receptor density. In fact, a change in median amplitude may represent an increased number of large amplitude events, as the average number of vesicles released at a synapse may be influenced by modulations of release probability (Huang, Bao, & Sakaba, 2010). Indeed, that is what we found in response to Ang II application. Peak frequencies for the spontaneous EPSC amplitude distributions arose at or near discrete multiples of the unitary amplitudes (about 15 pA) observed in our recording conditions (Figure 5.3). Amplitude histograms for VP and OT cells show differences in the peak frequency of events at these discrete amplitudes (Figure 5.3). Thus we interpret the change in median event amplitudes

between control and Ang II conditions as representing a change in release probability that results in an increased number of quanta released in response to a presynaptic action potential (increased multi-vesicular release).

5.2.1b Modulation of sEPSC frequency

Spontaneous EPSC frequency is increased in VP cells and reduced in OT cells in response to Ang II. These changes parallel those observed in asynchronous release frequency (section 4.2.4) for VP and OT cells, which suggests that modulations in asynchronous release contribute to the observed changes in sEPSC frequency. In VP cells, increases in miniature EPSCs (section 4.2.6) also likely contribute to the overall increase in sEPSC frequency. However, our observation that miniature EPSC frequency is also increased in OT cells suggests that in OT cells, sEPSC frequency modulation is influenced by decreases in asynchronous events but also increases in miniature events. This suggestion may explain why statistically significant changes in sEPSC frequency were less common in OT cells (5 out of 8 cells) than in VP cells (9 out of 9 cells).

Both frequency and amplitude changes represent aspects of the observed modulation of spontaneous events. To quantify an approximate summation of the observed changes in to both frequency and amplitude, we estimated the extent of modulation in charge transfer. Charge transfer estimates were obtained by multiplying the median amplitude by the median number of events in a ten second period for each cell. The value obtained in Ang II conditions was then divided by the control value. In a test case, we found that summing the charge integral (area

under the curve) for each event recorded under Ang II conditions, then dividing by the summed charge integral of control conditions yielded similar results to the above method. We estimate that in Ang II, the amount of charge transfer for VP cells was 173 +/- 27% of control, while charge transfer for OT cells was 86 +/- 2% of control.

5.2.2 Generation of an artificial synaptic template

We attempted to establish whether the changes in sEPSC frequency and amplitude (synaptic input) we observed in VP and OT cells in response to Ang II would result in a significant change in MNC firing frequency (spike output, the frequency of action potentials generated in response to a given stimulus). To that end, we selected a number of recorded sEPSC segments which will be "played back" to MNCs (i.e. used as a dynamic current command) in current clamp conditions, such that our current injection waveform resembles the synaptic events previously recorded in section 5.2.1. By recording the corresponding changes in spike output in Ang II naïve cells, we can assess the extent to which the cell responds to synaptic modulation in the absence of Ang II and any modulatory effects Ang II might produce in MNCs.

Two and three minute sections of sEPSC recordings in both control and Ang II treated conditions were selected as representing average responses by a comparison of median frequency and amplitude to the average values observed in section 5.2.1. Median values for an example input file are illustrated in Figure 5.4, set against the average values for VP cells. The 4 input files we chose to use were

all from VP cells, but the artificial alternating templates we generated (see section 2.5.4) simulates both increases and decreases in synaptic input as it moves from low to high frequency and amplitude or from high to low. The templates are composed of three 10 second epochs (i.e. periods of time) recorded in control conditions, followed by three 10 second epochs recorded in Ang II, followed again by control epochs and so on, for 4 to 6 minutes in total. By normalizing charge transfer for each 10 second epoch to the charge transfer for the preceding epoch, we simulate a range of modulations in synaptic activity that includes both increases (as in VP cells) and decreases (as in OT cells) in synaptic transmission.

5.2.3 Artificial synaptic modulation alters MNC spike output

In a sample play back experiment in which the artificial synaptic template were designed to resemble the synaptic changes evoked by Ang II in a VP cell, we observed an increase in spike output compared to control stimulus conditions. Examples of this response modulation for 12 cells subjected to one of the artificial synaptic templates are illustrated in Figure 5.5. This artificial synaptic template was generated from the VP cell whose response profile is illustrated in Figure 5.3. Here we compare 30 second epochs containing high synaptic activity to the preceding 30 second epoch of low synaptic activity, and thus simulate a VP neuron transitioning from control to Ang II conditions. The number of action potentials recorded for each 30 second epoch of high synaptic activity (Ang II condition) was divided by the number of action potentials for the preceding

(control condition) epoch. Average spike rates during the Ang II waveform were 34 +/- 12% greater than in the control condition ($p<0.005$ by z-test, n=12).

To simulate a range of modulations in synaptic activity that would include both increases (as in a VP cell transitioning into Ang II) and decreases (as in an OT cell transitioning into Ang II) in synaptic transmission, we normalized each 10 second sweep against its predecessor to generate a range of artificial synaptic activity modulations. Charge transfer (calculated here as the sum of the area under each EPSC for all EPSCs in each 10 sec sweep) for each 10 second epoch in the artificial synaptic template was divided by charge transfer in the preceding epoch. The number of action potentials generated in response to each of these 10 second epochs was also normalized by dividing by the action potentials generated in the previous epoch. These normalized action potential rates were then averaged over a number of cells.

A total of 4 artificial synaptic templates were tested, which gave us a wide range of frequency and amplitude modulations to play back to MNCs. In order to determine how MNC firing frequency would change in response to a change in these modulations, we plotted normalized spike frequency against normalized charge transfer. We find this generates a linear relation between input (change in charge transfer) and output (change in spike rate). The regression line for data compiled across all trials had a slope of 0.38 (r^2= 0.19, 33 cells, 118 data points), indicating a 38% change in spiking for every fold change in charge transfer (Figure 5.6). Regression lines for the various input files had an average slope of 32 +/- 10% ($p<0.001$ by z-test, n=4 input files, total of 33 cells tested). These data

confirm that increases or decreases in synaptic input will result in corresponding increases or decreases in MNC firing rate.

5.2.4 Quantifying the contribution of synaptic currents to MNC firing rate

The wide range of synaptic modulations plotted in Figure 5.6 allows us to speculate about the relative contribution of synaptic modulation to changes in MNC firing rates. Plotting our estimates (section 5.2.1b) for the average synaptic modulation observed in VP (173% of control charge transfer) and OT (86% of control charge transfer) cells against the slope (0.38) of the regression line of Figure 5.6, we can estimate that alterations in synaptic transmission account for an increase in spike frequency of about +28% in VP cells, and a reduction in spike frequency of about -5% in OT cells.

In addition to examining how changes in synaptic input alter firing rates, we also were able to plot the raw values for MNC firing rate against charge transfer for these cells. Regression lines (data not shown) of total charge transfer versus total spike output indicated that for every picocoulomb of charge transferred into a MNC by EPSCs in a 10 second period, the neuron would increase spiking by 0.06 +/- 0.01 Hz ($p<0.001$ by z-test, n=4 input files, Figure 5.6). Thus we find that synaptic modulation will alter action potential frequency in MNCs, and that synaptic potentials contribute a quantifiable amount to MNC action potential generation.

5.3 Discussion

Our assessment of spontaneous synaptic currents indicates that Ang II significantly alters release probability of the OVLT-SON synapse *in vitro*. In VP cells, Ang increased spontaneous event frequency and amplitude, while OT showed a depression in these parameters. These findings are consistent with the observations made in Chapter 4, in that Ang II facilitates release probability in VP cells while depressing probability of release in OT cells. Thus in every assessment we've made of presynaptic release probability, we see a consistent change in VP and OT cells that correlates with the changes in osmotic-induced excitation observed in Chapter 3, but these observations alone do not prove that modulation of synaptic transmission contributes to the observed alterations in MNC activity. The bidirectional modulation of MNC firing rate elicited in response to an artificial synaptic input confirms that synaptic modulation alters MNC activity.

5.3.1 Modulation of spontaneous activity

Examining the modulation of sEPSCs in VP cells more closely, we can make some interpretations about how alterations in release probability will impact the activity of VP neurons. Unlike the experimental findings in Chapter 3, we observed an increase in the amplitude of spontaneous events, which we attribute to the occurrence of multi-vesicular release events composed of greater numbers of vesicles relative to control conditions (section 5.2.1a). The multiple-Gaussian distribution of the events observed in Figure 5.3 is indicative of release events composed of increasing numbers of discrete quanta (Del Castillo & KATZ, 1954).

Note that the change in amplitude distribution occurs through an increase in the peak frequencies of events at regular multiples of the unitary event amplitude, rather than by globally shifting the amplitude distribution, as might be expected with a change in postsynaptic receptor number (Gordon & Bains, 2005). Additionally, the absence of any amplitude changes in miniature events (Figure 4.7) or asynchronous events (Figure 4.5) strongly suggest that the observed amplitude changes are occurring through an increased number of vesicles released by spontaneous presynaptic activity.

Such an interpretation may help explain how increased slope of the VP-osmolality relationship arises. If every presynaptic action potential produces more glutamate release in the presence of Ang II, this would generate a correspondingly larger excitation with increasing presynaptic activity. As both OVLT spike rate (Ciura & Bourque, 2006) and spontaneous synaptic release rate (Richard & Bourque, 1995) increase as a function of increasing osmolality, the presence of Ang II would have a multiplicative effect on the slope of the OVLT osmolality relationship, thereby increasing the slope of the VP-osmolality relationship (Figure 1.3).

Presumably the inverse interpretation holds true for OT cells. Although we lack experimental data directly showing a decrease in the slope of the OT-osmolality relationship under mild hypovolemic conditions, we might predict that such would be the case. More study is needed to determine if the blunted OT release observed in mild hypovolemia (Stricker et al., 1994; Koehler et al., 1994)

can be explained by a reduction in slope of the OT-osmolality relationship arising from presynaptic inhibition of OT cells in response to Ang II.

5.3.2 Modulation of synaptic transmission is sufficient to alter MNC activity

To determine if changes in synaptic transmission are sufficient to elicit changes in MNC spike frequency, we examined the responses of MNCs to an artificial synaptic template. We first demonstrate that current clamp injection of a waveform designed to mimic the synaptic alterations observed in a VP neuron responding to Ang II could increase MNC firing frequency. Next we confirmed that bidirectional synaptic modulations resulted in bidirectional changes in MNC firing rate. Increases in charge transfer induced increases in action potential frequency, while decreases in charge transfer reduced action potential frequency. We can therefore surmise that the synaptic facilitation observed in VP cells contributes significantly to increased spike frequency, while the synaptic depression observed in OT cells will decrease spike frequency. We may interpret these findings to suggest that in response to the increased synaptic activity generated by osmotic stimulation of the OVLT, Ang II modulation will make VP cells more responsive to the osmosensory synaptic activity, while OT cells will be less responsive, as we observed in Chapter 3 (Figure 3.1).

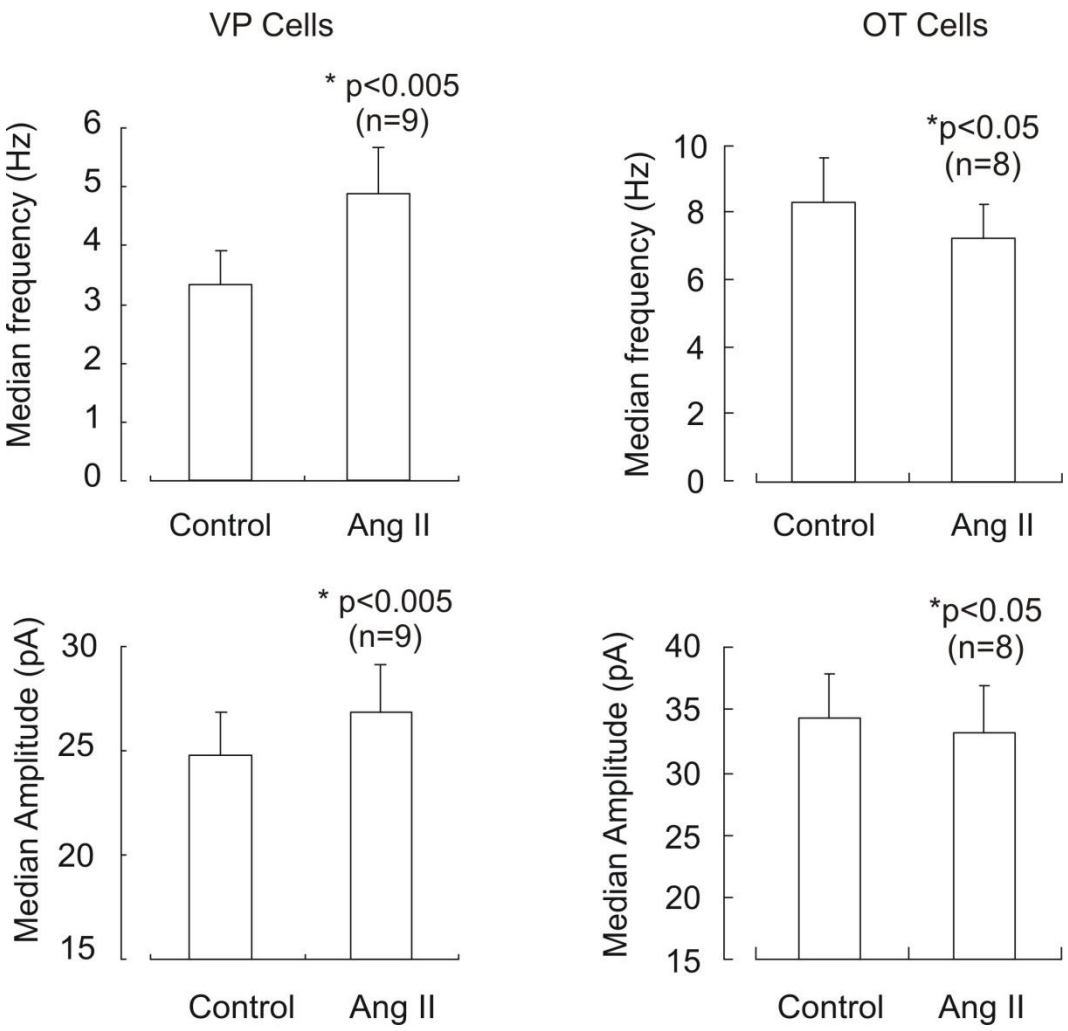

Figure 5.1 Ang II modifies median sEPSC frequency and amplitude

In VP cells (left), application of Ang II resulted in an increase in both the median frequency and amplitude of sEPSCs. In OT cells (right), application of Ang II resulted in a decrease in both sEPSC frequency and sEPSC amplitude.

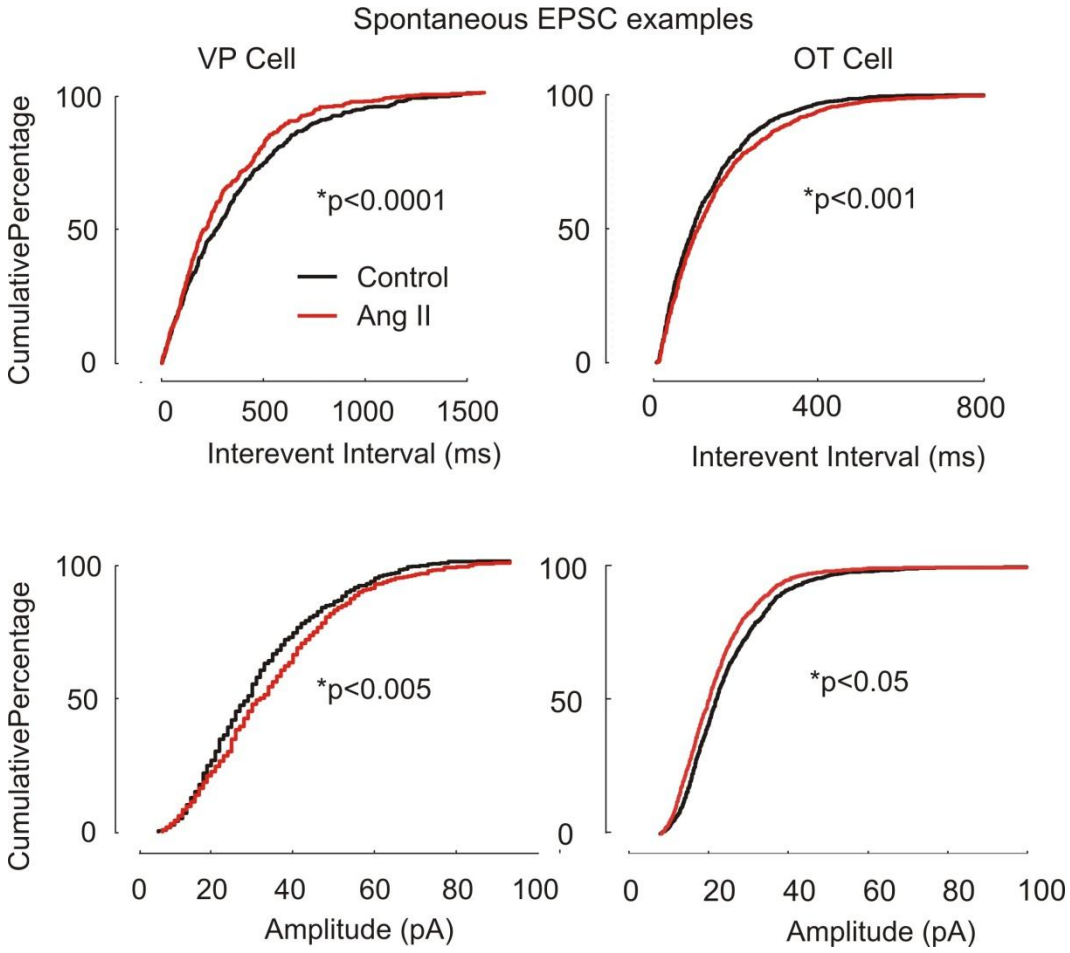

Figure 5.2 Ang II alters distribution of IEI and amplitude of spontaneous events Sample cumulative probability plots of IEI (top) and amplitude (bottom) from one VP cell (left) and one OT cell (right) are illustrated. In VP cells, application of Ang II (red) resulted in leftward shift in IEI distribution, indicating a reduction in IEI and increase in frequency compared to control (black). Ang II induced a rightward shift in amplitude distribution compared to controls, indicating an increase in amplitude. In OT cells, application of Ang II resulted in rightward shift in IEI distribution, indicating an increase in IEI and reduction in frequency. Ang II induced a leftward shift in amplitude distribution compared to controls, indicating a decrease in amplitude.

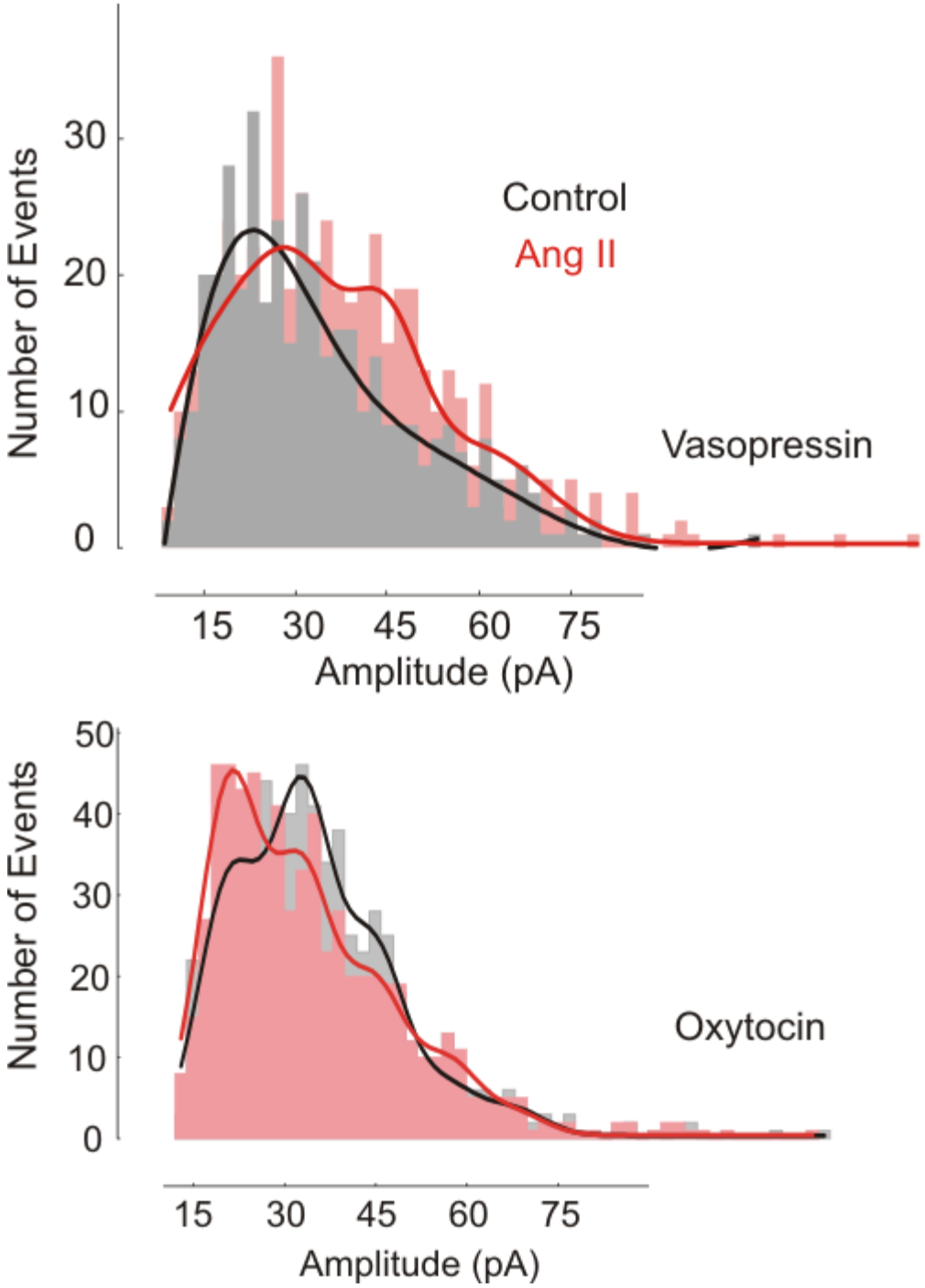

Figure 5.3 Ang II modulates spontaneous EPSC amplitude distribution

Amplitude distributions of sEPSCs show peaks at discrete multiples of the unitary EPSC amplitude (~15 pA). In the presence of Ang II, sEPSC amplitude distributions in VP cells show a greater number of events at higher amplitudes

(top, red) compared to in control conditions (top, black). In contrast, sEPSC amplitude distributions in OT cells show a greater number of events at lower amplitudes in Ang II (bottom, red) compared to in control conditions (bottom, black). Distributions in control and Ang II conditions are matched for event number.

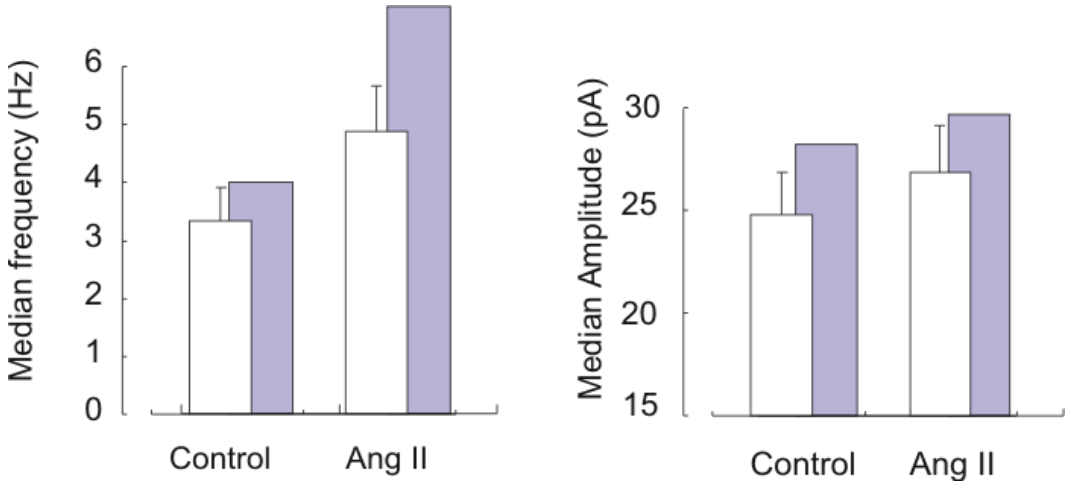

Figure 5.4 Comparing selected template to median values in VP cells

Median frequency and amplitude for an example artificial synaptic template file (blue) are juxtaposed to median values for VP cells (the same values as in Figure 5.1).

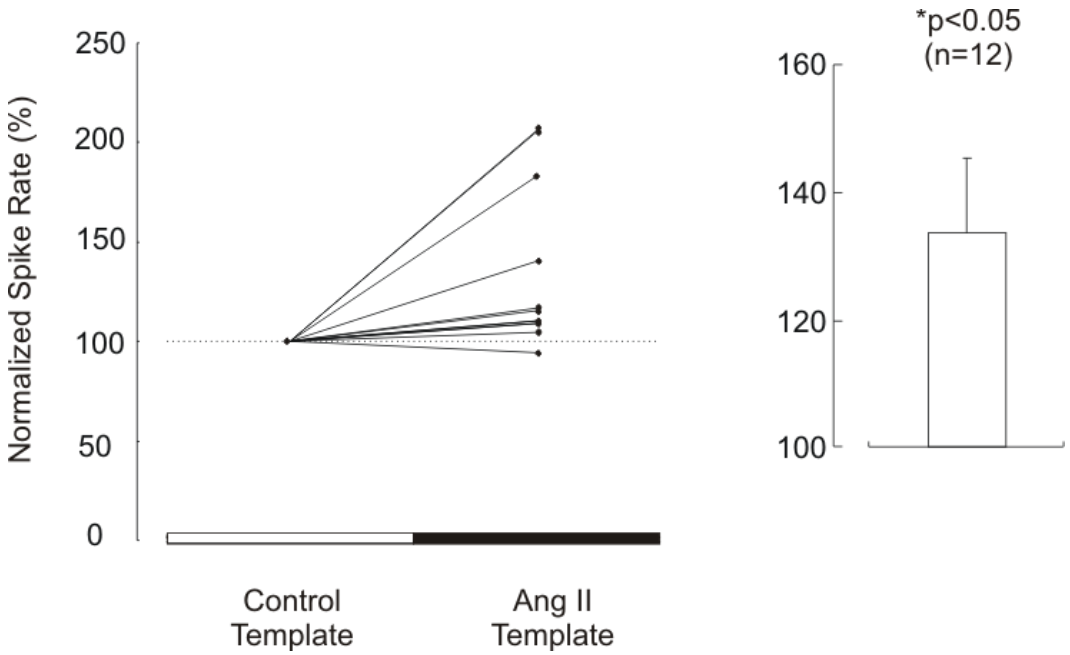

Figure 5.5 Play back of artificial synaptic template alters spike rate

Comparing spike output in response to an artificial synaptic template designed to simulate alterations in sEPSC frequency and amplitude experienced by a VP cell responding to Ang II, we find that spike rate is increased in response to the Ang II template (black bar), as compared to the control template (white bar). Quantification of the response reveals a significant facilitation of spike input in response to the Ang II template.

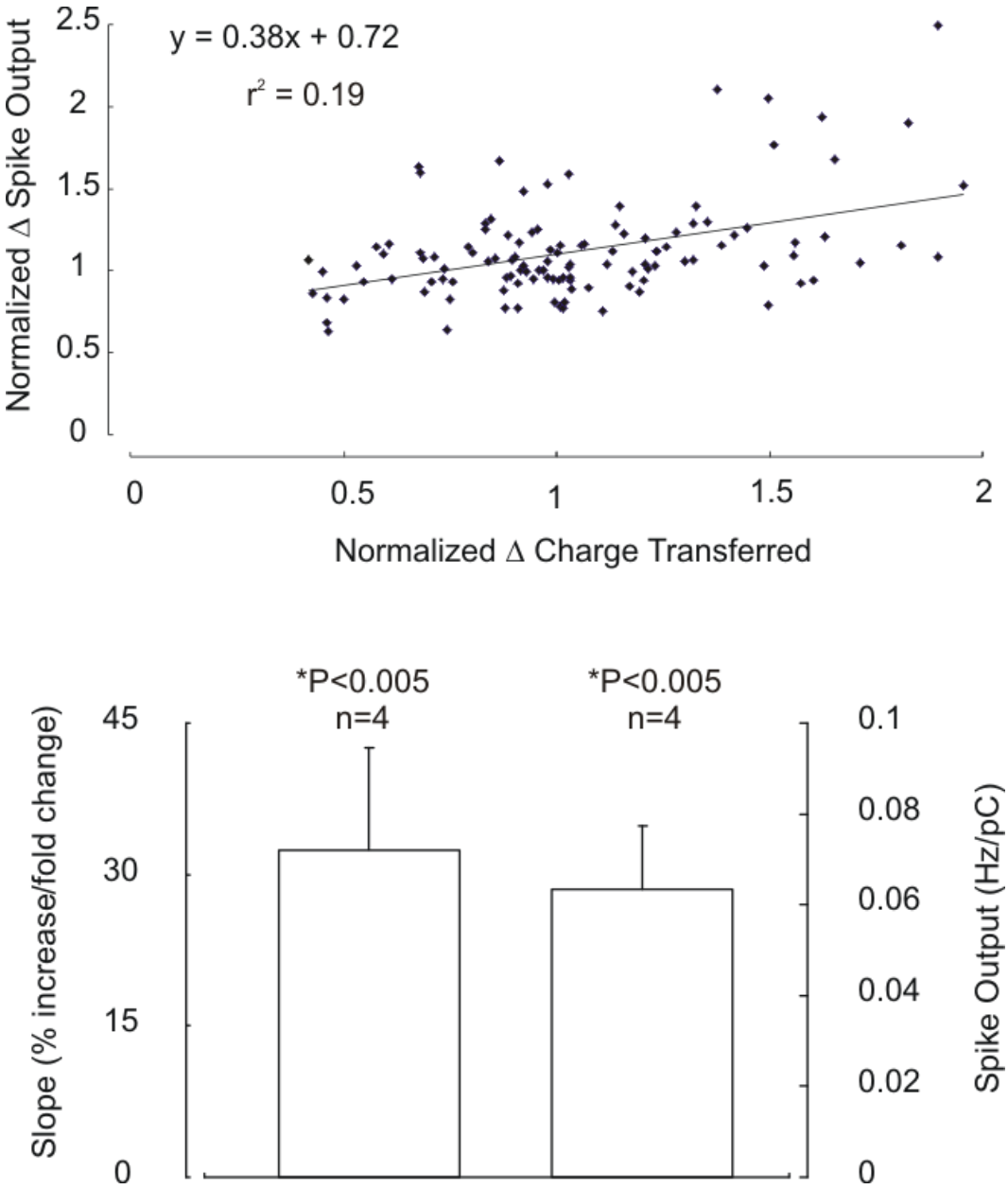

Figure 5.6 Changes in charge transfer alter spike output

Changes in charge transfer present in the artificial synaptic template elicit changes in spike output. (Top) Plotting normalized spike output against normalized charge transfer (4 artificial synaptic templates, 33 cells, 118 data points) generates a linear relationship. Thus increases in artificial synaptic transmission result in increases in MNC firing rate. A range of modulations in charge transfer are

plotted. VP cells show an increase in synaptic activity in response to Ang II, and this would be represented by a normalized change in charge transfer that is greater than 1. OT cells show a decrease in synaptic activity in response to Ang II, and this would be represented by a normalized change in charge transfer that is less than 1. (Bottom) The slope of the regression lines for the 4 artificial synaptic templates tested show an average slope of 0.32, indicating a 32% increase in MNC firing for every 100% increase in charge transfer. The regression lines for the plots of raw spike output versus raw charge transfer show an average of 0.06 Hz per picocoulomb, indicating that for every pC of charge transferred into an MNC in a ten second period, the neuron would increase firing by 0.06 Hz.

Chapter 6 General Discussion

The nearly overwhelming variety of neuropeptidergic signaling molecules capable of relaying information from the periphery to the central nervous system, and the wide variety of signaling mechanisms evoked there in response invokes chaotic images of swirling chemicals. However, we might propose that some underlying order must exist for the correct homeostatic responses to occur in a timely fashion. The successful integration of osmotic and volemic signals presents a complex challenge for the magnocellular neurosecretory system. This problem is not unique to the magnocellular system, but indeed represents an information processing problem common to all neurons. Wherever different cell types must respond differentially to a common input, a mechanism must exist to produce an appropriate modulation of synaptic gain. The experiments performed in this research project indicate that Ang II activity induces a differential response in synaptic signaling for VP and OT neurons. Although within the confines of one study we cannot definitively prove that differential signal modulation is common to multiple systems, identifying the mechanism by which MNCs address this particularly complex problem may nonetheless give us insights into how the nervous system integrates relevant information to produce an appropriate response.

6.1 Modulation of release probability alters spike output

Our findings indicate that osmosensory synaptic transmission is differentially regulated in VP and OT neurons by the hypovolemic peptide Ang II.

The enhancement of osmotic induced spike output observed in VP cells indicates that in response to osmotic stimulation of the OVLT, VP secretion will be facilitated by Ang II. Conversely, OT cell responsiveness to OVLT stimulation is reduced by Ang II. These changes in activity are partly attributable to changes in synaptic transmission, indicating that modulation of presynaptic probability of release alters MNC responsiveness to osmotic stimulation of OVLT.

The observed increase in presynaptic release probability is mediated by NO production, and results in increased frequency and amplitude of spontaneous synaptic events, while the converse effects arise from the activity of eCBs. An examination of spontaneous event distributions suggests that action potential mediated events are releasing greater numbers of quanta simultaneously in VP cells (section 5.2.1a), producing larger EPSCs. The larger amplitude events which result from this multivesicular release increase the probability of postsynaptic action potential generation (Huang et al., 2010). The inverse is true for OT cells.

Additionally the asynchronous events, which arise following action potential mediated synchronous release, show an increased frequency in VP cells and decreased frequency in OT cells. Iremonger and Bains demonstrate that asynchronous release events increase the probability of action potential generation in MNCs by prolonging the spike window following evoked synaptic release (Iremonger & Bains, 2007). Together, increases in event amplitude and asynchronous release will result in an increased depolarizing charge transfer (section 5.2.3) and therefore raise the probability of post synaptic spike discharge. By increasing the probability that OVLT-derived presynaptic activity will trigger

a postsynaptic spike, Ang II modulates the input-output relationship to increase the slope of the VP-osmolality relationship. Conversely, we expect a reduction in the slope of the OT-osmolality relationship in response to Ang II, which may explain the blunted or absent secretion of OT under mild hypovolemic conditions. Thus Ang II can modulate osmosensitivity by modulating synaptic gain through the cell type specific secretion of retrograde transmitters.

6.2 Can a single neuron exhibit selective attention?

Our observation that OT cells cease to respond to osmotic stimulation of the OVLT with a significant increase in activity is an intriguing one. It suggests that a cell may selectively attend to or ignore an excitatory input by regulating presynaptic transmission of that input. Other investigators have demonstrated that selective facilitation or depression of synaptic transmission in response to glucocorticoids (Di et al., 2009) or endothelin-1 (Zampronio et al., 2010) occurs in MNCs. These studies also found that synaptic depression was mediated by eCBs (Di et al., 2009; Zampronio et al., 2010), and facilitation was mediated by NO (Di et al., 2009). Our findings demonstrate that within a defined synaptic connection, presynaptic inhibition or excitation can reduce or increase the responsiveness of a neuron to incoming activity. Collectively, these data suggest a mechanism that allows a postsynaptic cell to govern the synaptic input it receives through the use of retrograde messengers that modulate presynaptic release probability.

The observation that such a modulation can be cell type specific leads to the idea that specific regulation of postsynaptic signaling controls presynaptic modulation. Di et al. found that differential release of eCB or NO is mediated by activation of specific G-protein signaling pathways. $G_{\alpha}s$ triggers cAMP (cyclic adenosine monophosphate) dependant synthesis of eCBs to inhibit presynaptic release, while activation of $G_{\beta\gamma}$ leads to nNOS activation and NO synthesis to facilitate release (Di et al., 2009). A parallel finding shows that activation of $G_{\beta\gamma}$ leads to increased synaptic transmission in the form of EPSC clustering, which promotes bursting activity in OT neurons during lactation (Wang & Hatton, 2007). The synaptic specificity which subsequently arises from these signaling pathways may be due to focal localization of the synthesis and secretion of these messengers (Di et al., 2009), while the presence of glial processes limits the diffusion of these molecules in the extracellular space (Di & Tasker, 2010). This notion is supported by the observation that nNOS is localized to the synapse through an interaction with postsynaptic density-95 protein (PSD-95), a postsynaptic scaffolding protein (Brenman et al., 1996). The eCB synthesis enzyme diacylglycerol lipase is also localized to synapses, in close proximity to the CB1 receptor (Yoshida et al., 2006). Synaptic localization of these messengers may permit focal activation and release to mediate synapse specific modulation of signaling.

The proposal that specific alterations in transmission can be elicited by retrograde messengers even from synapses in close proximity (Di & Tasker, 2008) begs the question of whether the presynaptic modulation observed in our

study may be specific to the osmosensitive neurotransmission arising from the OVLT. There remains a possibility that Ang II will alter all glutamatergic transmission in MNCs, rather than just affecting the synapses arising from the OVLT. Although we have not directly tested this possibility, the observation that OVLT lesions prevent Ang II induced secretion of VP (Thrasher & Keil, 1987) argues against such a notion.

The differential synaptic modulation by Ang II in MNCs represents a mechanism by which integration of neuroendocrine and neural signals may be accomplished. From an information processing perspective, the finding that a hypovolemic signal can enact an increase or decrease in osmosensory gain in a cell type specific fashion is very intriguing. We propose that the use of retrograde signaling molecules to determine synaptic gain represents a general mechanism for information processing. This theory is in accordance with observations in the neocortex, where activity dependant changes in excitatory and inhibitory transmission are differentially regulated in a cell type specific manner (Reyes et al., 1998).

6.3 Assessment of the contribution of synaptic modulation to MNC activity

The increased osmosensitivity conveyed by Ang II will likely interact positively with the increased osmosensory transduction gain of the MNCs (Zhang & Bourque, 2008) to further enhance osmosensitivity. Although it is difficult to predict how the two components will summate, we can make inferences about the relative contribution of synaptic modulation to the total osmotic gain by

comparing our results from Chapter 5 and Chapter 3. Our synaptic playback experiments predict an increase in osmotic evoked spiking of about +28% in VP cells. Comparing this to our osmotic evoked spiking experiments, which showed a facilitation of 221% in response to Ang II, we can estimate that about 12.7% of the change in osmotic evoked spiking can be attributed directly to presynaptic facilitation. Based on our findings for OT cells, which showed a depression of 81% in response to Ang II, we estimate that 6.2% of the evoked changed can be attributed directly to presynaptic depression. We note that our charge transfer estimates and artifical synaptic waveform included all synaptic events, rather than just the osmotic-evoked events. If osmotic-evoked currents from the OVLT are preferentially modulated as compared to other currents as we suggest in section 6.2, we expect that this comparison will have underestimated the total contribution of synaptic modulation.

In estimating the total contribution of synaptic input to spike output, we found that 1 pC of charge altered spike output by 0.06 +/- 0.01 Hz. Expressed another way, approximately 1.67 pC of charge input is necessary to generate a 1 Hz increase for 10 seconds under these conditions. For perspective, if we estimate that a miniature EPSC carries about 22 femtocoulombs of charge, it would take the release of 76 vesicles to increase spiking by 1 Hz over a 10 second time window (bearing in mind this calculation does not take into account effects of summation or temporal parameters).

Our horizontal angled slice inevitably lacks a great deal of connectivity and intrinsic activity which would also contribute to MNC spiking behavior *in*

vivo. The blockade of all inhibitory currents in our slice will also likely have an impact on MNC physiology. Inhibitory transmission increases spike train variability in OT cells (Li, Tripathi, & Armstrong, 2007). Inhibitory transmission also contributes to a more linear increase in action potential frequency to a given increase in excitatory transmission and acts to increase the dynamic range of responsiveness to excitatory potentials (Leng et al., 2001). Thus we can state that although synaptic modulation does make a finite contribution to the modulation of osmosensory gain, other factors also play a substantial role in determining spike output.

6.4 Synaptic and intrinsic modulation

In solidifying a role for synaptic inputs in the spike output of MNCs, we also note that the increase in intrinsic osmosensory transduction elicited by Ang II (Zhang & Bourque, 2008) likely interacts with the synaptic modulation we have outlined. In VP cells, the two mechanisms are likely to synergize, as both act to increase osmosensory gain in the regulation of VP cell activity. The response may be more complex in OT cells, as they stand in apparent opposition to each other and it is not clear whether presynaptic inhibition or SICC excitation would dominate. This opposition may explain, to a certain extent, the complexity of the response of OT to hypovolemic stimuli. If the relative contributions of intrinsic and synaptic mechanisms are altered under different conditions, OT responsiveness may change as well. Leng and colleagues demonstrate the importance of synaptic transmission for MNC osmosensitivity under basal

conditions, as AV3V lesioned animals showed little or no MNC activity (Leng et al., 1989). Conversely, Bourque et al. propose that the plateau potential generated from summation of DAPs in MNCs may reduce the necessity of synaptic input (Bourque et al., 1998). We might therefore propose that at low firing rates, synaptic activity dominates the control of MNC firing, while at high firing rates, intrinsic mechanisms dominate. This hypothesis could possibly explain the observations of Stricker et al. (Stricker et al., 1994) that OT secretion is negligible in response to mild hypovolemia (Ang mediated presynaptic inhibition), and substantial at greater levels of fluid deficits (Ang mediated intrinsic excitation). Nadeau et al. provide support for such a bi-modal activity in MNCs by the use of a computational model (Nadeau & Mouginot, 2011). These observations open an interesting avenue for future exploration.

6.5 Biphasic activity and release of VP and OT

As noted in section 1.2.1, VP activity is biphasic, in that it regulates diuresis at low concentrations (Robinson, 1985) and triggers vasoconstriction at high concentrations (Malayan et al., 1980). For low concentrations of VP, such as might be observed under mild hypovolemia, the increased slope of the VP-osmolality relationship serves to facilitate osmotic regulation of fluid balance (Verbalis, 2003). This leads us to ask whether a similar biphasic activity might exist for OT.

PEG induced secretion of VP and OT show similar secretion profiles in that they both follow an exponential relationship to increasing levels of volume

depletion (Stricker & Verbalis, 1986; Stricker et al., 1994). This comparable release activity might suggest that both peptides exhibit a form of biphasic behavior. If our observations regarding increased synaptic strength in VP cells account for the alterations in osmotic gain experienced by VP under mild hypovolemic conditions, we could also propose that the inverse alteration occurs in OT cells, resulting in blunted OT secretion in these conditions (see section 1.2.2). The net result of such a modulation would be increased antidiuresis and suppressed natriuresis. Thus we propose that mild hypovolemic conditions provide a physiological context in which the Ang II induced presynaptic modulation of OVLT-SON transmission results in a restoration of homeostasis.

Although it is not clear exactly what oxytocin might be doing under severe hypovolemic conditions, Huang et al. note that high levels of OT in the bloodstream can trigger renin secretion, resulting in elevated levels of Ang II in the blood (Huang, Sjoquist, Skott, Stricker, & Sved, 2000). Interestingly, this Ang II elevation was triggered by OT levels comparable to those found under hypovolemic conditions (~80 pg/mL), but not by OT levels comparable to those found under hypertonic conditions (~20 pg/mL). It is possible, then, that OT initiates a positive feedback loop under severe hypovolemia. This finding suggests that OT activity may serve two different functions at low release levels (natriuresis) and high release levels (Ang II secretion and subsequent antinatriuresis through Ang evoked aldosterone secretion (Tomaschitz et al., 2010)). It would also suggest that blunted OT secretion under mild hypovolemic

conditions, but prominent secretion under severe hypovolemic conditions could both function to reduce natriuresis and correct hypovolemia.

6.6 Evoked versus miniature potentials

Our finding that endocannabinoids mediate the Ang II induced suppression of transmission in OT cells is in accordance with suggestions made by Hirasawa et al. that OT may induce presynaptic inhibition in OT cells by triggering endocannabinoid release (Hirasawa et al., 2004). In an earlier study, the same lab had demonstrated that OT mediated suppression affected evoked EPSC release through the blockade of voltage gated calcium channels; altering action potential evoked calcium influx (Hirasawa et al., 2001). Notably, miniature EPSC activity was not suppressed. A similar observation applies to the facilitation of spontaneous EPSCs in OT cells by endothelin, in that application of TTX prevents any facilitation (Zampronio et al., 2010). This finding, along with our own observation that Ang II does not suppress mEPSC frequency in OT cells (in fact, enhancing frequency; section 4.2.6), support the notion that miniature event release relies on a different mechanism than evoked release. As miniature events occur in a calcium-independent fashion (Inenaga, Honda, Hirakawa, Nakamura, & Yamashita, 1998), we can reasonably conclude that our calcium dependant suppression of evoked EPSCs is mechanistically distinct from the modulation of mEPSCs. Thus Ang II may act at postsynaptic sites to modulate release through the use of retrograde messengers, as well as at presynaptic sites to enhance calcium independent release. Some recent findings support this notion by

demonstrating that Ang II facilitation of mEPSC frequency in MNCs occurs via the presynaptic modulation of TRPV1 channel activity, in a calcium-independent fashion (Yokoyama et al., 2010).

6.7 Conclusions and Perspective

The regulation of osmotic balance is a complex process involving the integration of numerous components contributing to the homeostatic regulation of water and salt in the body. The hypothalamic osmosensory system maintains osmotic balance in part through the regulation of VP and OT secretion. Our study outlines a mechanism by which neural integration combines osmotic and volemic signals to generate an appropriate homeostatic response. Additionally, the identification of retrograde messengers as differential regulators of synaptic gain suggests a common signaling pathway which may underlie the selective modulation of synaptic inputs. This finding has implications for all manner of homeostatic regulatory process within the hypothalamus, and perhaps even for more general functioning of the nervous system wherever the necessity of differential synaptic modulation arises.

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