COMPARISON OF PYRAMIDAL AND MAGNOCELLULAR
NEUROENDOCRINE CELL VOLUME RESPONSES TO OSMOTIC
STRESS AND STROKE-LIKE STRESS

by

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Abstract

Acute brain cell swelling (cytotoxic edema) can occur in the first minutes of stroke, presumably as a result of brain cells taking up water. In extreme hypo-osmotic situations such as excessive water-loading by patients, uptake by brain cells can expand the brain, causing seizures. But is ischemic brain cell swelling the same as hypo-osmotic swelling?

Water can passively diffuse across the plasma membrane. However the presence of water channels termed aquaporins (AQP) facilitates passive water diffusion by 10-100 times. Unlike astrocytes, there is no evidence of water channels on neuronal plasma membrane. However, there is still much debate about which cells (neurons or astrocytes) swell during over-hydration or during stroke and if neurons and astrocytes can volume-regulate during osmotic stress.

The purpose of this study was to examine and compare the volume responses of PyNs and magnocellular neuroendocrine cells (MNCs) to acute osmotic challenge and to OGD. We examined MNCs because they are intrinsically osmosensitive to small changes (2-3 mOsm) of plasma osmolality. We also examined if the same neurons behave similarly in brain slices or when dissociated and if they respond differently to acute osmotic stress and stroke-like stress.

Our results indicate that during acute osmotic stress (±40 mOsm) half of dissociated PyNs and MNCs tended to show appropriate responses. MNCs in brain slices showed similar responses to when they were dissociated, while brain slice PyNs
were less responsive than when dissociated. Exposure to OGD resulted in obvious differences between the two types of in vitro preparations. Dissociated PyNs and MNCs showed no consistency in their volume responses to 10 minutes of OGD. Dissociated neurons swelled, shrunk or were unchanged in about equal numbers. In contrast, brain slice PyNs underwent profound swelling whereas, brain slice MNCs showed minor volume decreases.

We conclude that about half of our dissociated neurons were too variable and unpredictable in their osmotic volume responses to be useful for osmotic studies. They also were too resistant to stroke-like stress to be good models for ischemia. Brain slice neurons were similar in their osmotic responses to dissociated neurons but proved to have consistent and predictable responses to stroke-like stress.
Co-Authorship

Nipuni Ranepura was responsible for the collection of the dissociated cell culture data and performed all of the data analysis for this thesis. Dr. Andrew collected the 2-photon laser scanning microscopy brain slice data and he also measured their cell volume change. Additionally, Devin Brisson conducted the electrophysiology recordings. The first draft of this thesis was written by Nipuni Ranepura. All subsequent drafts were written in collaboration with Dr. Andrew who provided thorough and essential suggestions.
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<tbody>
<tr>
<td>2PLSM</td>
<td>2-photon laser scanning microscopy</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>AP</td>
<td>Action potentials</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
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<tr>
<td>ADP</td>
<td>After-depolarization potential</td>
</tr>
<tr>
<td>AD</td>
<td>Anoxic depolarization</td>
</tr>
<tr>
<td>AQP</td>
<td>Aquaporin</td>
</tr>
<tr>
<td>Cd⁺²</td>
<td>Cadmium</td>
</tr>
<tr>
<td>Ca⁺²</td>
<td>Calcium</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>Chloride</td>
</tr>
<tr>
<td>CA</td>
<td>Cornu Ammonus</td>
</tr>
<tr>
<td>XS</td>
<td>Cross sectional area</td>
</tr>
<tr>
<td>DAP</td>
<td>Depolarizing after-potential</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
</tr>
<tr>
<td>ECF</td>
<td>Extracellular fluid</td>
</tr>
<tr>
<td>ECS</td>
<td>Extracellular space</td>
</tr>
<tr>
<td>FPP</td>
<td>Fast pre-potential</td>
</tr>
<tr>
<td>Hz</td>
<td>Frequency</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HS</td>
<td>Hypertonic saline</td>
</tr>
<tr>
<td>ICP</td>
<td>Intracranial pressure</td>
</tr>
<tr>
<td>ISM</td>
<td>Ion-selective microelectrodes</td>
</tr>
<tr>
<td>MNC</td>
<td>Magnocellular neuroendocrine cell</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Millivolts</td>
<td>mV</td>
</tr>
<tr>
<td>N-acetyl-L-aspartic acid</td>
<td>NAA</td>
</tr>
<tr>
<td>N-acetyl-aspartyl-glutamic acid</td>
<td>NAAG</td>
</tr>
<tr>
<td>Nucleus of the solitary tract</td>
<td>NTS</td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td>PVN</td>
</tr>
<tr>
<td>Peri-infarct depolarizations</td>
<td>PIDs</td>
</tr>
<tr>
<td>Peripheral nervous system</td>
<td>PNS</td>
</tr>
<tr>
<td>Population spike</td>
<td>PS</td>
</tr>
<tr>
<td>Potassium</td>
<td>K⁺</td>
</tr>
<tr>
<td>Pyramidal neuron</td>
<td>PyN</td>
</tr>
<tr>
<td>Osmolality</td>
<td>Osm</td>
</tr>
<tr>
<td>Organum vasculosum of the lamina terminalis</td>
<td>OVLT</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>OT</td>
</tr>
<tr>
<td>Oxygen glucose deprivation</td>
<td>OGD</td>
</tr>
<tr>
<td>Resting membrane potential</td>
<td>R_{mp}</td>
</tr>
<tr>
<td>Seconds</td>
<td>s</td>
</tr>
<tr>
<td>Sodium</td>
<td>Na⁺</td>
</tr>
<tr>
<td>Subfornical Organ</td>
<td>SFO</td>
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<tr>
<td>Standard error mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Supraoptic nucleus</td>
<td>SON</td>
</tr>
<tr>
<td>Tetraethylammonium</td>
<td>TEA⁺</td>
</tr>
<tr>
<td>Tetramethylammonium</td>
<td>TMA⁺</td>
</tr>
<tr>
<td>Tetrodotoxin</td>
<td>TTX</td>
</tr>
<tr>
<td>Tissue plasminogen activator</td>
<td>tPa</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>VP</td>
</tr>
<tr>
<td>Yellow fluorescent protein</td>
<td>YFP⁺</td>
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1.1 Stroke and Brain Cell Swelling

Stroke is caused by the interruption of oxygenated blood flow into the brain by either ischemia or hemorrhage. Worldwide 15 million people suffer from stroke each year and it is the third leading cause of death in Canada (Heart & Stroke Canada). Despite the prevalence, thrombolytic agents such as tissue plasminogen activator (tPa) are the only approved treatment for ischemic stroke patients. The narrow therapeutic window of four hours from stroke onset restricts the number of patients that can be treated. The complexities of drug development have been exacerbated by variations in pathology (global vs. focal ischemia or cytotoxic vs. vasogenic edema) and the necessity of appropriately matching the cellular mechanisms with the time-frame of the ischemic events.

Cytotoxic edema (cell swelling) is purportedly caused by interstitial water movement into brain cells and occurs soon after ischemia onset. Ultrastructural evidence show astrocytic end-feet swelling within five minutes of energy depletion (Dodson et al., 1977), lasting up to 24 hours after cerebral ischemia (Zhang et al., 1994). Evidence from a number of studies (Amiry-Moghaddam et al., 2003; Manley et al., 2000; Zador et al., 2009) have indicated that AQP4 (water channel) expression on peri-vascular astrocytes has a role in edema formation and progression, and therefore is a prime candidate for a possible treatment strategy. However, AQP4 is only located on glial membranes and does not account for water accumulation in the intracellular compartment of neurons. In fact, it is unclear how and why cortical neurons swell
within minutes of stroke-like conditions. The accepted explanation is that during depolarization sodium (Na\(^{+}\)) and chloride (Cl\(^{-}\)) influx osmotically draws water into neurons, but this is not a physiologically sound argument. First, opening ion channels allow ions to flow down their concentration gradients. So neuronal potassium (K\(^{+}\)) loss should compensate for Na\(^{+}\) that is gained, meaning there is no osmotic gradient for water to follow. It is estimated that intracellular Na\(^{+}\) increases roughly by 30 mM (millimoles), while intracellular K\(^{+}\) decrease by 20 mM (Hansen, 1985; Somjen, 2004). So Na\(^{+}\) and Cl\(^{-}\) increases during anoxic depolarization of 10 mM each would represent an intracellular osmotic increase of 20 mOsm. This cannot account for the massive PyN swelling post-anoxic depolarization (AD). Second, transmembrane water cannot quickly follow without AQPs. As well, Cl\(^{-}\), K\(^{+}\) and Na\(^{+}\) ions are stripped of their hydration shells as each transits their specific voltage- or ligand-gated channels. For the channel to ‘see’ the ion and let it pass, the ion must be stripped of its water shell as it enters the channel. Filter selectivity depends on ion dehydration. For example, the K\(^{+}\) ion just fits into its channel structure and the pore’s oxygen atoms comprises the ‘K\(^{+}\) filter’, which mimics the lost water shell. Na\(^{+}\) is too small for this pore to remove the Na\(^{+}\) shell, so Na\(^{+}\) is unable to pass. So it is unclear how water flows into ischemic neurons to cause swelling (Ganesh, et al., 2000; Hucho & Weise, 2001; Doyle et al., 1998; Dutzler, 2004).

Therefore, a better understanding of how neuronal volume changes are mediated during osmotic or ischemic stress is required to develop a treatment strategy to reverse post-ischemic cytotoxic edema that rapidly develops in the first minutes of stroke (Fraser & Andrew, 2008).
1.2 Neuroendocrine Nuclei of the Hypothalamus

Homeostasis is defined as the internal balancing of physiological processes that promotes long term survival of an organism. The hypothalamus, located in the ventral portion of the diencephalon is the main site in the central nervous system (CNS) responsible for the maintenance of homeostatic regulation. The hypothalamus functions as the link between the CNS and the endocrine system via the hypophysis (pituitary gland). By integrating ascending and descending neural information with hormonal signals, the hypothalamus is able to generate physiological changes to maintain internal balance around theoretical set points (Hatton, 1990; Tasker, 2006). The hypophysis is divided into two separate regions, the adenohypophysis (anterior pituitary) and neurohypophysis (posterior pituitary). The adenohypophysis is a major endocrine organ that regulates physiological processes such as, stress, growth and reproduction. Although the adenohypophysis has a key role in homeostasis, the remainder of this discussion will focus on the function of the neurohypophysis and its link with two specific hypothalamic nuclei the paraventricular (PVN) and the supraoptic (SON) (Hatton, 1990; Swanson & Sawchenko, 1983).

The SON is located in the lateral aspects of the optic chiasm and optic tracts. The PVN is found on either side of the dorsal third ventricle. Both nuclei regulate homeostatic processes involved in the stress response, feeding behaviour, fluid balance, cardiovascular function, body temperature and reproductive function (Hatton, 1990; Tasker, 2006). The majority of SON and PVN efferents terminate at the neurohypophysis where a number of peptides synthesized in MNCs are released into systemic circulation (Swanson & Sawchenko, 1983).
Paraventricular Nucleus

The PVN is an integrative site for neuroendocrine and autonomic functions. This complex nucleus is divided into eight subdivisions; three of which are MNC regions, and the remaining five are parvocellular regions (Armstrong & Hatton, 1980; Morris, 1983). The anterior and medial portions of the PVN are mostly oxytocin (OT)-expressing MNCs cells, whereas the vasopressin (VP)-expressing MNCs are concentrated to the posterior and dorso-lateral portions. In rats, the MNC dendrites are directed medially or ventro-medially, suggesting the majority of the dendrites remain within the subdivision in which the cell body lies. The MNC axons of the PVN leave the nucleus passing above and below the fornix, then arch towards and through the median eminence to reach the neurohypophysis (Swanson & Sawchenko, 1983).

Two subtypes of parvocellular neurons, pre-autonomic and neuroendocrine are also present in the PVN. Parvocellular neurons are smaller (10-15 μm) than MNCs and have 2-3 short primary dendrites. The dendrites branch out locally and contact dendrites of cells in both MNC and parvocellular divisions. As well as synthesizing and releasing OT and VP, pre-autonomic neurons synthesize somatostatin, corticotrophin, and thyrotropin-releasing hormone (Morris, 1983; Swanson & Sawchenko, 1983). Neuroendocrine cells are positioned medially long the third ventricle and synthesize somatosatin, corticotrophin, thyrotropin-releasing hormone, neurotensin, and dopamine among others, in addition to OT and VP. Both pre-autonomic and neuroendocrine parvocellular neurons project to the median eminence where the peptides are released to regulate adenohypophysis hormone release (Morris, 1983; Swanson & Sawchenko, 1980; Swanson & Sawchenko, 1983).
The PVN receives input from three main brain regions, the hypothalamus, extra-hypothalamic nuclei and the brainstem. The anterior and lateral hypothalamic, ventromedial, periventricular nuclei and the pre-optic area all send projections to the PVN. Two of the three circumventricular organs, the organum vasculosum laminae terminalis (OVLT) and the subfornical organ (SFO) (extra-hypothalamic nuclei), send projections that synapse with neurons in the PVN. The PVN (and SON) receive prominent catecholaminergic fibres from cell groups A1 (and C1), A2 and A6, which lie in the ventral medulla, nucleus of the solitary tract (NTS) and locus coeruleus, respectively (Swanson & Sawchenko, 1983).

Supraoptic Nucleus

The SON contains only MNCs anatomically segregated into OT and VP-expressing MNCs. OT-expressing MNCs are concentrated in the antero-dorsal SON and VP-expressing cells in postero-ventral SON (Swanson & Sawchenko, 1983). A small subpopulation of MNCs synthesizes both OT and VP. The synthesized OT and VP migrate to the neurohypophysis along the supraoptic-hypophysial tract. The majority of MNC axons in the SON contribute to this tract, whereas many dendrites of the MNCs extend into the ventral glial lamina of the SON (Hatton, 1990; Morris, 1983).

1.3 Magnocellular Neuroendocrine Cells

MNCs located in the PVN, SON and some small accessory nuclei, have a large cell body ranging from 25-40 μm in diameter with two or three simple dendrites. Each MNC sends a single axon into the neurohypophysis giving rise to 2,000-10,000 neurosecretory endings containing
neurosecretory granules of 150 nm in diameter (Theodosis & Poulain, 1993). Each granule is full of OT or VP and neurophysin (carrier protein), originally synthesized in the rough endoplasmic reticulum. After packaging of the neuropeptides at the cell body, the MNC’s axons transport the vesicles via fast axonal transport through the internal zone of the median eminence to terminate in the neurohypophysis (Renaud & Bourque, 1991). The release of the neuropeptides is triggered by action potentials (APs) generated at the MNC cell body that propagate down the axon, causing prolonged opening of voltage-dependent calcium (Ca²⁺) channels at the neurohypophysis. The influx of Ca²⁺ in the nerve terminal results in the granule membrane fusing with the nerve terminal membrane triggering exocytosis of the granules and release of OT or VP into the extracellular space (ECS). The neuropeptides diffuse into the fenestrated capillary vessels of the neurohypophysis and enter the systemic circulation, causing a peripheral response within seconds (Armstrong, 1995; Hatton, 1990; Voisin & Bourque, 2002).

Stimuli that induce long-term OT and VP release (acute or chronic) also cause retraction of astrocytic processes from between the somata, allowing large areas of soma-somatic contact. During astrocytic retraction, adjacent MNCs increase their electrical interaction (Tweedle & Hatton, 1980; Theodosis & Poulain, 1993; Theodosis et al., 1981) by sharing increased synaptic input from afferents to the cell bodies and by increased electronic coupling. This electrical coordination may explain AP pattern generation (phasic vs. bursts) and assist in OT and VP release from the neurohypophysis during stimulation. After cessation of the stimuli, the astrocytic processes reinsert between the somata over many hours (Perlmutter et al., 1985).
A number of cytological modifications occur within the MNC somata during increased secretory activity caused by stimulation (i.e., cardiovascular, osmotic, suckling). Specifically, there are increases in both cytoplasmic and nuclear volumes and enlargement of golgi and lysosomes organelles. These changes are associated with increased synthesis and release of OT and VP (Finan & Guilak, 2010; Theodosis & Poulain, 1993). In transgenic rats where green fluorescent protein (GFP+) is linked to VP expression, chronic dehydration increases the size of MNCs and the amount of fluorescent VP that they contain (Fujio et al., 2006).

MNCs are also influenced by non-synaptic inputs. The PVN is located near the third ventricle with dendrites projecting medially to the subependymal layer. These may be influenced by the diffusion of molecules from the cerebral spinal fluid (CSF) via the ECS. The SON has dendritic endings that are closely associated with the pial surface and the fluid compartment of the subarachnoid space. Therefore, both the PVN and SON receive indirect input from the extracellular fluid (ECF) microenvironment (Hatton, 1990; Morris, 1983).

**Oxytocin and Vasopressin Neuropeptide Release**

OT and VP are both nine amino acid peptides and are similar in structure, differing by two amino acids. Synthesized by a common precursor molecule, OT and VP along with their respective neurophysin are cleaved from their precursor molecule and packed separately into neurosecretory granules (Gimpl & Fahrenholz, 2001; Morris, 1983).

OT is distributed throughout the brain with the highest densities in the SON and PVN. In rats, OT release is accompanied with VP release except during suckling and parturition stimuli (Gimpl & Fahrenholz, 2001). OT acts on mammary and uterine tissue via a G-protein coupled
cell surface receptor. Suckling induces APs that are relayed to the OT-expressing MNCs, which in turn begin firing APs in a synchronized burst, resulting in pulsatile OT release from the neurohypophysis. In mammary tissue, OT stimulates the contraction of myoepithelial cells resulting in milk ejection. OT also induces the contraction of the uterine wall during parturition. Impulse firing triggers MNCs to release more OT, enhancing labor (Swanson & Sawchenko, 1983). OT also has a prominent role in cardiovascular regulation and is released in response to changes in plasma osmolality (osmol/kg or Osm) and blood volume (Gimpl & Fahrenholz, 2001).

During physiological situations where a large amount of OT is released into circulation, MNCs undergo structural changes. There are increases in space between neurosecretory terminals and perivascular space, and reduced astrocytic investiture. The changes are reversible within hours upon termination of stimulation. These changes may facilitate and maintain the characteristic synchronized electrical activity from MNCs during milk ejection (Gimpl & Fahrenholz, 2001).

Furthermore, MNCs show characteristic secretion patterns according to different stimuli. During hypertonic stimulation, OT neurons respond with small increases in spontaneous firing rate, whereas during lactation the same OT neurons respond with highly synchronized bursts of activity (Gimpl & Fahrenholz, 2001).

VP (anti-diuretic hormone) is released in response to fluctuations in plasma osmolality and is detected by peripheral receptors located near the portal vein and central receptors present near the third ventricle. VP is also released in response to decreased blood pressure, sensed by
baroreceptors located in the left heart, aortic arch and carotid sinus. VP exerts its physiological effects through second messenger G-protein coupled receptors located throughout the body and its release is stimulated by Na\(^+\) excretion, hemorrhage and increased plasma osmotic pressure (Leng et al., 1998).

Upon dehydration, released VP acts specifically in the distal convoluted tubules and collecting ducts of the kidney, where it stimulates water channels called AQPs to incorporate into the luminal membrane. This rapidly increases water permeability, increasing water retention and decreasing urine volume, thereby restoring plasma osmolality. In addition to effects of VP in the kidneys, the neuropeptide can also act as a constrictor in vascular smooth muscle, increasing systemic blood pressure. Furthermore, the anterior pituitary gland has VP receptors, allowing VP to influence the hypophysis itself (Amar & Weiss, 2003; Gimpl & Fahrenholz, 2001). Thus, VP is a key modulator of water regulation, cardiac output and the endocrine system that allows different systems of the body to work collectively to achieve homeostasis.

1.4 Pyramidal Neurons

Primarily located in the neocortex and hippocampus, PyNs are among the largest neurons in the brain. In rats, soma size can range from 20-40 \(\mu\text{m}\) in diameter and the total dendritic tree length can reach up to 790 \(\mu\text{m}\) (Garcia-Lopez et al., 2006). PyNs located in hippocampus and neocortex have the same range of resting membrane potentials (\(R_{\text{mp}}\)) of -60 to -68 mV (Andersen et al., 2007).
The hippocampus proper is a curved bi-lateral cortical structure comprised of three layers called the *Cornu Ammonus* (CA) regions, hence CA1, CA2 and CA3. The dentate gyrus, entorhinal cortex, subiculum, together with the hippocampus proper comprises the ‘hippocampal formation’. The CA regions are organized into layers, from the inside out they are: st. lacunsum, st. radiatum, st. pyramidale, st. oriens and the alveus. The st. pyramidale layer contains the cell bodies of the PyNs. Apical dendrites of CA1 PyNs are the major component of the st. radiatum and st. lacunosum layers and their basal dendrites make up the st. oriens layer. The alveus is the main output of the hippocampus proper and is comprised of PyNs axons that project to the fornix or the contralateral hippocampus. PyNs in the CA1 and CA3 both have pyramid-shaped somata and have apical and basal dendrites. CA3 PyNs have larger cell bodies and shorter dendritic trees than CA1 neurons. In rats, cell numbers reach over 300,000 CA1 and over 400,000 CA3 neurons (Amaral et al., 1990).

PyNs comprise the majority of neurons (60-70%) found in the neocortex. The neocortex has six layers and PyN somata can be found in layers II to VI. Neocortical PyNs are the output neurons of the neocortex. They have a high density of dendritic spines, prominent apical dendrites, excitatory synaptic function and their axon projects out of the cortex as well as locally. Intrinsically bursting PyNs are specifically located in layer V and have axons that mainly project to subcortical structures, but they also extend throughout layer V and VI projecting horizontally for several millimeters (Conners & Gutnick, 1990).

1.5 Osmosensation in the Central Nervous System
The hydration state of a cell has a profound influence on cell performance and cell survival. Fluctuations in intracellular or extracellular osmolality affect both the volume and the function of the cell. In mammals, an increase or decrease of 1% in plasma osmolality (2.9 mOsm/kg) is enough to induce physiological and behavioural responses that restore osmolality to the defined ‘set point’ over many minutes. Thus, the osmoregulatory system is highly sensitive since it is able to elicit a homeostatic response to the slightest changes in plasma water concentration (Baylis, 1987; Dunn et al., 1973). Only rarely are osmotic changes of ±20 mOsm induced over tens of minutes (i.e., acutely). Yet the majority of cell volume studies have used large osmotic shifts of ±60-180 mOsm induced over several minutes to produce responses that may not reflect physiological responses in vivo.

Osmosensation by specialized cells called osmoreceptors are located in the CNS and peripheral nervous system (PNS). These osmoreceptors are highly sensitive to the slightest change in plasma osmolality and collectively with other sensors transmit homeostatic information such as blood pressure, Na⁺ concentration and body temperature to different regions of brain to activate (or inhibit) osmoregulatory responses (Bourque, 2008).

The plasma osmolality in humans is ~285 mOsm/kg, in rats is ~295 mOsm/kg and in mice is ~305 mOsm/kg. (Rooke & Baylis, 1982; Dunn et al., 1973; Robertson et al., 1976). This value is a ‘set point’ around which plasma osmolality fluctuates. By either promoting or suppressing the release of VP and drinking behaviour, the plasma osmolality returns to the set point. VP is released in response to elevated plasma osmolality and functions to restore plasma osmolality to the set point by promoting water conservation. Basal concentration of VP in
humans is ~2.5 pg/mL (Robertson et al., 1976) and is similar to the concentration of 2.3 ±0.9 pg/mL found in rats (Dunn et al., 1973). In humans, a state of overhydration occurs when plasma osmolality drops below the set point to ~281 mOsm/kg and then minimal plasma VP (~0.25-0.45 pg/mL) is secreted, and maximal water diuresis occurs. As plasma osmolality increases above the set point, maximal plasma VP concentration nears ~3.7 pg/mL, causing progressive antidiuresis and initiates drinking behaviour. Both then lower plasma osmolality back to the set point (Rooke & Baylis, 1982).

A short period of water deprivation (4-24 hours) causes an acute increase in plasma osmolality, inducing maximal VP secretion. After 12 hours of water restriction in rats, the concentration of plasma VP increases in response to an elevated plasma osmolality (less than 2% increase) (Dunn et al., 1973; Perlmutter et al., 1985). In humans, a hypertonic saline (HS) injection will drive plasma osmolality levels to the antidiuresis threshold of 292 mOsm/kg. Within a few hours, maximal VP secretion occurs (Baylis, 1987; Dunn et al., 1973). As opposed to acute osmotic shifts (a change of ~10 mOsm over many minutes to several hours), chronic dehydration over 2-7 days dramatically increases plasma osmolality. Osmotic shifts of ~60 mOsm can be tolerated by mammals if there is a gradual rise in osmolality over several days (Fuijo et al., 2006). This is because brain cells have the time to adjust their internal osmolality to the level of the extracellular osmolality. Thus, cell volume is controlled (volume-regulation) over hours and days, as idiogenic osmoles (osmoles of uncertain origin such as amino acids) increase or decrease intracellular to match extracellular osmolality (Andrew, 1991).

**Osmoreceptors**
Osmoresponsive neurons of the CNS may display changes in firing frequency (Hz) or volume during osmotic stimulation but this does not necessarily classify them as an ‘osmosensitive’. Neurons that can intrinsically detect changes in extracellular fluid (ECF) osmolality from a set point and translate the osmotic stimuli into electrical signals are by definition, an osmosensitive neuron. In other words, they behave as a true osmometer. As ECF osmolality fluctuates, osmosensitive neurons show proportional changes in AP firing rate. Osmotic shifts caused by consumed food and liquids are first detected by peripheral osmoreceptors. Their osmosensory efferents project into the CNS to promote responses that will buffer the impact of osmotic shifts (Bourque, 1998; Bourque, 2008). Studies have shown neurons present in the OVLT and SFO neurons, in addition to MNCs, function as osmosensitive neurons.

Electrophysiological studies indicate that the rate of AP discharge in a subset of OVLT neurons varies positively with fluid osmolality (Sayer et al., 1984; Ciura & Bourque, 2006), a behaviour also seen in isolated OVLT neurons (Ciura & Bourque, 2006). Such behaviour is intrinsic to the neuron because it occurs when synaptic transmission is blocked (Vivas et al., 1990). In rats, when hypertonic (HS) is injected into the OVLT area, drinking is triggered and VP is released. Lesions to this area prevent VP release in response to ECF hyperosmolality (Buggy & Johnson, 1977; Thrasher & Keil, 1987).

Anderson et al. (2000) demonstrated the intrinsic osmosensitivity of dissociated SFO neurons. Electrophysiological recordings indicated neuron hyperpolarization and a decrease in firing rate during hypo-osmotic stimuli, whereas during hyperosmotic stimuli elicited an increase in firing rate and depolarization. Importantly, the recordings were tested at osmolalities within a
physiological range of 270-330mOsm, further indicating that SFO neurons behave as natural osmometers.

**Osmoresponsive Properties of MNCs**

Neuropeptide secretion from the SON and PVN is correlated with changes in electrical activity of the MNCs. The baseline AP firing rate is 1-3 Hz, inducing basal VP secretion. The firing rate varies as a positive function of ECF osmolality. MNCs are proportionally excited by hyperosmotic stimuli and inhibited by hypo-osmotic stimuli (Bourque, 1998). This is mediated by non-selective cation channels. A hyperosmotic stimulus increases MNC firing rate by the activation of non-selective cation current that depolarizes the membrane potential (Bourque, 1989). Studies using isolated MNCs showed hypo-osmotic stimuli inhibit firing by hyperpolarizing the membrane potential caused by a decrease in cation conductance (Oliet & Bourque, 1993).

As plasma osmolality rises, MNC firing increases, displaying characteristic ‘phasic bursts’. In vivo VP-expressing MNCs exhibit phasic bursts for 20-40 seconds (s) at a rate of 5-10 spikes/s and each burst is separated by 20 s silent periods. The phasic bursts are asynchronous between neurons, so the overall VP release from the neurohypophysis is continuous, not pulsatile (Leng et al., 1998).

Using recordings of MNCs in rat hypothalamic slices, Andrew & Dudek (1983) showed that after each AP there is a depolarizing after-potential (DAP). The DAPs summate temporally to form a plateau potential, bringing the membrane potential closer to the spike threshold and increasing the possibility of further spikes. The DAPs from each spike summate promoting
longer bursts. To show MNCs can intrinsically burst, synaptic transmission was blocked by incubating hypothalamic slices in high cadmium (Cd\(^{2+}\)). After synaptic communication was eliminated, MNCs still generated plateau potentials, indicating that the spikes were endogenously generated within the neuron by previous action potentials and did not require synaptic transmission. Further supporting that MNCs can intrinsically generate bursts, after tetrodotoxin (TTX) application which interrupts Na\(^{+}\) spike generation and blocks synaptic communication, the plateau potential was still present in hypothalamic slice when Ca\(^{2+}\) spikes were evoked (Andrew & Dudek, 1983). In a similar way, Mason (1980) showed that the firing patterns and depolarization of SON cells were evoked by increased osmolality and were reversible when cells were restored to control saline. Moreover, when synaptic input was blocked in hypothalamic slices, the SON cells were still osmosensitive.

Burst termination probably results from a gradual increase of endogenous inhibitory mechanisms involving Ca\(^{2+}\)-activated K\(^{+}\) conductance. The bursts progress through activity dependent inactivation of the plateau potential, reducing the probability of post-synaptic potentials causing more spikes to be generated (Andrew & Dudek, 1983; Brown et al., 2004).

The majority of research indicates MNCs are intrinsically osmosensitive as mentioned above. An alternate view or a supporting mechanism proposes a paracrine action from nearby astrocytes in combination with intrinsic properties of MNCs. Taurine (organic acid) is an agonist of glycine receptors (strychnine sensitive-chloride channels) and functions to dis-inhibit MNCs (Brown et al., 2004). The surrounding astrocytes release taurine as an inverse function of osmolality. Hyperosmotic stimuli can promote excitation of MNCs by eliminating the
hypermomlarizing effect of glycine receptors normally caused by taurine release (Hussy et al., 1997; Deleuze et al., 1998). Isolated SON neurons are highly sensitive to the increase of taurine release, which contributes to the inhibitory effect of hypo-osmotic stimuli. This effect can be blocked by Cl⁻ blockers. For instance in hydrated rats, strychnine blocks glycine receptors, increasing the basal activity of VP-expressing MNCs (Hussy et al., 1997; Deleuze et al., 1998).

**Osmoresponsive Properties of PyNs**

In a series of papers examining CA1, CA3 and neocortical PyNs in brain slices, Andrew and co-workers showed that the excitability of these neurons were increased by acute hypo-osmolality exposure (20-60 mOsm) and decreased by acute hyperosmolality. In particular, burst firing was inversely affected by the level of osmolality, helping to explain why epileptiform activity is promoted in patients where plasma osmolality drops acutely. So the osmotic effects on the excitability of the hippocampal CA1 neurons are not mediated by changes in the intrinsic properties of PyNs (Andrew et al., 1989; Ballyk et al., 1991). Azouz et al. (1997) confirmed that acute osmolality shifts of ±13% had no effect on resting membrane potential, cell input resistance, spike threshold and amplitude and several spike after-hyperpolarizations (AHPs). Therefore it is generally agreed that PyNs, like most neurons, are not classically osmosensitive (Ballyk et al., 1991; Azouz et al., 1997). However Azouz et al. (1997) also demonstrated the spike after-depolarization potential (ADP) was inversely affected by the level of osmolality, indicating that enhanced ADPs contributed to the epileptiform activity induced by hypo-osmolality. It is also clear that the elevated excitatory synaptic activity first reported during acute hypo-osmolality (Saly & Andrew, 1993) is the result of alteration to several unidentified currents.
pre- and post-synaptically (Barban & Schwartzkroin, 1998; Huang et al., 1997). Although PyNs do not display classic intrinsic osmosensitivity (osmotic stimuli does not induce proportional changes in their resting membrane potential), single PyNs are osmoreponsive by virtue of their ADPs. Moreover, altered synaptic input reinforces the effect on the ADP.

Osmotic effects on the excitability of hippocampal CA1 population are also indirectly mediated by altered field effects (ephaptic effects) during synchronous discharge (Ballyk et al., 1991). Field effects are mediated by passive current flow from adjacent cells and are considered a mode of cell to cell communication (Yim et al., 1986). Extracellular resistance has a major influence on field effect strength. Current generated by the firing neurons (the extracellular field potential) travels through the resistance of the ECS and pass into the neighbouring quiescent neurons which causes a brief depolarization, the so-called fast pre-potential (FPP). As the astrocytes swell during hypo-osmotic stimuli, there is an increase in extracellular resistance during which each field burst tends to increase population spike (PS) amplitude (Andrew et al., 2007). This increases the FPP in each cell, driving more PyNs to threshold. The converse is also true: during hyperosmotic stimuli, field effects are reduced by the decreased extracellular resistance as cells shrink (Fox et al., 2004).

1.6 Cell Swelling and In Vitro Stroke Models

Animal models are developed to mimic human stroke pathology, to allow investigation of molecular mechanisms responsible for ischemic tissue damage and to develop an effective and safe treatment. Numerous drugs have been found effective in rodent models but those
subsequently tested in a clinical setting are consistently unsuccessful. Clinical aspects of stroke involve high variability in localization, duration, severity of ischemia and co-existing systemic disease among patient populations, making it difficult to test the efficacy of a drug in a clinical trial (deCourten-Myers & Wagner, 1992; Durukan & Tatlisumak, 2007). The failure to develop an effective treatment is also due to the variability across experimental models and methodology. For example, a small decrease in brain temperature can result in a protective effect, whereas a small increase can exacerbate ischemic injury. Variations in cardiorespiratory function, anaesthesia type, age, gender and species also contribute to incorrect interpretation of data and conclusions. Despite the inconsistency among experiments, rodent models remain popular because of transgenic variations and technical simplicity.

In vitro techniques represent a less complicated approach to study the underlying molecular mechanisms of stroke. A brain region of interest is dissected and placed into a controlled environment allowing variables such as temperature, pH and oxygen to be continuously monitored and altered accordingly (Lipton, 1999). However, an environment outside the intact brain introduces conditions that are simulated and less physiological.

**Brain Slices**

Live brain slices (300-500 μm thick) are useful for studying some aspects of stroke and have many experimental advantages. The similarity between brain slices and in vivo tissue means the preservation of local cytoarchitecture and synaptic circuitry, glia-neuronal interactions, glia-glia coupling and intrinsic neuronal properties will generate more physiologically accurate events (Lipton, 1999). Additionally in osmotic studies, it is easier to
distinguish between true osmotic effects seen in clinical disorders from physiological processes since there is no influence from homoeostatic mechanisms such as VP secretion. Also, continuous saline superfusion of the brain slice allows for rapid exchange, thereby controlling the timing of treatment saline such as hyper-hypotonic saline or drugs (Andrew et al., 1989).

The preparation of the brain slice itself (dissecting and slicing) stimulates transcription of stress mRNA. But acutely prepared brain slices do not undergo genetic modification because there is not enough time between tissue preparation and testing. Some, but not all afferent and efferent projections are lost due to the thinness of the slice (300-500 µm). However, this is advantageous for imaging studies to view individual nuclei and neurons, and also for electrode placement.

Although brain slices may lead to a better understanding of morphological and physiological changes that occur in in vivo tissue, they are only able to provide evidence for underlying mechanisms and the same mechanisms must be implicated in vivo. Brain slices function at an altered metabolic state, with low adenosine-5'-triphosphate (ATP) levels and high aerobic glycolysis and are typically more sensitive to ischemia-like stress (Zhou et al., 1995). The most common method to simulate ischemia in the brain slice is to superfuse the brain slice in OGD saline, inducing a global-like ischemic event (Lipton, 1999; Richard et al., 2010). OGD induces AD in mouse brain slices (Joshi & Andrew, 2001) and in rat brain slices (Jarvis et al., 2001) within a time frame comparable to in vivo models of ischemia (Murphy et al., 2008).

However, focal ischemia is more prominent in patient population. Establishing a focal ischemia model to study the cellular mechanisms involved in the progression of an ischemic
event is more difficult compared to simulating global ischemia (Richard et al., 2010). A focal application of OGD saline onto the brain slice simulates a gradient of ischemic damage from the region of most severe damage (“core”) to the outermost boundary of the insult (“penumbra”). However, a better method may be to harvest live brain slices from animals stroked out only hours before testing. This method is now being carried out in the Andrew lab in collaboration with Dr. A. Jin.

**Isolated Neurons**

Cell cultures derived from embryonic and perinatal rats and mice are a reductionist but popular method to study ischemia-like damage (Rothman, 1984). Cell cultures allow investigation of a single cell (neuron or glia) or a heterogeneous population of cells and allow for direct visualization of morphological changes during live cell imaging. Easy manipulation and fewer uncontrollable variables are other advantages of cell cultures (Falkenburger & Schulz, 2006).

One major problem of cell dissociation is the loss of the astrocytic-neuronal relationship because during the dissociation procedure neurons lose their dendrites and axons. Loss of synaptic interaction with other neurons will not yield information on how the cell functions within its network. Consequently, dissociated neurons are less like live brain slices, with the exception of their intrinsic properties preserved in the cell body.

Another difference between cultured neurons and those in situ is that OGD-induced damage in cultured neurons requires a longer insult time to cause cell death. Cells grown in culture usually have a small drop in ATP production and as a result may develop protective
mechanisms that reduce ATP turnover during hypoxia (Hochachka, 1996). This is a plausible explanation because cultures are usually grown for days before testing, which is an ample time for changes in genetic expression to occur. Furthermore, the dissociation procedure uses enzymatic and mechanical treatments to breakdown the tissue and the resulting dissociated cells are suspended in media that do not mimic in vivo physiological conditions. For instance, HEPES-buffered solution, used during the dissociated procedure, lacks certain amino acids and bicarbonate, but has an excess of glucose (Aitken, 1998). Also, culture medium such as Neurobasal-A and Hibernate have an osmolality of ~260 mOsm which is often not corrected to ~290-300 mOsm by experimenters. Essentially, the dissociated cells are removed from ~290-300 mOsm environment (the brain), then grown and sustained in hypo-osmotic media for hours or days, inducing changes within the cell. Longer term changes must be ruled out by showing that cells only dissociated for a few hours behave similarly to those isolated over days.

1.7 Anoxic Depolarization, Peri-infarct Depolarizations and Stroke

As a consequence of the brains’ dependence on high glucose and oxygen levels and its’ low energy reserves, the brain is more susceptible to ischemic injury than any other tissue in the body. As energy stores (ATP) begin to deplete, ion pumps such as the Na\(^+\)- K\(^+\) ATPase pump fail, immediately leading to ion fluxes that degrade the brain cell’s metabolic function and structure (Hansen, 1985). This energy-draining AD initiates within minutes of ischemia and is characterized by a concurrent increase in extracellular K\(^+\) (from 3 to ~50-60 mM; Tasker, 1999) and a large decline in extracellular Na\(^+\) (from 130 to ~50 mM), Ca\(^{2+}\) and Cl\(^-\) (~2 to 0.01 mM)
concentrations (Lipton, 1999). Potassium increases are also due to Ca\(^{2+}\) – activated K\(^+\) channels opening as intracellular Ca\(^{2+}\) levels gradually increase prior to AD onset (Lipton & Lobner, 1990). The ion fluxes are probably mediated through voltage-gated Na\(^+\), Ca\(^{2+}\) and K\(^+\) channels, as well as ion exchangers that are dependent on Na\(^+\) concentrations. Reversal of their function during AD induces Na\(^+\) influx and water supposedly follows Na\(^+\) and Cl\(^-\) into the neuron, but this is unlikely, as discussed earlier (refer to 1.1 Stroke and Brain Cell Swelling).

Minutes and hours after stroke onset, recurring waves of per-infarct depolarizations (PIDs) originating near the initial site of injury spread across the cerebral gray matter. The PIDs migrate into regions of compromised blood flow, consuming energy stores (ATP levels decrease to ~50%) and possibly increasing the damaged area. As a result, neuronal cell bodies are left swollen and their processes beaded, as observed in brain slices (Jarvis et al., 2001), whole isolated cortical preparations (Davies et al., 2007) and in vivo (Murphy et al., 2008). Although both neurons and astrocytes undergo AD, astrocytes are capable of recovery to near resting potential and are less depolarized. Astrocytes in cortical slices rapidly swell in response to OGD for 10 minutes. But upon re-exposure to oxygen and glucose, they quickly regain their volume, whereas PyNs remain swollen with beaded dendrites (Risher et al., 2009).

PyNs in the CA1 region are highly prone to damage caused by OGD. Within six minutes of OGD, a wave of AD passes through the CA1 pyramidal layer (Jarvis et al., 2001). In rats, by 10 minutes of ischemia in vivo, CA1 PyN neurons are completely damage and unable to recover (Murphy, et al., 2008). In contrast, MNCs in hypothalamic slices undergo a weaker AD and do not show soma swelling or beading of their dendrites (Brisson & Andrew, 2010 – SfN abstract).
1.8 Measuring Volume Changes of Brain Cells

There are several ways to measure changes in brain cell volume. First, ion-selective microelectrodes (ISM) can be used to measure brain extracellular volume via ion concentration measurements. Ion marker molecules such as tetraethylammonium (TEA\(^+\)) and tetramethylammonium (TMA\(^+\)) are both impermeable to cell membranes and are used to study ECS volume. The TMA\(^+\) marker is injected into ECS by applying current (+80 nA) for 40-80 s and using ISM, TMA\(^+\) activity is recorded. Then the TMA\(^+\) activity is fitted onto a diffusion curve and the values of volume fraction (\(\alpha\) = ratio between the volume of the ECS and the total volume of the tissue), tortuosity (\(\lambda\) = the hindrance imposed by cellular structures for diffusion in that medium), and concentration of TMA\(^+\) is derived to show ECS volume changes under pathophysiological states (Sykova, 2004).

In healthy adult brain tissue, the ECS \(\alpha\) is 0.20, indicating that ECS composes 20% of total tissue volume and the \(\lambda\) is \(~1.6\). During ischemia and anoxia, \(\alpha\) decreases to \(~0.05\) in rat spinal cord and cortex, and \(\lambda\) increases to \(~2.1\) (Nicholson et al., 1979; Sykova & Nicholson, 2008). This implies an increase in brain cell volume. Limitations of ISM include damage from direct contact of the micropipette onto the measurement site on the cell, which can cause more K\(^+\) release. Also, TMA\(^+\) can be taken up by astrocytes and TMA\(^+\) may diffuse way from the area of interest into surrounding tissue or bath solution thereby obscuring true ECS measurement (Holthoff & Witte, 1996; Binder et al., 2004).

Electrical resistance measurement is a second method for measuring cellular volume changes. A constant current pulse is applied to the ECS and the measured resistance will indicate
an ECS increase or decrease. During hypo-osmotic conditions, the increase in resistance between the cells indicates ECS shrinkage caused by cell swelling. Conversely, hyper-osmotic conditions tissue decrease resistance as cells shrink and ECS increases. It cannot be determined if neurons or astrocytes or both are changing volume.

Neither technique above can measure the volume change of single cells. Measuring single cell capacitance is an effective method. Capacitance can be calculated from the rate and amplitude of voltage or current responses, under current and voltage clamp, respectively. As the cell membrane surface area increases, the ability of the cell membrane to hold charge increases thereby, increasing the measured membrane capacitance. In cell volume studies, capacitance is measured by injecting a hyperpolarizing pulse. A measured high capacitance means a large volume and a measured low capacitance indicates a small volume (O’Connor et al., 1993; Gentet et al., 2000). However, capacitance measurements will be obscured if the membrane stretches.

Morphological measurement of cell volume using cross sectional area (XS) or diameters is effective but only semi-quantitative because actual volume changes occur in three dimensions (O’Connor et al., 1993; Somjen et al., 1993). However, cross-sectional measurement is a straight-forward technique that can easily be conducted on isolated cells and is possible in brain slices by using a 2PLSM or differential interference contrast (DIC), microscopy near the slice surface.

1.9 The Aquaporin 4 Water Channel
The recovery and survival of patients with stroke, hemorrhage and traumatic brain injury depends on the reduction and reversal of cerebral edema. Pharmaceutical targeting of AQPs has shed new opportunity for therapeutic intervention of cerebral edema. To date, thirteen AQP channels are found throughout the human body. Separated into two classes, AQPs are selective for water, while aquaglyceroporins conduct glycerol, urea, and water. In brain tissue AQP1 is expressed in the choroid plexus and sensory afferents; AQP4 in astrocytes, ependymal and endothelial cells; AQP9 in astrocytes, tanycytes, endothelium and catecholaminergic neurons, and AQP11 is present intracellularly in hippocampal, cortical and cerebellar neurons (Jung et al., 1994; Nielson et al., 1997; Badaut et al., 2002; Fraser & Andrew, 2008). However, there is no evidence yet of AQP11 present on the neuronal membrane.

Interestingly, AQP4 mRNA expression is at least ten times higher in brain tissue compared to other tissues (Jung et al., 1994), with the highest concentration at astrocyte perivascular end-feet. The end-feet are in close contact with cerebral arterioles and capillaries which suggest AQP4 may have an active role in water exchange between the brain and blood (Nielson et al., 1997; Fraser & Andrew, 2008). Immuno-gold labelling of AQP4 in astrocytes shows a high expression in perivascular processes specifically in SON, PVN and accessory/circularis nuclei, all of which have a role in osmotic pressure regulation (Badaut et al., 2000). Furthermore, the glia lamina associated with osmosensory brain regions (SON and SFO) show strong AQP4 expression (Nielson et al., 1997).

The robust AQP4 staining in astrocytes within MNC nuclei (Badaut et al., 2002) and the median eminence (Frigeri et al., 1995) suggests water channels allow changes in plasma osmotic
pressure to be transferred between blood and glia. Thus, AQP4 expression in astrocytes explains water exchange between brain and blood during pathophysiological conditions that cause rapid cell swelling (Badaut et al., 2002).

AQP4 is a potential therapeutic target for pathological conditions involving cellular swelling. The reduction of AQP4 in perivascular astrocyte end-feet will decrease and delay edema formation during the initial phase. Since water flow through AQP4 is bi-directional, up regulating AQP4 expression during the late phase will reduce cellular swelling. Therapeutics that regulates AQP4 expression may affect the final outcome of the pathology (Fraser and Andrew, 2008).

1.10 Research Rationale

Cultured and dissociated neurons have been used for years to investigate cell volume change in response to acute osmotic or ischemic stress. But how similar are these responses to the same neurons when observed in live brain slices? The question is important because the different preparations provide differing answers. Until recently it was only possible to image neuronal cell bodies in brain slices very near to the cut surface using contrast optics. With the development of 2PLSM, fluorescent neurons and their dendrites can be viewed deep to the cut brain slice surface to examine cell volume changes in real-time in cell bodies, dendrites and axons (Andrew et al., 2007).
Research Aims

I. To investigate how dissociated PyNs and live brain slice PyNs volume respond to acute osmotic challenges and to OGD.

II. To investigate how dissociated MNCs and live brain slice MNCs volume respond to acute osmotic challenges and to OGD.

III. To investigate if these volume responses differ depending on cell type.

IV. To examine how volume responses of dissociated cells compare with their responses in live brain slices.

V. To gather evidence for or against the hypothesis that ischemic neuronal swelling is osmotically driven.
Chapter 2: Materials & Methods

All animal protocols were in agreement with guidelines of the Canadian Council on Animal Care and were approved by the Queen’s University Animal Care Committee.

2.1 Dissociated Cell Experiments

Sprague-Dawley rats (30-90 day-old, Charles River, QC) and C57 black mice of the B6.Cg-Tg (Thy1-YFP) 16Jrs/J strain (30+ day-old) were housed in a controlled environment (22°C, 12hr light-12hr dark). An animal was placed in a rodent restrainer and guillotined.

The brain was removed in 1-3 minutes and placed in ice-cold oxygenated (95% O₂ and 5% CO₂) sucrose artificial cerebrospinal fluid (aCSF). The sucrose aCSF contained (in mM) sucrose 240, KCl 3.3, NaHCO₃ 26, MgSO₄ 1.3, NaH₂PO₄ 1.2, D-glucose 11, and CaCl₂ 1.8 (pH 7.3-7.4).

All solutions in this study were measured to have an osmolality of ~290 mOsm, if the neurons were isolated from rats and ~300 mOsm (by adding 10 mOsm of mannitol to normal aCSF), if the neurons were isolated from mice. The osmolality of the solutions and media were measured using an osmometer.

The excised brain was mounted on a stage and the brain slices were cut in the coronal plane (300-400 μm thick) using a vibrating blade microtome (Leica VT1200S). Slices containing the SON, PVN and hippocampus were placed in steri-filtered Hibernate media (Brain Bits, Springfield, IL) supplemented with B27 (Gibco, Invitrogen, Burlington, ON).
The SON, PVN and CA1 region were micro-dissected from the slices and placed in steri-filtered Hibernate media containing 2 mg/ml of papain (Worthington, Lakewood, NJ), and then placed in a circulating water bath at 30°C for 30 minutes. After incubation the dissected tissue was triturated in steri-filtered Hibernate/B27 media and then the dissociated cells were centrifuged at 200 rpm for 4 minutes at 4°C. The supernatant was removed and the pellet re-suspended in steri-filtered Neurobasal A media supplemented with B27 (Invitrogen) and 100U/ml penicillin-streptomycin (Invitrogen). The media (Hibernate and Neurobasal A) was measured to have an osmolality of ~260 mOsm. This was usually corrected to ~290 mOsm or ~300 mOsm by adding the appropriate amount of mannitol.

The dissociated cells were plated on 35 mm or 50 mm uncoated glass bottom culture dishes (MatTek, Ashland, MA) at a low density to prevent synaptic contacts from forming. After plating, the cells dishes were incubated at 37°C in 5% CO₂ (Thermo Scientific, Forma Series II) for 2 hours. After, ~2 ml of steri-filtered Neurobasal A media supplemented with B27/penicillin-streptomycin were added to the dishes which were returned to the incubator. All osmotic and OGD experiments were performed between 3 hours to 3 days after dissociation. The majority of data was collected within 12 hr of dissociation.

**Identification of Cells**

To verify the identification of the neurons, some experiments were conducted using fluorescent neurons. Twenty-nine PyNs from the CA1 region was extracted from mice expressing yellow fluorescent protein (YFP⁺) (Feng et al. 2000). Eighty-two MNCs were
extracted from the SON of transgenic rats expressing a VP-enhanced green fluorescent protein (GFP⁺) fusion gene.

The PyNs recorded were the largest in the dish at 15-25 μm in width. Most pyramidal neurons were dissected from the CA1 region of the hippocampus, although a few were from the layer VI of the cortex. If the mouse was YFP⁺, only fluorescent PyNs of that diameter range were observed.

MNCs measured from the SON/PVN dishes were assumed to be MNCs if they were 20-30 μm in diameter. Neurons that were large and oval-like without any processes were used. If the rat was from the VP-GFP⁺ strain, only fluorescent SON neurons of that diameter range were observed.

**Experimental Paradigms**

All experiments were conducted at a constant aCSF flow rate of 2-3 ml/min at 35-37°C. The cells were viewed using an Axio Examiner D1 (Zeiss) microscope. Using Imaging Workbench 6.0 software (Indec Biosystems Inc.) the cells were imaged. The fluorescence of the cells was checked after the experiment was conducted to ensure no damage occurred from the UV light. Auto-fluorescence was checked using SR-101 and YFP⁺ filter set.

In all osmotic experiments the neurons (both PyNs and MNCs) were subjected to +40 or -40 mOsm saline by adding either mannitol or distilled water, respectively, to normal aCSF (NaCl 120, KCl 3.3, NaHCO₃ 26, MgSO₄ 1.3, NaH₂PO₄ 1.2, D-glucose 11, and CaCl₂ 1.8). The osmotic stress paradigm entailed a sequence of 10 min exposures to control aCSF, +40 and then -
40 mOsm, followed by a return to control aCSF. Alternatively, in some experiments the order of the +40 and -40 mOsm aCSF exposures were reversed.

OGD solution was made by lowering normal aCSF glucose from 11 mM to 1mM (equimolar NaCl was added to replace glucose) and by replacing 95% O₂/5 % CO₂ gas mixture with 95% N₂/5 % CO₂. The cells were subjected to normal aCSF, OGD saline and then normal aCSF, each for 10 minutes.

Using Imaging workbench, the perimeters of each neuron at specific time points were traced and then the software calculated the cross-sectional area (XS area). For each cell, the percent (%) change in XS area was determined between a treatment condition and its preceding treatment condition. In addition, % change in XS area was determined between the beginning control aCSF1 and the end control aCSF2 to determine ‘recovery’. Swelling was defined as a >5% increase in XS area, conversely, shrinking was defined as >5% decrease. ‘No change’ was defined as change in XS area between ±5%.

2.2 Brain Slice Experiments

Neocortical slices taken from YFP⁺ mice and hypothalamic slices taken from GFP⁺ rats were dissected and sliced as described for dissociated neurons. After incubation for 1 hr in aCSF at 31°C, the brain slices were imaged using a 2PLSM.

Seventy-four brain slice MNCs tested were isolated from VP-GFP⁺ rats subjected to chronic dehydration. The rats were given 2% NaCl solution instead of tap water for 3 to 5 days to increase the size and fluorescence of MNCs (Fujito et al., 2006). Because these rats plasma
osmolalities were ~40 mOsm higher than normal, all aCSF solutions were elevated by 40 mOsm with mannitol. Otherwise, the brain slices were prepared and tested using the same protocols as described for the non-dehydrated rats. The remainder of the neurons were extracted from non-dehydrated rats (n=85) and mice (n=33).

All neurons tested in these experiments were fluorescent and were imaged in live brain slices using the 2PLSM. The imaging chamber that was mounted on a fixed stage of an upright Axioscope II FS microscope (Carl Zeiss, Jena, Germany). Imaging was at an excitation wavelength for YFP+ of 920 nm using the Zeiss LSM 710 NLO meta multi-photon system directly coupled with a Coherent Ti: Sapphire laser. Three-dimensional image stacks were taken at 3.0-µm increments with a Zeiss 40 X water-immersion objective (Carl Zeiss). Data acquisition and analyses were controlled by Zeiss LSM software.

Brain slice PyN and MNC osmotic experiments entailed exposure to control aCSF, +40 or -40 mOsm stress, followed by a return back to control aCSF, each for 10 minutes. Some experiments were conducted with the order of the osmotic stress reversed. In addition, the same osmotic paradigm was used to test brain slice MNCs from dehydrated rats. These brain slices were exposed to ±40, as well as ±10 mOsm stress. The brain slice OGD paradigm consisted 10 minutes each of control aCSF, OGD saline and then control aCSF.

Then using Zeiss LSM software, the perimeter of each neuron at specific time points was traced and the XS area was calculated. The % change for each neuron was calculated. Swelling was defined as a >5% increase in XS area. Conversely, shrinking was defined as >5% decrease. ‘No change’ was defined as change in XS area between ±5%. For the ±10 mOsm experiments,
swelling was defined as >3% increase in XS area. Shrinking as >3% a decrease and ‘no change’ in between ±3%.

2.3 Electrophysiology Recordings

MNCs were prepared as described above using the dissociated cell protocol. MNCs were visualized through a 40X objective mounted to an Axioscope 2 FS microscope (Zeiss). All electrophysiological recordings were performed at 36°C at an aCSF flow rate of 2-5 ml/min.

Patch micropipettes were pulled from borosilicate glass capillaries to a resistance of 3-8 MΩ using a Flaming/Brown micropipette puller (P-87; Sutter Instrument Co., Novato CA, USA). The internal recording solution consisted of (mM) 140 K⁺-gluconate, 2 MgCl₂, 0.1 CaCl₂ (calculated free intracellular Ca²⁺: 15 nM), 1.1 EGTA, 10 HEPES, 2 NaATP adjusted to a pH of 7.3 with KOH.

All recordings were performed in whole cell current clamp using the ‘Bridge’ mode of an Axoclamp 2A amplifier, sampled using a Digidata 1322A A/D converter (Axon Instruments) and analyzed with Clampex software (version 10.1). During whole-cell recordings, the MNCs (n=8) were simultaneously imaged while exposed to OGD for 15 minutes followed by at least 10 minutes of aCSF recovery.

2.4 Statistical Analysis
The mean and standard deviation (SD) of the % change for each neuronal type when dissociated and brain slice was calculated. The osmotic data for dissociated PyNs and MNCs were compared using the Mann-Whitney-U test. The dissociated PyNs and MNCs OGD data were compared using an unpaired t-test. The brain slice PyN and MNC osmotic and OGD data were compared using the Mann-Whitney-U test. The same test was used to compare dissociated and brain slice data.
Chapter 3: Results

3.1 Dissociated Cell Experiments

Dissociated PyN Responses to Osmotic Challenge

We are confident the majority of our dissociated neurons isolated from layers II-V and the hippocampi are pyramidal because first, we only tested the largest diameter cells (15 to 20 μm) of which PyNs make up the majority (Fig 1A). Second, larger neurons of this size range taken from YFP⁺ mice were fluorescent (n=7). Previous studies have shown that only PyNs are positive in neocortex or CA1 region from these animals.

The majority of our dissociated PyNs directionally responded appropriately to ±40 mOsm stress (Fig 2B &E), but with a wide variability ranging among the 33 neurons (Fig 2A, D). However, many of the neuronal responses did not satisfy our somewhat arbitrary criterion of swelling or shrinking by >5% to be considered a convincing response. The transitions between the two osmotic extremes of -40 and +40 mOsm caused the greatest % change in XS area (Fig 2C, F). As well as, in the hypo-osmotic paradigm, this transition showed the most consistency in terms of the number of neurons (Fig 2B) that displayed the expected response (Fig 2C).

Nineteen of 33 dissociated PyNs (Fig 5A) returned to volumes in control saline (aCSF2) that were similar to the original saline (aCSF1) as shown in Figure 2C & F (aCSF 2 vs. 1), indicating about half of dissociated PyNs show recovery.
Figure 1: Examples of typical dissociated PyNs observed in this study using Dodt-gradient contrast optic microscopy

(A) Image of dissociated PyN during osmotic stress.
   Left: A PyN during 10 minutes of aCSF1 (control).
   Middle - Left: A PyN swelling in response to -40 mOsm.
   Middle - Right: A PyN shrinking in response to +40 mOsm.
   Right: A PyN during 10 minutes of aCSF2 (recovery)
   NOTE: All images are of the same neuron.

(B) Image of dissociated PyN following 10 minutes of OGD.
   Left: Low magnification of fluorescent YFP+ dissociated PyN post-OGD.
   Middle: Image of the dissociated PyN post-OGD.
   Right: High magnification of fluorescence YFP+ dissociated PyN post-OGD.
   NOTE: All images are of the same neuron.
A.  

<table>
<thead>
<tr>
<th>aCSF1</th>
<th>-40 mOsm</th>
<th>+40 mOsm</th>
<th>aCSF2</th>
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</table>

B.  

![Images](image1.png)
Figure 2: Dissociated PyN responses to ±40 mOsm stress

(A & D) Distribution of % change in cell body XS area. Each point of the graph represents the % change XS area of a single neuron.
(B & E) Directional responses of each PyN. Swelling is defined as >5% increase in XS area. Shrinking is >5% decrease. Between ±5% is considered no change.
(C & F) Mean % change in cell body XS area of appropriate responses. Only neurons that appropriately responded (i.e., swelled in hypo-osmotic saline and shrunk in hyper-osmotic saline) were averaged and plotted.

Panels A, B, and C show responses when -40 mOsm aCSF (hypo) is presented first, followed by +40 mOsm (hyper). Panels D, E, and F show responses with this order reversed. NOTE: *p <0.01 compared to brain slice MNCs from non-dehydrated rats; ^p <0.05 compared to brain slice MNCs from dehydrated rats during ±10 mOsm stress.
Distribution of Dissociated PyN Responses to ±40 mOsm

A. Directional Responses by Dissociated PyNs to ±40 mOsm

B. Mean of Appropriate Responses by Dissociated PyNs to ±40 mOsm

C. Post-OGD
Dissociated MNC Responses to Osmotic Challenge

We tested only the largest diameter cells (20 to 30 μm) that were isolated from the SON and PVN. Therefore, the majority of our dissociated neurons were considered to be MNCs (Fig 3A). There were 14 fluorescent MNCs isolated from VP- GFP⁺ rat.

Similar to PyNs, a large percentage of dissociated MNCs exposed to hypo-osmotic (-40 mOsm) and hyperosmotic (+40 mOsm) saline for 10 minutes were appropriately volume responsive (Fig 4A & D). However, it seems that in the hypo-osmotic paradigm (Fig 4A), MNCs tended to stay shrunken upon return to control aCSF from hyper-osmotic saline (Fig 4B). Also, in the hyper-osmotic paradigm, MNCs tended to display shrinking throughout the whole paradigm (Fig 4E). Despite many being osmoresponsive, the majority of MNCs were unable to recover (Fig 5B). Most MNCs (n=12/19) shrank, while the remaining seven MNCs returned to their volumes observed during pre-osmotic stress.

We analyzed a subset of the dissociated PyNs (Fig 2C, F) and MNCs (Fig 4C, F) responding as expected to the osmotic shifts. That is, we examined the neurons that displayed a XS area >5% increase in hypo-osmotic saline and >5% decrease in hyper-osmotic saline. This allowed us to compare only the neurons that appropriately swelled or shrunk (“osmo-responders”) between the two neuronal types. Surprisingly, these results indicate that PyNs and MNCs (Fig 2C, F & Fig 4C, F) were similar (p >0.05) in their direction and in the degree of volume change.
Figure 3: Examples of typical dissociated MNCs observed in this study using Dodt-gradient contrast optic microscopy

(A) Example of typical dissociated MNCs in response to osmotic stress.
   Left panel: GFP* fluorescent image of dissociated MNC after ± 40 mOsm stress.
   Middle panel: Dissociated MNC shrinking in response to +40 mOsm stress.
   Right panel: Dissociated MNC swelling in response to -40 mOsm stress.

(B) Example of typical dissociated MNCs in response to 10 minutes of OGD.
   Left: GFP* fluorescent image of dissociated MNC post-OGD.
   Middle: Dissociated MNC pre-OGD.
   Right: Dissociated MNC following a 10 minute exposure to OGD. Cell body size appears unchanged in comparison to pre-OGD cell body size.

Each row of images represents the same MNC during the experimental paradigm.
A. Post-osmotic Stress

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B. Post-OGD

<table>
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<tbody>
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<td>![Image 5]</td>
<td>![Image 6]</td>
</tr>
<tr>
<td>![Image 7]</td>
<td>![Image 8]</td>
</tr>
</tbody>
</table>

39
Figure 4: Dissociated MNC responses to ±40 mOsm stress

(A & D) Distribution of % change in cell body XS area.
    Each point of the graph represents the % change XS area of a single neuron
(B & E) Directional responses of each MNC.
    Swelling is defined as >5% increase in XS area. Shrinking is >5% decrease.
    Between ±5% is considered no change.
(C & F) Mean % change in cell body XS area of appropriate responses.
    Only neurons that appropriately responded (i.e., swelled in hypo-osmotic saline
    and shrunk in hyper-osmotic saline) were average and plotted.

Panels A, B, and C show responses when -40 mOsm aCSF (hypo) is presented first, followed by
+40 mOsm (hyper). Panels D, E, and F show responses with this order reversed.
NOTE: *p <0.05 compared to brain slice MNCs from dehydrated rats during ±10 mOsm stress.
Distribution of **Dissociated MNC** Responses to ±40 mOsm

A. Directional Responses by **Dissociated MNCs** to ±40 mOsm

B. Mean Appropriate Responses by **Dissociated MNCs** to ±40 mOsm

C. Distribution of **Dissociated MNC** Responses to ±40 mOsm

D. Directional Responses by **Dissociated MNCs** to ±40 mOsm

E. Mean Appropriate Responses by **Dissociated MNCs** to ±40 mOsm

F. Distribution of **Dissociated MNC** Responses to ±40 mOsm
Figure 5: Recovery of dissociated neurons following ±40 mOsm stress

(A) Dissociated PyNs.
(B) Dissociated MNCs.

The histogram indicates the number of neurons that showed no change, swelling and shrinking when comparing their % change in XS area at the end of the experiment (aCSF2) compared to their % change in XS area at the beginning of the experiment (aCSF1). This is a measure of ‘recovery’. Swelling is defined as >5% increase in XS area. Shrinking is >5% decrease. Between ±5% is considered no change.
A. Dissociated PyNs

B. Dissociated MNCs

No Change  |  Swelling  |  Shrinking
---|---|---
33 Neurons

No Change  |  Swelling  |  Shrinking
---|---|---
19 Neurons
3.2 Dissociated Cell Volume Changes in Response to OGD

Our dissociated neurons were considered to be pyramidal (Fig 1B) because we selected neurons of the largest diameter (15 to 20 μm) isolated from the neocortex and CA1 regions of the brain slices. In addition, 22 fluorescent cells were isolated from YFP+ mice neocortex or CA1 region, confirming that cells of this size were PyNs.

Our dissociated neurons derived from hypothalamic slices were considered to be MNCs (Fig 3B) because we selected the largest diameter cells (20 to 30 μm) isolated from the SON and PVN. Sixty-eight fluorescent MNCs were isolated from GFP+ rat, confirming that cells of this size were MNCs. In the Andrew lab, preliminary patch clamp studies have shown that dissociated MNCs (n=8) subjected to 15 minutes of OGD are still able to generate action potentials (Appendix I; Fig 16). The average $R_{mp}$ after OGD insult was -48 mV (Table 1; Appendix II), indicating that our freshly isolated MNCs are electrophysiologically healthy in culture and have not undergone drastic intracellular changes induced by the stress from the dissociation procedure. Also, this suggests that dissociated MNCs are capable of surviving a prolonged ischemic-like insult.

As shown in Fig 6A, the volume responses of dissociated PyNs and MNCs to 10 minutes of OGD are quite unpredictable. The majority of PyNs (60/108; Fig 6B) and MNCs (106/162; Fig 6D) did not swell in response to OGD. The remaining dissociated neurons of both types either swelled or shrank during OGD in almost equal numbers (Fig 6B, D). However, PyNs showed a significantly (p <0.05) greater increase in volume than MNCs. Furthermore, when
comparing only the responders to OGD, PyNs displayed a dramatically larger swelling response
of 12.1% (Fig 6C) compared (p <0.0001) to MNCs swelling response of 8.3% (Fig 6E).

The post-OGD period was analyzed to see if any volume recovery was apparent. The
number of ‘no change’, swollen and shrunken neurons did not change significantly upon return
to control aCSF for 10 minutes (Fig 6B, D).
Figure 6: Dissociated PyN and MNC responses to OGD

(A) Distribution of the % change in cell body XS area of PyNs and MNCs following 10 minutes of OGD.
(B) Directional responses to OGD of each PyN.
(C) Mean % change in cell body XS area of PyNs. The mean of the no change, swelling, and shrinking groups are plotted.
(D) Directional responses to OGD of each MNC.
(E) Mean % change in cell body XS area of MNCs. The mean of the no change, swelling, and shrinking groups are plotted.

Each histogram in (B) showing the number of neurons responding corresponds to its histogram in (C) showing the amount of change by the group. Swelling is defined as >5% increase in XS area. Shrinking is >5% decrease. Between ±5% is considered no change.

NOTE: *p <0.0001 compared between dissociated neuronal types.
A. Distribution of **Dissociated PyN and MNC Responses to OGD**

[Graph showing % Change in XS Area for 108 PyNs and 162 MNCs.]

**Directional Responses to OGD**

**B. Dissociated PyNs**

- **No Change**
- **Swelling**
- **Shrinking**

<table>
<thead>
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<th>Swelling</th>
<th>Shrinking</th>
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</tr>
<tr>
<td>30</td>
<td>108</td>
<td>10</td>
<td>2</td>
</tr>
</tbody>
</table>

**C. Mean Responses of Dissociated Neurons to OGD**

- **Dissociated PyNs**
- **No Change**
- **Swelling**
- **Shrinking**

<table>
<thead>
<tr>
<th>Mean % Change in XS area</th>
<th>No Change</th>
<th>Swelling</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Response to OGD</td>
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<td>2</td>
</tr>
<tr>
<td>Recovery from OGD</td>
<td>108</td>
<td>10</td>
<td>2</td>
</tr>
</tbody>
</table>

**D. Dissociated MNCs**

- **No Change**
- **Swelling**
- **Shrinking**

<table>
<thead>
<tr>
<th>% Change in XS Area</th>
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<th>Swelling</th>
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<tr>
<td>30</td>
<td>162</td>
<td>10</td>
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</tbody>
</table>

**E. Mean % Change in XS area**

- **Dissociated MNCs**
- **Response to OGD**
- **Recovery from OGD**

- **No Change**
- **Swelling**
- **Shrinking**

**Graph showing Mean % Change in XS area for 108 PyNs and 162 MNCs.**
3.3 Brain Slice Experiments

Responses to Osmotic Challenges

Using 2PLSM, we examined neuronal volume changes in brain slices by imaging fluorescent PyNs in YFP\(^+\) mice and fluorescent MNCs in GFP\(^+\) rats (Fig 8). Neurons were imaged at least 60 \(\mu\)m deep to the cut slice surface to minimize the possibility of damage from slicing.

Brain slice PyNs showed a trend to being osmoreponsive to \(\pm 40\) mOsm stress, as shown in Fig 7A. However, the majority did not show change in XS area beyond \(\pm 5\%\) and thereby were classified under ‘no change’ (Fig 7B). Brain slice PyNs showed primarily smaller changes in cell volume (Fig 7C) than their dissociated counterparts (Fig 2F).

Brain slice MNCs from non-dehydrated rats (Fig 8) were mostly responsive to \(\pm 40\) mOsm osmotic stress (Fig 9A, D), regardless of whether the order of hypo-osmotic stress was presented before or after hyper-osmotic stress (Fig 9C, F). Although MNCs responded appropriately to the osmotic shifts, almost half of these neurons displayed changes small changes in XS area between \(\pm 5\%\) (Fig 9B, E).

Half of the brain slice PyNs (n=6) recovered from \(\pm 40\) mOsm stress (Fig 10A), whereas the remaining half of the PyNs were shrunken. Similarly, almost half of the MNCs (15/33; Fig 10B) recovered from exposure to osmotic stress. However of the remaining MNCs, half shrunk and the other half swelled. Taken together, it appears that close to half of brain slice neurons tested were not good osmo-responders.
Figure 7: Brain slice PyN responses to ±40 mOsm stress

(A) Distribution of the % change in cell body XS area.
   Each point of the graph represents the % change XS area of a single neuron.
(B) Directional responses of each PyN.
   Swelling is defined as >5% increase in XS area. Shrinking is >5% decrease. Between ±5% is considered no change.
(C) Mean % change in cell body XS area of appropriate responses.
   Only neurons that appropriately responded (i.e., swelled in hypo-osmotic saline and shrunk in hyper-osmotic saline) were averaged and then plotted.
A. Distribution of **Brain Slice PyN** Responses to ±40 mOsm

![Graph 1]

B. Directional Responses by **Brain Slice PyNs** to ±40 mOsm

![Graph 2]

C. Mean of Appropriate Responses by **Brain Brain Slice PyNs** to ±40 mOsm

![Graph 3]
Figure 8: 2PLSM imaging of typical brain slice MNCs observed in this study during ±40 mOsm stress

This brain slice was from a non-dehydrated rat. Brain slice GFP+ MNCs imaged during the sequence of +40 to -40 mOsm stress. The red line indicates the perimeter of the cell body. The area (µm²) of each neuron is also indicated. Each MNCs outlined is appropriately volume responding.
Control

+40 mOsm

-40 mOsm

Wash

\[ \text{Area} = \ \mu m^2 \]
Figure 9: Brain Slice MNC responses to ±40 mOsm stress

(A & D) Distribution of the % change in cell body XS area. Each point of the graph represents the % change XS area of a single neuron.

(B & E) Directional responses of each MNC. Swelling is defined as >5% increase in XS area. Shrinking is >5% decrease. Between ±5% is considered no change.

(C & F) Mean % change in cell body XS area of appropriate responses. Only neurons that appropriately responded (i.e., swelled in hypo-osmotic saline and shrunk in hyper-osmotic saline) were averaged and plotted.

Panels A, B, and C show responses when -40 mOsm aCSF (hypo) is presented first, followed by +40 mOsm (hyper). Panels D, E, and F show responses with this order reversed.

NOTE: * p<0.01 compared to the expected mean response of dissociated PyNs.
Distribution of **Brain Slice MNC** Responses to ±40 mOsm (Non-dehydrated rats)

A. Directional Responses by **Brain Slice MNCs** to ±40 mOsm (Non-dehydrated rats)

B. Mean of Appropriate Responses by **Brain Slice MNCs** to ±40 mOsm
**Figure 10: Recovery of dissociated neurons following ±40 mOsm stress**

(A) Brain slice PyNs.
(B) Brain slice MNCs.

The histogram indicates the number of neurons that showed no change, swelling and shrinking when comparing their % change in XS area at the end of the experiment (aCSF2) compared to their % change in XS area at the beginning of the experiment (aCSF1). This is a measure of ‘recovery’. Swelling is defined as >5% increase in XS area. Shrinking is >5% decrease. Between ±5% is considered no change.
A. Brain Slice PyNs

B. Brain Slice MNCs

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<tr>
<td>33 Neurons</td>
<td>16</td>
<td>8</td>
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</table>
Responses of MNCs from Dehydrated Rats

We tested if these brain slice MNCs, which have larger cell bodies and produce more VP, show increased osmotic responses under the same levels of ±40 mOsm stress. In Figure 12A-C, the same experiments were analyzed as presented using MNCs from non-dehydrated rats (Fig 9). Here, data was obtained from hypo-osmotic stress presented first (Fig 12A). We did not additionally test hyper-osmotic stress first because the previous experiments indicated that the order of presentation did not make a difference, although this was not analyzed in depth. One obvious difference was that a greater proportion of ‘dehydrated’ MNCs osmo-responded (Fig 12B) compared to ‘non-dehydrated’ MNCs (Fig 9B). In fact, these neurons were the best osmo-responders of our study, where 9 of 12 swelled in -40mOsm aCSF (Fig 12B). However, looking closely at Fig 12A, all neurons tended to swell. Responses were directionally appropriate and ranged between -19% and 17%.

We also examined if we could detect volume responses of dehydrated MNCs to a more physiological range of stresses between ±10 mOsm (Fig 11). We predicted that the extent of volume changes should be smaller than the ±40 mOsM responses. This proved to be the case where the % change in XS area ranged from -11% to +13% with ±10 mOsm (Fig 12D) compared to -19% to +17% with the ±40 mOsm stress (Fig 12A). The responders to ±10 mOsm stress showed surprisingly robust changes (Fig 12F). On the other hand, a lower percentage of neurons responded (compare Fig 12E with 12B) as expected for a lower level of osmotic stress.

In regards to recovery from osmotic stress, 11 out of 12 MNCs (Fig 12B) were able to recover from ±40 mOsm exposure. Interestingly, dehydrated MNCs displayed a much more pronounced recovery response (Fig 12B). The MNCs from non-dehydrated rats were more
variable in their response (Fig 10B) further indicating that these MNCs from dehydrated rats are good osmo-responders. Recovery from ±10 mOsm osmotic experiments were more variable, where similar number of MNCs were unchanged, swollen or shrunken after osmotic stress exposure compared to their original volumes (Fig 12E).
Figure 11: 2PLSM imaging of brain slice MNCs during ±10 mOsm stress

This brain slice was from a *dehydrated* rat. Brain slice GFP$^+$ MNCs imaged during the sequence of -10 to +10 mOsm stress. The red line indicates the perimeter of the cell body. The area (µm$^2$) of each neuron is also indicated. Each MNCs outlined is appropriately volume responding, with the exception of the top-left neuron that maintained its volume at ~775µm$^2$. 
**Figure 12: Brain slice MNC responses to ±40 and ±10 mOsm stresses**

(A & D) Distribution of the % change in cell body XS area.
Each point of the graph represents the % change XS area of a single neuron.

(B & E) Directional responses of each MNC.
For the ±40 mOsm experiments swelling is defined as >5% increase in XS area.
Shrinking is >5% decrease. Between ±5% is considered no change. For ±10 mOsm experiments swelling is defined as >3% increase in XS area. Shrinking is >3% decrease. Between ±3% is considered no change.

(C & F) Mean % change in cell body XS area of appropriate responses.
Only neurons that appropriately responded (i.e., swelled in hypo-osmotic saline and shrunk in hyper-osmotic saline) were averaged and plotted.

Comparisons of neuronal changes in cell volume to a sequence of ±40 mOsm challenge (A, B, C) and ±10 mOsm (D, E, F).
**NOTE:** #p <0.05 compared to dissociated MNCs; *p <0.05 compared to dissociated PyNs.
Distribution of **Brain Slice MNC** Responses to Osmotic Challenge (Dehydrated Rats)  

A. Osmotic Challenge ±40 mOsm  

![Graph showing % change in XS area for 12 neurons](image)

- aCSF1 → Hypo
- Hypo → Hyper
- Hyper → aCSF2

- n=12

B. Osmotic Challenge ±40 mOsm  

![Graph showing % change in XS area for 12 neurons](image)

- aCSF1 → Hypo
- Hypo → Hyper
- Hyper → aCSF2

- n=12

C. Osmotic Challenge of ±40 mOsm  

![Graph showing mean % change in XS area](image)

- aCSF1 → Hypo
- Hypo → Hyper
- Hyper → aCSF2

- n=12

Brain Slice MNC Directional Responses to Osmotic Challenge (Dehydrated Rats)  

E. Osmotic Challenge ±10 mOsm  

![Graph showing % change in XS area for 23 neurons](image)

- aCSF1 → Hyper
- Hyper → Hypo
- Hypo → aCSF2

- n=23

F. Osmotic Challenge of ±10 mOsm  

![Graph showing mean % change in XS area](image)

- aCSF1 → Hyper
- Hyper → Hypo
- Hypo → aCSF2

- n=23

Mean of Appropriate Brain Slice Responses (Dehydrated Rats)  

- aCSF 2 vs. 1

- n=12

- Mean % Change in XS Area

- aCSF1 → Hypo
- Hypo → Hyper
- Hyper → aCSF2

- n=12

- n=11

- n=4

- n=11

- n=11

- n=16

- n=8

- n=9

53
3.4 Volume Responses to OGD in Brain Slices

The responses to 10 minutes of OGD (Fig 14A) show dramatic differences between PyNs and MNCs (whether from dehydrated and non-dehydrated rats). The 21 PyNs examined in YFP+ mice brain slices (Fig 13A) all displayed a pronounced swelling on average of 34.5% (Fig 14D). No recovery was observed in control aCSF post-OGD (not shown). In sharp contrast, MNCs (Fig 13B, C) either did not change volume or slightly shrunk (Fig 14C, D). There was no significant difference found between non-dehydrated and dehydrated MNC responses to OGD with regard to distribution (Fig 14A), direction (Fig 14B) and amount of volume change (Fig 14C).
Figure 13: 2PLSM imaging of brain slice neurons during exposure to OGD

(A) Top: Brain slice of YFP⁺ PyNs pre-OGD. 
   Bottom: Brain slice pyramidal cell bodies and dendrites swelling following a 10 minute exposure to OGD.

(B) Top: Brain slice of GFP⁺ MNCs pre-OGD. 
   Bottom: Brain slice of MNC after 15 minutes of OGD. No visible swelling of the cell bodies or dendrites.

(C) Left panel: (top) Example of a single MNC in brain slice pre-OGD and (bottom) following 15 minutes of OGD. The MNC is not swelling in response to OGD. 
   Right panel: (top) High magnification of brain slice MNCs pre-OGD and (bottom) following 15 minutes of OGD. The MNCs are not swelling in response to OGD.
Figure 14: Brain Slice PyN and MNCs responses to OGD

(A) Distribution of the % change in cell body XS area of PyNs and MNCs following 10 minutes of OGD.
(B) Responses to OGD of each MNC from non-dehydrated rats.
(C) Responses to OGD of each MNC from dehydrated rats.
(D) Mean % change in cell body XS area of PyNs and MNCs in response to OGD.

Swelling is defined as >5% increase in XS area. Shrinking is >5% decrease. Between ±5% is considered no change.
NOTE:* p <0.001 compared to MNCs from dehydrated and non-dehydrated rats.
A. Distribution of **Brain Slice PyN** and **MNC** Responses to OGD

![Graph showing the distribution of Brain Slice PyN and MNC responses to OGD](image)

B. **Brain Slice MNC** Directional Responses to OGD

- **Non-dehydrated Rats**
- **Dehydrated Rats**

![Graph showing directional responses of Brain Slice MNC](image)

C. **Mean Response to OGD by Brain Slice Neurons**

![Bar graph showing mean response to OGD by Brain Slice Neurons](image)
Chapter 4: Discussion

4.1 Overview

During early ischemia or acute hypo-osmotic conditions, neurons swell. However, the cellular events that lead to neuronal swelling are poorly documented. It is generally accepted that the permeability of the neuronal membrane allows some water to flow down its osmotic gradient. However compared to plasma membranes with water channels, the water permeability of lipid bilayer alone is insufficient to mediate the rapid movement of water seen during acute ischemic conditions. Therefore, the large and rapid accumulation of intracellular water in PyNs must be attributed to some other mechanism other than osmotic influx.

Neuronal responses to osmotic stress and ischemic insults (Somjen et al., 1993; Aitken, et al. (1998); Shulyakova et al., 2010) have been examined primarily in cultured and dissociated neurons. Although there are numerous osmotic studies conducted on dissociated and brain slice PyNs, osmolarity stress levels are always extremely high. There are two major problems in particular with dissociated cell studies. First, neurons are usually grown in physiologically stressed conditions for hours or days, before testing begins. Therefore, most of the neurons that are tested are those that survived these conditions. Second neurons are usually subjected to unphysiological high osmotic stresses. In light of these problems, the majority of our experiments were conducted within 3-12 hours after the dissociation procedure and our experimental paradigm consisted of more physiologically relevant levels of osmotic stress.
Interestingly, there is very little known about MNC volume responses to osmotic stress despite the fact that one study has indicated that they volume respond to physiological shifts (Oliet and Bourque, 1993). The present study investigates both dissociated and brain slice PyNs and MNCs subjected to physiological osmotic stress and simulated ischemia.

Based on previous research leading into this study we had three assumptions. First, brain slice PyNs and MNCs would not be osmoresponsive because they lack water channels (Andrew et al., 2007). Second, brain slice MNCs would dramatically swell during OGD as previously observed in brain slice PyNs (Andrew et al. 2007). Third all dissociated neurons would respond similarly to osmotic stress. These preconceptions were not validated in our study.

4.2 Osmotic Volume Responses - Dissociated Neurons

Our data show that about half of dissociated PyNs and half of MNCs respond to overhydration (-40 mOsm) and dehydration (+40 mOsm) by changing volume appropriately. These findings are in contrast to observations by Somjen et al. (1993) that dissociated hippocampal PyNs maintained their original shape and size under strong hypo-osmotic conditions. However, they observed PyNs that grew dendrites ~30 µm long. Since neurons lose their processes during the dissociation procedure, this implies that the cultures matured for a few days before testing. The cells may have become volume resistant over time. More generally, differences in dissociation and incubation procedures between labs may change fundamental properties of the neurons, a major drawback for using isolated cells in osmotic studies. This
surprised us because our neurons appeared healthy in the dish where they could survive over several days; intracellular recordings showed good resting potentials with robust action potentials (Appendix I). Yet it was clear that there was a general `die-back` of cells starting at 3-hr post-dissociation and over the following 48 hr. Thus we may have included neurons that appeared healthy but were in fact compromised. This may have widened the variability of our response range.

When the astrocytic investiture is lost during the dissociation procedure, both PyNs and MNCs in our study may become less able to maintain volume when subjected to osmotic stress, possibly because astrocytes are not present to directionally pump water to oppose the passive neuronal volume change. However this does not explain why dissociated PyNs in Somjen et al.`s study were osmoresistant and why many in our investigation did not significantly osmorepond. **We must conclude that both of our populations of dissociated neurons behaved too inconsistently and unpredictably to obtain consistent data involving osmotically induced volume change.**

If neurons lack water channels, then why do we see significant volume changes in half our dissociated neurons that did respond appropriately? Passive osmotic water flow across plasma membrane results from overall concentration differences in macromolecules, osmolytes and ions between the internal and external environment of the neuron (Fullerton et al., 2006). Some cells, with membranes that lack AQP, can resist volume change under high osmotic gradients because they have very low permeability to passive water diffusion (reviewed in Andrew et al., 2007). These `barrier membranes` not only lack aquaporins but also have outer leaflets whose lipid components confer both low fluidity and high resistance to water permeation.
(Krylov and others 2001). We subjected our neurons to ±40 mOsm stress, which are lower and more physiologically relevant than the plethora of studies conducted using larger ranges of ±60-180 mOsm. Since many of our dissociated neurons osmotically swelled and shrunk appropriately, this study has ruled out that these neurons have barrier-like plasma membrane.

4.3 Osmotic Volume Responses - Brain Slice Neurons

Our data show that approximately two thirds of brain slice PyNs are osmotically unresponsive to overhydration (-40 mOsm) and dehydration (+40 mOsm). This supports previous reports that PyNs in cortical slices are unresponsive to a range of osmolalities (-60 to +60mOsm) during several minutes of osmotic stress (Rosen & Andrew 1990, 1991; Saly & Andrew, 1993; Andrew et al., 2007). That is, brain slice PyNs fail to show changes in cell volume or resting membrane potential during exposure to 10-15 minutes of osmotic stress. Our data also show that more than half of brain slice MNCs are volume responsive to osmotic stress and to a greater degree than brain slice PyNs. This is perhaps expected because MNCs are known to be intrinsically osmosensitive neurons (Oliet & Bourque, 1993; Bourque, 1998) and one study using DIC microscopy has reported appropriate volume responses by MNCs imaged near the slice surface (Oliet and Bourque, 2003).

Importantly, our data also show brain slice MNCs from dehydrated rats exposed to ±10 mOsm stress display volume changes that were appropriate and smaller than ±40 mOsm induced volume changes. This provides support that MNCs are capable of detecting more discrete and
physiologically relevant changes in osmolality not usually tested in osmotic studies (Andrew et al. 1997, 2007).

Under conditions of water restriction and hyper-osmolality, glial retraction from between the magnocellular cell bodies and dendrites coincides with increases in MNC excitability and release of VP into the blood (Hatton, 1990; Theodosis & Poulain, 1993). Glial retraction allows increased soma-somatic contact between MNCs and so may enhance co-ordination between adjacent neurons during periods of dehydration to increase VP production/ release to induce water conservation (Gamrani et al., 2010, in press). Appropriately then, chronic salt loading of VP-GFP+ transgenic rats for 3-5 days causes: 1) a marked increase in GFP expression in MNCs, 2) larger cell bodies and 3) increased numbers of GFP+ MNCs (Fujio et al., 2006), reflecting up-regulation of VP synthesis. We observed a greater degree of both swelling and shrinking to ±40 mOsm stress by MNCs from dehydrated rats, compared to MNCs from non-dehydrated rats. We propose the following explanation. During chronic dehydration, retraction of astrocyte processes between MNCs renders the neurons more susceptible to passive osmotic swelling or shrinking. We also suggest a greater astrocytic investiture surrounding PyNs and their dendrites compared to MNCs may help account for their reduced volume changes induced by osmotic stress as observed in neocortical slices (Andrew et al., 2007). Validation requires a morphometric study utilizing electron microscopy.

4.4 Oxygen Glucose Deprivation and Neuronal Volume Responses
The current study shows that pyramidal cell bodies display *no swelling in dissociation* but *extreme swelling in brain slices following 10 minutes of OGD*. This supports a previous report in slices by Andrew et al. (2007) demonstrating that pyramidal cell bodies swell and their dendrites bead during OGD with little recovery. What is striking in the current study is the *complete lack of swelling by MNCs in comparably treated brain slices*.

So why do MNCs in brain slices also not swell in response to OGD? Other research in the Andrew lab has shown that AD propagates through the hypothalamus, but it only weakly invades the SON and PVN. Current clamp recording of single neurons show that MNCs only slightly depolarize and easily recover from 15 minutes of OGD. In contrast, PyNs completely depolarize and cannot recover. The massive swelling of PyN cell bodies and beading of dendrites are a result of an overwhelming AD event. The healthier MNCs, as we show in this study, do not swell post-AD. Rather, most actually shrink slightly. The 2PLSM evidence in the current study provides further convincing evidence that MNCs resist stroke injury.

### 4.5 Astrocytic Involvement in Neuronal Osmotic Stress-induced Volume Changes

In response to an induced osmotic gradient, the swelling and shrinking observed in our experiments is likely the result of slow and passive water flow across neuronal plasma membrane. Over the 10 minutes of osmotic stress, half of the MNCs and some PyNs were able to passively change their volume accordingly to some extent. Dissociated neurons may be more susceptible to swelling because they lack the astrocytic investiture if it works by facilitating...
water movement to and from neurons. Expression of AQP4 is concentrated on the astrocyte membrane facing blood vessels, implying that perivascular astrocytic processes are a crucial site of water flux (Nielson et al., 1997). In fact, there are numerous studies in rats that demonstrate the clinical importance of perivascular AQP4 pool mediating water exchange between brain-blood interface (Zeyalov et al., 2008; Zeng et al., 2010). This is probable since astrocytes are able to mediate rapid shifts of water under osmotic stress, reflecting expression of AQPs in their plasma membrane.

But what accounts for the swelling seen in brain slice PyNs upon exposure to 10 minutes of OGD? Acute ischemic swelling as induced by OGD is universally regarded as ‘osmotic’ swelling. But passive water flow seems unlikely to account for the profound increase in volume observed in brain slice PyNs. Our dissociated and brain slice MNCs were volume responsive to hypo-osmotic stress (-40 mOsm and -10mOsm) but did not swell in response to OGD. Conversely, brain slice PyNs responded with dramatic swelling to OGD compared to their volume responses during osmotic stress. This leads to our hypothesis that OGD-swelling and osmotic swelling are mediated by separate mechanisms.

### 4.6 OGD-induced Swelling

The current study shows that PyNs swell when energy-deprived in brain slices but not when dissociated. The established concept is that during AD, Na\(^+\) and Cl\(^-\) influx osmotically draws water into neurons, evoking neuronal swelling. This is wrong for the following reasons.
The neuronal gain of Na\(^+\) during depolarization is mostly counter-balanced by the loss of K\(^+\) as these ions flow down their concentration gradients. Also water cannot easily follow the osmotic gradient without AQP channels. Moreover when Na\(^+\), K\(^+\) and Cl\(^-\) pass through their respective channels, their hydration shells are stripped off. So how does water build up inside neurons?

To sustain prolonged depolarization, the neuron must replenish its energy stores and remove waste by-products. In normal conditions, the main function of the N-acetyl-L-aspartic acid/N-acetyl-aspartyl-glutamic acid (NAA-NAAG) molecular water pump is to continuously remove neuronal metabolic water into the ECF against the water gradient (Figure 15A). During ischemia-like events when ATP stores become depleted, the NAA-NAAG water pump begins to fail (Figure 15B). Intracellular water accumulates and without water channels, swelling ensues. With continued energy depletion, breakdown of cellular macromolecules into smaller and more numerous osmotically active components increase transmembrane water diffusion. Eventually, the osmotic gradient between the neuron and ECF equalizes and the swelling stops. However over several minutes, the chances of recovery without damage decreases. Thus, we support the proposal by Baslow & Guilfoyle (2007) that neurons swell during stroke mainly because they are suddenly deprived of energy required to pump accumulating intracellular water out.

MNCs from dehydrated rats should show more OGD-induced swelling than MNCs from non-dehydrated rats because glial retraction allows more passive water influx. However, MNCs from dehydrated and non-dehydrated rats showed similar volume change in response to OGD. MNCs passively conduct water as shown by our osmotic studies yet resist OGD swelling. Again, osmotic swelling and OGD swelling must be induced by two separate mechanisms; if they
involved the same mechanism there should be more swelling in dehydrated brain slice MNCs. This also implies that MNCs are inherently resistant to OGD independent of the extent of astrocytic investiture because brain slice MNCs from dehydrated rats (presumably with less glia) survive a 10 minute exposure to OGD as well as non-dehydrated.

Why are both dissociated PyNs and MNCs resistant to OGD swelling? Numerous studies have reported (but underplayed) the fact that cultured and dissociated neuronal preparations exposed to stroke-like conditions can survive tens of minutes (or even hours) of ischemia-like conditions. A very long duration of OGD is needed to kill dissociated/cultured neurons than to kill same neurons in brain slices (Strasseur, et al., 1995). Isolated neurons depolarize slowly and show minimal, if any, swelling because they do not undergo a strong and coordinated AD (Perez-Velazquez et al., 1997). The result is that dissociated neurons are not metabolically stressed enough to swell to the extent that PyNs swell in brain slices.

4.7 Conclusions and Future Directions

All three of our preconceptions leading into this study were invalidated by our results. We assumed that neurons would be unresponsive to osmotic stress since neuronal membranes lack water channels. In fact, about half of dissociated PyNs and MNCs and brain slice MNCs swelled and shrunk appropriately during osmotic stress. This was presumably caused by passive trans-membrane water movement. We also assumed that if dissociated PyNs and MNCs
responded, they would respond predictably. Instead, we observed that about half of our
dissociated neurons were inconsistent and unpredictable.

We further expected brain slice MNCs to swell in response to OGD as previously
reported in brain slice PyNs (Andrew et al., 2007). However the most significant finding of our
study is the remarkable resistance of brain slice MNCs to OGD compared to the severe OGD-
induced swelling in brain slice PyNs. We also provided evidence against the hypothesis that
ischemic neuronal swelling is osmotically driven, since we also found that brain slice MNCs
swell in response to hypo-osmotic stress. Therefore, a separate mechanism must be responsible
for osmotic swelling and OGD-induced swelling.

Further 2PLSM experiments investigating osmotic responses of PyNs in neocortical
slices must be conducted deeper (> 60 µm) into the brain slice in young rats to better coincide
with our previous results showing almost negligible volume change to osmotic stress in PyNs
(Andrew et al., 2007; Risher et al., 2008). As well as, further investigation of MNCs during
ischemic-like stress to explore the properties of MNCs that may protect them from ischemia-like
events.
Figure 15: Proposal as to how neurons can resist swelling until stroke occurs

(A) Neuronal water pump during normal physiological conditions.
(B) Neuronal water pump failure during ischemia.

Diagram by Dr. David Andrew, modified from Baslow and Guilfoyle (2007).
A. Neuronal Water Pump

- Passive efflux with shells of hydration
- $\text{H}_2\text{O}$ drawn in

B. Pump Failing During Ischemia

- $\text{H}_2\text{O}$ drawn in
- $\text{H}_2\text{O}$ efflux
- Neuronal stores drop

Both actively concentrated intraneuronally
References


Swanson, L. W., and Sawchenko, P. E. (1980). Separate neurons in the paraventricular nucleus project to the median eminence and to the medulla or spinal cord. Brain Res. 198,190-195.


Figure 16: Whole-cell recording of dissociated MNC following 15 minutes of OGD

(A) Baseline whole-cell recording (20 s) pre-OGD application.
Resting membrane potential was -48 mV.

(B) Whole-cell recording of dissociated MNC after 15 minutes of OGD.
Appendix II

Table 1. Whole-cell recording data of MNCs in response to 15 minutes of OGD. $R_{mp}$, resting membrane potential; $A_{mpl}$, amplitude; $R_{in}$, input resistance; SD, standard deviation.

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