ACUTE AND CHRONIC ADAPTATION OF SUPRAOPTIC NEURONS TO CHANGES IN OSMOLALITY

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ABSTRACT

Vasopressin (VP) is an antidiuretic hormone that is synthesized and released by osmosensitive magnocellular neurosecretory cells (MNCs) to regulate water homeostasis in the body. The rate and firing pattern of MNCs determines the amount of VP release, which is elevated during physiological stress particularly dehydration. During acute osmotic changes the MNCs shrink and swell due to hypertonic and hypotonic stimuli, respectively. In contrast to hippocampal neurons, which display regulatory volume increases (RVI) and regulatory volume decreases (RVD) in response to hypertonic and hypotonic stimuli, MNCs do not have compensatory mechanisms. The MNCs undergo hypertrophy as a part of their physiological structural and functional plasticity during chronic dehydration. These changes are thought to be important during long term osmotic changes for the sustained and high level releases of hormone. However, the mechanism of hypertrophy is still unclear and it is difficult to address this issue *in vivo*. We therefore undertook studies on acutely isolated MNCs to test hypertrophy in MNCs. We observed that acutely isolated MNCs treated with hyperosmolar solution (325 mOsmol kg^{-1}) for 150 minutes *in vitro* showed hypertrophy (a 9% increase in CSA) and recovered their original size when returned to isotonic solution (295 mOsmol kg^{-1}) for another 60 minutes. Whole cell patch clamp experiments showed a 34% increase in cell membrane capacitance following treatment with hypertonic solution for 90-150 minutes. The osmotically-evoked hypertrophic response was blocked by using a TAT (human immunodeficiency virus transactivator of transcription) peptide (TAT-NSF700) that prevents SNARE-mediated exocytotic fusion by blocking the function of NSF (*N*-ethylmaleimide-sensitive factor). The hypertrophic response did not appear to be altered by a scrambled version of the peptide, showing that osmotically-evoked hypertrophy depends on SNARE-mediated exocytotic fusion. The VP and OT-MNCs exposed to hyperosmolar solution for two hours showed increased immunofluorescence for L-type Ca^{2+} channels (both $Ca_v1.2$ and $Ca_v1.3$). Our data suggest that the osmotically-evoked hypertrophy is associated with an increase in the total membrane surface area due to the exocytotic fusion of intracellular granules with the plasma membrane and with increased expression of L-type Ca^{2+} channels. This study will be helpful in understanding of the adaptation that MNCs undergo during long term dehydration and pathological conditions that lead to increased plasma osmolality.

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DEDICATION

I dedicate this thesis to my husband, Aftab Kasana, for his love, support and trust that always gave me confidence to make my dreams come true.

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LIST OF ABBREVIATIONS

AAA ATPases Associated with a variety of cellular Activities

AHP Afterhyperpolarizing afterpotential

AQP Aquaporins

ARC Arachidonate- regulated Ca^{2+} channel

$[Ca^{2+}]_i$ Intracellular Ca^{2+}

CRAC Ca^{2+} release-activated Ca^{2+} channel

CREB cAMP response element binding

CSA Cross sectional area

DAP Depolarizing afterpotential

ECF Extracellular fluid

ER Endoplasmic reticulum

HAP Hyperpolarizing afterpotential

HIV Human immuno-deficiency virus

HNS Hypothalamo-neurohypophysial system

ICF Intracellular fluid

IP_3 Inositol 1, 4, 5-triphosphate receptor

MARCKS Myristoylated alanine-rich C kinase substrate

MNC Magnocellular neurosecretory cell

MnPO Median preoptic nucleus

NI Neurophysin I

NII Neurophysin II

NMDAR N-methyl D-aspartate receptor

NSF *N*-ethyl-maleimide–sensitive factor

OT Oxytocin

OVLT Organum vasculosum lamina terminalis

PIPES Piperazine-1, 4-bis (2-ethanesulfonic acid)

PKC Protein kinase C

PVN Paraventricular nuclei

RuR Ruthenium red

RVI	Regulatory volume increase
RVD	Regulatory volume decrease
SERCA	Sarcoendoplasmic reticulum Ca^{2+}-ATPase
SICs	Stretch inactivated cation channels
SNARE	Soluble NSF attachment receptor
SNAP25	Synaptosome-associated proteins-25
SON	Supraoptic nuclei
Syt IV	Synaptotagmin IV
TAT	Transactivating regulatory protein
TRPV	Transient receptor potential vanilloid
TTX	Tetrodotoxin
VAMP	Vesicle associated membrane protein
VGCCs	Voltage-gated Ca^{2+} channels
VP	Vasopressin

CHAPTER 1

INTRODUCTION

1.1 Osmoregulation and extracellular fluid (ECF)

Body fluid is divided into two compartments, extracellular fluid (ECF) and intracellular fluid (ICF) (Guyton & Hall, 1996; Cecil *et al*., 2008). The ECF, whose main cations and anions are Na^+ and Cl^- respectively (Guyton & Hall, 1996), is located outside the cell and divided into sub-compartments blood plasma, interstitial fluid and transcellular fluid while the ICF is found in cytoplasm (Nanovic, 2005) and has K^+ as its main cation (Boron & Boulpaep, 2009). ECF and ICF are separated by a selectively permeable membrane called the plasma membrane that is freely permeable to water (Guyton & Hall, 1996). All mammals maintain the osmolality of the ECF, which is the measure of all the osmolytes or solutes (ions, proteins etc) in solution expressed in moles per kilogram of the solvent (units for osmolality is mOsmol kg^{-1}). Osmolality is similar to osmolarity which is the measure of total moles of solutes per litre of the solution (Guyton & Hall, 1996; Bourque, 2008). Changes in extracellular osmolality make water flow in and out across the plasma membrane through osmosis in the form of passive diffusion i.e. water moves from the area of higher concentration to the area of lower concentration through the plasma membrane to balance extracellular and intracellular or cytoplasmic osmolality (Alberts *et al*., 1994; Russell *et al*., 2008). This happens because where the osmolytes are present in lower concentration (either inside or outside of membrane), water concentration is higher and this drives water to move to the other area (Strange, 1994; Boron & Boulpaep, 2009). Mammals share a common set point of osmolality of about 300 mOsmol kg^{-1} (Bourque, 2008). Osmotic pressure is created by the difference of osmolalities across the membrane. The set point of osmotic pressure is maintained at a stable point by the regulation of both water and sodium balance in both compartments (Guyton & Hall, 1996; Bourque & Oliet, 1997). A rise in plasma osmolality is termed as hyperosmolality and a decrease as hypo-osmolality. Changes in the concentrations of both water (which occurs through water intake, evaporation and diuresis) and sodium (which occurs through Na^+ intake and excretion) cause fluctuations in osmolality. Water

deprivation or salt intake can cause hyperosmolality, which stimulates the thirst sensation and also leads to antidiuretic hormone release to enhance water reabsorption in kidneys. ECF hypo-osmolality suppresses the release of antidiuretic hormone and enhances diuresis (an increase in the flow of urine by the kidney) (Bourque, 2008).

Cell volume changes are significant not only in brain but in all body organs. The expansion of brain cells is limited by our rigid skull and due to their fragility, they can not afford much osmotically-evoked cell volume changes compared to other organs. In extreme osmotic conditions these volume changes can be lethal (Bourque *et al*., 1994; Ordaz *et al*., 2004). The hypo-osmotic changes of ECF can cause swelling of brain that leads to ischemia, infarct, excitotoxicity and neuronal death (Ordaz *et al*., 2004). When cells are exposed to hypertonic solution (a solution having more solutes and less water), the water will tend to come out of the cells to equilibrate and cells shrink in size. On the contrary if cells are placed in hypotonic solution (a solution having less solutes and more water), the cells will swell due to water travelling inside of the cell (Alberts *et al*., 1994). This osmotically-induced swelling and shrinking as a result of water transport through water channels on the plasma membrane is regulated physiologically by regulatory volume increase (RVI) and regulatory volume decrease (RVD), which are compensatory mechanisms to maintain adequate cell volume (Lang *et al*., 1998; Lang, 2007). These mechanisms regulate the internal osmolality by ion transport across the plasma membrane, leading to influx and efflux of various osmolytes as well as metabolites following volume changes (Lang *et al*., 1998).

1.1.1 Central osmoreceptors

All mammalian tissues experience changes in individual cell volume and their metabolic functions due to changes in ECF osmolality (Strange, 1994). The systemic infusion of NaCl triggered the thirst sensation in dogs, which gave an idea of cellular dehydration and volume reduction due to water efflux as a result of hyperosmolality or salt intake (Gilman, 1937). In 1947, Verney confirmed Gilman's findings and further suggested that cells that are specially sensitized to osmotic changes are located in the brain. When different blood vessels perfusing anterior hypothalamic region of dogs' brains were infused with hyperosmolar solution, it triggered antidiuresis by the release of antidiuretic hormone (Verney, 1947). These specialized

sensory cells were thought to be neurons located in the anterior region of hypothalamus (Jewell & Verney, 1957). As reviewed by Bourque & Oliet in 1997, the hypothalamus possesses osmoreceptor neurons from which the central pathway for osmoregulation originates. The supraoptic and paraventricular nuclei of hypothalamus and the area between these two nuclei contain somata of primary osmoreceptors. These cells send their projections through the median eminence to the neurohypophysis (Poulain and Wakerley, 1982). Moreover, there are other important structures in the central nervous system controlling osmoregulation that are associated with the lamina terminalis. They are located near the anterior part of the hypothalamus and include the subfornical organ (SFO), the median preoptic nucleus (MnPO) and the organum vasculosum lamina terminalis (OVLT) (Bourque *et al*., 1994; Bourque & Oliet, 1997). Peripheral osmoreceptors also play an important part in body fluid homeostasis (Bourque *et al*., 1994). Peripheral osmoreceptors are found in the upper regions of the alimentary tract, blood vessels innervating intestines, the oropharyngeal cavity, the hepatic portal vein, the liver, the gastrointestinal tract and the splanchnic mesentery, but their cellular and molecular anatomy is still unknown (Bourque, 2008).

1.1.2 The magnocellular neurosecretory cells (MNCs)

The primary osmoreceptors are known as magnocellular neurosecretory cells (MNCs), which are large neuroendocrine cells found in the supraoptic and paraventricular nuclei (SON & PVN) of the hypothalamus and also the area between these two nuclei (Bargmann & Scharrer, 1951; Bourque & Oliet, 1997). The terminals of MNCs reside in the neurohypophysis while the somata of MNCs are located largely in the SON and PVN and each of them contain two paired parts which are bilaterally located and found lateral to the optic chiasm and adjacent to the third ventrical respectively. It was estimated using Nissl staining of rats that each of the SON paired parts contains 4400 to 7000 MNCs while each part of the PVN contains 1300 to 2000 MNCs (Swanson & Sawchenko, 1983; Armstrong *et al*., 1994). The SON and PVN together contain 50% of total MNCs of hypothalamus (Bourque & Oliet, 1997; Hatton & Li, 1998). MNCs have one to three dendrites extending towards the ventral glial lamina in the case of the SON and the third ventricle for PVN (Armstrong *et al*., 1994). A single axon projecting from the MNC somata

or primary dendrite leads to the neurohypophysis for the release of hormones to the blood stream (Armstrong *et al*., 1994).

1.1.2.1 Hormones of MNCs

MNCs synthesize and secrete either vasopressin (VP) or oxytocin (OT), which are neuropeptide hormones of special importance in mammals (Swaab *et al*., 1975). VP plays a significant role in osmoregulation and acts as an antidiuretic hormone controlling water excretion by water reabsorption in the kidney. VP has effects not only on osmotic regulation but also on blood pressure by the regulation of circulating blood volume and baroreflex sensitivity (Koshimizu *et al*., 2006) and thermoregulatory cooling by preemptive renal water reabsorption (Sharif-Naeini *et al*., 2008a). OT has mostly female reproductive functions in parturition during uterine smooth muscle contraction and in lactation for the milk let down by contraction of myoepithelial cells of mammary glands. OT is not only a female reproductive hormone but also has roles in male reproduction for sperm transport (Studdard *et al*., 2002) and in osmoregulation in some species (including rats) by causing natriuresis (Bourque & Oliet, 1997). Both hormones are synthesized and released through the hypothalamo-neurohypophysial system (HNS). They are synthesized in the somata of MNCs and packaged into secretory granules, which are transported to the neurohypophysis or posterior lobe of the pituitary gland and stored in axon terminals (Theodosis *et al*., 1978; Bicknell, 1988; Fisher & Bourque, 2001). Hormone release depends on Ca^{2+} triggered depolarization and exocytotic fusion of granules with the plasma membrane of the MNC terminals. The secretory large dense core granules contain either VP or OT, their specific associated carrier proteins (neurophysin II and neurophysin I, respectively) and precursors (Brownstein *et al.,* 1980). Immature secretory granules are synthesized in the trans-Golgi network (TGN) and transformed from unregulated to regulated exocytotic carriers with the help of the SNARE proteins VAMP4 and synaptotagmin IV (Syt IV) while undergoing maturation (Eaton *et al*., 2000).

VP plays an important role in various types of physiological regulations through acting on three G-protein coupled VP receptors, V_{1a}, V_{1b} and V_2 receptors (Ball, 2007). The reabsorption of water from kidney is enhanced by VP binding with V_2 receptors on the principal cells in the collecting ducts of kidney. Seven out of a total of eleven water channels called aquaporins (AQP)

are found in the kidney of mammals (Ball, 2007) and three of these water channels, aquaporin-2 (AQP2), aquaporin-3 (AQP3) and aquaporin-4 (AQP4), are important in the VP- regulated water reabsorption. VP translocates the AQP2 channel by binding with V_2 receptors in the principal cells of collecting ducts of kidneys for reabsorption of water (Petersen, 2006). The AQP2 water channels are translocated from cellular vesicles to the apical membrane of principal cells by exocytotic fusion and water enters the cell through pores present on AQP2. Then water exits through AQP3 and AQP4 on the basolateral plasma membrane of the collecting duct principal cells to the ECF (Petersen, 2006; Ball, 2007).

1.1.2.2 Electrophysiology of MNCs

1.1.2.2.1 Rate and firing pattern of MNCs

MNCs are capable of displaying different rates and patterns of firing with changes in the osmolality of the ECF (Bourque, 1998). The amount of release of hormones is determined by the rate and pattern of action potentials generated in the somata of MNCs (Bicknell, 1988). MNCs in rats exhibit resting membrane potentials of -58 mV to -68 mV and the threshold for action potentials ranges from -44 mV to -55mV. MNCs display three types of firing patterns (Bourque, 1989; Bourque, 1998) and their pattern and frequency of firing regulate the release of VP (Bicknell, 1988). When the osmolality of the ECF is normal, both types of MNCs (VP & OT) fire slowly, irregularly and infrequently at a rate of 1-3 Hz. When the ECF osmolality increases only by 1%, cells start firing faster and can fire in a fast continuous manner with more than three spikes per second (Bourque & Oliet, 1997; Poulain & Wakerley, 1982). Further increases in osmolality tend to cause VP-MNCs to fire in phasic bursts. VP-MNCs show periodic burst activity consisting of bursts of action potentials lasting for tens of seconds and having a firing rate of 5-15 Hz and interburst intervals or silent periods of the same length (Poulain & Wakerley, 1982). Most OT-MNCs do not adopt phasic bursting in response to increased osmolality, but during lactation or parturition they do fire rapidly with bursts of 30-50Hz, lasting for 2-4 seconds and recurring every 3-15 minutes (Brown & Moos, 1997). The increase in firing rate for the increased hormone release during conditions brought by osmotic changes is also triggered by continuous excitatory drive from other osmosensitive cells in the OVLT and this is required for the sustained phasic bursts (Richard & Bourque, 1995; Brown & Bourque, 2004). It has been

observed from electrophysiological recordings that after dehydration of 6 hours or more, phasic firing was displayed by 84-100% of VP-MNCs while only 4% OT-MNCs showed phasic firing even after 24 hours of dehydration (Poulain *et al*., 1977; Wakerley *et al*., 1978). Other neuroendocrine modulators like VP & OT are released in an autocrine fashion from somatodendritic regions (Ludwig & Pittman, 2003) along with peptides such as apelin (De Mota *et al.,* 2004) and dynorphin (Brown & Bourque, 2004.

MNCs fire action potentials in different firing patterns and rates as a result of intrinsic and extrinsic factors (synaptic inputs). The intrinsic factors include osmosensitive channels (stretch inactivated cation channels; SICs) and activity dependent currents e.g. depolarizing afterpotentials (DAPs) (Oliet & Bourque, 1993a; Oliet & Bourque, 1993b). The action potentials propagate to the terminals, depolarize the membrane and activate voltage-gated Ca^{2+} channels (VGCCs), and ultimately release hormone into the blood circulation from the neurohypophysis (Bicknell, 1988; Fisher & Bourque, 2001; Bourque *et al*., 1994). I will now discuss some of the intrinsic factors in greater detail.

1.1.2.2.2 Depolarizing afterpotentials (DAP)

In MNCs the phasic bursts are triggered by long-lasting (1-3s) depolarizing afterpotentials (DAPs), which are independent of synaptic inputs (Andrew & Dudek, 1983). Following spikes, the summation of these DAPs results in long-lasting plateau potentials that last for tens of seconds and causes a generation of a burst (Andrew & Dudek 1983; Li & Hatton, 1997). The DAP is an activity-dependent current that plays a significant role in release of hormones from VP and OT-MNCs. For the sustained release of VP for water conservation, the MNCs adopt phasic pattern of bursts while the phasic pattern of firing in MNCs is regulated by membrane's intrinsic properties (Andrew and Dudek, 1983). Both VP and OT- MNCs exhibit DAPs but VP-MNCs differ in pattern and rate of firing from OT-MNCs and also have different magnitude of expression for DAP. More VP-MNCs (75%) display DAP as compared to OT-MNCs (32%) (Oliet & Bourque, 1992; Armstrong *et al*., 1994). The electrical activity of MNCs is regulated by many Ca^{2+} dependent pathways and increases or decreases of intracellular Ca^{2+} $[Ca^{2+}]_i$ regulate DAP generation, plateau potentials and phasic bursts in MNCs (Li *et al*., 1995).

Continuous firing in MNCs causes secretory fatigue which brings progressive decrease in VP secretion (Bicknell, 1988). MNCs undergo a silent phase of same length of burst for increased hormonal secretion. To avoid secretory fatigue, phasic bursts in VP-MNCs are terminated by the activation of some inhibitory currents including hyperpolarizing afterpotentials (HAP) and an afterhyperpolarizing afterpotential (AHP) (Armstrong *et al*., 1994). The exact mechanism of termination of phasic bursts is not completely known but these inhibitory currents may be involved in the regulation of DAP to sustain plateau potentials or phasic bursts (Andrew & Dudek, 1984; Roper *et al*., 2003). Both of the HAP & AHP are mediated by Ca^{2+} dependent K^{+} channels and their main function is to regulate MNC firing rate and pattern (Andrew & Dudek, 1984; Armstrong *et al*., 1994).

Dynorphin and apelin, κ-opioid peptides are co-packaged in neurosecretory granules and co-released with VP during exocytotic fusion of granules with the plasma membrane from the somatodendritic regions of VP MNCs (Brown *et al*., 2006) by exocytosis (Pow and Morris, 1989). Dynorphin may regulate VP release by interfering with phasic firing in the MNCs (Brown & Bourque, 2004; Brown *et al.*, 2006).

1.1.2.2.3 Stretch inactivated cation channels (SICs) in MNCs

MNCs are intrinsically osmosensitive and regulate fluid homeostasis by releasing VP. Changes in osmolality cause the cells to shrink or swell in hyperosmolality or hypo-osmolality respectively (Oliet & Bourque, 1992). An increase in osmolality depolarizes the plasma membrane by the activation of stretch inactivated cation channels (SICs) and this causes the cells to fire (Oliet & Bourque, 1992; Sharif-Naeini *et al*., 2006). SICs are mechanosensitive channels expressed on the plasma membrane of MNCs and the osmotic changes in cell volume regulate these channels (Oliet & Bourque, 1993a; Oliet & Bourque, 1993b). Osmotically induced swelling or pipette pressure during patch clamp increases the membrane tension and closes the SICs, which leads to membrane hyperpolarization and makes firing of MNCs less likely. The SICs are opened due to the shrinkage of cells when exposed to hyperosmotic stimuli thereby increasing the likelihood of firing and increasing the hormone release caused by Ca^{2+} influx in

MNC terminals (Oliet & Bourque, 1992). The SICs are responsible for 60% of the input conductance of the MNCs at the physiological set point of osmolality (Bourque & Oliet, 1997). The SICs of MNCs belong to the transient receptor potential vanilloid (TRPV) family, a family of cation channels. MNCs express an N-terminal splice variant of the TRPV1 gene that is found to be involved in the osmosensory transduction mediated by VP MNCs (Sharif-Naeini *et al*., 2006). All TRPV channels are blocked by ruthenium red (RuR) and the osmosensitivity of MNCs was inhibited when the channels were blocked with RuR. The MNCs of TRPV1 knock out mice do not display osmotically- evoked depolarization. This clearly demonstrated the role of SICs in the osmosensory transduction in the MNCs (Sharif-Naeini *et al*., 2006; Sharif- Naeini *et al*., 2008b; Sudbury *et al*., 2010).

MNCs are intrinsically thermosensitive and fire rapidly in response to increases in temperature to release more VP, but the central thermosensory mechanism for this regulation is not completely known. The thermosensitivity of VP neurons is regulated by the SICs (Sharif- Naeini *et al*., 2008a). TRPV1 are temperature sensors (Benham et al., 2003) and expression of TRPV1 gene is required for the thermosensory transduction of VP release for the thermoregulation during hyperthermia (Sharif-Naeini *et al*., 2008a). A specific antagonist of TRPV1 channels to which they are sensitive is SB366791 and heat activated currents in VP-MNCs are blocked by this compound (Sharif-Naeini *et al*., 2008a). TRPV1 knock out mice displayed low thermosensitivity in VP-MNCs (Kanai *et al*., 2007; Sharif- Naeini *et al*., 2008a)

SICs behave mechanically in response to osmosensation and this has been explained by observing the role of actin filaments on the mechanosensitivity of SICs in MNCs. Whole cell patch clamp recordings have showed the direct effect of actin- cytoskeleton on the SICs activation and inhibition because an actin depolymerizing compound called cytochalasin-D reduced the SICs mechanosensitivity and the actin filament polymerizing compound jasplakinolide increased it. The osmosensitivity of SICs therefore appears to depend on their interaction with the cytoskeleton (Zhang *et al*., 2007b).

1.2 Calcium channels

Many cellular events are Ca^{2+} dependent and Ca^{2+} influx is brought about by specific ion channels called Ca^{2+} channels. There are different Ca^{2+} channels present on cellular plasma membranes including voltage gated calcium channels (VGCCs), transient receptor potential (TRP) channels, store operated channels or Ca^{2+} release-activated Ca^{2+} channels (CRACs), arachidonate- regulated Ca^{2+} channels (ARC) and N-methyl D-aspartate receptors (NMDARs). Intracellularly, Ca^{2+} is released by two types of receptors, which are ryanodine receptors and inositol 1, 4, 5-triphosphate receptors (IP_3) (Striggow & Ehrlich, 1996). Ca^{2+} is a second messenger (Petersen *et al*., 2005) and Ca^{2+} signaling is essential for cellular functions. Ca^{2+} influx from the extracellular compartment and Ca^{2+} storage and its release have important roles in various cell processes including health and disease (Berridge *et al*., 2003). Ca^{2+} signaling is triggered by external stimuli in form of growth factor, hormone secretion, neurotransmitter release and membrane excitation in excitable cells (Siegel *et al*., 2006). Intracellular Ca^{2+} is stored in the endoplasmic reticulum (ER) and contributes to the integrity of ER, which has a pump for its release called sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump for its storage and release for different signaling pathways (Koch, 1990) and its concentration varies greatly from intracellular (~100 nM) to extracellular (mM) compartments. This concentration gradient is responsible for enhanced signaling events in cytosol (Clapham, 2007). Moreover the mitochondria, which is another store house for Ca^{2+}, is also capable of regulating its important functions e.g. oxidative phosphorylation by the storage of Ca^{2+} (Zamponi, 2005). The depolarization of plasma membrane brings changes in $[Ca^{2+}]_i$ by Ca^{2+} influx through Ca^{2+} channels and its rise in concentration acts as a chemical signal to trigger secretion and release of hormones and neurotransmitters and muscle contraction (M*c*Cleskey *et al.*, 1986).

1.2.1 Voltage-gated Ca^{2+} channels (VGCC)

Ca^{2+} entry into cells through the plasma membrane occurs mostly by VGCC, which are found in excitable cells like myocytes, neurons, endocrine and neuroendocrine cells (but not in non-excitable cells such as epithelial or most blood cells) and are activated by membrane depolarization (Siegel *et al*., 2006). These channels have high selective permeability to Ca^{2+}

(Catterall, 2000). Upon depolarization of the plasma membrane, the VGCCs are opened causing Ca^{2+} influx, which acts as a second messenger of electrical signaling and initiates intracellular biochemical events through Ca^{2+} signaling pathways. This signaling is essential for different functions; in neurons for neurotransmission, in muscles for excitation-contraction, in neuroendocrine for excitation-secretion and gene expression (Catterall, 2000). These channels are heteromultimeric protein structures and each channel consists of a complex of four subunits an α_1- subunit and three auxillary subunits (an intracellular β- subunit which serves for trafficking of ions, γ-subunit and a disulphide linked complex of α_2 -δ). These four subunits are present in a 1:1:1:1 stoichiometry. The α_1, largest amongst all subunits, is 190-250 KDa in its molecular mass and makes the Ca^{2+} selective conductance pore, a voltage sensitive gate for Ca^{2+} influx. There are four homologous domains within each α_1 subunit and each domain further comprises six transmembrane segments (S1 – S6). The function of voltage sensor is served by segment 4 (S4) and ion conductance is determined by the pore loop between segment S5 and S6. Electrophysiological studies have showed different types of VGCC which include L-, N-, P-, Q-, R- and T- types. Ten genes of α_1 have been discovered that fall into three major families, Ca_v1, Ca_v2 and Ca_v3. Similar biophysical and pharmacological characteristics of these families divide them in subfamilies; L-type ($Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$), P/Q-type ($Ca_v2.1$), N- type ($Ca_v2.2$), R-type ($Ca_v2.3$) and T-types ($Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$) (Hofmann *et al*., 1999; Catterall, 2000; Jarvis & Zamponi, 2007). Formerly these channels were named as α_{1S} ($Ca_v1.1$), α_{1C} ($Ca_v1.2$), α_{1D} ($Ca_v1.3$), α_{1F} ($Ca_v1.4$), α_{1A} ($Ca_v2.1$), α_{1B} ($Ca_v2.2$), α_{1E} ($Ca_v2.3$), α_{1G} ($Ca_v3.1$), α_{1H} ($Ca_v3.2$) and α_{1I} ($Ca_v3.3$). The original nomenclature depended on the functional properties of different Ca^{2+} currents e.g. the L-type Ca^{2+} channels stands for long- lasting, T-type for tiny or transient, N-type for neither L nor T. P-type current was first discovered in Purkinje cells of mammals and in the presynaptic terminals of squid giant and R-type is named for resistant or residual, because it was insensitive to all current blockers at that time (Catterall, 2000; Fisher & Bourque, 1995; Hofmann *et al*., 1999; Joux *et al*., 2001).

MNCs express all these channels either on somata or terminals or both. The N-type Ca^{2+} channels are present on the somata and terminals of MNCs and they contribute 26- 40% of the total VGCC current, P-type Ca^{2+} channels are expressed only on the somata of MNCs which

makes a 20% of the total VGCC while Q- type are found only in the terminals, R-type channels are expressed on both somata and terminals and the T- types are expressed only in somata and not in the axon terminals (Fisher & Bourque, 1996; Catterall, 2000; Fisher *et al*., 2000; Joux *et al*., 2001).

1.2.1.1 The L-type Ca^{2+} channels

The long lasting L-type voltage gated Ca^{2+} (L-type Ca_v) channels belong to family Ca_v1 (Ca_v 1.1, Ca_v 1.2, Ca_v 1.3, Ca_v 1.4) of the α_1 subunit. They are high voltage-activated Ca^{2+} channels and activate at voltages more positive than -40mV except for $Ca_v1.3$, which activates at voltages above -50mV (Fox *et al.*, 1987; Koschak *et al.*, 2001). Primarily, the L-type Ca^{2+} channels are regulated by second-messenger regulated protein phosphorylation cascades. The four isoforms of Ca_v1 are found in various tissues of the body mainly muscles and neuroendocrine cells, where they regulate excitation-contraction and excitation-secretion coupling respectively. The L-type Ca^{2+} channels are blocked by various organic antagonists such as benzothiazepines, phenylalkylamines and dihydropyridines. (Catterall, 2000; Fisher & Bourque, 2001; Triggle, 2006).

L-type Ca_v channels have roles in brain relating to neuronal development and survival, injury, synaptic plasticity, gene expression and neurotransmitter/hormone release (Lipscombe *et al*., 2004). $Ca_v1.2$ and $Ca_v1.3$ are expressed predominantly in CNS and play significant roles in neuronal functions such as long term signal transduction events (Ahlijanian *et al*., 1990; Silver *et al*., 1990; Tanaka *et al*., 1995; Striessnig *et al*., 2006). Neuronal gene expression may be regulated by L-type Ca_v channels ($Ca_v1.2$ & $Ca_v1.3$) by the regulation of Ca^{2+}/calmodulin-activating transcription factors or by increased nuclear Ca^{2+} (Chung & Jan, 2006). $Ca_v1.2$ also acts as a transcriptional regulator when its C terminal is translocated as a signal to nucleus (Gomez-Ospina *et al*., 2006). Recent studies of Ca^{2+} imaging have confirmed the localization of $Ca_v1.2$ and $Ca_v1.3$ in the mitotic apparatus of cells confirming their role in cell division (Loechner *et al*., 2009). Single cell RT-PCR and immunocytochemistry have identified both $Ca_v1.2$ and $Ca_v1.3$ in somata and dendrites of MNCs (Glasgow *et al*., 1999; Joux *et al*., 2001) and they contribute 23-28% of total VGCC currents observed in MNCs somata (Fisher &

Bourque, 1995, Fisher & Bourque, 1996; Foehring & Armstrong, 1996). L- type Ca^{2+} channels are also expressed on the internal membranes of cytoplasm other than plasma membrane of MNCs (Fisher *et al*., 2000; Joux *et al*., 2001). L-type Ca^{2+} channels regulate gene expression in MNCs (Burbach *et al*., 2001) because the C-termini part of both $Ca_v1.2$ and $Ca_v1.3$ contain the sequences that specifically cause the channels to activate a transcription factor known as the CREB (cAMP response element binding) protein (Burbach *et al*., 2001). L-type Ca^{2+} currents mediate the somatodendritic release of dynorphin in hippocampal granule cells and this may be functionally similar to the secretion coupling in neuroendocrine cells (Simmons *et al*., 1995; Fisher & Bourque, 2001)

1.3 SNARE mediated exocytotic fusion

Cellular communication requires vesicle trafficking by the fusion of membranes, which requires a soluble NSF attachment receptor (SNARE) protein (Jahn & Scheller, 2006). NSF is *N*-ethyl-maleimide–sensitive factor, an essential element of the protein machinery for exocytosis, endocytosis and transcytosis of vesicle fusion with the plasma membrane (Furst *et al*., 2003). NSF belongs to the ATPase family known as AAA, which stands for ATPases Associated with a variety of cellular Activities and is an 85 kDa protein inhibited by *N*-ethyl-maleimide (Hoyle *et al*., 1996). NSF is linked to a protein called soluble NSF attachment protein or α-SNAP, which causes NSF to disassemble the cellular machinery required for vesicle fusion (Matsushita *et al*., 2005). There are three domains of NSF comprising the N-terminal domain, which facilitates the binding of NSF with α-SNAP, ATPase domain D1, which hydrolyses the ATP and brings conformational changes in the NSF, and ATPase domain DII, which is necessary for the hexamerization of NSF (Furst *et al*., 2003). The cellular machinery for vesicle fusion is a complex of various proteins including SNARE proteins, NSF and α-SNAP, Rab and Rab effectors, and members of the Sec1/Munc18 protein family (Matsushita *et al*., 2003; Matsushita *et al*., 2005). They together make a SNARE proteins complex and the disassembly of SNARE complex by NSF and α-SNAP is essential for its recycling (Furst *et al*., 2003; Matsushita *et al*., 2003; Matsushita *et al*., 2005). The role of the SNARE protein complex has been observed as an essential element in almost all intracellular membrane fusions (Jahn & Scheller, 2006).

VP and OT secretion and release to the blood circulation is regulated by Ca^{2+} dependent exocytotic fusion of peptide containing intracellular granule membrane with the plasma membrane of MNCs (Fisher & Bourque, 2001). The SNARE protein complex also regulates the exocytotic fusion of AQP2 channels with the apical membrane of renal collecting tubes on the release of VP for water reabsorption (Procino *et al*., 2008). The core of SNARE protein complex is made by three proteins, which are syntaxin-1, SNAP25 (synaptosome-associated proteins-25) and VAMP (vesicle associated membrane protein or synaptobrevin) and these three proteins together transport the exocytotic vesicle to the target plasma membrane (Sollner *et al*., 1993). There are two types of SNARE proteins, v-SNAREs (found on the secretory vesicle) and t-SNAREs (present on target membrane) and both opposing SNAREs fuse the membranes together by assembling into a four helix bundle by using free energy released during bundle formation (Sutton *et al*., 1998; Jahn & Scheller, 2006). Calcium dependent exocytotic fusion of membranes for neuropeptide release is also regulated by some other proteins such as synaptotagmin (a Ca^{2+} binding protein), complexin and Munc18 or Sec1, which binds to the syntaxin at its N-terminus and detaches from it for syntaxin bonding with SNAP25 and VAMP for the formation of SNARE core complex (Dulubova *et al*., 2003; Procino *et al*., 2008). In neurotransmission, synaptotagmins catalyze the SNARE mediated exocytotic fusion by supplying energy and force but its exact role is not known in hypothalamic neurons for release of neuropeptides (VP and OT) (Gustavsson *et al*., 2009; Zhang *et al*., 2011).

It was observed that L-type Ca^{2+} channels are selectively found in the lipid rafts of plasma membrane of the bovine chromaffin cells (Park & Kim, 2009). SNARE mediated exocytosis depends on the association of SNARE proteins with lipid rafts. L-type Ca^{2+} channels are involved in the exocytosis of large dense core vesicle for increase in neurotransmitter release by the regulation of protein kinase C (PKC) translocation and phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) (Park & Kim, 2009). The L-type Ca^{2+} channels including $Ca_v1.2$, $Ca_v1.3$ have been found interacting with some of the proteins of SNARE complex such as syntaxin and SNAP-25 (Fisher & Bourque, 2001). The coexpression of syntaxin and synaptotagmin with L-type Ca^{2+} channels in *Xenopus oocytes* supported the involvement of $Ca_v1.2$ in the secretory process (Wiser *et al*., 1999). Immunocytochemistry has showed the

coexpression of syntaxin 1 and other vesicle-associated proteins with the Cav1.3 at the basal pole of hair cells (Song *et al*., 2003) and recently it has been presented that II & III interdomains of $Ca_v1.2$ and $Ca_v1.3$ interact with the exocytotic secretory machinery involved in glucose-mediated insulin secretion (Jacobo *et al*., 2009).

It has been observed in endothelial cells that NSF-regulated exocytosis can be inhibited by fusion polypeptides known as TAT-NSF polypeptides composed of 11 amino acid human immuno-deficiency virus (HIV) transactivating regulatory protein (TAT) factor (Matsushita *et al*., 2005). The ATPase activity is inhibited by a membrane permeant NSF700-722 peptide of domain 2 of NSF by interfering with NSF hexamerization (Matsushita *et al*., 2003; Matsushita *et al*., 2005). NSF700 is fused to the 11-amino protein transduction domain of the HIV TAT protein to inhibit the exocytosis in cells (Morrell *et al*., 2005; Trang *et al*., 2009). This inhibiting protein domain showed permeability for the endothelial cell membrane where it is fused with 22 amino acid of NSF to block its action for exocytosis by inhibiting the NSF-ATP hydrolysis and NSF-disassembly activity (Matsushita *et al*., 2003; Matsushita *et al*., 2005). The scrambled version of NSF700–722 (TAT–NSF700scr) is used as control peptide that does not inhibit SNARE-mediated exocytosis (Trang *et al*., 2009; Morrell *et al*., 2005).

1.3.1 Neuropeptide secretion

In MNCs, peptide secretion is achieved through regulated exocytotic fusion of large dense core granules (100-200 nm in diameter) like other neuroendocrine cells that are specialized to secrete large amount of neurosecretory products (Burgoyne & Morgan, 2003). The secretory granule exists in two forms before exocytosis, one is immature secretory granules with unregulated exocytotic carriers and the second is mature secretory granules with regulated exocytotic carriers. The secretory peptide is synthesized in the cisternae of rough endoplasmic reticulum (RER), packed and processed in *cis*- Golgi domain and finally in *trans*-Glogi network where it becomes a mature membrane- bound secretory granule or vesicle and ready for the fusion with plasma membrane for the hormone release. SNARE proteins such as Synaptotagmin IV (Syt IV)

and VAMP4 enter the immature granule but are removed during the switch from immature to mature large dense core granules (Boron & Boulpaep, 2009; Eaton *et al*, 2000).

The hormones or neuropeptides are synthesized in the somata of MNCs in the form of secretory vesicles, then they are packed and released on the occasion of cell firing followed by the rise of $[Ca^{2+}]_i$ by the Ca^{2+} influx through VGCC (Robinson & Martin 1998; Fisher & Bourque, 2001). The interaction between Ca^{2+} channels and the exocytotic protein apparatus is an important determinant of activity-secretion coupling for neuropeptide release (Fisher & Bourque, 2001). N and P/Q channels ($Ca_v2.1$ and $Ca_v2.1$ respectively) interact with SNARE proteins such as syntaxin, SNAP-25, and synaptotagmin for neuropeptide release (Catterall, 1999) while L-type Ca^{2+} channels may be involved in the somatodendritic release of VP through exocytotic fusion (Shibuya *et al*., 1998; Fisher & Bourque, 2001). Microdialysis studies have showed that neuropeptides (VP & OT) are released in an activity-dependent manner from somatodendritic regions of MNCs and may have a significant role in the regulation of MNCs function (Ludwig *et al*., 2005; Ludwig & Leng, 2006). The somatodendritic regions of MNCs have been shown to have many large dense core granules through electron micrographs, and they can be induced to fuse with the plasma membrane (Pow & Morris, 1989). The somatodendritic release of neuropeptides has an autocrine role in hormone release as VP and OT-MNCs express their respective receptors and binding of peptides with their receptors cause increases in intracellular Ca^{2+} (Dayanithi *et al*., 1996). Structural remodeling of the SON in non-lactating rats can be induced by giving them prolonged intraventricular infusion of OT, which suggests a role for somatodendritic release of OT during lactation and parturition (Theodosis *et al*., 2008). The somatodendritic release of both VP and OT do not parallel axonal release but it is important in regulation of cellular activity and can modulate synaptic inputs on MNCs (Ludwig *et al*., 2002; Ludwig *et al*., 2005). Endogenous peptides like dynorphin and apelin are co-stored and co-released with VP and have inhibitory effects on VP release (Brown *et al*., 1999) and dynorphin may have a role in termination of phasic bursts in VP-MNCs (Brown & Bourque, 2004). Immunocytochemistry studies have shown coexpression of dynorphin with VP secretory granules (Shuster *et al*., 2000). Dynorphin inhibits the VP release through acting on kappa-opioid receptors, which are co-localized on VP-MNCs in hypothalamic nuclei as well as in the

posterior pituitary. These receptors are translocated to the plasma membrane during exocytotic fusion of large dense granule in a stimulus-dependent fashion (Shuster *et al*., 1999).

1.4 Morphological identification of MNCs after isolation

Methods have been developed to isolate MNCs (Armstrong *et al*., 1994; Oliet & Bourque, 1992) and it was seen that isolated MNCs contain short processes (<30µm), are oblong shaped, and look bright under phase contrast (Silverman & Zimmerman, 1983). It was estimated that 100 to 500 MNCs are isolated from each tissue block of SON after enzymatic digestion, which is 2-10% of the total number of cells (Oliet and Bourque, 1992). To determine whether neurons isolated from SON are MNCs, a criterion depending on the cross sectional area (CSA) of cells was developed (Oliet and Bourque, 1992). It was observed that more than 96% of cells had CSA >160µm² were specifically immunoreactive to either OT or VP carrier proteins (NI or NII, respectively) (Oliet & Bourque, 1992).

1.5 Osmotic changes in MNCs

1.5.1 Short term osmotic changes and cell volume

Osmotically-evoked cell volume changes always remain a challenge for the brain. MNCs are intrinsically osmosensitive and change their cell volume as a function of change in external osmolality (Oliet & Bourque, 1992). Also mechanosensitive SICs are activated by changes in osmolality and this enables the MNCs to respond electrically to changes in membrane tension (Oliet & Bourque, 1993b). The osmotically-induced cell shrinkage releases membrane tension, activates SICs and thereby depolarizes the cell membrane and increases cell firing and neuropeptide release (Fig 1) (Oliet & Bourque, 1993a). While the swelling of cells inhibits cell firing and hormone release by hyperpolarizing the membrane potential as SICs are closed due to increase in membrane tension (Fig 1) (Bourque & Oliet, 1997).

Cell conductance is the ease with which ions flow across the plasma membrane through ion channels (Rhoades & Bell, 2009). Shrinkage of MNCs on exposure to a hyperosmolar stimulus

increases the conductance of the cell and swelling in response to a hypo-osmolar stimulus decreases the conductance of MNCs (Bourque & Oliet, 1997). A brief exposure of MNCs to hypertonic stimulus evoked conductance changes in only 90 sec by regulating the cell volume through mechanosensitive channels (SICs) (Bourque & Oliet, 1997). This has been observed in both osmotically-induced cell volume changes and also changes evoked by pipette suction or inflation. These volume and conductance changes are reversible as soon as the stimuli are removed (Bourque & Oliet, 1997).

In most cells, the osmotically-evoked cell volume changes (shrinkage & swelling) are regulated by compensatory mechanisms. Such regulations include regulatory volume increases (RVI) or regulatory volume decreases (RVD) on shrinking or swelling respectively, and regulate internal osmolality by ion transport and accumulation of organic osmolytes. These regulations are essential for increasing or decreasing internal osmolality with respect to changes in external osmolality to avoid potentially deleterious cell volume changes (Lang, 2007).

Cell capacitance is the charge holding capacity of the plasma membrane, which acts as a capacitor to separate the ions of inside and outside compartments (Rhoades & Bell, 2009). The input capacitance of MNCs is directly proportional to the total membrane surface area of cell (Fernandez *et al*., 1984). Zhang & Bourque, 2003 studied short term osmotic changes during slow and fast osmotic ramps in MNCs. Slow osmotic ramps for 60 minutes caused slow and gradual shrinkage and swelling in response to hypertonic and hypotonic stimuli respectively, while fast osmotic changes of 10 minutes evoked volume changes within a minute and were maximal within 4 min (Zhang & Bourque, 2003). MNCs undergo shrinkage or swelling after acute hyper or hypo-osmotic stimuli exposure respectively and maintain these changes in volume during a 10 minute step change in osmolality. Hippocampal neurons also show acute osmotically-induced volume changes but they either recover their volume with osmotic decrease or respond slowly than the MNCs with an osmotic increase. This suggests that unlike MNCs, hippocampal neurons exhibit volume compensatory mechanisms (RVI and RVD) in response to osmotic changes (Zhang & Bourque, 2003). Whole cell patch clamp recordings showed changes in input capacitance in hippocampal neurons in response to osmotic changes but not in MNCs, which suggests that hippocampal neurons regulate their total membrane surface area either by

exocytotic fusion or endocytotic removal of membranes during osmotic stress. During short term osmotic changes, the sustained osmotically-evoked volume changes in MNCs are not the result of changes in total membrane surface area, as there is no change in input capacitance (Zhang & Bourque, 2003). This may be explained by the presence of membrane reserves in MNCs that increase in size when MNCs shrink and decrease in size when MNCs swell (Zhang & Bourque, 2003). MNCs lack volume regulation in response to acute osmotic changes and only cell size expands or contracts (due to presence of elastic compartments within membrane) when the membrane tension increases or decreases, respectively without changing the total membrane surface area (Zhang & Bourque, 2003). On the basis of these observations it was suggested that membrane reserves may regulate cell volume changes (shrinking & swelling) in response to osmotically-evoked changes in membrane tension which ultimately activate SICs in cytoskeleton-dependent fashion (Zhang & Bourque, 2003; Zhang *et al*., 2007b; Bourque, 2008).

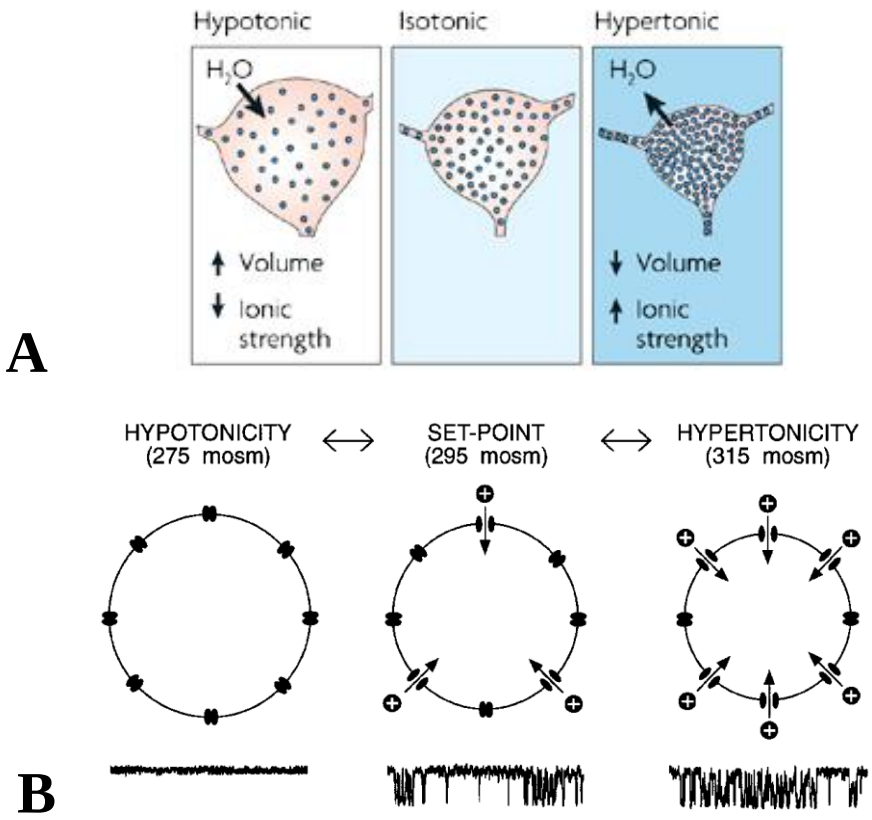


Figure 1: Change in cell volume in response to change in osmolality of MNCs. MNCs are intrinsically osmosensitive and changes in osmolality regulate their cell volume as they do not have RVI or RVD. A: Hypo-osmolar solution causes the MNCs to swell, stretch inactivated channels are closed due to increase in membrane tension and there is less ions transport; MNCs regulate normal flow of ions when osmolality is normal and maintain their size; MNCs shrink in volume due to hyperosmolar solution and there is increase ions flow. B: Stretch inactivated channels are expressed on MNCs which regulate the MNCs volume in response to change in osmolality. MNCs exhibit slow irregular firing when osmolality is normal or isotonic, there is slow firing when cell undergo hypoosmolar solution and fire rapidly when exposed to hypertonic solution. (Bourque, 2008; Bourque & Oliet, 1997) (Reproduced with permission from Copyright Clearance Center)

1.5.2 Long term osmotic changes

The HNS undergoes sustained osmotically-induced structural and functional plasticity occurring on minutes to hours time scale during sustained dehydration (Hatton, 1997). These changes include retraction of astrocytic processes surrounding the MNCs, increased synaptic innervations, increased synaptic inputs of excitatory noradrenergic and glutamatergic neurotransmitters and decreases in gliotransmitter and taurine release and in GABAergic inputs. MNCs adopt direct and tight apposition with each other and also exhibit hypertrophy. These changes reverse when the external osmotic stimulus is removed or hormone secretion (VP or OT) returns to its basal level (Hatton, 1997).

At basal conditions when the osmolality of external ECF is normal, both VP and OT- MNCs somata and dendrites are separated by fine astrocytic processes but hyperosmotic stimuli remove these processes. This glial retraction causes the direct and tight apposition of MNCs with each other. The juxtaposed neurons experience increased synaptic transmission with an increased number of multiple synapses (Theodosis *et al*., 2004). MNCs undergo hypertrophy (Hatton, 1997, Theodosis *et al*,. 2004; Di & Tasker, 2004; Zhang *et al*., 2007a; Theodosis *et al*., 2008) during chronic dehydration, lactation and parturition. The interaction of astrocytes and neurons in the HNS is a very important feature for structural and functional changes to occur in response to chronic changes in HNS and the retraction is more extensive depending on the duration of stimuli. The cellular mechanism involved in this structural remodeling remains unclear (Theodosis *et al*., 2008).

Previous literature showed a wide variety of experiments to see the response of HNS exposed to osmotic stimuli for chronic or longer term (Hatton, 1997). It has been observed that stress-mediated synaptic events also regulate the structural plasticity during long term osmotic changes and MNCs respond to osmotic changes by the regulation of cell volume during dehydration or salt loading (Hatton, 1997). The ultrastructural changes can be evoked in the experimental animals either by addition of salt to the drinking water for 7-10 days or by water deprivation. Both of these treatments take hours to days for the development of structural changes but

injection of hypertonic solution can evoke structural plasticity within 5 hours (Beagley & Hatton, 1992; Hatton, 2002; Hatton, 1997) and increased dendritic bundles and glial retraction can be observed in 30 minutes after the transcardial perfusion of hypertonic solution (325 mOsmol kg^{-1}) (Tweedle *et al*., 1993). The MNCs showed a 25% increase in capacitance after 16-24 hours of water deprivation, which suggests an increase in total membrane surface area through exocytotic membrane fusion (Zhang *et al*., 2007a). Electron microscopy of SON showed that somatic surface area of MNCs is increased by 70% during chronic dehydration of 10 days with 2% NaCl in rats (Modney & Hatton, 1989). This increase in surface area is also accompanied by a 15 fold increase in membrane apposition of adjacent MNCs during dehydration. This data also suggested a significant increase in the multiple synapses made between soma-somatic and somatodendritic contacts (Modney & Hatton, 1989). Electrophysiological and immunohistochemical findings showed a 20% increase in neuronal surface area and a 30% increase in glutamatergic and GABAergic innervation on VP and OT-MNCs during chronic dehydration caused by 2% NaCl for 7 days in rats and VP and (Di & Tasker, 2004). Whole cell patch clamp recording showed 33% increase in cell capacitance of rats dehydrated for 7 days with addition of 2% salt in their drinking water (Tanaka *et al*., 1999). All these changes due to dehydration are reversible with rehydration for similar time periods (Hatton, 1997).

Long term dehydration causes up-regulation of VP genes (Zingg *et al*., 1986) and an increase in central VP receptors (V_{1a}) (Hurbin *et al*., 2002). Water deprivation of 3 days in rats caused an increase in plasma osmolality and a 16% decrease in plasma volume as well as an increase in gene transcription of VP (Hayashi *et al*., 2006). Chronic dehydration caused increased expression of different receptors and channels e.g. dynorphin receptors (Shuster *et al*., 1999), L-type Ca^{2+} channels (Zhang *et al*., 2007a) and Na^{+} channels (Tanaka *et al*., 1999). Dynorphin binding kappa- opioid receptors are translocated from large dense granules to plasma membrane through exocytotic fusion in response to hyperosmotic stimulus (Shuster *et al*., 1999).

A whole cell patch clamp experiment carried out in our laboratory showed an 80% increase in nifedipine-sensitive (L-type Ca^{2+}) currents in both VP & OT-MNCs acutely isolated from rats dehydrated for 16-24 hours, while other types of Ca^{2+} currents were not affected (Zhang *et al*.,

2007a). Radioligand binding showed an increase in the density of L-type Ca^{2+} channels (Zhang *et al*., 2007a). This study could not resolve whether Ca_v 1.2 or Ca_v 1.3 or both is responsible for the increase in L-type Ca^{2+} current increase (Zhang *et al*., 2007a). An increase in L-type Ca^{2+} channel, Ca_v1.2 was observed in pituicytes of rats which were chronically dehydrated (Wang *et al*., 2009). The mechanism underlying the increase of L-type Ca^{2+} currents and increased density of L-type Ca^{2+} channels is not clear. The increase of L-type Ca^{2+} current that occurs during long term osmotic increases may be important for the sustained high level of hormone release in several ways. The increase in L-type Ca^{2+} currents may affect the cell firing patterns (making them shorter and faster) through enhanced activation of Ca^{2+} dependent currents (DAP, slow and medium AHP). MNCs respond to changes in dehydration with increase in gene expression and L-type Ca^{2+} channels are involved in the activation of a transcription factor (CREB) (Burbach *et al*., 2001) which suggests that the increase in L-type Ca^{2+} currents could result in increased gene expression. It has been hypothesized as well that somatodendritic release may be significant for long term osmotically-evoked changes in MNCs and the increase in L-type Ca^{2+} channels may enhance the somatodendritic release of VP & OT and other co-released peptides (dynorphin & apelin) during physiological osmotic stress. The increased density of L-type Ca^{2+} channels could be explained by an increase in the synthesis of channels or the translocation of existing channels (Zhang *et al*., 2007a).

1.5.2.1 Hypertrophy

Many cells regulate their surface membrane area in response to osmotic changes. This regulation tends to resist changes in membrane tension and is known as surface area regulation (Morris & Homann, 2001). Increase in membrane tension due to hypo-osmolar stimuli causes an insertion of new membrane from cytoplasmic reserves while a decrease in tension in hyperosmolar stimuli tends to result in uptake of membrane into internal reserves. MNCs undergo hypertrophy when exposed to long term hyperosmolality or chronic dehydration, which is opposite to this phenomenon. In lactation and parturition only OT neurons show these ultrastructural changes while in chronic dehydration both OT and VP neurons undergo hypertrophy (Hussy, 2002).

Preliminarily findings in our lab suggest that MNCs acutely isolated from normally hydrated rats can show hypertrophy in response to a 30 mOsmol kg^{-1} rise of external osmolality over the course 2.5 hours *in vitro* (unpublished data). Increased osmolality initially decreased the CSA of MNCs as it was predicted according to Zhang & Bourque, 2003 but this followed by an enlargement of CSA over the following two hours. This change was reversed when the bathing solution was replaced with isotonic solution. This enlargement in CSA was not found in the hippocampal neurons or dorsal root ganglion cells suggesting that hypertrophy is a unique adaptation of MNCs. Different experiments have been done to see how hypertrophy in MNCs is regulated. For that purpose MNCs were treated with different blockers before exposure to hyperosmolar solution. Hypertrophy was prevented by pretreatment of the cells with tetrodotoxin (TTX), which blocks Na^+ channels and therefore blocks action potential firing. Hypertrophy was also blocked by SB366791 (SICs blocker; Sharif-Naeini *et al*., 2008a), nifedipine (L-type Ca^{2+} channel blocker) or BAPTA-AM (which chelates intracellular Ca^{2+}) (unpublished data). This data suggests that hypertrophy of MNCs is dependent on the activation of Na^+ channels, SICs and Ca^{2+} influx through L-type Ca^{2+} channels (unpublished data). Our lab also observed that hypertrophy is reversed if hypertrophied cells incubated in hyperosmolar solution are treated with TTX or SB366791 or nifedipine. These data suggest that hypertrophy occurs through the following sequence of events. An increase in osmolality shrinks the cells, which leads to the activation of SICs, which increases the cell firing and Ca^{2+} influx through L-type Ca^{2+} channels and ultimately results in hypertrophy of MNCs (unpublished data). There is still need to know the physical and biochemical changes to understand mechanism of this hypertrophy in MNCs that may facilitate the sustained and high level of hormone release.

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

The structural and functional adaptations that occur in MNCs in response to sustained increases in osmolality may be important for maintaining high VP concentrations when its demand is increased (Hatton, 1997). One part of these adaptations is hypertrophy of the MNCs. Electrophysiological and radioactive ligand binding studies showed that increases in osmolality also cause an increase in L-type Ca^{2+} channels (Zhang *et al*., 2007a). Little is known about the mechanisms of either of the hypertrophy or the increase in L-type Ca^{2+} channels because it is difficult to address these questions in whole animal experiments. Most of the observations have been taken from *in vivo* studies in which induction of hyperosmolality takes a long time and the mechanism of hypertrophy cannot be studied easily. We therefore decided to study hypertrophy in *in vitro* experiments and found that this phenomenon may be observed in MNCs acutely isolated from rats in tissue culture. On the basis of this preliminary information we designed two hypotheses;

1. Hypertrophy, a structural and functional adaptation of MNCs to sustained increases in external osmolality, may be due to the insertion of new membrane through the exocytotic fusion of intracellular granules with the plasma membrane of MNCs.
2. Sustained increases in osmolality increase expression of L-type Ca^{2+} ($Ca_v1.2$ and $Ca_v1.3$) channels in isolated MNCs.

The objectives of this study are to understand the mechanisms of osmotically-induced hypertrophy in MNCs and of the increase in the L-type Ca^{2+} channel density in response to increased osmolality.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

All chemicals, unless stated otherwise, were from Sigma (St. Louis, MO). The TAT-NSF700 peptide and its scrambled version were purchased from AnaSpec, Inc. (Fremont, CA). Primary antibodies including Neurophysin I, II were from Santa Cruz Biotechnology, CA while Cav1.2, 1.3 were from Alomone laboratories, Israel. Secondary antibodies were Cy3-labeled antigoat from Jackson ImmunoResearch laboratories, PA and Alexa Fluor 555 antirabbit were from Invitrogen, CA.

3.2 Animal and cell preparation

Normally hydrated Male Long Evans rats (Charles River, QC, Canada) weighing 250-300g, were used for all experiments. According to the guidelines of the Canadian Council on Animal Care and our University Ethics committee, the rats were anesthetized by halothane inhalation and killed by decapitation. The brain was then removed from the skull carefully, placed onto Petri dish and moistened with PIPES (piperazine-1, 4-bis (2-ethanesulfonic acid) solution composed of (in mM, pH 7.1): PIPES 20, NaCl 110, KCl 5, $MgCl_2$ 1, glucose 25 and $CaCl_2$ 1 (abbreviated as PGC). The osmolality of the PGC was maintained at 295 mOsmol kg^{-1} ±3 throughout using a VAPRO pressure osmometer (WESCOR, Logan, UT, USA) by adding mannitol as required. Hyperosmotic solution was maintained at 325 mOsmol kg^{-1} in our experiments. Increases of osmolality of this magnitude were used to characterize the SICs (Oliet & Bourque, 1993a; Oliet & Bourque, 1993b) and have been used in our lab to cause osmotic changes in MNCs isolated from rats (Zhang *et al*., 2009; Wang *et al*., 2009). A brain slice of approximately 1.0 mm thickness containing most of the supraoptic nuclei was excised and shifted to a dish in isotonic PGC. The slice was fixed with pins and two tissues containing most of the SON were cut from the slice using an inverted microscope. Tissue blocks were put into 10 ml of PGC with trypsin (Type XI, 0.6 mg/ml) in a 15 ml glass tube and bubbled with 100% O_2 in a water bath at 30^{o}C for

90 minutes and then shifted to trypsin free PGC at room temperature for 30 minutes with the continued supply of O_2. Tissue blocks were triturated by a series of fire polished Pasteur glass pipettes with decreasing diameters. Finally, the MNCs were plated on the glass bottomed culture dishes (Fisher & Bourque 1995; Fisher *et al.*, 2000). To avoid evaporation the dishes were covered and cells were allowed to settle down and adhere to the bottom of dishes for half an hour at room temperature before doing experiments.

3.3 Hypertrophy response

Acutely isolated MNCs were photographed at 0 minute with a cooled CCD camera attached to a Zeiss Axiovert 200 using a 40X objective in a solution that is close to the normal set point of extracellular osmolality in the rat (295 mOsmol kg^{-1}) and then rapidly shifted to a hyperosmolar solution (325 mOsmol kg^{-1}). The MNCs were photographed at a time interval of 15, 30, 60, 90, 120 and 150 minutes. Finally the cells were returned to media of normal osmolality and photographed at 180 and 210 minutes. The maximal circumference of the cell somata was traced using the software Image J (NIH) which then gives the cross sectional area (CSA) of the cells in pixels which can then be converted to area in μm^2.

3.4 Whole-cell patch clamp recording

An EPC-9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) was used for performing whole cell patch clamp recording at room temperature. The micropipettes (1.2 x 0.68 mm, A-M Systems Inc., Carlsborg, WA, USA) were prepared by using a P-97 horizontal pipette puller (Sutter Instrument Company, Novato, CA, USA). A microforge (Narashige, Tokyo, Japan) was used to fire-polish the micropipettes. The recording electrodes (4-6 MΩ) were filled with an internal solution (pH 7.34) containing (in mM): K-gluconate 120, $MgCl_2$ 1, EGTA 1, HEPES 10, Na_2ATP 4, and Na-GTP 1 (adjusted to 265 mOsmol kg^{-1}). There were two groups of MNCs used for whole cell patch clamp. In one group the cells were incubated with isotonic PGC (295 mOsmol kg^{-1}) and for second in hyperosmolar (325 mOsmol kg^{-1}) for 90-150 minutes.

3.4.1 Measurement of cross sectional area (CSA) of MNCs

Cells were photographed before whole cell patch clamp for taking C_{slow} with a cooled CCD camera attached to a Zeiss Axiovert 200 using a 40X objective. In the software Image J (NIH), the maximal circumference of the cell somata was traced with the mouse which then gives the cross sectional area (CSA) of the cells in pixels which can then be converted to area in μm^2.

3.4.2 Cell capacitance

Whole cell capacitance was measured using the C_{slow} function of the PULSE software (HEKA, Germany). The C_{slow} represents the input capacitance of cell according to the software while C_{fast} is for capacitance of the electrode which was cancelled before taking the C_{slow}. The cells having a CSA greater than 160 μm^2 were considered as MNCs (Oliet &Bourque, 1992) for taking capacitance through whole cell patch clamp recording.

3.5 Hypertrophy with TAT peptide

A recently developed TAT (human immunodeficiency virus transactivator of transcription) peptide (TAT-NSF700; H-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Gly-Gly-Leu-Leu-Asp-Tyr-Val-Pro-Ile-Gly-Pro-Arg-Phe-Ser-Asn-Leu-Val-Leu-Gln-Ala-Leu-Leu-Val-Leu-OH) that prevents SNARE-mediated exocytotic fusion by blocking the function of NSF (*N*-ethylmaleimide-sensitive factor) (Matsushita *et al*., 2003; Matsushita *et al*., 2005) was used in hypertrophy experiments. MNCs were preincubated with TAT peptide (5µg/ml) for an hour prior to take photographs. Cells were photographed at 0 minute in isotonic PGC (295 mOsmol kg^{-1}) similary as done in hypertrophy experiments and then shifted to hyperosmolar solution (325 mOsmol kg^{-1}) and photographed at 15, 30, 60, 90, 120 and 150 minutes. Then cells were shifted to isotonic solution and photographed at 180 and 210 minutes. Other dishes of MNCs were preincubated with scrambled TAT peptide (TAT-NSF700scr; H-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Gly-Gly-Ile-Pro-Pro-Val-Tyr-Phe-Ser-Arg-Leu-Asp-Leu-Asn-Leu-Val-Val-Leu-Leu-Leu-Ala-Gln-Leu-OH) with the same procedure to act as control.

3.6 Immunocytochemistry

All procedures used in these experiments were in accordance with the guidelines of the University of Saskatchewan Animal Care Committee. MNCs were isolated from Male Long Evans rats by using the protocol described earlier, except poly-L-lysine coated dishes were used. After isolation the cells were treated with hyperosmolar solution (PGC= 325 mOsmol kg^{-1}) for 2 hours and washed briefly with PBS. The cells were fixed for 20 minutes at room temperature with ice cold methanol (100%), which also serves to permeablize the cells. The cells were rinsed three times with PBS and treated with blocking solution containing 10% donkey serum in antibody diluent i-e; 1% BSA in PBS for 30 minutes. Then cells were incubated with primary antibody overnight at 4˚C following three washes with PBS. Goat antibodies directed against neurophysin I (1: 100) and neurophysin II (1: 100), which identify OT and VP releasing MNCs respectively, were used as primary antibodies (Santa Cruz Biotechnology). To target L-type Ca^{2+} channels (both $Ca_v1.2$ & $Ca_v1.3$), rabbit antibodies $Ca_v1.2$ (1:200) and $Ca_v1.3$ (1:200) from Almone laboratories were utilized. Next day the dishes were rinsed three times with PBS and then incubated with secondary antibodies for 1 hour. The secondary antibodies used were Alexa fluor 488 donkey anti-goat (1:500) and Alexa fluor 555 donkey antirabbit (1:200) from Invitrogen. The cells were again rinsed with PBS for three times and then stained with Hoechst stain (2µl/1ml of PGC) for 10 minutes followed by PBS washing. A mounting solution (Citifluor, Marico) was added at the end. Cells were viewed using a Zeiss inverted Axiovert- 200 microscope with 40x objective using DIC and fluorescence imaging with appropriate filter sets. Images were captured using a cooled CCD camera and the mean intensity of fluorescence was measured using software Image J software by drawing an outline around the circumference of cell bodies. Mean grey value was selected in image J to measure mean intensity of immunofluorescence which was then converted to intensity in arbitrary units per μm^2 for analysis. The negative controls were having no primary antibodies. Six different types of combinations were used to target L-type Ca^{2+} ($Ca_v1.2$ & $Ca_v1.3$), VP-MNCs (N II) and OT (NI) MNCs in control and dehydrated cells. Combinations and concentration of antibodies, treated with control and hypertonic PGC are given in table 3.1 below.

TABLE 3.1 Combination of antibodies used in immunucytochemistry

	Combinations	**Concentration of primary antibodies**	**Concentration of secondary antibodies**
1	$Ca_v1.2$, NI, NII	$Ca_v1.2$ = 1:200 NI= 1: 100 NII= 1:100	Donkey anti rabbit 1:200 Donkey anti goat 1:500
2	$Ca_v1.2$ NI	$Ca_v1.2$ = 1:200 NI = 1: 100	Donkey anti rabbit 1:200 Donkey anti goat 1:500
3	$Ca_v1.2$, NII	$Ca_v1.2$ = 1:200 NII = 1: 100	Donkey anti rabbit 1:200 Donkey anti goat 1:500
4	$Ca_v1.3$, NI, NII	$Ca_v1.3$= 1:200 NI= 1: 100 NII= 1:100	Donkey anti rabbit 1:200 Donkey anti goat 1: 500
5	$Ca_v1.3$, NI	$Ca_v1.3$= 1:200 NI= 1: 100	Donkey anti rabbit 1:200 Donkey anti goat 1: 500
6	$Ca_v1.3$, NII	$Ca_v1.3$= 1:200 NII = 1: 100	Donkey anti rabbit 1:200 Donkey anti goat 1: 500

Table3.1: L-Type Ca^{2+} channels ($Ca_v1.2$ & $Ca_v1.2$), Neurophysin I (NI) and Neurophysin II (NII**)**

3.7 Data analysis

Quantitative data were entered to Graph Pad Prism or Excel worksheets and the Student t-test was used to determine statistical significance with a confidence level of $p < 0.05$. All results are displayed as a mean ± standard error of the mean (SEM).

CHAPTER 4

RESULTS

MNCs undergo hypertrophy as a result of long term hyperosmotic stimulation of experimental animals caused by either water deprivation or salt loading (Hatton, 1997). Previous work in our lab showed that MNCs isolated from rats following overnight water deprivation had a whole cell capacitance that was 25% larger than that of MNCs isolated from normally hydrated rats (Zhang *et al*., 2007a) and recently an *in vitro* study in our lab showed that treatment of isolated MNCs with hyperosmolar solution for 2.5 hours displayed hypertrophy (unpublished data). My work had four objectives; First of all we tested for osmotically-induced hypertrophy in acutely isolated MNCs from normally hydrated rats to replicate lab findings. Second, whole cell patch clamp was used to test whether 2.5 hours of cells treatment with hypertonic solution causes a change in total cell capacitance. The third objective was to test whether hypertrophy could be prevented with TAT-NSF peptide, which blocks SNARE- mediated exocytotic fusion (Matsushita *et al*., 2005) and to confirm presence of new membrane insertion during osmotic stress in MNCs. Fourth and last; because L-type Ca^{2+} current is increased after dehydration in MNCs (Zhang *et al*., 2007a), we tested whether there is increase in immunoexpression of L-type Ca^{2+} channels ($Ca_v1.2$ and $Ca_v1.3$) by using specific antibodies targeted against these channels in MNCs acutely isolated from normally hydrated rats treated with isotonic or hypertonic solution.

4.1 Osmotically-induced hypertrophy in isolated MNCs

Acutely isolated MNCs (n = 12; from three separate preparations) from normally hydrated rats were rapidly shifted from a solution that is close to the normal set point of extracellular osmolality in the rat (295 mOsmol kg^{-1}) to a hyperosmolar solution (325 mOsmol kg^{-1}) and the CSA was monitored. After 15 minutes, CSA was found to be reduced from 455 ± 15 μm^2 to 409 ± 16 μm^2; $P < 0.001$ with a paired t-test. This represents a decrease of about 10%, which is consistent with the predicted shrinkage in cell size in response to acute hyperosmotic treatment (Zhang & Bourque, 2003). Over the following 135 minutes MNCs displayed an expansion to 496 ± 16 μm^2, which was found statistically significant ($P < 0.001$ with a paired t-test; compared to t = 0). This is an increase of 9% compared to their original CSA. Cell size was compared to the

shrinkage state (t=15 minutes), which showed a 21% increase in CSA that was extremely significant $P < 0.001$). When the MNCs were returned to media of normal osmolality at 150 minutes, the cells shrunk (452 ±14 μm^2 at 180 minutes to 438 ± 16 μm^2 at 210 minutes $P > 0.05$) to a size not significantly different than that at time 0 minute. The results are plotted below in Figure 4.1. This suggests that MNCs undergo hypertrophy in hypertonic solutions and that this process reverses when the cells are returned to solutions of normal osmolalities. Our *in vitro* results of hypertrophy may correspond to the process *in vivo* (Hatton, 1997; Tanaka *et al*., 1999; Di & Tasker, 2004; Zhang *et al*., 2007a) and allow us to study the mechanism.

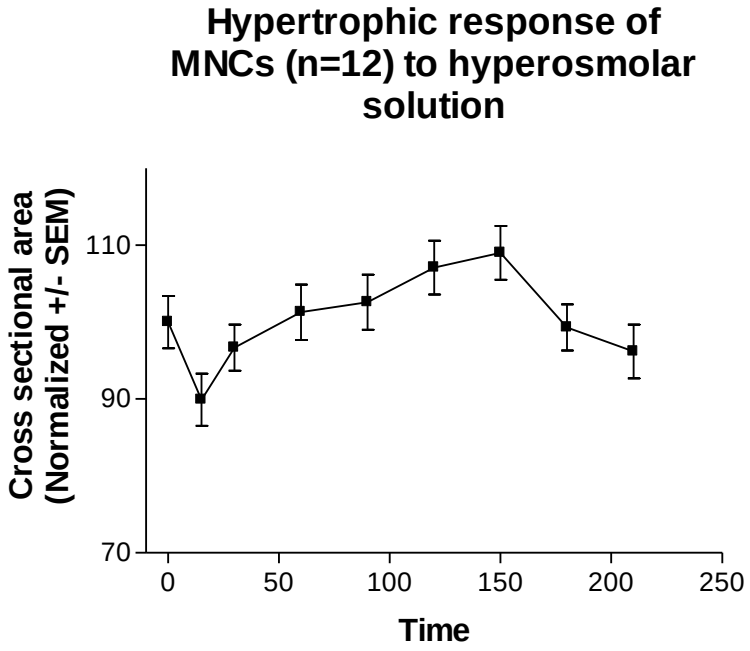


Figure 4.1 Hypertrophic Response of MNCs. The cells were photographed at 0 minute in normal osmolality solution (295 mOsmol kg^{-1}) and then subjected to hyperosmolar solution (325 mOsmol kg^{-1}) for 150 minutes. Photographs were taken at the indicated times and then shifted to normal osmolality solution for 60 minutes. Images were analyzed using Image J (NIH) software. The cells showed rapid drop in CSA at 15 minutes but then there was a gradual increase in size that was maximum at 150 minutes. The hypertrophy was recovered when the cells were returned to the solution of normal osmolality.

4.2 Effect of increased osmolality on whole cell capacitance

In order to observe the association between hypertrophy of MNCs and their total increase in the membrane, we tested whether MNCs treated with hypertonic solutions showed an increase in observed CSA and total cell capacitance. MNCs acutely isolated (Figure 4.2) from 6 normally hydrated rats were incubated in normal (295 mOsmol kg^{-1}; n=21) and hyperosmolar (325 mOsmol kg^{-1}; n=23) solutions. The MNCs were photographed prior to whole cell patch clamp with a cooled CCD camera attached to a Zeiss Axiovert 200 using a 40X objective and the CSA was measured and analyzed using Image J (NIH). The mean CSA of the MNCs that were subjected to whole cell patch clamp was 276 ± 11 μm^2 (in normal solution) and 305 ± 17 μm^2 (in hyperosmolar solution) (Figure 4.3), which was not found to be statistically significant $P > 0.05$ with an unpaired t-test. The CSA was found to be 10.5% higher for the hyperosmolar group as compared to the control group. The increase in CSA was observed immediately before measuring capacitance of the MNCs incubated in hyperosmolar solution for 90-150 minutes. This small increase in CSA of MNCs after treatment with hyperosmotic solution is consistent with the results shown in Figure 4.1, in which we found 9% increase in CSA at 150 minutes after treatment of cells with hyperosmotic solution. This difference was significant because those experiments followed the change in size of individual MNCs (and therefore we used a paired t-test) whereas the unpaired data did not show a significant difference due to large range of sizes of MNCs.

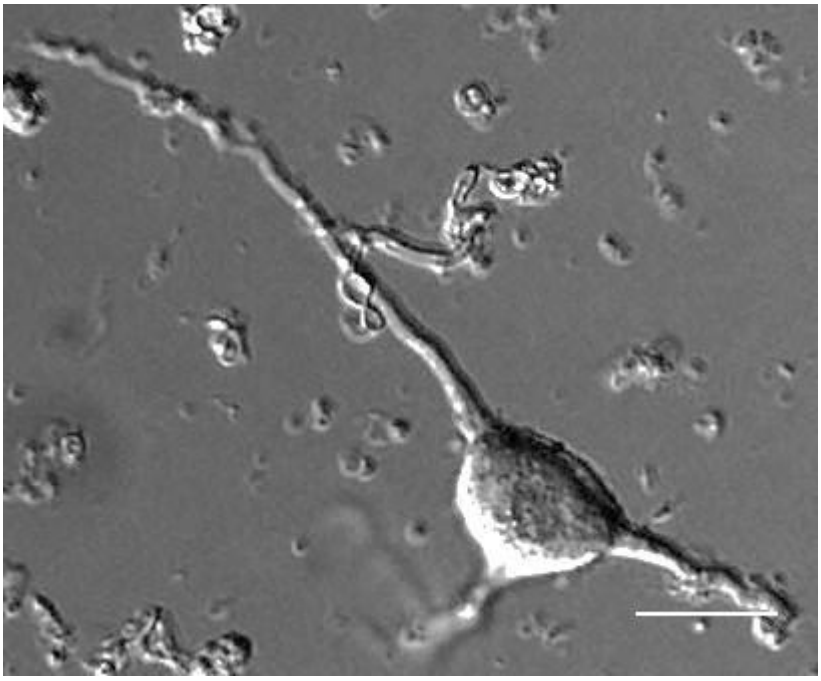


Figure 4.2 An acutely isolated MNC from SON of male Long Evans rat showing a typical image of a cell with three dendrites and somata in hyperosmolar solution (325 mOsmol kg^{-1}) before patch clamp recording. Scale bar is 15 µm.

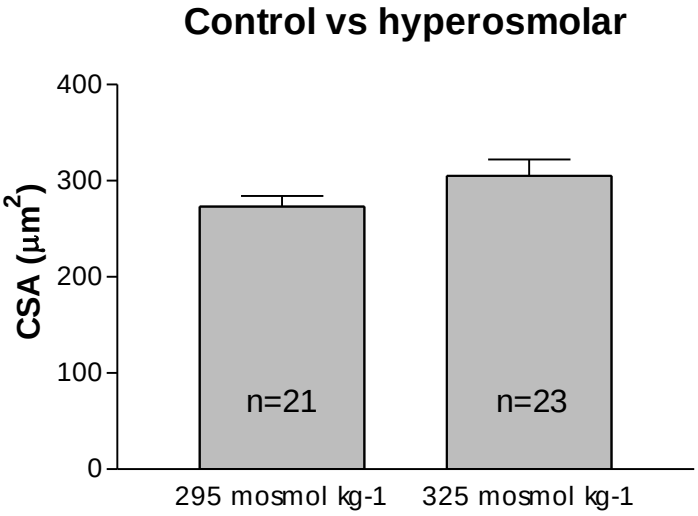


Figure 4.3 Cross sectional area (CSA) of MNCs treated with normal and hyperosmolar solution. The mean CSA of MNCs was measured from two groups of MNCs treated with control (295 mOsmol kg^{-1}) and hyperosmolar (325 mOsmol kg^{-1}) solution for 90-150 minutes. The mean CSA of cells (n=23) in hyperosmolar solution was not significantly different than cells (n=21) in isotonic solution.

After taking photographs, the cells either incubated in isotonic solution (n=21) or hyperosmolar solution (n=23) were patch clamped and the capacitance was measured after 90-150 minutes of incubation. There was a marked difference in the total cell capacitance between the two groups. Figure 4.4 shows that MNCs exposed to high osmolality had a mean capacitance significantly larger than that of the control MNCs (from 11.2 ± 0.5 pF to 15.0 ± 0.6 pF; $P < 0.001$ with an unpaired t-test), which represented a 34% increase for hyperosmolar versus control group. Our findings of an increase in cell capacitance from MNCs culture are similar in magnitude to the increase in cell capacitance observed in MNCs isolated from dehydrated rats by 33% with salt loading in their water for 7 days (Tanaka *et al*., 1999) and 25% with water deprivation for 16-24 hours (Zhang *et al*., 2007a). This supports our hypothesis that MNCs hypertrophy is associated with an increase in total membrane surface area by the addition of new membrane through exocytotic fusion.

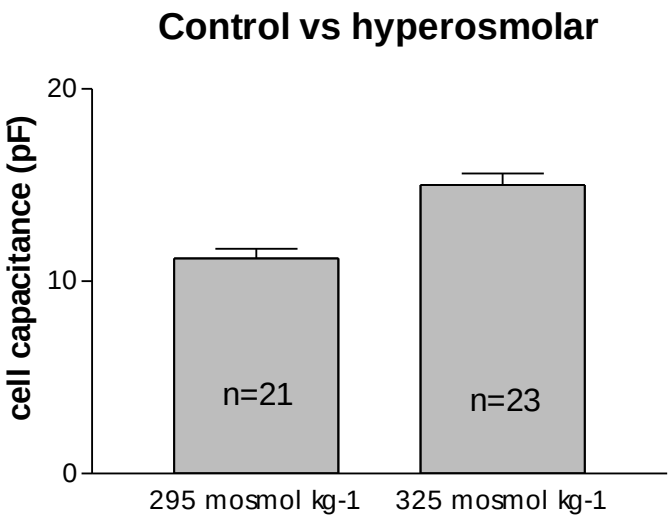


Figure 4.4 Cell capacitance of MNCs treated with normal and hyperosmolar solution. Whole cell patch clamp was performed on two groups of MNCs treated with control (295 mOsmol kg^{-1}) and hyperosmolar (325 mOsmol kg^{-1}) solution for 90-150 minutes. The capacitance (pF) was recorded from each cell of both groups. The mean capacitance of cells (n=23) in hyperosmolality (15.0 ± 0.6 pF) was significantly higher then the capacitance of cells (n=21) in extracellular osmolality (11.2 ± 0.5 pF).

4.3 Prevention of hypertrophic response with TAT-NSF peptide

To test whether the observed osmotically-evoked increase in CSA depends on exocytotic fusion of granules with the plasma membrane, I tested the response of cells to increases in osmolality in the presence of an agent (TAT-NSF700). TAT (human immunodeficiency virus transactivator of transcription) peptide (TAT-NSF700) that prevents SNARE-mediated exocytotic fusion by blocking the function of NSF (*N*-ethylmaleimide-sensitive factor) (Matsushita *et al*., 2005). Acutely isolated MNCs (n=67; from 15 preparations) were treated with hyperosmolar solution as described above in the presence of TAT-NSF700 peptide, while other MNCs (n=27; from 5 preparations) were treated with scrambled version of the peptide (TAT-NSF700scr). The hypertrophy was observed by taking photographs at different intervals and the CSA (μm^2) of cells was measured. It was observed that treated cells shrunk (349 ± 9 μm^2 t=0 to 331± 8 μm^2 t=15 n = 67; $P < 0.001$ with a paired t-test) but showed no detectable hypertrophy (329± 8 μm^2 t=150; t=15 to t= 150 $P > 0.05$ with a paired t-test) (Figure 4.5). The hypertrophic response did not appear to be altered by the scrambled version of the peptide. The CSA first shrunk from 310 ± 14 μm^2 to 292 ± 13 μm^2 and then increased to 329 ± 15 μm^2 (a 6% increase from t=0 to t= 150 minute; n = 27; $P < 0.001$ with a paired t-test) as shown in Figure 4.5. Hypertrophy was however, virtually eliminated by preincubation with TAT-NSF700, suggesting that osmotically-evoked hypertrophy depends on SNARE-mediated exocytotic fusion and hypertrophy is a result of insertion of new membrane.

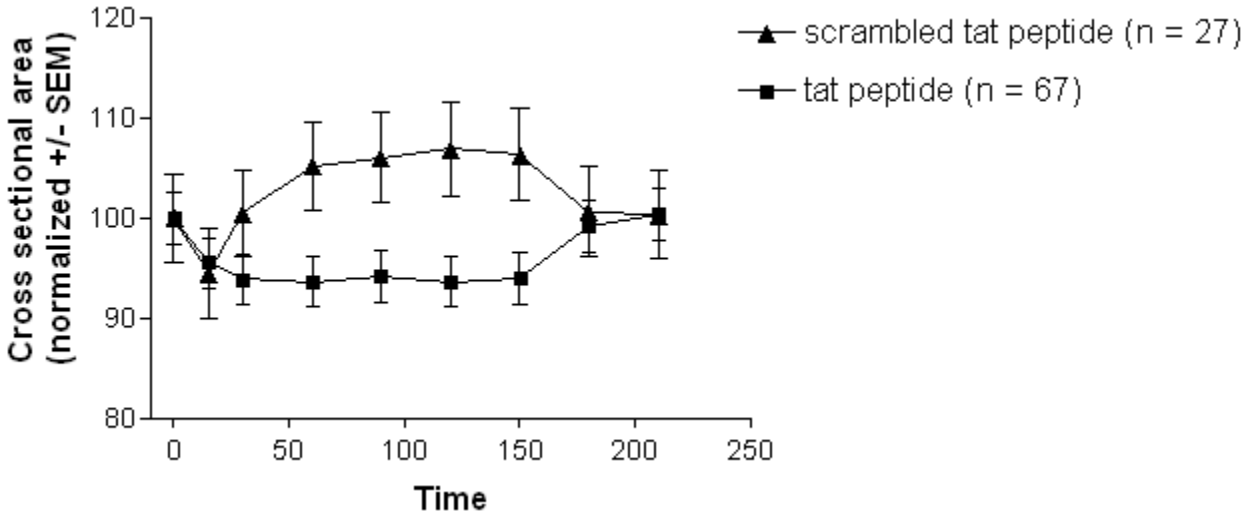
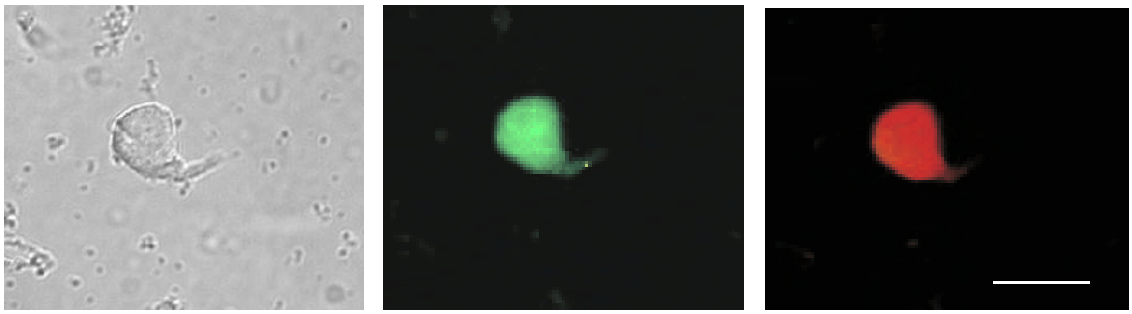


Figure 4.5 Effect of MNCs on hypertrophic response with TAT peptide and scrambled version of TAT peptide. The hypertrophic response was blocked by the preincubation of MNCs (n=67) with TAT peptide (TAT-NSF700) while the cells (n=27) treated with scrambled version of the peptide (TAT-NSF700scr) showed hypertrophy.

4.4 Immunocytochemistry of L-type Ca^{2+} channels following treatment with hyperosmolar solution

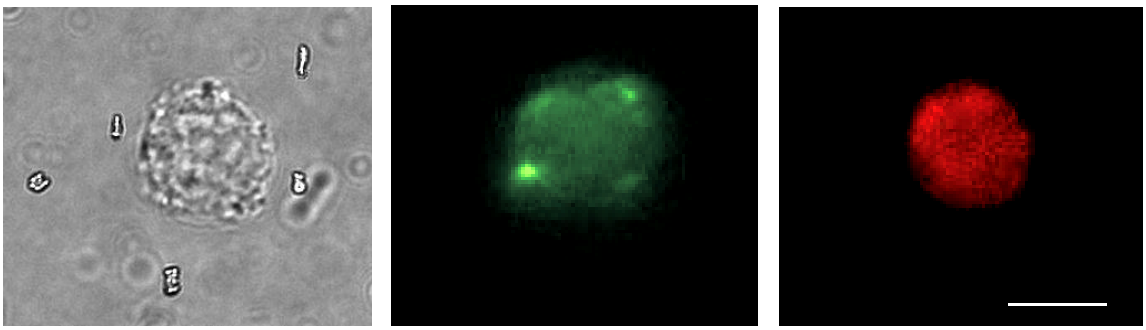
We performed immunocytochemistry to test for changes in expression of L-type Ca^{2+} channels ($Ca_v1.2$ & $Ca_v1.3$) on the somata of VP-MNCs and OT-MNCs in response to increases in osmolality. Chronic dehydration causes increase in L-type Ca^{2+} channels density and L- type Ca^{2+} currents (Zhang *et al*., 2007a). Both $Ca_v1.2$ and $Ca_v1.3$ are expressed in the somata and dendrites of MNCs (Joux *et al*., 2001) and mRNA for both channels are expressed in SON (Zhang *et al*., 2007a). Therefore both $Ca_v1.2$ and $Ca_v1.3$ may contribute to L-type Ca^{2+} channels in MNCs. We isolated MNCs acutely from 14 well hydrated rats and treated with either hyperotonic solution (325 mOsmol kg^{-1}) or isotonic solution (295 mOsmol kg^{-1}) for 2 hours. The difference in expression of L-type Ca^{2+} channels in both groups of cells was observed by using antibodies against $Ca_v1.2$ and $Ca_v1.3$. We also labeled both groups with antibodies markers against OT and VP- MNCs (neurophysin I and II, respectively) to differentiate the immunoexpression of L-type Ca^{2+} channels in both types of MNCs). Immunoreactivity to NI & NII is represented by green fluorescence (Fig 4.6 and 4.7) and to L-type Ca^{2+} channels $Ca_v1.2$ & $Ca_v1.3$ is represented by red fluorescence (Fig 4.6 and 4.7). The immunofluorescence of $Ca_v1.2$ and $Ca_v1.3$ showed intense and diffuse staining throughout the cell cytoplasm and plasma membrane suggesting a distribution of L-type Ca^{2+} channels in the cytoplasm and plasma membrane of the MNCs. It was found that MNCs treated with hyperosmolar solution had higher immunoreactivities for both $Ca_v1.2$ (Fig 4.8) and $Ca_v1.3$ (Fig 4.9). When plotted and analyzed, the mean intensities (intensity in arbitrary units per μm^2) of $Ca_v1.2$ (Fig 4.10) and $Ca_v1.3$ (Fig 4.11) was found significantly higher for the cells incubated for 2 hours with hypertonic solution compared to the cells treated with normal solution. The mean intensity of $Ca_v1.2$ was found to be; control=15 ±1 arbitrary units/μm^2, hypertonic 30 ± 2 arbitrary units/μm^2 $P<0.001$ with unpaired t test and $Ca_v1.3$; control = 13±1 arbitrary units/μm^2, hypertonic 26 ±2 arbitrary units/μm^2 $P< 0.001$ with unpaired t test. This suggests an increase in number of L-type Ca^{2+} channels due to the increased synthesis of channel proteins during sustained hyperosmotic stimulation. These results do not rule out the other explanations for increase in L-type Ca^{2+} channels i.e. it could be the translocation of channels from internal membranes of cytoplasm to surface of cells, which we could not observe because of the diffuse staining in the cytoplasm and

plasma membrane. Our results of increases in intensity of immunoreactivity to L-type Ca^{2+} channels $Ca_v1.2$ & $Ca_v1.3$ in response to increased osmolality are consistent with radioligand binding results and whole cell patch clamp recordings in which the density of L-type Ca^{2+} channels and total L-type Ca^{2+} currents, respectively were increased after dehydration (Zhang *et al*., 2007a). Six combinations were designed to see any difference between OT MNCs (NI) and VP- MNCs (NII) for the expression of L-type Ca^{2+} channels; $Ca_v1.2$ (Figure 4.10) and $Ca_v1.3$ (Figure 4.11) but the difference was statistically not significant and VP-MNCs showed only a little higher intensity for expression of L-type Ca^{2+} channels as compared to OT-MNCs in normal and hypertonic solutions. We did not observe a significant change in intensity of immunostaining for NI and NII in control and hyperosmolar groups. The negative control showed no significant difference in immunoreactivity in hyperosmotically treated cells compared to control. This supports the hypothesis that hypertrophy observed in response to increased external osmolality is associated with the up-regulation of synthesis of L-type Ca^{2+} channels ($Ca_v1.2$ & $Ca_v1.3$).



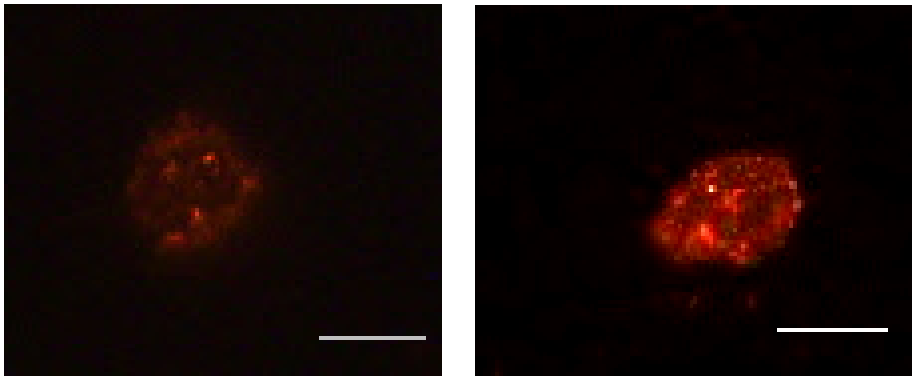
A: DIC **B: NI&NII** **C: $Ca_V1.2$**

Figure 4.6: Immunofluorescence in MNC using antibodies directed against its specific carrier proteins (NI and NII) and L-type Ca^{2+} channel: $Ca_v1.2$ Immunostaining results showing an MNC soma with one dendrite. A: A differential interference contrast (DIC) image of MNC, B: Green colour representing specific carrier protein targeted by using primary antibodies; Neurophysin I (NI) and Neurophysin II (NII), C: Red colour showing result of immunofluorescence by using primary antibody directed against L-type Ca^{2+} channel $Ca_v1.2$ to observe change in intensity of channels during hypertonic stimuli. Scale bar is 20 µm.



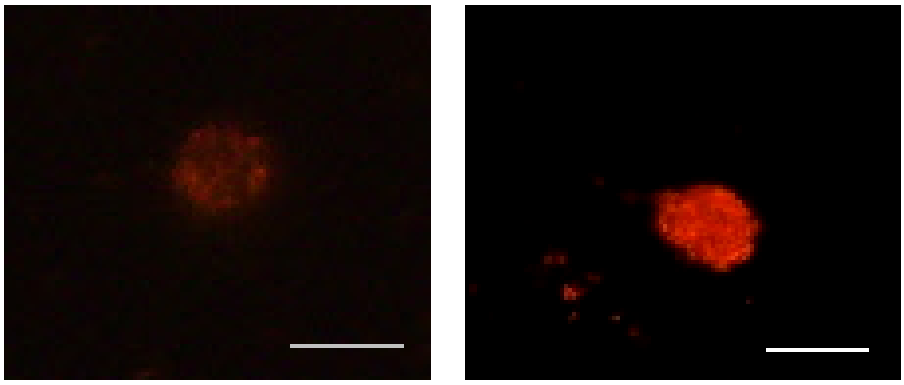
A: DIC **B: NI&NII** **C: $Ca_V1.3$**

Figure 4.7 Immunofluorescence in MNC using antibodies directed against its specific carrier proteins (NI and NII) and L-type Ca^{2+} channel: $Ca_v1.3$ Immunostaining results showing an MNC soma. A: differential interference contrast (DIC) image of MNC, B: Green colour representing specific carrier protein targeted by using primary antibodies; Neurophysin I (NI) and Neurophysin II (NII), C: Red colour showing result of immunofluorescence by using primary antibody directed against L-type Ca^{2+} channel $Ca_v1.3$ to observe change in intensity of channels during hypertonic stimuli. Scale bar is 20 µm.



A: Control **B: Hyperosmolar**

Figure 4.8 The increase in immunofluorescence for $Ca_v1.2$ in acutely isolated MNCs due to increase in external osmolality Images are showing immunofluorescence towards $Ca_v1.2$ in MNCs acutely isolated from hydrated rats, treated with control (normal) and hyperosmolar (high osmolality) and cells were also identified as MNCs with specific carrier markers; NI and NII (not shown). A: The MNC treated with control solution (295 mOsmol kg^{-1}) showing immunofluorescence for $Ca_v1.2$ and B: showing increased intensity of immunofluorescence for $Ca_v1.2$ after treatment of MNCs with hyperosmolar solution (325 mOsmol kg^{-1}). The scale bar is 20 µm.



A: Control **B: Hyperosmolar**

Figure 4.9 The increase in immunofluorescence for $Ca_v1.3$ in acutely isolated MNCs due to increase in external osmolality Images are showing immunofluorescence towards $Ca_v1.3$ in MNCs acutely isolated from hydrated rats, treated with control (normal) and hyperosmolar (high osmolality) and cells were also identified as MNCs with specific carrier markers; NI and NII (not shown). A: The MNC treated with control solution (295 mOsmol kg^{-1}) showing immunofluorescence for $Ca_v1.3$ and B: showing increased intensity of immunoflourescence for $Ca_v1.3$ after treatment of MNCs with hyperosmolar solution (325 mOsmol kg^{-1}). The scale bar is 20 µm.

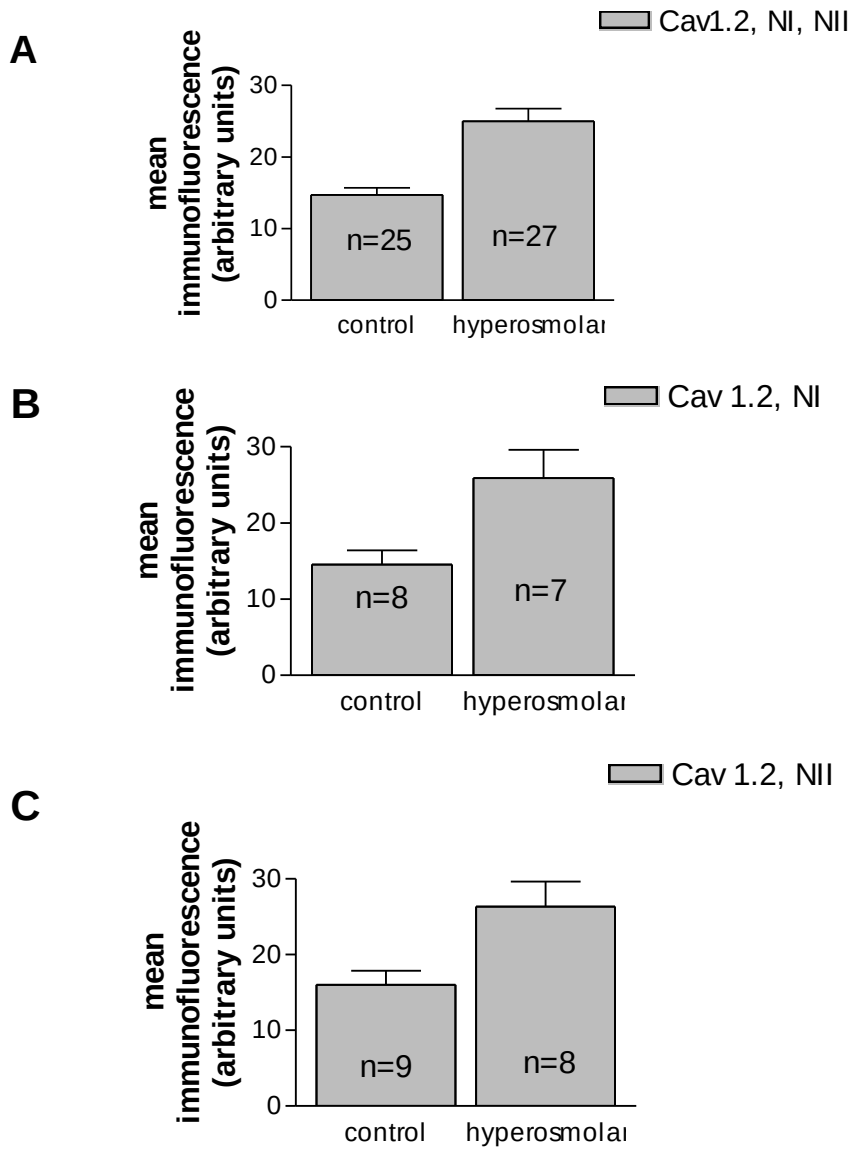


Figure 4.10 Immunofluorescence for L-type Ca^{2+} channel; $Ca_v1.2$ (A) MNCs immunoreactive to a mixture of antibodies directed against NI and NII showed higher mean intensity of immunofluorescence for $Ca_v1.2$ in the group of MNCs that were incubated with hyperosmolar solution. (B) OT-MNCs were identified by using their specific protein marker; NI and (C) VP-MNCs were identified by using their specific protein marker; NII to see the difference of L-type Ca^{2+} immunoexpression: $Ca_v1.2$ in both control and hypertrophied MNCs. The mean intensity of immunofluorescence was higher in cells in hyperosmolar solution in both types of cells (B&C) and there was no significant difference in expression of $Ca_v1.2$ between OT and VP-MNCs.

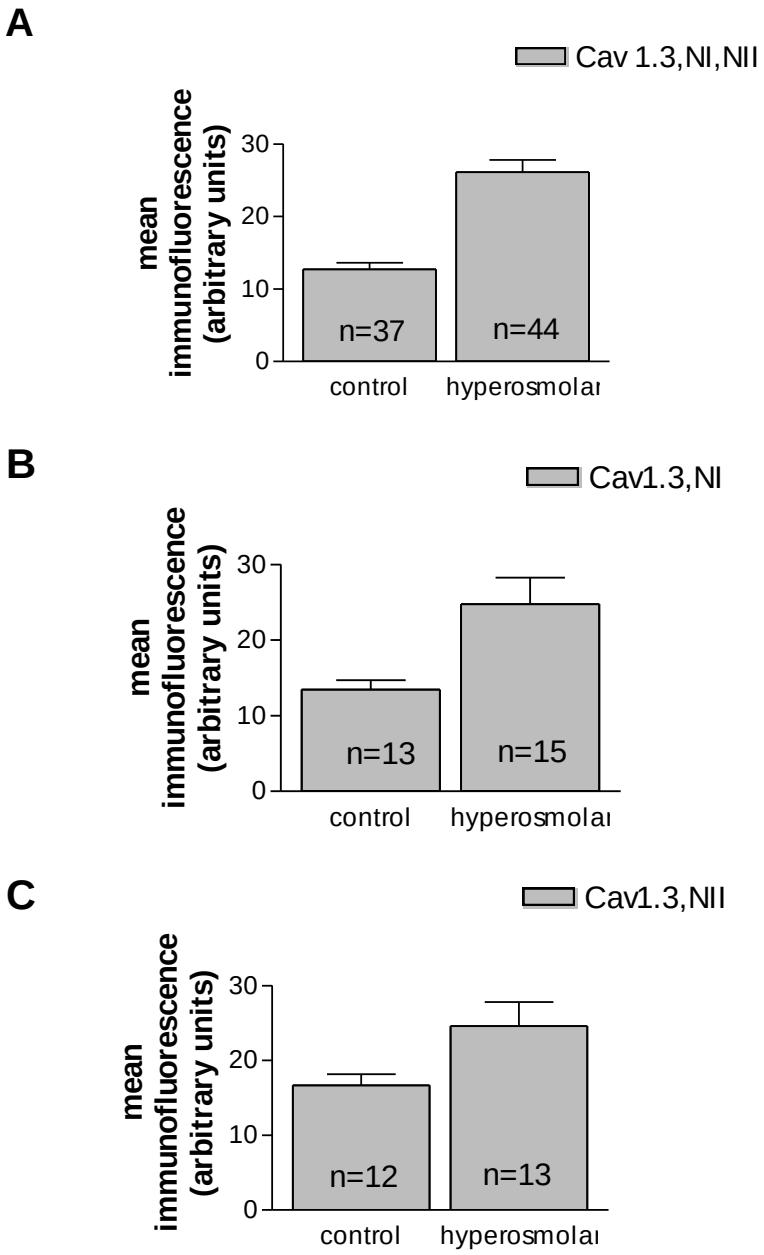


Figure 4.11 Immunofluorescence for $Ca_v1.3$ (A) MNCs immunoreactive to a mixture of antibodies directed against NI&NII showed higher mean intensity of immunofluorescence for $Ca_v1.2$ in MNCs treated with hyperosmolar solution. (B) OT- and (C) VP-MNCs were identified by using their specific protein marker; NI & NII, respectively. (B&C) The mean intensity of immunofluorescence was higher in cells in hyperosmolar solution in both types of cells OT & VP-MNCs and there was no significant difference in expression of $Ca_v1.2$ between both types of cells.

CHAPTER 5

DISCUSSION

During chronic dehydration, MNCs undergo hypertrophy, which is associated with increases in cell capacitance and L-type Ca^{2+} current (Zhang *et al*., 2007a). This hypertrophy is opposite to the shrinkage of cells observed in MNCs culture in response to acute increases in osmolality (Zhang & Bourque, 2003). The mechanisms underlying the osmotically-induced hypertrophy of MNCs, which is likely to be a part of long term adaptation to increases in osmolality, is not clear. We therefore subjected MNCs in culture to hyperosmolar solutions for tens of minutes and observed a significant increase in the cell size (cross sectional area). Whole cell patch clamp recordings showed that osmotically-evoked hypertrophy is associated with an increase in cell capacitance of about 34%. This reflects the increase in total membrane surface area of MNCs and suggests insertion of new membrane into the cell surface. We observed the association of the hypertrophic increase in total membrane surface area with exocytotic fusion and also we observed the immunoexpression of L-type Ca^{2+} channels in normal and hypertrophied MNCs ($Ca_v1.2$ & $Ca_v1.3$). Our hypertrophy experiments and whole cell capacitance recordings confirmed that increases in external osmolality for 90-150 minutes cause an increase in the size of MNCs by increasing the total membrane surface area and that this increase is dependent on the exocytotic fusion of intracellular membrane with the plasma membrane. Our data from immunocytochemistry displayed a significant increase in expression of L-type Ca^{2+} channels (both $Ca_v1.2$ and $Ca_v1.3$) in MNCs treated with hyperosmolar solution as compared to control cells, suggesting increased synthesis of channels during sustained hyperosmotic stimulation. I will discuss first the difference between short term and long term changes in MNC size in response to increases in external osmolality.

5.1 Short and long term osmotic changes

Acute exposure to hyperosmolar and hypo-osmolar solutions rapidly causes shrinkage and swelling of cells, respectively with reversal to their original size upon removal of stimuli (Zhang & Bourque, 2003). In contrast, hippocampal cells compensate for osmotically-induced volume

changes (shrinking and swelling) because they exhibit volume regulatory compensatory mechanisms known as regulatory volume increase (RVI) and regulatory volume decrease (RVD). RVI and RVD are the mechanisms to regulate the internal osmolality by the regulation of ion transport and accumulation of organic osmolytes to avoid the deleterious changes in cell volume due to changes in external osmolality (Lang, 2007). Due to the absence of compensatory mechanisms, osmotically-induced volume changes cause the greatest possible changes in the membrane tension which are indicators of external osmolality in MNCs. Mechanosensitive channels (SICs) are activated in response to osmotically-evoked changes in cell volume and respond electrically to changes in membrane tension (Oliet & Bourque, 1993a). The plasma membrane of MNCs is depolarized when the hyperosmotic stimuli cause shrinkage of cell, release the membrane tension, activate SICs in a cytoskeleton-dependent manner and initiate the firing of MNCs for the release of hormone (Oliet & Bourque, 1993a; Oliet & Bourque, 1993b and Zhang *et al*., 2007b). MNCs do not show a change in total membrane surface area when they undergo acute shrinkage and swelling (Zhang & Bourque, 2003). Acute shrinkage and swelling of MNCs upon exposure to osmotic changes may be regulated by the folding and unfolding of membrane reserves and not the total membrane surface area (Zhang & Bourque, 2003). This is how MNCs respond to acute hyperosmotic change but the scenario is different in long term hyperosmotic perturbations.

Studies showed that the hypothalamo-neurohypophysial system (HNS) undergoes structural and functional plasticity during chronic dehydration, one aspect of which is hypertrophy of MNCs (Hatton, 1997; Zhang *et al*., 2007a). Similar changes were seen during lactation and parturition (Theodosis *et al*., 2004; Theodosis *et al*., 2008). Long term exposure to increases in osmolality causes an increase in MNC size and capacitance. Hypertrophy appears to be part of broader adaptation to high osmolality that enables sustained high level release of VP (Hatton, 1997). Different responses of MNCs to acute (Zhang & Bourque, 2003) and long term changes (Hatton, 1997; Zhang *et al*., 2007a) in external osmolality led us to study osmotically-induced hypertrophy. The mechanism of this hypertrophy is unknown and difficult to study *in vivo* and we therefore attempted to study osmotically-induced hypertrophy *in vitro*.

We observed hypertrophy in MNCs culture, treated with hyperosmolar solution over tens of minutes. MNCs shrunk by about 10% of their original size as an acute response to hypersomolar

solution (325 mOsmol kg^{-1}) after 15 minutes and this was observed by measuring the CSA of MNCs. The magnitude of shrinkage was consistent with acute shrinkage observed by Zhang & Bourque, 2003. We observed that cells gradually expanded in size after 15 minutes and attained a maximum size about 9% greater than their original size at 150 minutes. These data suggest that hypertrophy is a structural adaptation of MNCs in response to long term increases in external osmolality and may be required as part of structural plasticity for the facilitation of hormone release when there is need to regulate the change in external osmolality in MNCs.

5.2 Hyperosmolality causes increase in total membrane surface area

Chronic dehydration increases the capacitance of MNCs (Tanaka *et al*., 1999; Di & Tasker, 2004; Zhang *et al*., 2007a). We therefore attempted to observe an association between osmotically-evoked hypertrophy with the increase in total cell capacitance in MNCs acutely isolated from normally hydrated rats. Whole cell patch clamp showed that MNCs treated with hyperosmolar solution (325 mOsmol kg^{-1}) demonstrated a significant increase of 34% in capacitance as compared to control cells (295 mOsom kg^{-1}). Our findings are consistent with the whole cell patch clamp results of capacitance taken from MNCs acutely isolated from rats either dehydrated for 16-24 hours by water deprivation or due to salt loading for 7-10 days which showed 20-33% increase in capacitance (Zhang *et al*., 2007a; Tanaka *et al*., 1999; Di & Tasker, 2004). OT-MNCs displayed similar increases in capacitance during lactation (Teruyama & Armstrong, 2005). Total cell capacitance is not regulated by short term osmotic changes in MNCs (Zhang & Bourque, 2003). Cell capacitance directly reflects the total membrane surface area (Fernandez *et al*., 1984; Zhang & Bourque, 2003). This suggests that osmotically-evoked hypertrophy regulates the total membrane surface area as a result of exocytotic fusion of intracellular membrane with plasma membrane.

We measured the cell CSA of MNCs before subjecting the cells to whole cell patch clamp. MNCs were identified on the basis of their CSA from non MNCs and we selected only cells which had CSA > 160μm^2 (Oliet & Bourque, 1992). The mean CSA of the hypertrophic group was slightly larger than the control group (10.5%), but this difference was not significant. This difference is similar to the results in Figure 4.1, but those results were significantly different because the data was paired. The data was not found significant also because of the large variation in cell size. For instance, we found the mean CSA of MNCs in control osmotic solution

was 276 μm² (n=21) with min and max values of 160 μm² and 390 μm² respectively while hyperosmolar group had mean CSA of 305 μm² (n=23) with min and max values of 196 μm² to 493 μm², respectively.

Our imaging experiments showed that MNCs increase in CSA (9% in Figure 4.1 and 10.5% in Figure 4.3) during two hours of incubation with high osmolality solutions. In order to see whether that treatment caused an increase in capacitance we tested other MNCs and treated them in same way. The capacitance of those cells was on average higher (34%) than control cells. This suggests that treatment of MNCs with high osmolality solutions for two hours caused an increase in CSA and also an increase in total membrane surface area. We can conclude from these results that increase in CSA is likely to be caused by insertion of new membrane in to plasma membrane. This raises the question of whether the size of the increase in CSA is appropriate for the increase in capacitance. The MNCs shrunk after exposure to increase in osmolality (t=15) as observed in hypertrophy experiments (Figure 4.1) by 10%. But over the next 135 minutes the increase in size was 21% (t=15 to t=150) (Figure 4.1) and this increase reflects the addition of new membrane.

If the MNCs were perfect spheres and there was a 20% increase in CSA, then we would expect that there would be an 80% increase in capacitance. This is because the CSA would be equal to πr^2 whereas the total membrane surface area would be equal to $4\pi r^2$. So if CSA is 20% larger, the total membrane surface area would be increased by 4 times as much. This suggests that 34% increase in capacitance is smaller than we would expect for a 20% increase in CSA. The MNCs are not perfect spheres and as we have discussed it was not possible for us to get even a rough estimate of the true increase in membrane because of the odd shape of the MNCs, the presence of processes that may or may not increase in size and the presence of an unmeasureable amount of membrane reserves inside the cell that is invisible to us. Electron microscopy could be used to measure membrane reserves and total membrane surface area.

5.3 Hypertrophy is regulated by SNARE-mediated intracellular exocytotic fusion

We tested whether the hyperosmotically-mediated increase in total membrane surface area of MNCs leading to hypertrophy is dependent on the SNARE-regulated exocytotic fusion of intracellular granules with the plasma membrane of cells or not. It was observed that MNCs did not undergo hypertrophy when pre-treated with cell membrane permeant TAT-NSF peptide in hyperosmolar solution. The TAT-NSF peptide blocks the SNARE complex of proteins that is responsible for SNARE mediated exocytotic fusion by interfering with the function of NSF (Matsushita *et al*., 2005). MNCs shrunk normally after 15 minutes but then did not show hypertrophy, whereas cells treated with the scrambled version of the peptide showed hypertrophy with 6% increase in cell CSA observed after 150 minutes. Therefore, our data confirms that increase in cell size (hypertrophy) is due to the insertion of new membrane into the cell surface. This data suggest that osmotically-evoked hypertrophy is regulated by an insertion of new membrane depending on the SNARE-mediated exocytotic fusion. The fusion of granules with plasma membrane could be a mechanism of translocation of receptors or channels for the sustained release of hormone during long term hyperosmolality.

VP release from MNCs depends on the SNARE-mediated exocytotic fusion of large dense core granules (containing hormone) with the plasma membrane (Fisher & Bourque, 2001). Our data could not find whether the intracellular granules fusing with plasma membrane contain neuropeptide or not. VP is released when MNCs get signals to release for the reabsorption of water by acting on peripheral receptors in kidneys (Procino *et al*., 2008). Other than axons, VP is also released from somatodendritic regions of MNCs along with endogenous co-peptides, dynorphin and apelin. Somatodendritic release exerts autocrine feedback inhibition on VP output after binding with their receptors on MNCs (Brown *et al*., 2006) and it is a very slow process that takes from 2-5 hours to respond to osmotic changes (Gillard *et al*., 2007). MNCs adopt a phasic burst pattern of firing, which enhances for the sustained release of VP (Oliet & Bourque, 1993a; Oliet & Bourque, 1993b). It was observed during chronic hyperosmotic stimulation that dynorphin receptors (kappa-opioid receptors) are translocated to the MNCs plasma membrane (Shuster *et al*., 1999). Dynorphin may also have a role in the modulation of phasic bursts (Brown & Bourque, 2004). This suggests that osmotically-evoked hypertrophy of MNCs more likely reflects the fusion of a distinct population of intracellular granules and that fusion may

result in translocation of receptors and channels to membrane surface of MNCs to regulate the cell firing for sustained hormone release.

5.4 The activity dependence of osmotically-evoked hypertrophy

Preliminary unpublished data suggest that osmotically-evoked hypertrophy of isolated MNCs is activity dependent. Hypertrophy can be reversed by blocking electrical activity (Na^+ channels), SICs or Ca^{2+} influx through L-type Ca^{2+} channels in hyperosmotically treated MNCs (unpublished data). It was observed in our lab that action potentials preventing by using Na^+ channel blockers (TTX) in MNCs treated with hyperosmolar solution prevented hypertrophy. Pretreatment of cells with SB366791 (SIC channel blocker) and nifedipine (which blocks Ca^{2+} influx through L-type Ca^{2+} channels) also prevented hypertrophy (unpublished data). We observed increase in cell size and capacitances of MNCs in response to increase in external osmolality for tens of minutes suggesting increase in total membrane surface area, which is mediated by exocytotic fusion of intracellular granules with plasma membrane. Our findings and our lab results suggest that hypertrophy does not alter membrane tension or SIC activation itself and hypertrophic response may follow a sequence of events: hyperosmolality causes shrinkage of cells which activates the SICs leading to cell firing, increases in Ca^{2+} influx through L-type Ca^{2+} channels and the resultant increase in Ca^{2+} is necessary for the activation of hypertrophic response (unpublished data). This suggests that cells continue to fire following hypertrophy and this firing is necessary for the maintenance of MNCs hypertrophy and this response does not alter the activation of SICs by altering membrane tension. Exocytotic fusion may involve the granules containing neuropeptides and lead to the somatodendritic release of hormones or co-peptides for the regulation of long term adaptation in response to osmotic stress.

5.5 Sustained hyperosmolality causes increase in immunoexpression of L-type Ca^{2+} channels

Our immunocytochemistry results showed that hyperosmotically-evoked hypertrophy is associated with an increase in the total immunoreactivity to specific antibodies against L-type Ca^{2+} channels including $Ca_v1.2$ and $Ca_v1.3$ in VP and OT-MNCs. Acutely isolated MNCs from normally hydrated rats were incubated either in normal osmolality solution or hyperosmolar solution for 2 hours. Our hypertrophy experiments showed that MNCs experience hypertrophy

after 150 minutes of treatment with hyperosmolar solution. We observed intensity of immunofluorescence in control and treated cells for $Ca_v1.2$ (15 ±1 arbitrary units/μm^2, 30 ± 2 arbitrary units/μm^2) and $Ca_v1.3$ (13 ±1 arbitrary units/μm^2, 26 ± 2 arbitrary units/ μm^2) and found an overall increase in both types of channels in treated cells. Both Cav1.2 and $Ca_v1.3$ contribute to L-type Ca^{2+} currents in neurons and have already been identified in MNCs through immunocytochemistry (Joux *et al*., 2001) and also through single cell RT-PCR (Glasgow *et al*., 1999). L-type Ca^{2+} channels; $Ca_v1.2$ and $Ca_v1.3$ are predominantly located on the somata and proximal dendrites of MNCs (Glasgow *et al*., 1999; Joux *et al*., 2001) and expression of voltage-gated Ca^{2+} channels on internal membranes was observed in the cytoplasmic pool of MNCs (Fisher *et al*., 2000). Our results are consistent with the increase in total L-type Ca^{2+} currents observed through whole cell patch clamp recordings, which demonstrated 80% increase in total L-type Ca^{2+} currents in both with no change in other Ca^{2+} currents and an increase in L-type Ca^{2+} channels binding sites observed through radio-ligand binding, which demonstrated 32% increase in L-type Ca^{2+}channel density in acutely isolated MNCs from 16-24 hours of chronically dehydrated rats (Zhang *et al*., 2007a). Increases in osmolality do not cause any immediate change in total Ca^{2+} currents (Liu *et al*, 2005). Our data also showed a comparison of increased expression of these channels in VP and OT-MNCs and it was observed that there was no significant difference in expressions of VP (NII) and OT (NI) MNCs. Thus, our data from osmotically-induced increase in expression of L-type Ca^{2+} channels suggest that sustained increases in external osmolality causes increase in number of Ca^{2+} channels in hypertrophied MNCs (VP & OT) by increasing synthesis of channel proteins.

MNCs not only undergo a structural plasticity (hypertrophy) but also many other functional events including signal transduction, gene transcription and translation occur during this long term osmotic stress (Hatton, 1997). The dramatic increase in immunoreactivity of L-type Ca^{2+} channels (Cav1.2 and $Ca_v1.3$) in response to hyperosmotic treatment of MNCs for tens of minutes suggests that an increase in channel synthesis may be a part of this structural plasticity. *In situ* hybridization of MNCs acutely isolated from dehydrated rats demonstrated increase in Na^+ channel mRNA levels suggesting remodeling of electrogenic machinery of MNCs (Tanaka *et al*., 1999) and possibly new L-type Ca^{2+} channels are also synthesized during chronic osmotic stress. Whole cell patch clamp recordings showed increase in total L-type Ca^{2+} currents while RT-PCR to evaluate the increase in the synthesis of any type of Ca^{2+} channels showed that there

was no increase in the mRNA coding for these channels in SON after water deprivation in rats (Zhang *et al*., 2007a) and these results were consistent with the microarray study that suggested no increase in mRNA level for any type of Ca^{2+} channels following sustained increase in external osmolality (Hindmarch *et al*., 2006). Immunocytochemical study at electron microscopic level could find out the increase in synthesis of channels in hypertonic solution and protein synthesis inhibitors such as cycloheximide or emetine could also be used to block synthesis to confirm whether hyperosmotically-induced increases in L-type Ca^{2+} channels ($Ca_v1.2$ & $Ca_v1.3$) immunoreactivity is due to increase in channel synthesis or not.

We could not observe immunolabeling of L-type Ca^{2+} channels selectively in cytoplasmic membranes in our results because of the diffuse staining through out the cytoplasm and plasma membrane so we could not find the translocation of channels on the basis of immunocytochemistry. We cannot rule out the translocation of existing L-type Ca^{2+} channels from internal stores to the plasma membrane during sustained dehydration or long term incubation of MNCs in hyperosmolar solution. L-type Ca^{2+} channels are expressed on the internal membranes of MNCs (Fisher *et al*., 2000) and dynorphin receptors are translocated from internal membrane to cell surface during sustained dehydration (Shuster *et al*., 1999). L-type Ca^{2+} channels are involved in the somatodendritic release of dynorphin in hippocampal neurons, which suggests that L-type Ca^{2+} channels may also be involved in the somatodendritic release of neuropeptides (VP/OT) and co-released peptides (e.g. dynorphin) during osmotically-evoked long term adaptation in MNCs. The translocation of channels could be detected by using fluorescently labeled dihydropyridines or receptor/channel binding ligands and by use of electron microscopy.

5.6 Physiological consequences of hypertrophy and increase in L-type Ca^{2+} channels

Chronic dehydration causes VP-MNCs to undergo hypertrophy, adopt phasic bursts (action potentials that last for tens of seconds with equal silent phases), increase in L-type Ca^{2+} and Na^{+} currents and their respective channels densities and increased expression of VP and dynorphin receptors (Bicknell, 1988; Zhang *et al*., 2007a; Tanaka *et al*., 1999). Structural changes occur selectively in OT-MNCs during lactation and parturition (Hussy, 2002). However, it was recently observed in our lab that osmotically-induced hypertrophy depends on the electrical activity (Na^{+} channels) of MNCs and Ca^{2+} influx through L-type Ca^{2+} channels (unpublished

data). We observed that MNCs hypertrophy in response to hyperosmotic stimuli is associated with increases in total membrane surface area dependent on the exocytotic fusion of intracellular granule with plasma membrane. This suggests that hypertrophy may be involved in the regulation of structural and functional plasticity occurring in HNS for the sustained and high increase of hormone.

L-types Ca^{2+} channels ($Ca_v1.2$ and $Ca_v1.3$) play significant roles including neuropeptide release, secretion and gene transcription in MNC somata and terminals (Fisher & Bourque, 1995; Fisher & Bourque, 1996; Fisher & Bourque, 2001; Foehring & Armstrong, 1996; Joux *et al*., 2001; Glasgow *et al*., 1999). L-type Ca^{2+} channels may be involved in the long term adaptation of MNCs as they play significant roles in gene transcription in neurons. They regulate CREB (cAMP response element binding) protein activation, which is a transcription factor controlled by the Ca^{2+} influx through L-type Ca^{2+} channels and NMDA receptors (Bading *et al*., 1993; Ginty *et al*., 1993; Komendantov *et al*., 2007). Phosphorylation and activation of CREB occurs because Ca^{2+} influx translocates the activated calmodulin to the nucleus (Deisseroth *et al*., 2003). L-type Ca^{2+} channels play this role because of a sequence that enables them to interact with calmodulin and sequences in the C- termini of the $Ca_v1.2$ and $Ca_v1.3$ that enable them to interact with post synaptic adaptor proteins that anchor the channels at the specific location to initiate CREB activation (Dolmetsch *et al*., 2001, Weick *et al*., 2003; Zhang *et al*., 2006). Long term hyperosmotic stress regulate gene expression in MNCs through L-type Ca^{2+} channels ($Ca_v1.2$ & $Ca_v1.3$) (Burbach *et al*., 2001) and causes increase in expression of VP receptors (V_{1a}) (Hurbin *et al*., 2002), Na^+ channels on the plasma membrane and increase in Na^+ and L-type Ca^{2+} currents (Tanaka *et al*., 1999; Zhang *et al*., 2007a). This suggests that increase in L-type Ca^{2+} channels could be important in the long term adaptation for enhancement of gene expression that occur in the development of hypertrophy for the sustained release of hormone.

L-type Ca^{2+} channels may be involved in the somatodendritic release of neuropeptides (VP/OT) and co-peptides (dynorphin & apelin) for the regulation of firing pattern in MNCs during sustained increase in external osmolality. Somatodendritic release have autocrine feed back control on the hormone release (Sladek, 2004; Ludwig & Leng, 2006) and can regulate synaptic inputs on MNCs (Ludwig et al., 2005) while dynorphin may regulate the phasic bursts in VP-MNCs (Brown & Bourque, 2004). Phasic bursts are significant for the sustained release of VP

during chronic dehydration (Hatton, 1997). L-type Ca^{2+} currents regulate dendritic release of dynorphin in hippocampal neurons (Simmons *et al*., 1995). The Ca^{2+} dependent potentials such as DAP, HAP and AHP regulate firing rate and patterns in MNCs, which may be enhanced by the increase in L-type Ca^{2+} currents/channels by depolarizing the plasma membrane due to Ca^{2+} influx through these channels. Increase in L-type Ca^{2+} channels may regulate the phasic bursts in MNCs by somatodendritic release of dynorphin for the sustained release of hormone and in maintenance of hypertrophy of MNCs during dehydration for the fluid homeostasis.

L-type Ca^{2+} channels may regulate the exocytotic fusion of intracellular granule with the plasma membrane for the somatodendritic release of hormones in MNCs. VP/OT release is dependent on SNARE mediated exocytotic fusion (Fisher & Bourque, 2001) and synaptotagmin (a Ca^{2+}sensor) which catalyzes this process (Gustavsson *et al*., 2009). We know that $Ca_v1.2$ and $Ca_v1.3$ have been found associated with the SNARE protein complex during different studies (Wiser *et al*., 1999; Song *et al*., 2003). But there is lack of information on the association of synaptotagmin and L-type Ca^{2+} channels in hypothalamic neurons (Gustavsson *et al*., 2009; Zhang *et al*., 2011). Exocytotic fusion of large dense core granules with the plasma membrane of chromaffin cells is mediated by Ca^{2+} influx through L-type Ca^{2+} channels where these channels enhance the neurotransmitter release by protein kinase C (PKC) translocation and MARKS phosphorylation (Park & Kim, 2009). Osmotically-evoked hypertrophy depends on the Ca^{2+} influx through L-type Ca^{2+} channels (unpublished data) and exocytotic fusion of intracellular granules which suggests that increase in L-type Ca^{2+} channels during sustained increase in external osmoality may regulate the long term adaptation in MNCs.

5.7 Significance of findings to osmotic regulation

Structural and functional adaptations occur in HNS in response to long term osmotic changes and MNCs are sensitive to small changes in external osmolality (Bourque & Oliet, 1997). MNCs undergo hypertrophy as a function of long term external hyperosmolality (Hatton, 1997). We also demonstrated that hypertrophy causes increases in total cell capacitance that reflects an increase in total membrane surface area of MNCs and such finding may lead to further investigation of morphometrical changes in cells during sustained increase in osmolality. Our study indicates that osmotically-mediated hypertrophy in MNCs involves insertion of new membrane on the plasma membrane through SNARE mediated exocytotic fusion. Such finding

may contribute to identify the nature of fusing membrane and relationship of exocytotic SNARE proteins (synaptotagmins) with the hormone release in MNCs during sustained dehydration. Our results of hyperosmolality-evoked increase in immunoexpression of L-type Ca^{2+} channels suggested the increase in synthesis of L-type Ca^{2+} channels that may be significant in finding the role of L-type Ca^{2+} channels in long term adaptation of MNCs for hormone secretion and gene regulation during sustained osmotic stress. Therefore, our studies will be helpful in finding the physiology of MNCs in fluid homeostasis during long term dehydration and pathological conditions that cause increased plasma osmolality.

5.8 Future directions

Our findings will further lead to study and explore the mechanism of hypertrophy and biochemical or physical factors involved in osmotically-induced hypothalamic plasticity. The hypertrophy experiments will lead to use of fluorescence microscopy with membrane specific dyes to see the movements of membranes during hypertrophic response of MNCs to confirm whether hypertrophy depends on the exocytotic fusion of intracellular granules or due to inhibition of endocytosis. Our observations of increase in L-type Ca^{2+} channels during stimulated conditions will direct interest to see whether increase in the synthesis of channels can be prevented by blocking protein synthesis. Translocation of channels from the MNC cytoplasm to plasma membrane during increase in osmolality can be observed by using specific fluorescently labeled ligands of L-type Ca^{2+} channels. Identification of isoforms of synaptotagmin (Ca^{2+} sensor) and syntaxin, which are important members of SNARE protein complex and their association with hypertrophic response and L-type Ca^{2+} channels will also be an important step in exploring osmotically-induced plasticity.

CHAPTER 6

CONCLUSION

MNCs undergo hypertrophy as a result of increases in external osmolality lasting for tens of minutes. This hypertrophy involves an increase in the total membrane area of the MNCs, which suggests insertion of new membrane. The increase in surface area depends on the exocytotic fusion of intracellular vesicle membranes with the plasma membrane. Preliminary findings also suggest that osmotically-evoked hypertrophy is associated with an increase in the number of L-type Ca^{2+} channels in MNCs. The osmotically-evoked hypertrophy and increase in L-type Ca^{2+} channels in MNCs may contribute to the structural and functional adaptation in response to chronic treatment of cells with hyperosmolar solution.

CHAPTER 7

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