ION CURRENTS REGULATED BY ACUTE AND CHRONIC OSMOTIC STIMULI IN RAT SUPRAOPTIC NUCLEUS NEURONS

A Thesis

Submitted to

The College of Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the Department of Physiology

University of Saskatchewan

Saskatoon

By

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ABSTRACT

The magnocellular neurosecretory cells (MNCs) of the hypothalamus are able to change their firing rate and pattern in response to small changes in external osmolality due to the involvement of osmosensitive ion channels. The firing rate and pattern determine the release of vasopressin (VP), a primary hormone regulating osmolality by controlling water excretion from the kidney. Both VP- and oxytocin (OT)-MNCs display irregular and infrequent fire when plasma osmolality is near normal, and they progressively increase the frequency of firing to fast continuous firing with increases in osmolality. VP-MNCs also respond to osmotic stimulation by adopting a phasic pattern of firing, which maximizes neuropeptide secretion. Sustained dehydration also causes structural and functional adaptations in MNCs.

Voltage-dependent Ca^{2+} channels play many important roles not only in the regulation of cell excitability but also in intracellular signal transduction, and L-type Ca^{2+} channel-mediated Ca^{2+} signals initiate intracellular signal transduction events that activate long-lasting changes in brain function and behavior. Our electrophysiological and immunocytochemical studies demonstrate that 16-24 h of water deprivation causes a significant increase in the amplitude of L-type Ca^{2+} current (from -55.5 ± 6.2 to -99.1 ± 10.0 pA) but not in other types of Ca^{2+} current. This increase occurred in both VP- and OT-MNCs. Such an increase in L-type Ca^{2+} current may contribute to modulation of firing rate and pattern, regulation of vasopressin release, structural adaptation in MNCs during sustained dehydration.

The mechanisms underlying the transition of the electrical behaviour are not completely understood. Ion channels, especially osmosensitive ion channels, play key roles in the modulation of MNC firing. A voltage-gated, 4-AP- and TEA-insensitive slowly activating

outward current displayed a significant increase in about 66% of MNCs when the osmolality of the external solution was acutely increased from 295 to 325 mosmol kg^{-1}. The responding cells showed an increase in net outward current from 12.3 ± 1.3 pA/pF to 21.4 ± 1.8 pA/pF. The reversal potential of this current was near the equilibrium for K^+ and shifted with changes of K^+ concentrations in external solution, suggesting that this current is a K^+-selective current. The KCNQ/M current selective blockers linopirdine (150 μM) and XE991 (5 μM) suppressed this current. The IC_{50} of XE991 blockade was 3.9 μM. The KCNQ/M channel openers retigabine (10 μM) and flupirtine (10 μM) significantly increased the current and shifted its activation curve toward more negative potentials. E4031, a specific blocker of ERG K^+ channels, did not significantly block this current. The results from immunocytochemistry suggest that MNCs express KCNQ2, KCNQ3, KCNQ4, and KCNQ5, but not KCNQ1. These data suggest that this osmosensitive current could be a KCNQ/M current. Studies using single unit extracellular recording in hypothalamic explants showed that 10 μM XE991 increased MNC firing rate and that 20 μM retigabine decreased firing rate or caused a cessation of firing. These data suggest that a KCNQ/M current contributes to the regulation of MNC firing. KCNQ/M channels play key roles in regulating neuronal excitability in many types of central neurons. Slow activation of this current during firing might suppress activity by hyperpolarizing the cells and thus contribute to a transition between fast continuous and burst firing.

Our studies will be beneficial to understand the mechanisms that control VP and OT in response to acute changes in osmolality and also the mechanisms underlying MNC adaptation during sustained dehydration.

ACKNOWLEDGEMENTS

A number of people kindly offered support during my graduate studies. I would like to specifically thank Dr. Thomas E Fisher, my supervisor, not only for offering me an opportunity to pursue my Ph D study in neuroelectrophysiology but also for his encouragement, advice, and careful guidance during my studies. He very kindly shared his research experience, professional expertise, and research insight with me, and I would like to thank him not only for his patience but also for his support in applying for my scholarship, in submission of manuscripts, and in my travel to several scientific conferences. I would also like to thank him for improving my confidence in my research career and for helping me to become an independent neuroscience researcher. Such happy research experiences have been greatly helpful for my professional development.

I would like to thank our graduate chair, Dr. Nigel West, my advisory committee members, Drs. Michel Desautels, Wolfgang Walz, and John Howland, and previous committee members, Drs. Prakash Sulakhe and John Tuchek, for their help and guidance during my studies and the writing of this thesis. In addition, I would like to thank my External Examiner, Dr. Alastair Ferguson from Queen's University.

I would like to thank Xuan Vo, Dr. Kosala Rajapaksha, Xiaohong Liu, Blanc Star, Xiaoyu Xu, Daoyi Wang, and other people in Dr. Fisher's laboratory and in the Department of Physiology, for their help. I would like to thank my wife, Xuelian Wang, for encouragement and support.

I would also like to extend thanks to Dr. Charles W. Bourque and Jessica Sudbury for their expert assistance with the extracellular recording experiments, Drs. Bechara Kachar of

NIDCD/NIH and Ken Mackie of Indiana University for generously providing antibodies (directed against $K_V7.4$, and $K_V7.2$ and $K_V7.3$, respectively), and Valeant Research and Development for kindly providing us with a sample of retigabine.

I would like to thank the College and Medicine, University of Saskatchewan, the Heart and Stroke Foundation of Saskatchewan, and the Canadian Institute of Health Research for their financial support for my scholarships and for travel to several scientific conferences.

DEDICATION

This thesis is dedicated to my family, especially to my wife, Xuelian Wang. They have been giving motivation and inspiration to me and offering me unconditional love and support throughout my graduate studies.

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LIST OF ABBREVIATIONS

ACSF: artificial cerebrospinal fluid

AHP: afterhyperpolarizing potential

4-AP: 4-aminopyridine

AQP: aquaporin

BAPTA: 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraaecetic acid

BFNC: benign familial neonatal convulsion

BK: large-conductance Ca^{2+}-activated K^+ channel

$[Ca^{2+}]_i$: intracellular Ca^{2+} level

CAN: calcium-activated non-specific

CREB: cAMP-responsive element binding

DAG: diacylglycerol

DAP: depolarizing afterpotential

DFNA2: dominant progress hearing loss

DHP: dihydropyridine

DIC: differential contrast interference

diC8-PIP_2: dioctanoyl PIP_2

ECF: extracellular fluid

E_K: the equilibrium potential for potassium

ERG: *ether-a-go-go*-related gene

fAHP: fast afterhyperpolarizing potential

FFA: flufenamic acid

HAP: hyperpolarizing afterpotential

IK: intermediate-conductance Ca^{2+}-activated K^+ channel

IP_3: inositol 1, 4, 5-trisphosphate

K_{Ca}: Ca^{2+}-activated K^+ channel

LTP: long-term potentiation

mAHP: medium afterhyperpolarizing potential

MNC: magnocellular neurosecretory cell

MnPO: median preoptic nucleus

NMDA: N-methyl-D-aspartate

NMDG: N-methyl-D-glucamine

NP: neurophysin

OC: osmosensitive current

OT: oxytocin

OVLT: organum vasculosum of the lamina terminalis

PBS: phosphate-buffered saline

PDZ: postsynaptic density-95 (PSD-95)/Discs large/zona occludens-1 (ZO-1)

PI: phosphoinositide

PIP: phosphatidylinositol phosphate

PIP_2: phosphatidylinositol (4,5)-bisphosphate

PIP_3: phosphatidylinositol (3,4,5)-trisphosphate

PIPES: piperazine-1,4-bis(2-ethanesulfonic acid)

PLC: phospholipase C

PVN: paraventricular nucleus

RT-PCR: reverse transcription-polymerase chain reaction

RVD: regulatory volume decrease

RVI: regulatory volume increase

S1-6: transmembrane segments 1-6

sAHP: slow afterhyperpolarizing potential

SFO: subfornical organ

SIC: stretch inactivated cation channel

SK: small-conductance Ca^{2+}-activated K^+ channel

SNARE: soluble N-ethylmaleimide-sensitive factor attachment receptor

SON: supraoptic nucleus

TASK: TWIK-related acid-sensing K^+ current

TEA: tetraethylammonium

TRAAK: TWIK-related arachidonic acid activated K^+ current

TREK: TWIK-related K^+ current

TRP: transient receptor potential

TRPA: transient receptor potential (ankyrin)

TRPC: transient receptor potential (canonical)

TRPM: transient receptor potential (melastatin)

TRPML: transient receptor potential (mucolipin)

TRPP: transient receptor potential (polycystin)

TRPV: transient receptor potential (vanilloid)

TTX: tetrodotoxin

VDCC: voltage-dependent Ca^{2+} channel

VP: vasopressin

XE991: 10, 10-bis (4-pyridinylmethyl)-9(10H)-anthracenone

1. INTRODUCTION

1.1. Body fluid homeostasis and the central osmoreceptors

The extracellular fluid (ECF) compartment, one of two major compartments containing body water, includes three major subcompartments: blood plasma, interstitial fluid, and transcellular fluid (Nanovic, 2005), and it contains many cations and anions. Na^+ and Cl^- are the major cation and anion (Guyton & Hall, 1996), respectively. The cell, a fundamental element of all life, has a surrounding plasma membrane, which forms a relatively separated intracellular environment. The cell membrane as biological membrane is a lipid bilayer which is a permeability barrier due to its property of semipermeability and thus causes great differences in the concentrations of particles across the plasma membrane (Guyton & Hall, 1996). Thus, the differences in the concentrations of substances as osmoles across the membrane will lead to a difference in pressure across the membrane (Guyton & Hall, 1996), which is called osmotic pressure and is dependent on the numbers of solutes in the ECF (Guyton & Hall, 1996). The osmoles per kilogram of solvent determine the value of osmolality (Guyton & Hall, 1996). Mammals, through the regulation of sodium and water balance, are able to keep their osmotic pressure at a stable set point, which varies between types of organisms (Bourque, 1998; Candela & Yucha, 2004). Osmoreceptors in the central nervous system play a crucial role in the body fluid homeostasis (Bourque & Oliet, 1997). The site of central osmoreceptors was explored sixty years ago, and the anterior region of the hypothalamus was first thought to be the central osmoreceptors that control the release of vasopressin (VP; Verney, 1947; Jewell & Verney, 1957). VP, as an antidiuretic hormone, is the primary hormone that regulates plasma osmolality by binding to the receptors on the cells in the collecting ducts of the kidney to control water excretion from the kidney (Chinard, 1964; Nielsen *et al.*, 1995; Bourque & Oliet, 1997). VP

plays many physiological roles by acting on G protein-coupled VP receptors, which include three types (Petersen, 2006; Ball, 2007), V_{1a}, V_2, and V_{1b} receptors. The primary locations of the V_{1a} receptors, which contribute to muscle contraction, glycogenolysis, platelet adhesion, and brain functions such as learning and memory, social behaviour, and thermoregulation, are in vascular smooth muscle, uterus, liver, platelets, and central nervous system (Petersen, 2006; Ball, 2007). The pituitary corticotroph is the primary location of the V_{1b} receptors, which regulate the release of adrenocorticotropic hormone (Ball, 2007). Osmoreceptors of the hypothalamus express V_{1a} and V_{1b} receptors, which may mediate inhibitory or excitatory effects of VP on the osmoreceptors (Hurbin *et al.*, 1998). The primary location of the V_2 receptors are in the basolateral membrane of the collecting duct in the distal nephron (Ball, 2007). VP can bind to V_2 receptors to cause translocation of aquaporin-2 (AQP2) water channels from cellular vesicles to the apical plasma membrane, which enables the reabsorption of water into the cells (Petersen, 2006). Water exit from the cells is then mediated by AQP3 and AQP4 water channels expressed in the basolateral plasma membrane of the collecting duct principal cells (Petersen, 2006). Mutations of the V_2 receptors cause insensitivity of the collecting duct to VP and ultimately lead to nephrogenic diabetes insipidus (Spanakis *et al.*, 2008).

The level of plasma VP is dependent on osmolality of the plasma (Bourque, 1998). VP, a peptide of 9 amino acids (van Kesteren *et al.*, 1992), is synthesized in magnocellular neurosecretory cells (MNCs), which are central osmoreceptors (Bourque & Oliet, 1997). The supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus are primary locations of the somata of the MNCs (Figure 1.1; Bourque & Oliet, 1997). Both the SON and the PVN contain two paired parts. Each of the SONs contains about 4400 to 7000 MNCs in rats, and each of the PVNs contains approximately 1300 to 2000 MNCs (Swanson &

Sawchenko, 1983). In addition, the MNCs are also found in the region between the SON and the PVN (Swanson & Sawchenko, 1983). The MNCs also synthesize another hormone, oxytocin (OT), which causes the uterus to contract at birth and milk ejection during lactation (Bourque & Oliet, 1997). In some cases, OT can also play a role in osmoregulation by natriuresis, and changes in the plasma osmolality can lead to alteration of plasma levels of both VP and OT (Bourque & Oliet, 1997).

In addition, the neurons located in the lamina terminalis (Figure 1.1), including the median preoptic nucleus (MnPO), the subfornical organ (SFO), and the organum vasculosum of the lamina terminalis (OVLT), target the MNCs through synaptic transmission and also sense small changes in osmolality (Bourque & Oliet, 1997). These neurons therefore are also central osmoreceptors and play a central role in the regulation of body fluid homeostasis (Bourque *et al.*, 1994). Some peripheral osmoreceptors (Figure 1.1), which are situated primarily in the hepatic portal vein, the gastrointestinal tract, the oropharyngeal cavity, and so on, also play a role in the regulation of body fluid homeostasis (Bourque, 2008).

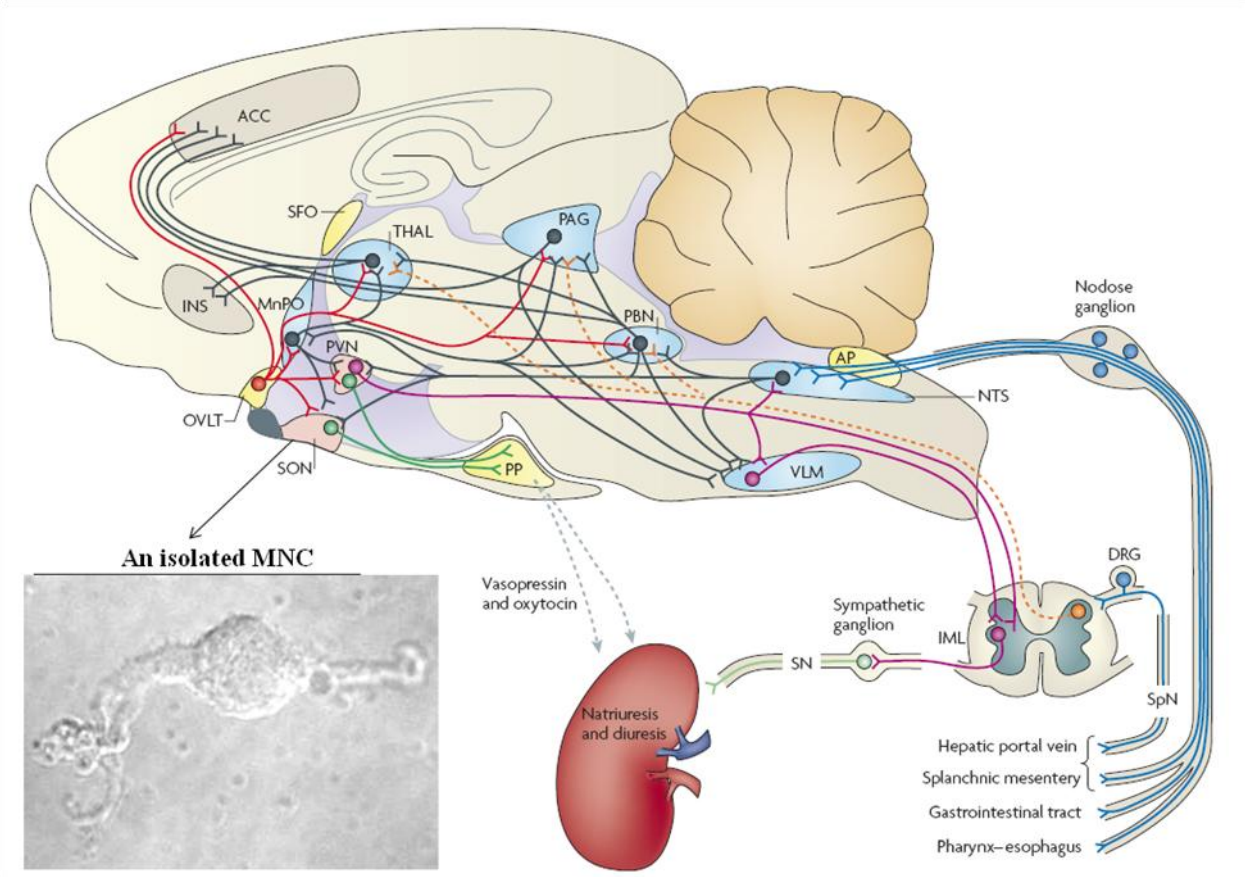


Figure 1.1 Central and peripheral osmoreceptors. ACC, anterior cingulate cortex; AP, area postrema; DRG, dorsal root ganglion; IML, intermediolateral nucleus; INS, insula; MnPO, median preoptic nucleus; NTS, nucleus tractus solitarius; OVLT, organum vasculosum laminae terminalis; PAG, periaqueductal grey; PBN, parabrachial nucleus; PP, posterior pituitary; PVN, paraventricular nucleus; SFO, subfornical organ; SN, sympathetic nerve; SON, supraoptic nucleus; SpN, splanchnic nerve; THAL, thalamus; VLM, ventrolateral medulla. (Bourque, 2008) (Reproduced with permission from Copyright Clearance Center)

1.2 The magnocellular neurosecretory cells (MNCs) and vasopressin (VP) release

1.2.1 VP secretion is a Ca^{2+}-dependent event

VP and OT are produced by the MNCs. Axons from the MNCs extend through the median eminence and terminate in the neural lobe of the neurohypophysis (Hatton, 1997). VP and OT are packed into secretory vesicles after synthesis in the somata of the MNCs. These vesicles are transported through axons to the terminals in the neurohypophysis and the secretory granules are then translocated to the plasma membrane. The secretory vesicle membrane will fuse with the plasma membrane and the hormone and other contents in the secretory granules, following an increase in intracellular Ca^{2+} level ($[Ca^{2+}]_i$), are then released into the extracellular space (Theodosis *et al.*, 1978; Bicknell, 1988; Lim *et al.*, 1990; Lindau *et al.*, 1992). Thus, VP and OT are released into the blood stream by Ca^{2+}-dependent exocytosis and the release is a Ca^{2+}-dependent event (Fisher & Bourque, 2001). Ca^{2+} entry across the plasma membrane in MNCs is conducted primarily by voltage-dependent Ca^{2+} channels (VDCCs), which play many crucial roles not only in the regulation of membrane excitability but also in cellular signal transduction (Fisher & Bourque, 2001). The voltage sensitivity is an important feature of these channels, and such a property enables them to respond to depolarization in the MNC terminals induced by propagation of excitatory signals from the cell body. Thus, the depolarization in the terminals leads to the VDCCs to open and causes Ca^{2+} influx and subsequent hormone release. Na^+/Ca^{2+} exchangers in a reverse mode can also contribute to the Ca^{2+} entry across the plasma membrane (Saris & Carafoli, 2005). In addition, sequestration of intracellular Ca^{2+} in neurons plays an important role in the regulation of $[Ca^{2+}]_i$ and the endoplasmic reticulum contributes to the regulation of $[Ca^{2+}]_i$ by using internal membrane Ca^{2+}-ATPase to pump Ca^{2+} into stores or by using Ca^{2+} release channels to release Ca^{2+} from the stores into the cytosol (Carafoli, 2002). Ca^{2+}

release from the internal Ca^{2+} stores can also contribute to hormone release in the MNCs (Li & Hatton, 1997a; Sladek & Kapoor, 2001).

1.2.2 Voltage-dependent Ca^{2+} channels

The VDCCs, which are broadly expressed in various tissues including excitable cell types, are composed of a superfamily of ion channels sensitive to membrane potentials and selectively permeable to Ca^{2+}, and they have been reviewed in detail in MNCs (Fisher & Bourque, 1996) and in other types of cells (Hofmann *et al.*, 1999; Catterall, 2000; Fisher & Bourque, 2001; Jeziorski & Greenberg, 2006). Ca^{2+} signaling plays crucial roles in cellular functions such as excitation-contraction coupling in muscle cells, excitation-secretion coupling in neuroendocrine cells, synaptic transmission, gene expression, and other Ca^{2+}-dependent events. The VDCC is a multimeric complex (Figure 1.2) containing an α_1 subunit, which forms the Ca^{2+}-selective pore of the channel and contains the binding site for the channel blockers, and three auxiliary subunits including a transmembrane α_2-δ subunit, an intracellular β subunit, and a transmembrane γ subunit (Hofmann *et al.*, 1999; Catterall, 2000; Jeziorski & Greenberg, 2006). Ten individual genes for α_1 subunits have been identified (Table 1.1), and they form three families, Ca_V1, including $Ca_V1.1$ (α_{1S}), $Ca_V1.2$ (α_{1C}) $Ca_V1.3$ (α_{1D}), and $Ca_V1.4$ (α_{1F}), Ca_V2, including $Ca_V2.1$ (α_{1A}), $Ca_V2.2$ (α_{1B}), and $Ca_V2.3$ (α_{1E}), and Ca_V3, including $Ca_V3.1$ (α_{1G}), $Ca_V3.2$ (α_{1H}), and $Ca_V3.3$ (α_{1I}). The α_1 subunit is composed of four homologous domains from domain I to domain IV, each of which contains six transmembrane segments from S1 to S6. The S4 segment has positively charged residues and is a voltage sensor. The VDCCs, due to the differences in biophysical and pharmacological properties, are divided into several subtypes including L- ($Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$, and $Ca_V1.4$), P/Q- ($Ca_V2.1$), N- ($Ca_V2.2$), R- ($Ca_V2.3$),

and T-types ($Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$) (Table 1.1), and specific Ca^{2+} entry from these different subtypes play individual roles in Ca^{2+}-dependent events (Fisher & Bourque, 2001). Several types of the VDCCs, including N-, P/Q-, and L-types, are functionally expressed in the somata and/or axon terminals of the MNCs (Table 1.1; Fisher & Bourque, 1995, 1996; Foehring & Armstrong, 1996; Glasgow *et al.*, 1999; Joux *et al.*, 2001).

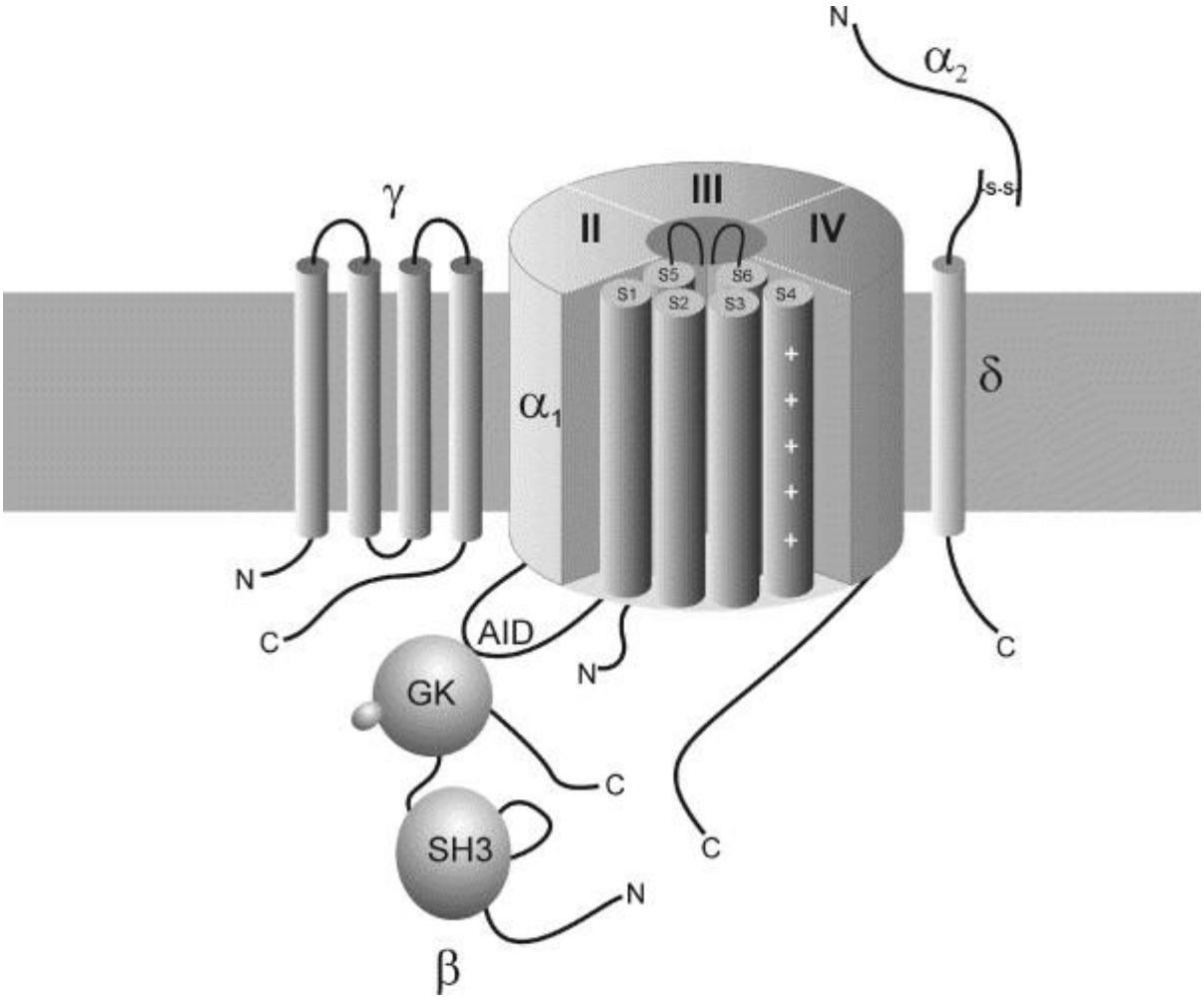


Figure 1.2 Structure of the voltage-gated Ca^{2+} channels. AID: alpha interaction domain, GK: guanylate kinase, SH3: Src homology 3. (Jeziorski & Greenberg, 2006) (Reproduced with permission from Copyright Clearance Center)

Table 1.1 Subtypes, pharmacology, and roles of voltage-dependent Ca^{2+} channels. (Fisher & Bourque, 1996, 2001)

Voltage-dependent Ca^{2+} channel	High voltage-activated Ca^{2+} channel				Low voltage-activated Ca^{2+} channel
Channel subtype	L-type	P/Q-type	N-type	R-type	T-type
α_1 subunit designation (old/new nomenclature)	$\alpha_1 1.1/\alpha_{1S}$ $\alpha_1 1.2/\alpha_{1C}$ $\alpha_1 1.3/\alpha_{1D}$ $\alpha_1 1.4/\alpha_{1F}$	$\alpha_1 2.1/\alpha_{1A}$	$\alpha_1 2.2/\alpha_{1B}$	$\alpha_1 2.3/\alpha_{1E}$	$\alpha_1 3.1/\alpha_{1G}$ $\alpha_1 3.2/\alpha_{1H}$ $\alpha_1 3.3/\alpha_{1I}$
Antagonists	Dihydropyridines	ω-agatoxin-IVA	ω-conotoxin-GVIA	SNX482	kurtoxin
Expression in MNCs	Soma/Terminal	P-type: Soma Q-type: Terminal	Soma/Terminal	Soma	Soma
Function in secretion	For $\alpha_1 1.2$ and $\alpha_1 1.3$: Endocrine and neuroendocrine secretion, slow release from neuronal terminals and dendrites. For $\alpha_1 1.1$ and $\alpha_1 1.4$: None known	Neurotransmission, endocrine and neuroendocrine secretion			None known

1.2.2.1 L-type Ca^{2+} channels

1.2.2.1.1 Biophysics and pharmacology of the L-type Ca^{2+} channels

L (for long-lasting)-type Ca^{2+} channels belong to Ca_V1 family. The channel activates at voltages more positive than -40 mV and are therefore high voltage-activated Ca^{2+} channels (Nowycky *et al.,* 1985; Fox *et al.,* 1987) except for the $Ca_V1.3$, which activates at more negative potentials (> -50 mV) and mediates a low threshold Ca^{2+} current (Koschak *et al.,* 2001). Peak current can be obtained when the voltages are beyond 0 mV (Nowycky *et al.,* 1985; Fox *et al.,* 1987). The L-type Ca^{2+} channel has a conductance of 25 pS and the channel shows very slow inactivation with a $t_{1/2}$ of over hundreds of milliseconds (Nowycky *et al.,* 1985; Fox *et al*., 1987), which is dependent on intracellular Ca^{2+} level and membrane potentials (Lee *et al.*, 1985; Morad & Soldatov, 2005). One of important features of the L-type Ca^{2+} channel is that the channel shows sensitivity to dihydropyridine (DHP) because the S5 and S6 segments of domain III and the S6 segment of the domain IV in the α_1 subunits contain DHP binding sites (Striessnig *et al.,* 1998), and the channel can therefore be activated by agonists such as Bay K 8644, but selectively blocked by its antagonists such as nifedipine. The selective blockers of the L-type Ca^{2+} channel from DHP derivatives have been shown to have a therapeutic role and are broadly used in the clinic (Triggle, 2006), specifically in the treatment of hypertension.

1.2.2.1.2 Distribution, physiology, and pathophysiology of the L-type Ca^{2+} channels

Four members of the L-type Ca^{2+} channels have been identified (Table 1.2) and they are $Ca_V1.1$ (α_{1S}), $Ca_V1.2$ (α_{1C}), $Ca_V1.3$ (α_{1D}), and $Ca_V1.4$ (α_{1F}) (Hofmann *et al.,* 1999). The L-type Ca^{2+} channels have been found to be expressed in various tissues. $Ca_V1.1$ is specifically expressed in regions of the T-tubular membrane of the skeletal muscle in which contraction is

triggered by membrane depolarization and plays a key role in the excitation-contraction coupling of the skeletal muscle (Schneider & Chandler, 1973; Tanabe *et al.*, 1988). Thus, mutations at charged residues in the S4 segment of the channel lead to thyrotoxic hypokalaemic periodic paralysis, a heritable muscle disorder (Dias da Silva *et al.*, 2002). In addition, mutations (R1086H and R1086C) of the proteins mediating the $Ca_V1.1$ channel gene are associated with malignant hyperthermia (Striessnig *et al.*, 2004), a potentially lethal inherited autosomal dominant disorder and also linked with a mutation of ryanodine receptor type 1 of muscle sarcoplasmic reticulum (Striessnig *et al.*, 2004). $Ca_V1.4$ is specifically expressed in the retina with a role in synaptic transmission between photoreceptors and bipolar cells (Strom *et al.*, 1998), and in immune cells with unknown roles. Mutations of the proteins mediating the $Ca_V1.4$ channel gene in the retina lead to congenital stationary night blindness, an inherited non-progressive eye disorder (Strom *et al.*, 1998). Both $Ca_V1.2$ and $Ca_V1.3$ are broadly expressed in many cells and play several physiological roles such as the regulation of learning and memory, blood pressure, heart development and rhythm, hair cell generation, insulin secretion, and bladder contraction (Table 1.2; Bourinet *et al.*, 2004; Moosmang *et al.*, 2005). The L-type Ca^{2+} channel signaling system, therefore, has physiological and pathophysiological significance and offers several therapeutic targets (Striessnig *et al.*, 2006). Mice with homozygous knockout of the $Ca_V1.2$ gene only survive till day 14.5 post coitum because of the dysfunction of cardiomyocytes (Seisenberger *et al.*, 2000). Ca^{2+} currents mediated by the $Ca_V1.2$ channels contribute to about 50% of phenylephrine-induced vascular contraction (Moosmang *et al.*, 2003), and the relaxant effects of the DHP in vascular smooth muscle totally disappear in the knockout mice of the $Ca_V1.2$ gene (Sinnegger-Brauns *et al.*, 2004). In addition, effects of DHP on inhibiting the L-type Ca^{2+} current and insulin secretion in pancreatic β cells are absent in those

mice (Sinnegger-Brauns *et al.*, 2004). These studies suggest that the $Ca_V1.2$ Ca^{2+} channel plays an essential role in regulating contractility of vascular smooth muscle, blood pressure, and insulin secretion. The knockout of the $Ca_V1.3$ channel gene causes congenital deafness and dysfunction of the cardiac pacemaker (Platzer *et al.*, 2000), suggesting that the Ca^{2+} channels formed by the $Ca_V1.3$ play crucial physiological roles in cochlear and outer hair cells and cardiac pacemaker cells.

Table 1.2 Distribution and function of the L-type Ca^{2+} channel-subtypes (Moosmang *et al.*, 2005; Striessnig *et al.*, 2004; Strom *et al.*, 1998; Bourinet *et al.*, 2004)

L-type Ca^{2+} channel				
Channel subtype	$Ca_V1.1$	$Ca_V1.2$	$Ca_V1.3$	$Ca_V1.4$
α_1 subunit family (Old/New nomenclature)	$\alpha_1 1.1/\alpha_{1S}$	$\alpha_1 1.2/\alpha_{1C}$	$\alpha_1 1.3/\alpha_{1D}$	$\alpha_1 1.4/\alpha_{1F}$
Expression	Skeletal muscle specific	Ubiquitous and coexpressed in many cells	Ubiquitous and coexpressed in many cells	Retina and immune cells specific
Function	Excitation-contraction coupling of the skeletal muscle	Controlling blood pressure. Regulating heart development. Regulating mood, modulating LTP, spatial learning, and other roles in brain function. Insulin secretion. Bladder contractions.	Sinus node rhythm. Hair cell generation. Regulating mood, modulating LTP, and other roles in brain function.	Synaptic transmission between photoreceptors and bipolar cells in the retina. Unknown roles in immune cells.

1.2.2.1.3 L-type Ca^{2+} channels in gene expression and brain function

Although Ca^{2+} can influx through all types of VDCCs, Ca^{2+} influx through L-type Ca^{2+} channels preferentially activates some cellular signal transduction pathways and plays a crucial role in transcriptional responses (Cartin *et al.*, 2000; West *et al.*, 2001; Spangenburg *et al.*, 2004; Wamhoff *et al.*, 2004). Specifically in the central nervous system, the L-type Ca^{2+} channel-mediated Ca^{2+} signals initiate intracellular signal transduction events that activate long-lasting changes in brain function and behaviour (Hetzenauer *et al.*, 2006; Striessnig *et al.*, 2006). Both $Ca_V1.2$ and $Ca_V1.3$ are broadly and functionally expressed in the central nervous system (Moosmang *et al.*, 2005). However, $Ca_V1.3$, unlike $Ca_V1.2$, can mediate a low threshold Ca^{2+} current and thus regulate neuronal excitability and neurotransmitter release by subthreshold Ca^{2+} signaling (Koschak *et al.*, 2001). The L-type Ca^{2+} channels not only contribute to N-methyl-D-aspartate (NMDA) receptor-independent long-term potentiation (LTP; Morgan & Teyler, 1999) but also downregulate NMDA receptor-dependent LTP by the β3 subunits of the Ca_V channels (Jeon *et al.*, 2008). Ca^{2+} influx from the L-type Ca^{2+} channels of the $Ca_V1.2$ and $Ca_V1.3$ subfamily in the brain can preferentially activate transcription factors such as cAMP-responsive element binding (CREB) proteins (Figure 1.3; Hardingham *et al.*, 1999; Weick *et al.*, 2003; Zhang *et al.*, 2006) and c-Fos (Hetzenauer *et al.*, 2006; Zhao *et al.*, 2007). Formation of LTP and various forms of memory are tightly associated with activity of the CREB proteins (Frank & Greenberg, 1994; Yin & Tully, 1996; Silva *et al.*, 1998). Ca^{2+} entry can cause phosphorylation of the CREB proteins by two pathways (Rajadhyaksha & Kosofsky, 2005), a calmodulin kinase IV-dependent pathway and a mitogen-activated protein kinase signaling pathway. The molecular and cellular mechanisms underlying this role of the L-type Ca^{2+} channel in brain function are not completely known. However, some specific structures of the L-type Ca^{2+} channel are important.

The L-type Ca^{2+} channel expresses an isoleucine-glutamine motif in the C-terminus of the channel, which is a binding site of Ca^{2+}-calmodulin and also necessary for activation of the pathways (Dolmetsch *et al.*, 2001). In addition, phosphorylation of the CREB proteins induced by Ca^{2+} entry from the L-type Ca^{2+} channel needs the involvement of PDZ, postsynaptic density-95 (PSD-95)/Discs large/zona occludens-1 (ZO-1), domain proteins in the C-termini of both $Ca_V1.2$ and $Ca_V1.3$ subunits (Figure 1.3; Weick *et al.*, 2003; Zhang *et al.*, 2005). The $Ca_V1.3$ subunits, through the PDZ domain protein, also connect with Shank (SH3 domain and ankyrin repeat-containing synaptic scaffolding protein), which also interacts with an adaptor protein (Figure 1.3; Zhang *et al.*, 2005), and this interaction is critical for the phosphorylation of the CREB proteins (Zhang *et al.*, 2006; Calin-Jageman *et al.*, 2007).

In addition to activating the CREB proteins, Ca^{2+} entry from the L-type Ca^{2+} channels can also activate other important transcription factors. Ca^{2+} entry specifically from the L-type Ca^{2+} channels activates calcineurin (Rajadhyaksha & Kosofsky, 2005), which is a protein phosphatase also known as protein phosphatase 2B. Calcineurin can control nuclear factor of activated T cell, a cytoplasmic component of transcription factors, and thus regulate expression of several genes (Rajadhyaksha & Kosofsky, 2005). Thus, calcineurin plays important roles in learning and memory (Groth *et al.*, 2003). The L-type Ca^{2+} channel in rat hippocampus also mediates activation of nuclear factor-kappa B, a protein complex of transcription factors (Shen *et al.*, 2002). Therefore, the activation of these transcription factors by Ca^{2+} entry from the L-type Ca^{2+} channels contributes to formation of various types of memory and processes of synaptic plasticity and thus causes long-lasting changes in brain function and behaviour.

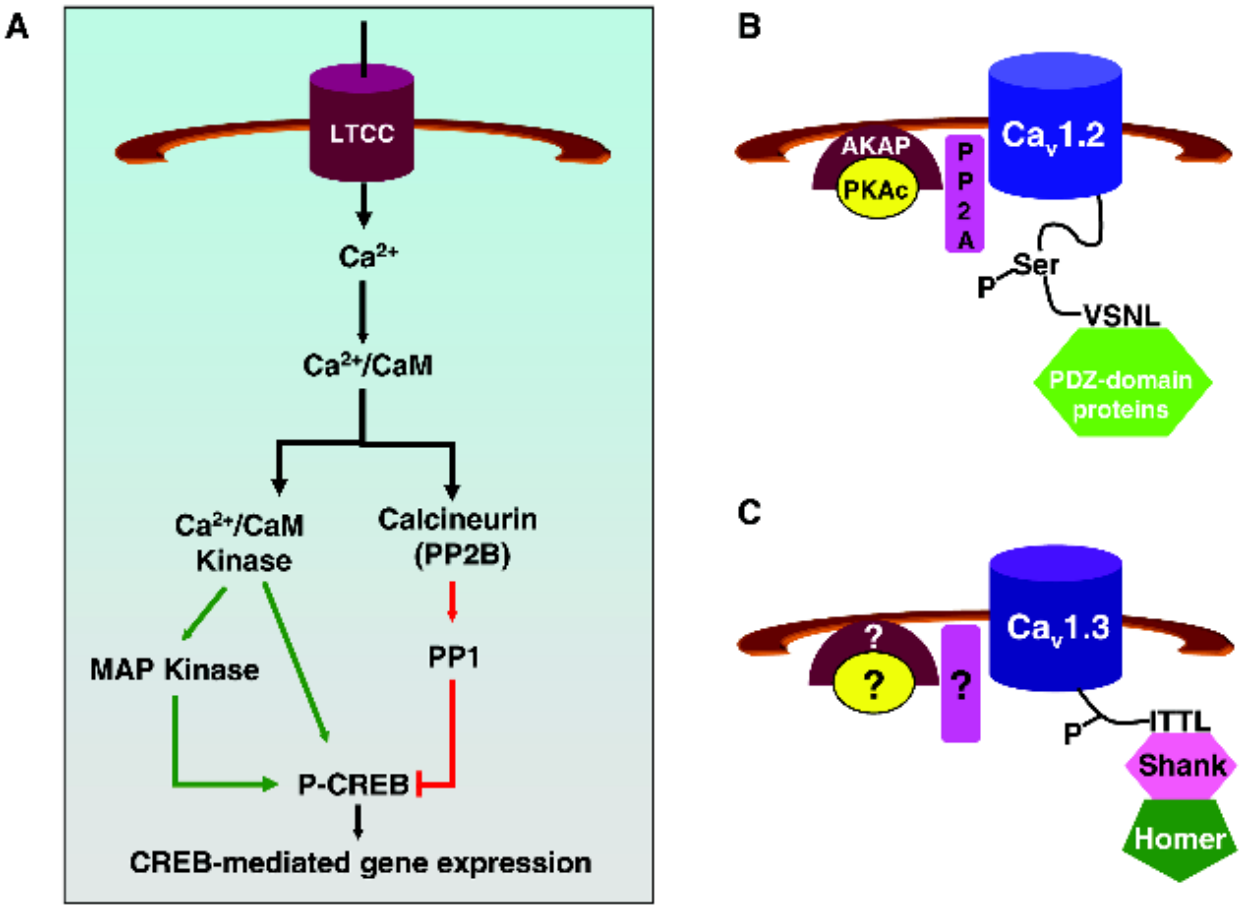


Figure 1.3 L-type Ca^{2+} channel (LTCC)-mediated Ca^{2+} pathways on activation of the transcription factor, cAMP-responsive element binding (CREB) proteins. CaM, calmodulin; ITTL, Ile-Thr-Thr-Leu; MAP, mitogen-activated protein; PDZ, postsynaptic density-95 (PSD-95)/Discs large/zona occludens-1 (ZO-1); PP2A, protein phosphatase 2A; PP2B, calcineurin; VSNL, Val-Ser-Asn-Leu;, Shank, SH3 domain and ankyrin repeat-containing protein. (?, unknown proteins) (Rajadhyaksha & Kosofsky, 2005) (Reproduced with permission from Copyright Clearance Center)

1.2.2.1.4 Expression of the L-type Ca^{2+} channels in MNCs

L-type Ca^{2+} channels are expressed not only in the somata of the MNCs but also in the terminals (Fisher & Bourque, 1996) and the proportion of the L-type Ca^{2+} channels is about 23%-28% of the total VDCC currents in the somata of the MNCs (Fisher & Bourque, 1995; Foehring & Armstrong, 1996). Both $Ca_V1.2$ and $Ca_V1.3$ are expressed in the somata and dendrites of the MNCs (Joux *et al.*, 2001).

1.2.2.2 T-type Ca^{2+} channels

T (for transient and tiny)-type Ca^{2+} channels belong to Ca_V3 family and mediate tiny Ca^{2+} currents with single channel conductance of 3.6-9 pS (Huguenard, 1996). The T-type Ca^{2+} current is a transient current with a time constant of inactivation of between 10-100 ms, which is dependent on voltages, channel subtypes, and cell types expressing the channel (Huguenard, 1996). The T-type Ca^{2+} current activates at voltages more positive than -60 mV (Nowycky *et al.*, 1985) and is therefore a low voltage-activated Ca^{2+} current. The T-type Ca^{2+} channel can be selectively blocked by a scorpion toxin (kurtoxin; Chuang *et al.*, 1998). The T-type Ca^{2+} channel has been identified in many neurons, and its physiological roles such as regulating sleep rhythm, neuropathic pain, and absence epilepsy have been reviewed in detail (Shin *et al.*, 2008). In some excitable cells, like cardiac pacemaker cells**,** the low threshold T-type Ca^{2+} channel activates at voltages below the threshold of action potentials and contributes to generation of spontaneous action potentials (Noble, 1984). In addition, the T-type Ca^{2+} channel contributes to a low threshold-activated afterdepolarizing potential to initiate burst firing in dorsal root ganglion neurons and thus regulates the generation of neural information (White *et al.*, 1989). The T-type Ca^{2+}-channel also has a physiological significance in endocrine cells such as pancreatic β cells

(Bhattacharjee *et al.*, 1997). The T-type Ca^{2+} current is expressed in the somata of the MNCs but not in the axon terminals, and may play an important role in modulating MNC firing rate and pattern (Fisher & Bourque, 1995, 1996).

1.2.2.3 N-type Ca^{2+} channels

N (for neither L nor T)-type Ca^{2+}-channels belong to the $Ca_V2.2$ family and have intermediate biophysical properties between L-type Ca^{2+} channels and T-type Ca^{2+} channels (Nowycky *et al.*, 1985; Fox *et al*., 1987). The N-type Ca^{2+} channel can be selectively blocked by a cone snail toxin ω-conotoxin-GVIA (McCleskey *et al.*, 1987). The N-type Ca^{2+} channel is broadly expressed in neurons and endocrine cells, interacts with SNARE (soluble N-ethylmaleimide-sensitive factor attachment receptor) proteins, participates in the coupling between excitation and secretion, and thus regulates neurotransmitter release (Fisher & Bourque, 2001; Fox *et al.*, 2008). In addition, the N-type Ca^{2+} channel has also been found to play an important role in modulating long-term changes in brain function by regulating LTP (Jeon *et al.*, 2007). The N-type Ca^{2+} channel is expressed in both the somata of the MNCs and in the terminals (Fisher & Bourque, 1996) and the proportion of the N-type Ca^{2+} current is about 26%-40% of the total VDCC currents in the somata of the MNCs (Fisher & Bourque, 1995; Foehring & Armstrong, 1996).

1.2.2.4 P/Q-type Ca^{2+} channels

The P-type Ca^{2+} channel was first identified in mammalian Purkinje cell neurons and the presynaptic terminals in the squid giant synapse, and has a conductance of about 10-12 pS (Llinas *et al.*, 1989). The Q-type Ca^{2+} channel was first isolated from rat cerebellar granule

neurons and its proportion in total Ca^{2+} currents is the largest in these cells (Randall & Tsien, 1995). Like the N-type Ca^{2+} channel, the P/Q-type Ca^{2+} channel is also a high voltage-activated Ca^{2+} channel, but belongs to $Ca_V2.1$. The P/Q-type Ca^{2+} channel can be selectively blocked by a funnel web spider toxin ω-agatoxin-IVA. However, the differences between the P-type Ca^{2+} channel and the Q-type Ca^{2+} channel are that the Q-type Ca^{2+} channel shows inactivation and is much less sensitive to ω-agatoxin-IVA (Randall & Tsien, 1995). The P/Q-type Ca^{2+} channel also interconnects with the SNARE proteins, participates in the coupling between excitation and secretion, and thus triggers neurotransmitter release (Fisher & Bourque, 2001; Fox *et al.*, 2008). The biophysical and pharmacological properties of the current expressed in the MNCs suggest that the P-type Ca^{2+} current is present only in the somata of the MNCs with a proportion of about 17%-20% of total Ca^{2+} currents and that the Q-type Ca^{2+} currents is present only in the terminals (Fisher & Bourque, 1995, 1996; Foehring & Armstrong, 1996).

1.2.2.5 R-type Ca^{2+} channels

R (for resistance)-type Ca^{2+} channels are high voltage-activated Ca^{2+} channels but decay more rapidly than other high voltage-activated Ca^{2+} channels (Zhang *et al.*, 1993). The R-type Ca^{2+} channel-mediated current can be significantly detected at voltages more positive than -40 mV (Zhang *et al.*, 1993). The R-type Ca^{2+} channel has a single channel conductance of about 14 pS and the channel-mediated peak Ca^{2+} current can be obtained at about 0 mV (Zhang *et al.*, 1993). The R-type Ca^{2+} channel is resistant to the selective blockers of L-, N-, P/Q-, and T-type Ca^{2+} channels (Zhang *et al.*, 1993), but selectively blocked by SNX482, a peptide purified from the venom of an African tarantula (Arroyo *et al.*, 2003). In addition, the R-type Ca^{2+} channel, compared with the T-type Ca^{2+} channel, displays much more rapid deactivation (Randall & Tsien,

1997). These properties above suggest that the R-type Ca^{2+} channel may not afford a steady Ca^{2+} current; however, the R-type Ca^{2+} channel can cause a transient but large rise of $[Ca^{2+}]_i$ (Randall & Tsien, 1997). Thus, the R-type Ca^{2+} channel can also play a role in neurotransmitter release (Waterman, 2000; Fisher & Bourque, 2001). In addition, a recent study showed that the R-type Ca^{2+} channel in dendritic spines plays a specific role in activation of small-conductance Ca^{2+}-mediated K^+ (SK) channels (Bloodgood & Sabatini, 2007), which is important in controlling neuronal firing frequency (Faber & Sah, 2003).

1.3 Electrical Activity in MNCs

1.3.1 Firing rate and pattern determine hormone release

Studies have revealed that the MNCs are able to change their firing rate and pattern in response to small changes in external osmolality (Bourque, 1998; Figure 1.4). However, VP- and OT-MNCs can display a great difference in firing patterns during the stimuli. Both the VP-MNCs and the OT-MNCs display infrequent and irregular firing (<3 Hz) under normal conditions, and both can progressively increase the frequency of firing to fast continuous firing (>3 Hz) with increases in osmolality (Figure 1.5) induced by hyperosmotic injection, dehydration, carotid occlusion, or haemorrhage (Poulain & Wakerley, 1982; Bicknell, 1988). Under normal conditions, 73% and 10% of MNCs show slow irregular and fast continuous firing, respectively (Poulain *et al.*, 1977). After haemorrhage, the VP-MNCs increase firing frequency to 6.4 ± 0.6 Hz in four minutes. The OT-MNCs, however, gradually activate over 20 minutes and only increase firing rate to 3.7 ± 0.7 Hz (Poulain *et al.*, 1977). The VP-MNCs can also respond to osmotic stimuli by adopting a phasic pattern of firing (Figure 1.5) characterized by burst discharges of action potentials (5-15 Hz for about 20 seconds) divided by rest intervals lasting 5-

25 seconds (Poulain & Wakerley, 1982; Bicknell, 1988). About 90% of the VP-MNCs after haemorrhage and 84-100% of the VP-MNCs following 6 h dehydration show phasic firing, which is absent in other hypothalamic neurons (Poulain *et al.*, 1977; Wakerley *et al.*, 1978). Although the OT-MNCs can also increase their firing rate during dehydration and haemorrhage, only 3-4% of the OT-MNCs display phasic firing after 24 h dehydration. During a suckling-induced milk ejection, however, the OT-MNCs can show burst firing, a discharge of action potentials with a high frequency of 30-60 Hz (Poulain *et al.*, 1977). VP-MNCs do not respond to the suckling-induced milk ejection (Poulain *et al.*, 1977).

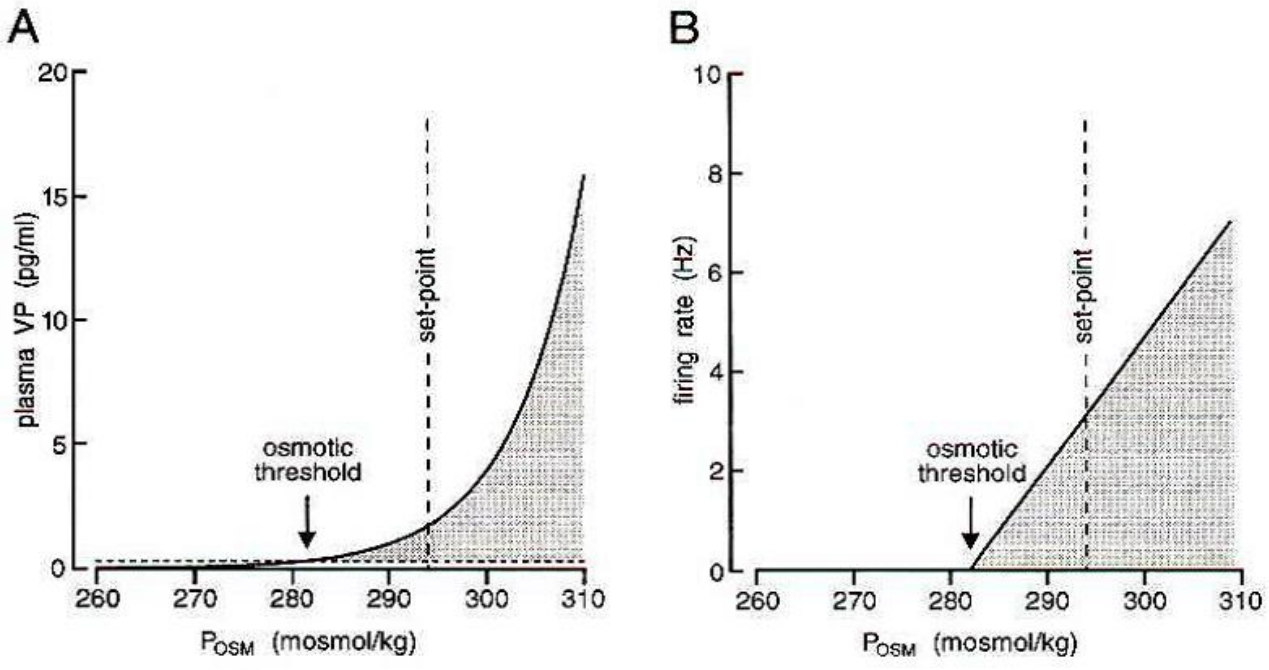


Figure 1.4 Effects of plasma osmolality on vasopressin secretion and firing rate in VP-MNCs. (Bourque, 1998) (Reproduced with permission from Copyright Clearance Center)

Action potentials initiated in the somata of the MNCs depolarize the membrane in the terminals through spike propagation and thus activate the VDCCs of the terminals to cause VP release from the neurohypophysis (Bicknell, 1988; Fisher & Bourque, 2001). Therefore, VP release is dependent on the frequency of MNC firing. The contribution of each action potential to the amount of released VP increases with increases in the firing frequency, but constant firing will cause neuronal fatigue and ultimately lead to a decrease in VP release (Bicknell, 1988). Studies have shown that VP release over a given period is maximized at 10-15 Hz, and an increase in firing frequency will no longer cause more secretion of VP and will even lead to a decrease in secretion (Bicknell, 1988). In addition, VP release during an optimal firing rate starts decreasing when the duration of the stimulation is over 20 s (Bicknell, 1988), and the time for significant recovery from the fatigue is about 10-20 s. Thus, the release of VP is determined primarily by the firing rate and pattern in the somata of the MNCs (Bicknell, 1988). The phasic pattern of firing with a burst of 20 s duration at 12-13 Hz followed by rest intervals of about the same length allows secretory response of the MNCs to recover during the rest intervals and thereby maximizes the secretion of VP (Poulain & Wakerley, 1982; Bicknell, 1988).

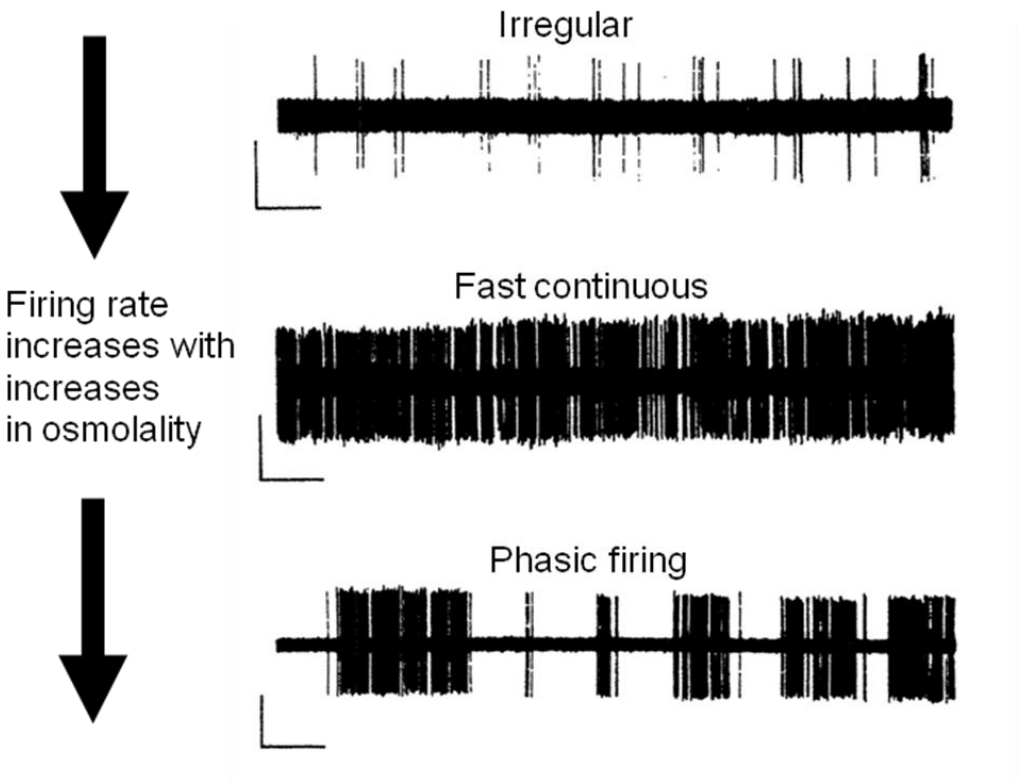


Figure 1.5 MNCs can display three types of electrical activity. The mechanisms of the shift in electrical activity from slow irregular to fast continuous and eventually phasic firing are still unclear. Scale mark is 200 μV and 10 s. The figure was modified from Poulain *et al*.. (Poulain *et al.*, 1977)

1.3.2 Initiation of firing and burst

Plasma osmolality regulates the level of membrane polarization and the firing rate in the MNCs (Bourque, 1998). When the plasma osmolality is near the set point, the resting membrane potential of the MNCs is between -58 mV and -68 mV in rats (values vary with species; Bourque, 1998). Extrinsic (synaptic inputs) and intrinsic factors can cause a small increase in the membrane potential to reach the threshold of an action potential, which is between -44 mV and -55 mV, and initiate firing in the MNCs (Mason, 1980; Bourque, 1989; Gribkoff & Dudek, 1990; Bourque, 1998). The former mainly depolarizes the neurons by excitatory postsynaptic potentials due to the activation of excitatory receptors; the latter is attributed to intrinsic properties of the MNCs such as osmosensitive channels and activity dependent currents, which play key roles in regulating firing rate and pattern of the MNCs. An osmosensitive stretch inactivated cation channel has been identified in the MNCs (Oliet & Bourque, 1993). The activity dependent currents contribute to a depolarizing afterpotential (DAP), which follows action potentials and can summate into plateau potentials in the MNCs (Andrew & Dudek, 1983).

1.3.2.1 Stretch inactivated cation channels (SICs)

1.3.2.1.1 Molecular nature and biophysical properties of the SICs

MNCs are intrinsically osmosensitive because increases in osmolality can depolarize the cell membrane and initiate spontaneous firing in isolated MNCs (Oliet & Bourque, 1992). This is attributed to an osmosensitive stretch inactivated cation channel (SIC; Oliet & Bourque, 1993). The SIC belongs to the transient receptor potential vanilloid (TRPV) family of cation

channels, and is a splice variant of TRPV1 with a truncated N-terminal domain (Sharif-Naeini *et al.*, 2006). TRPV1, which is also known as a capsaicin receptor and is highly expressed in the central nervous system (Tóth *et al.*, 2005), is a nonselective ligand-gated cation channel that can also be activated by heat and low pH (Tominaga *et al.*, 1998; Jhaveri *et al.*, 2005). The SIC was also found to sense changes in temperature in the VP-MNCs and hyperthermic stimuli activated the SIC (Sharif-Naeini *et al.*, 2008a), suggesting that the SIC may play a role in regulating VP release by a thermal control.

Current-voltage analysis has indicated that the open channel conductance of the SIC is about 32 pS (Oliet & Bourque, 1993). The trivalent inorganic cation gadolinium (Gd^{3+}), which can block mechanosensitive cation channels, can dose-dependently block the SIC with an IC_{50} of about 30 μM (Oliet & Bourque, 1996). Ruthenium red, a blocker of the TRPV channels, can completely inhibit the SIC at 10 μM (Sharif-Naeini *et al.*, 2006). In addition, the SIC in the MNCs is sensitive to SB366791 (Sharif-Naeini *et al.*, 2008a), a potent and selective antagonist of the TRPV1 channels (Gunthorpe *et al.*, 2004).

The SIC is a mechanosensitive cation channel, which is reversibly regulated by altering pipette pressure or osmolality of the extracellular solution (Oliet & Bourque, 1993). When the extracellular solution is hypertonic, the MNCs shrink, which causes the SIC to open and cations to flow into the cells (Figure 1.6). The influx of the cations then depolarizes the cell. The mechanism underlying the osmosensitivity of the SIC in the MNCs has recently been revealed in part, and this is due to the interactions between the cellular cytoskeleton and the channel proteins (Zhang *et al.*, 2007b). The SIC displays no mechanosensitivity after the MNCs are treated with cytochalasin-D, which disrupts cellular actin microfilaments. Jasplakinolide, which promotes actin polymerization, increases the osmosensitivity of the channel in the MNCs.

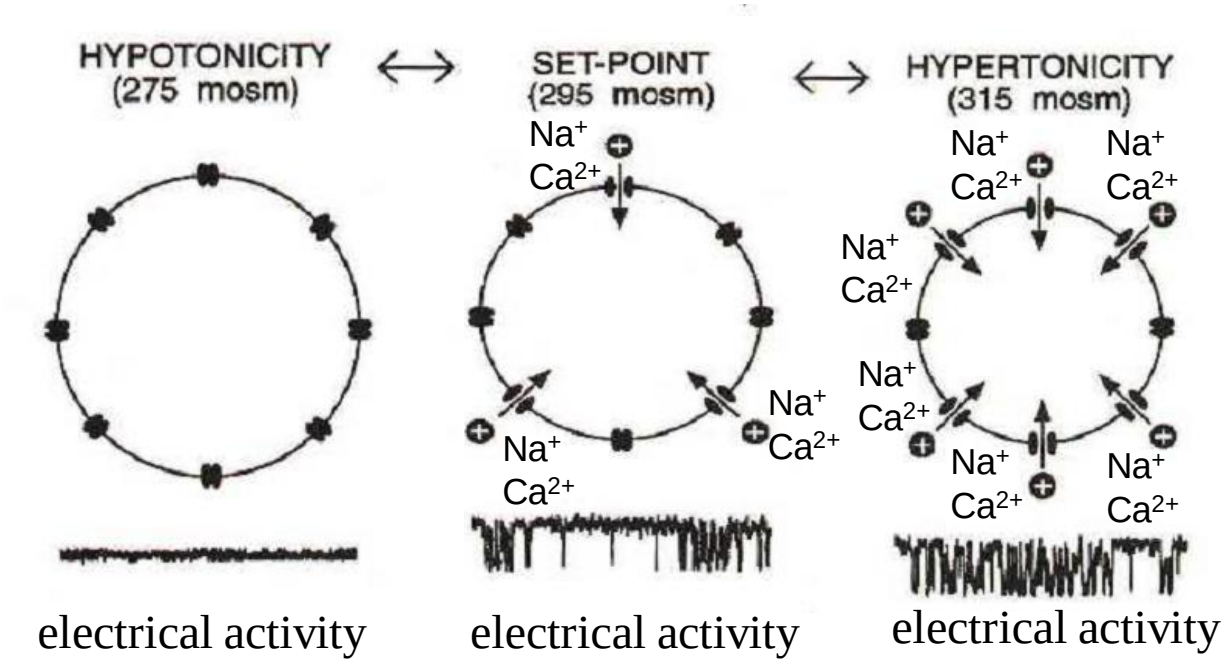


Figure 1.6 MNCs are intrinsically sensitive to changes in osmolality of the extracellular fluid. A stretch inactivated cation channel (SIC) has been demonstrated to play an important role in the increase in MNC firing during elevations in osmolality. (Bourque, 1998; Liu, 2004) (Reproduced with permission from Copyright Clearance Center)

1.3.2.1.2 Ionic selectivity of the SICs

Under physiological conditions, studies from voltage-clamp experiments have indicated that the current mediated by the SIC has a reversal potential of -41.5 mV and this is affected by concentrations of both the external Na^+ and K^+ but not Cl^-, suggesting that the SIC is a non-selective cation channel (Oliet & Bourque, 1993). Subsequent research has indicated that the SIC is permeable not only to monovalent cations with a selectivity sequence of $K^+ > Cs^+ > Na^+$, but also to divalent cations. The ratio of K^+ to Na^+ permeability (P_K/P_{Na}) is about 3.6 (Zhang & Bourque, 2006). However, an increase in the concentration of Na^+ of the extracellular solution causes an increase in the permeability of the channel to Na^+ (Bourque *et al.*, 2002; Zhang & Bourque, 2006). Thus, these properties of the SIC enable it to be considered as an intrinsic Na^+ sensor (Bourque *et al.*, 2002). In addition, the SIC is also highly permeable to Ca^{2+}, and the ratio of Ca^{2+} to Na^+ permeability is about 5 (Zhang & Bourque, 2006). Thus, Ca^{2+} may flow through the SIC and this activation may cause subsequent Ca^{2+}-induced Ca^{2+} release from internal Ca^{2+} stores in the MNCs. Ca^{2+} imaging studies have also shown a significant increase in intracellular Ca^{2+} level after the activation of the SIC in the MNCs (Zhang & Bourque, 2006). It is interesting that external Ca^{2+} can also block the SIC in the MNCs by an unknown mechanism with an IC_{50} of 4.9 mM (Zhang & Bourque, 2006).

1.3.2.1.3 Contribution of the SICs to osmosensory transduction

The SIC can sense small changes in osmolality and mediate the influx of cations such as Na^+, and it may therefore play an important role in the increase in MNC firing during elevations in osmolality (Figure 1.6). Since the SIC current is modulated by osmotic stimuli, its

contributions to osmosensory transduction in the MNCs have been investigated. Studies from the whole-cell patch-clamp recording have shown that the SIC contributes about 60% of the input conductance of the MNCs when osmolality is near normal (Bourque & Oliet, 1997). Furthermore, both ruthenium red and Gd^{3+} block macroscopic osmoreceptor currents and the increase in membrane conductance in response to hyperosmolality in the MNCs at the same concentration that they block the SIC (Bourque & Oliet, 1997). This suggests that the SIC contributes to osmoreception in the MNCs (Bourque & Oliet, 1997). Excitatory peptides such as angiotensin II, cholecystokinin, and neurotensin cause excitatory effects in the MNCs by activating the SIC (Chakfe & Bourque, 2000, 2001). These excitatory peptides may act on their respective receptors to activate $G_{q/11}$ proteins to modulate the second messenger system and thus regulate osmolality by modulating the open probability of the SIC in the MNCs (Chakfe & Bourque, 2000, 2001; Bourque *et al.*, 2002).

Since the SIC underlies osmosensitivity through to the involvement of actin filaments, cytochalasin-D significantly reduces the excitatory effect caused by hypertonic stimuli in the MNCs by disrupting cellular actin microfilaments. Jasplakinolide, in contrast, greatly increases the excitatory effect by promoting actin polymerization (Zhang *et al.*, 2007b). Although hypertonic stimuli cause increases in membrane conductance and depolarizing potentials by regulating the SIC (Bourque & Oliet, 1997) in wild-type mice, ruthenium red-sensitive increases in membrane excitability are absent in mice in which the TRPV1 gene has been knocked out (Sharif-Naeini *et al.*, 2006). Furthermore, knockout of the TRPV1 gene in mice causes dysfunction in the regulation of VP release and plasma osmolality (Sharif-Naeini *et al.*, 2006). The SIC is also highly expressed in OVLT neurons, which are also intrinsically sensitive to changes in osmolality of the ECF (Bourque, 1998), and the channel also contributes to

osmoregulation in these neurons. Ruthenium red-sensitive osmosensory signal transduction in the OVLT neurons disappears in the knockout mice of the TRPV1 gene (Ciura & Bourque, 2006). Furthermore**,** water intake is decreased in the knockout mice of the TRPV1 gene in response to water dehydration (Ciura & Bourque, 2006). The studies above suggest that the SIC plays key roles in osmosensory transduction in central osmoreceptors and thus regulates VP release and body fluid homeostasis (Sharif-Naeini *et al.*, 2008b).

1.3.2.2 Depolarizing afterpotentials

In addition to the SIC, which plays an important role in the increase in MNC firing during elevations in osmolality (Bourque *et al.*, 2002), a depolarizing afterpotential, which is an activity dependent current, plays key roles in regulating firing rate and pattern of the MNCs (Li *et al.*, 1995; Li & Hatton, 1997a).

1.3.2.2.1 Properties of the depolarizing afterpotentials

The depolarizing afterpotential (DAP) was first reported in MNCs by Andrew *et al.* in 1983 (Andrew & Dudek, 1983). The DAPs can sum and lead to a transient elevation of the membrane potential for tens of seconds to form a plateau potential (Figure 1.7), which causes generation of a burst (Andrew & Dudek, 1983). The DAP is not dependent on axonal conduction or chemical synapses but independently generated by an endogenous mechanism (Andrew & Dudek, 1983). Both VP-MNCs and OT-MNCs can exhibit the DAP in rat hypothalamic slices and also in acutely isolated MNCs (Oliet & Bourque, 1992; Erickson *et al.*, 1993; Armstrong *et al.*, 1994). However, there is a different percentage in expression of the DAP between the VP-

MNCs and the OT-MNCs since they display different firing patterns in response to osmotic stimuli. The studies using intracellular recording in the hypothalamic explants have shown that individual spikes evoked with current pulses is followed by a DAP in about 55% of VP-MNCs and 32% of OT-MNCs (Armstrong *et al.*, 1994). After brief trains of spikes, however, even more VP- and OT-MNCs will display the DAP (Armstrong *et al.*, 1994). Therefore, there is a greater tendency for the VP-MNCs to express the DAP than the OT-MNCs.

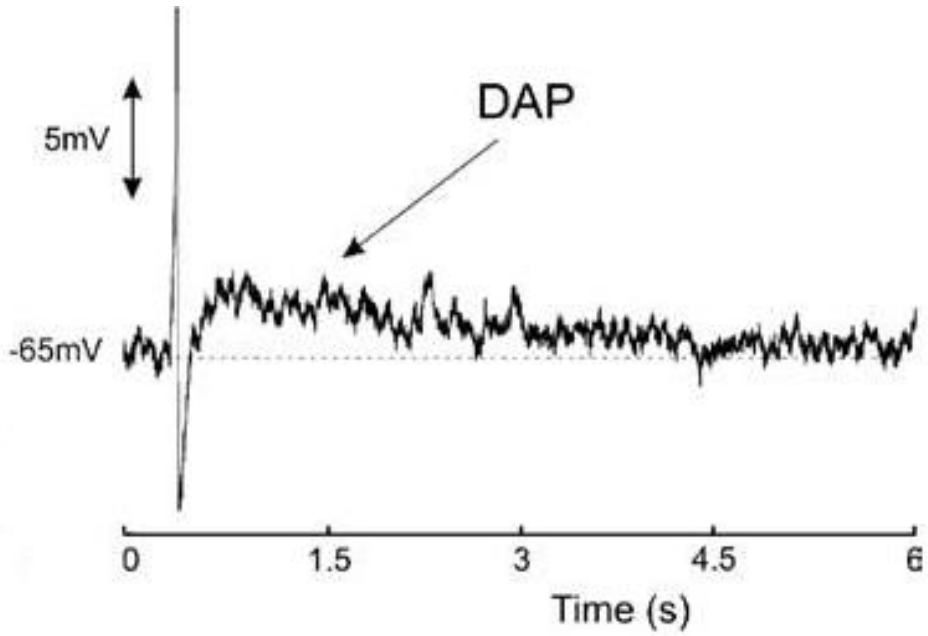


Figure 1.7 Depolarizing afterpotential (DAP). The traces are taken from sharp electrode recordings in the explants, and DAP is evoked by current pulses. (Roper *et al.*, 2003) (Reproduced with permission from Copyright Clearance Center)

1.3.2.2.2 Voltage- and Ca^{2+}-dependence of the depolarizing afterpotentials

The DAP is greatly dependent on the level of membrane polarization and the internal concentration of Ca^{2+} (Bourque, 1986; Andrew, 1987; Li & Hatton, 1997a, b; Li *et al.*, 1999). The DAP appears when the membrane potentials are over its activation threshold of -65.7 ± 0.7 mV (Li & Hatton, 1997b). The amplitude and duration of the DAP are determined by the numbers of action potentials evoking the DAP and the levels of membrane potential (Andrew, 1987; Li & Hatton, 1997b, a). A larger DAP can be induced by twin pulses than by single pulse (Li & Hatton, 1997b). The DAP induced by a single spike in the MNCs clamped at about -63 mV has an amplitude of 3.00 ± 0.19 mV, a duration of 1.02 ± 0.06 s, and a time to peak amplitude of 0.32 ± 0.04 s, while the DAP induced by three spikes has an amplitude of 5.76 ± 0.26 mV, a duration of 2.19 ± 0.11 s, and a time to peak amplitude of 0.51 ± 0.06 s (Li & Hatton, 1997a). Therefore, increases in membrane potentials will cause increases in the amplitude of the DAP in the MNCs (Li & Hatton, 1997b). The cell membrane potential can be lifted by 3 mV for a period lasting several seconds due to the activation of the DAP (Figure 1.7; Roper *et al.*, 2003).

As with those identified in molluscan pacemaker neurons (Thompson & Smith, 1976), the DAP in the MNCs is also strongly dependent on the level of intracellular Ca^{2+} (Bourque, 1986; Li *et al.*, 1995; Li & Hatton, 1997a; Li *et al.*, 1999). The studies examining the relationship between the DAP and intracellular Ca^{2+} level in the MNCs have shown that Cd^{2+} and removal of extracellular Ca^{2+} reduce or abolish the DAP (Bourque, 1986; Li *et al.*, 1995). In addition, Ca^{2+} in internal Ca^{2+} stores also plays an important role in the modulation of the DAP in the MNCs. Ryanodine or ruthenium red, which block ryanodine receptor-induced Ca^{2+} release channels, cause decreases of the DAP amplitude by 50% and duration by 45% (Li & Hatton, 1997a). In addition, thapsigargin and cyclopiazonic acid, which specifically inhibit the internal

sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase and thereby deplete Ca^{2+} stores (Seidler *et al.*, 1989; Thastrup *et al.*, 1990), also cause decreases of the DAP amplitude by 50% and duration by 45% in the MNCs (Li & Hatton, 1997a). Moreover, the internal application of BAPTA [1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraaecetic acid, a Ca^{2+} chelator], abolishes the DAP in the MNCs (Li *et al.*, 1995). Caffeine-stimulated Ca^{2+} release from Ca^{2+}-induced Ca^{2+} release channels (Leijten & van Breemen, 1984) can greatly increase the amplitude and duration of the DAP in the MNCs (Li & Hatton, 1997a). In contrast, heparin-induced block of Ca^{2+} release from inositol 1, 4, 5-trisphosphate (IP_3)-induced Ca^{2+} release channels (Nilsson *et al.*, 1988) does not reduce the amplitude and duration of the DAP in the MNCs (Li & Hatton, 1997a). These studies suggest that the DAP is greatly dependent on the concentration of intracellular Ca^{2+} and that both Ca^{2+} influx via the VDCCs and Ca^{2+} release from Ca^{2+}-induced Ca^{2+} release channels (but not from IP_3-induced Ca^{2+} release channels) increase the DAP and contribute to phasic firing in the MNCs (Li & Hatton, 1997a).

1.3.2.2.3 Ionic mechanisms of the depolarizing afterpotentials

Although the DAP plays a key role in the promotion of phasic firing and thus modulates MNC firing patterns (Li *et al.*, 1995; Li & Hatton, 1997a), the studies exploring the ionic mechanisms of the DAP have not reached a clear conclusion. DAPs are mediated by a subthreshold persistent sodium channel in rat subfornical organ neurons (Washburn *et al.*, 2000), by calcium-activated non-specific (CAN) cation channels in the medial lateral habenula (Partridge & Swandulla, 1988; Chang & Kim, 2004; Hung & Magoski, 2007), in *Aplysia* bag cell neurons (Hung & Magoski, 2007), in olfactory bulbar neurons (Pressler & Strowbridge, 2006), and in thyroid follicular cells (Partridge & Swandulla, 1988), and by other Ca^{2+}-activated

channels in mouse sympathetic neurons (Martinez-Pinna *et al.*, 2000). CAN channels are encoded by TRP genes (Hardie & Minke, 1993). The TRP superfamily includes six main subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin; Nilius *et al.*, 2007). One of important characteristics of the CAN channels is that they do not display any inactivation caused by intracellular Ca^{2+} or by membrane potential, and they can therefore afford a persistent excitatory effect and play important physiological roles in excitable cells (Partridge *et al.*, 1994). CAN channels are expressed in neurons and non-neurons and are permeable to Na^+, K^+, and Ca^{2+}, and even Cs^+, Li^+, Cl^-, and tetraethylammonium (TEA; Partridge *et al.*, 1994). Two unique subtypes of CAN channels from the TRPM family, TRPM4 and TRPM5, are selective for monovalent cations but impermeable to Ca^{2+} (Nilius *et al.*, 2007). This is very different from other TRP channels, which are highly permeable to Ca^{2+}. TRPM4 and TRPM5 are also Ca^{2+}- and voltage-dependent and can be blocked by flufenamic acid (FFA; Gottlieb *et al.*, 2006), which is a blocker of the CAN channels and can block the DAP, suggesting that the CAN channels mediated by TRPM4 and TRPM5 could mediate the DAP in the MNCs (Teruyama & Armstrong, 2007).

The ionic mechanisms of the DAP in the MNCs are not completely understood. Li & Hatton reported (using whole-cell patch-clamp recording in the MNCs) that the current mediating the DAP, which was evoked by three brief depolarizing pulses, had a reversal potential of -87.4 ± 1.6 mV, which nears the expected the equilibrium potential for potassium (E_K, -89.4 mV), and that the reversal potential shifted with changes in the concentrations of K^+ in the external solution (Li & Hatton, 1997b). Moreover, under their conditions, the DAP in the MNCs could be reversibly blocked by TEA, a non-selective blocker of K^+ channels. These data suggest that generation of the DAP is dependent on a reduction of outward K^+ currents in the

MNCs. In addition, Cs^+, a blocker of K^+ channels, can also suppress the DAP, which is evoked by trains of a constant number of action potentials, by directly blocking the currents contributing to the DAP but not by interfering with $[Ca^{2+}]_i$ (Ghamari-Langroudi & Bourque, 1998). In contrast, another study has shown that TEA cannot block the DAP induced by evoking trains of action potentials in the MNCs during intracellular recording (Greffrath *et al.*, 1998). Moreover, both DAP and phasic firing are inhibited by FFA during intracellular recording in the MNCs (Ghamari-Langroudi & Bourque, 2002), with no changes in the membrane potential or Ca^{2+}-dependent parameters of the spikes evoking the DAP. Thus, FFA also causes a direct blockade of the channel mediating the DAP (Ghamari-Langroudi & Bourque, 2002), suggesting that the DAP may be mediated by a CAN channel in the MNCs. In addition to this well known Cs^+-sensitive DAP, a Cs^+-resistant fast DAP, which is evoked by a train of action potentials and has a much faster time course than the Cs^+-sensitive DAP, has recently been reported using whole-cell patch-clamp recordings in the MNCs (Teruyama & Armstrong, 2007). This fast DAP is expressed in all VP-MNCs (but in only 20% of OT-MNCs) and is also Ca^{2+}-dependent. The fast DAP can be affected by concentrations of both the external Na^+ and K^+ but not Cl^-, and is blocked by FFA, suggesting that it may also be mediated by a CAN channel, possibly by TRPM4 and/or TRPM5 (Teruyama & Armstrong, 2007). DAPs are evoked under different experimental conditions in the studies above and have different properties. These studies also demonstrate that although the DAP plays a key role in generation of phasic firing in the MNCs, the ionic mechanisms of the DAP are unclear and that it may be mediated by multiple currents.

1.3.2.2.4 The depolarizing afterpotentials in phasic firing

Only VP-MNCs normally display phasic firing in rats (Armstrong *et al.*, 1994), but OT-MNCs could also possess the machinery for phasic bursting because they can be induced to fire by a phasic pattern when $[Ca^{2+}]_i$ is increased (Li *et al.*, 1995). Since OT-MNCs can sometimes exhibit a DAP in rat hypothalamic slices but only VP-MNCs normally display phasic firing (Armstrong *et al.*, 1994), the appearance of the DAP does not mean that phasic firing will subsequently be initiated in the MNCs. The DAP does however play a key role in initiating the phasic burst and the reasons are summarized below. Phasic firing can be terminated because of abolishment of the DAP by depletion of intracellular Ca^{2+} (Li *et al.*, 1995; Li & Hatton, 1997a) or by blockade of the CAN channels (Ghamari-Langroudi & Bourque, 2002). Moreover, a reduction in $[Ca^{2+}]_i$ in the MNCs displaying phasic firing can eliminate phasic firing (Li *et al.*, 1995). An elevation of $[Ca^{2+}]_i$ in the MNCs, in contrast, facilitates generation of the DAP and the plateau potential and thus promotes phasic firing (Li *et al.*, 1995). In addition, histamine by acting on H1 receptors to activate G-proteins and Ca^{2+}-independent pathways can enhance the DAP and thus promote phasic firing in the MNCs (Li & Hatton, 1996), while dynorphin, by acting on κ-opioid receptors to inhibit the DAP, can cause burst termination (Brown *et al.*, 2006). Furthermore, a depolarizing current pulse in cat MNCs (which have resting potentials similar to that of the rat) cannot elicit a DAP or a plateau potential (Fagan & Andrew, 1991), which can be evoked by injecting currents in the rat MNCs. Phasic firing is absent in the cat MNCs (Fagan & Andrew, 1991). These studies suggest that the DAP is a requisite but not sufficient feature for initiating and sustaining phasic bursts in the MNCs (Erickson *et al.*, 1993).

1.3.2.3 Plateau potentials

Plateau potentials, which were originally identified in cultured MNCs of foetal mice (Legendre *et al.*, 1982), are due to summation of DAPs. The DAP can summate to form a much larger, slow, persistent depolarizating plateau potential because of two possible mechanisms, as suggested by Roper *et al*. (Roper *et al.*, 2004). One is that an increase in intracellular Ca^{2+} after action potentials leads to suppression of K^+ currents and thus causes further depolarization of membrane. The other is that the depolarization causes further activation of the VDCCs and the Ca^{2+}-dependent DAP. The plateau potential, which has a threshold of about -45 mV, can rise to peak at about -20 mV and then gradually and slowly repolarize to a steady phase at -30 mV (Legendre *et al.*, 1988). The plateau potential can last over one hundred seconds and then quickly fall down with a fast repolarizing phase followed by a refractory period, which takes 10-30 s and is mediated by calcium-activated K^+ channels (Legendre *et al.*, 1982; Legendre *et al.*, 1988). The plateau potential, like the DAP, also displays voltage-sensitivity and can be inactivated by a hyperpolarizing pulse (Legendre *et al.*, 1982; Andrew, 1987). The plateau potential can be totally inhibited by hyperpolarizing potentials below -90 mV. The plateau potential is sensitive to the application of TTX or to low-Na^+ recording solutions, but not to alterations of extracellular K^+ (Andrew, 1987). In addition, a decrease or removal of extracellular Ca^{2+} also suppresses or abolishes the plateau potential, respectively, suggesting that it is also Ca^{2+}-dependent (Bourque, 1986; Andrew, 1987). Sr^{2+}, an agonist of Ca^{2+} channels, greatly enhances the plateau potential or allows cells to regain plateau potentials to sustain phasic firing (Andrew, 1987).

1.3.3 Termination of firing and burst

Phasic bursts can last tens of seconds in MNCs, and then the cells cease firing during rest intervals (Bicknell, 1988). The specific mechanisms underlying the termination of phasic bursts are complicated and not completely understood (Roper *et al.*, 2004). However, the DAP summates into the plateau potential that causes an initially high firing rate that then progressively decreases because of adaptation mediated by some inhibitory currents (Figure 1.8), which include a hyperpolarizing afterpotential (HAP) and an afterhyperpolarizing potential (AHP; Andrew & Dudek, 1984; Roper *et al.*, 2003). These inhibitory currents might cause a decrease in the ability of the DAP to sustain the plateau potential and phasic firing.

1.3.3.1 Hyperpolarizing afterpotentials and afterhyperpolarizing potentials

1.3.3.1.1 Hyperpolarizing afterpotentials

The membrane potential of the MNCs, after single action potential, becomes more negative than that in the resting state to form an undershoot phase merging into a HAP (Andrew & Dudek, 1984; Bourque *et al.*, 1985; Roper *et al.*, 2003). The HAP is expressed in both VP-MNCs and OT-MNCs, and there are no significant differences in amplitude between the two types (Armstrong *et al.*, 1994). The HAP can briefly hyperpolarize the cells for 25-125 ms and has amplitude of 7.4 ± 0.8 mV (Figure 1.8; Andrew & Dudek, 1984; Bourque *et al.*, 1985). The HAP is transient, and it mono-exponentially decays with a time constant of 17.5 ± 6.1 ms (Bourque *et al.*, 1985). Data from intracellular recordings have shown that the HAP in the MNCs is voltage dependent and has a reversal potential of -85 mV (Bourque *et al.*, 1985), which is near E_K. This reversal potential is not significantly modulated by either the level of intracellular Cl^- or

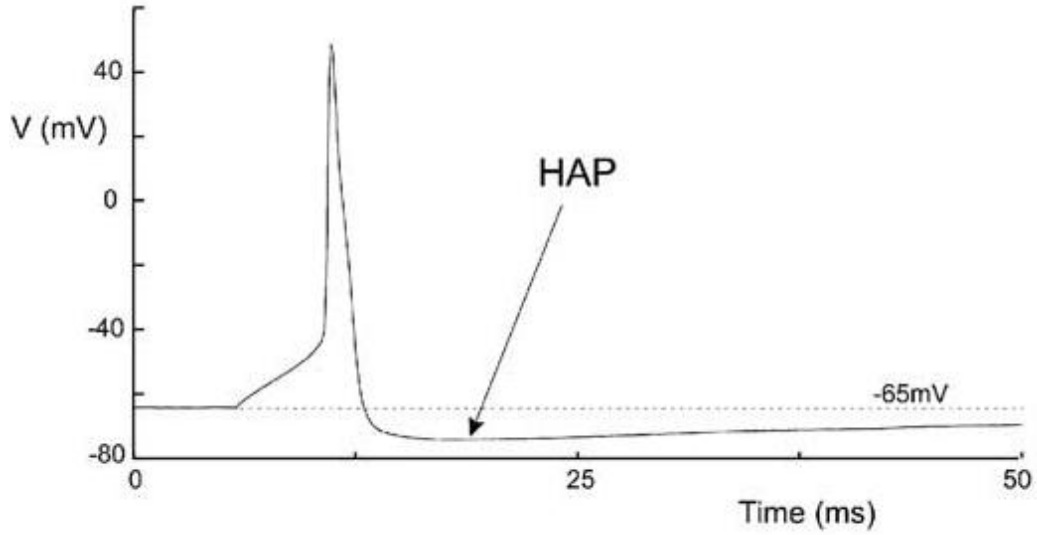
extracellular Mg^{2+}, suggesting that the HAP is mediated by a subtype of voltage-dependent K^+ channel (Bourque *et al.*, 1985). In addition, the magnitude and duration of the HAP is greatly affected by the level of intracellular Ca^{2+} (Bourque *et al.*, 1985), suggesting that the HAP is mediated by a large-conductance Ca^{2+}-activated K^+ (BK) channel (Andrew & Dudek, 1984; Bourque *et al.*, 1985; Roper *et al.*, 2003). A transient, Ca^{2+}-dependent outward K^+-selective current, which is sensitive to 4-aminopyridine (4-AP; a non-selective blocker of K^+ channels) but not to TEA, also contributes to the HAP and modulates firing rate in the MNCs (Bourque, 1988; Bourque *et al.*, 1998). The HAP is unlikely to be involved in the termination of phasic bursts in the MNCs because its transient property suggests that it would not be able to contribute to an inhibitory rest interval that remains over hundreds of milliseconds (Andrew & Dudek, 1984; Bourque *et al.*, 1985). However, the nature of the HAP suggests that it can influence the interspike interval so that the cell can transiently stay below critical threshold voltage and thus limit firing frequency in the MNCs (Andrew & Dudek, 1984).

1.3.3.1.2 Afterhyperpolarizing potentials

Unlike the HAP, activation of an AHP in the MNCs requires a brief spike train of high frequency or a prolonged stimulation, in which case it is followed by a DAP (Andrew & Dudek, 1984). There is therefore a partial overlap among the DAP, the HAP, and the AHP. The amplitude and duration of the AHP, which is expressed in both VP-MNCs and OT-MNCs (Armstrong *et al.*, 1994), are greatly dependent on both the strength and duration of the depolarizing pulses that activate them (Andrew & Dudek, 1984) and not significantly different between the two types of MNCs (Armstrong *et al.*, 1994). The AHP can hyperpolarize the MNCs

by 12.5 mV for hundreds of milliseconds (Figure 1.8) and then decay with a time constant of about 500 ms (Andrew & Dudek, 1984; Roper *et al.*, 2003). The AHP in the MNCs may contain multiple components and the currents contributing to the AHP are not completely known. The AHP is generated by an increase in a K^+ conductance and is Ca^{2+}-dependent, suggesting that the AHP is also mediated by Ca^{2+}-activated K^+ channels (Andrew & Dudek, 1984). The generation of this type of AHP is dependent on electrical activity in the cells and a spike train with fifteen action potentials can cause the maximal amplitude of the AHP (Kirkpatrick & Bourque, 1996). This AHP can be selectively blocked by apamin and d-tubocurarine (Bourque & Brown, 1987; Armstrong *et al.*, 1994; Kirkpatrick & Bourque, 1996), selective blockers of small-conductance Ca^{2+}-mediated K^+ (SK) channels, and this indicates that the AHP is conducted by SK channels. This apamin-sensitive AHP follows a train of action potentials and is referred to as the mAHP (Kirkpatrick & Bourque, 1996). There is also an apamin-insensitive AHP, which follows a single spike and is referred to as the fast AHP (fAHP; Kirkpatrick & Bourque, 1996). The nature of the post-train AHP (the mAHP) suggests that it could contribute to an inhibitory rest interval and be involved in the termination of phasic bursts by suppressing the DAP and the plateau potential, and thus play an important role in controlling firing rate and pattern in the MNCs (Andrew & Dudek, 1984; Bourque *et al.*, 1985). Another study has indicated that although the mAHP may be able to contribute to the termination, the mAHP is unnecessary for the burst termination because the MNCs, after blockade of the mAHP by apamin, still display phasic firing but increase the rate and duration of the bursts (Kirkpatrick & Bourque, 1996).

A



B

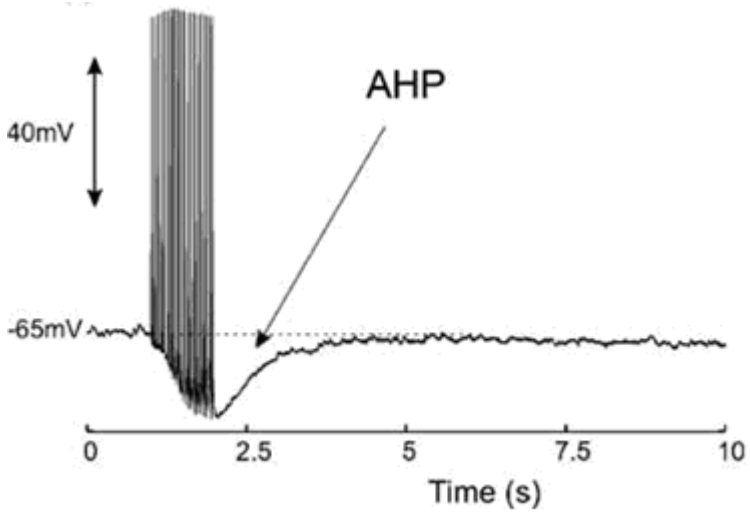


Figure 1.8 Hyperpolarizing afterpotentials (HAP) and afterhyperpolarizing potentials (AHP). The traces are taken from whole-cell recordings in the slice, and AHP and HAP are evoked by current pulses. (Roper *et al.*, 2003) (Reproduced with permission from Copyright Clearance Center)

Greffrath *et al.* also reported that 26% of MNCs express a slow Ca^{2+}-dependent AHP (sAHP), which decays with a time constant of 1010.3 ± 311.30 ms and could be mediated by intermediate-conductance Ca^{2+}-activated K^+ (IK) channels (Greffrath *et al.*, 1998). This sAHP could regulate the firing rate and pattern of the MNCs by attenuating the DAP (Greffrath *et al.*, 1998). A non-BK and non-SK-mediated, calcium-activated slow AHP, which decays with a time constant of 1.4 ± 0.1 sec and is inhibited by carbachol, has been reported in principal neurons in the lateral nucleus of the amygdala (Sah & Faber, 2002). A muscarine-sensitive slow AHP that decays with a time constant of 2031 ± 244 ms and does not appear to be mediated by BK, IK, or SK channels, is expressed in the MNCs and modulates phasic firing by attenuating the DAP and the plateau potential (Ghamari-Langroudi & Bourque, 2004). In addition, an apamin-insensitive slow AHP has been reported in the MNCs and shows a significant increase in peak amplitude in OT-MNCs during lactation (Teruyama & Armstrong, 2005). These types of slow AHPs are possibly generated by a long train of action potentials in the MNCs. Such a sAHP is also reported in other excitable cells, and it is interesting that several types of sAHP can be regulated by various neurotransmitters (Sah & Faber, 2002). The physiological roles of the sAHP, specifically in the termination of the phasic burst or in the transition from fast continuous to phasic firing, are unclear because of the absence of selective modulators of the currents contributing to the sAHP.

1.3.3.1.3 Ca^{2+}-dependent K^+ channels

Ca^{2+}-activated K^+ (K_{Ca}) channels belong to a subfamily of K^+ channels, the opening and closing of which are controlled by intracellular Ca^{2+} (Faber & Sah, 2003). The K_{Ca} channels were first reported in human erythrocytes (Gárdos, 1958). They were first identified in neurons

by Meech in *Aplysia* nerve cells where they caused a TEA-sensitive, Ca^{2+}-dependent hyperpolarization (Meech, 1972). The K_{Ca} channel is composed of two distinct types of subunits (Faber & Sah, 2003): four α subunits and a β subunit. The α subunit contains six transmembrane domains from S1 to S6 (Faber & Sah, 2003). There is a specific site in the C-terminus of the α subunit in the channel and the site is linked to calmodulin, which can bind Ca^{2+}. The binding leads to a conformational change of the structure in the K_{Ca} channel and thus causes the opening of the channel (Fanger *et al.*, 1999).

K_{Ca} channels are broadly expressed in various types of excitable and non-excitable cells. The K_{Ca} channels have been divided into three families based on their biophysical and pharmacological properties: BK, IK, and SK channels (Faber & Sah, 2003). The BK channel, which contains a calcium-binding motif known as the "Ca^{2+} bowl" in the cytoplasmic C-terminus (Schreiber & Salkoff, 1997), is much more sensitive to intracellular Ca^{2+}, possibly because it is closer to the Ca^{2+} source than the SK channel using calmodulin as a Ca^{2+} sensor, and the BK channel can therefore rapidly respond to $[Ca^{2+}]_i$ on the cytosolic face to alter its activity (Roper *et al.*, 2003; Fakler & Adelman, 2008). In addition, there are also positively charged residues in the S4 segment of the α subunit of the BK channel, and therefore activation of the channel is also dependent on the level of membrane depolarization (Jan & Jan, 1997). The BK channel is highly selective to K^+, and current-voltage analysis has indicated that it has a big channel conductance (about 200-300 pS; Blatz & Magleby, 1984; Latorre *et al.*, 1989; McManus, 1991). The BK channel can be selectively blocked by iberiotoxin and paxilline (Galvez *et al.*, 1990; Sanchez & McManus, 1996). In addition, TEA and charybdotoxin can also block the BK channel (Blatz & Magleby, 1984; Smith *et al.*, 1986). The BK channel contributes to the generation of the fAHP in neurons and thus modulates spike broadening, which may regulate

neurotransmitter release (Shao *et al.*, 1999; Faber & Sah, 2003). The IK channel has an intermediate single channel conductance of about 20-85 pS (Latorre *et al.*, 1989; Ishii *et al.*, 1997). Different from the BK channel, the IK channel is not voltage dependent and is blocked by clotrimazole. Apamin does not have any effect on the current conducted by the IK channel. The IK channel can mediate a sAHP and thus control excitability and firing pattern of cells (Greffrath *et al.*, 1998; Vogalis *et al.*, 2002). The SK channel has a small conductance of about 2-20 pS and is also voltage-independent (Latorre *et al.*, 1989; Ishii *et al.*, 1997). Three types of SK channels, SK1, SK2, and SK3, have been identified (Kohler *et al.*, 1996). The SK channel is insensitive to TEA at low concentrations, charybdotoxin, and iberiotoxin but is specifically sensitive to apamin and tubocurarine. A study have also shown that the SK2 and the SK3 are more sensitive to apamin and contribute to the mAHP (Stocker *et al.*, 1999), and that the SK1 is much less sensitive to apamin and may contribute to the sAHP (Kohler *et al.*, 1996). MNCs express BK and SK3 (Dopico *et al.*, 1999; Stocker & Pedarzani, 2000; Greffrath *et al.*, 2004), and IK is possibly expressed in the MNCs (Greffrath *et al.*, 1998). The SK channel contributes to the generation of the mAHP and thus regulates neuronal firing rate and pattern in the MNCs (Kirkpatrick & Bourque, 1996). In addition, a recent study showed that the SK channel, which, unlike the BK channels, is more sensitive to Ca^{2+} entry through the NMDA receptors than through the VDCCs and plays a role in a Ca^{2+}-mediated feedback loop, which is generated due to activation of NMDA receptors (Ngo-Anh *et al.*, 2005; Gu *et al.*, 2008). Thus, the SK channel may play a role in regulating LTP and memory formation (Ngo-Anh *et al.*, 2005; Gu *et al.*, 2008).

1.3.3.2 Other inhibitory currents

Besides the inhibitory K^+ currents discussed above, MNCs also express other inhibitory K^+ currents, such as a voltage-dependent delayed rectifier K^+ current (Cobbett *et al.*, 1989). This delayed rectifier K^+ current activates at potentials more positive than -40 mV with a sigmoidal time course. After activation during voltage steps, the current can be maintained for about 300 ms and then displays time-dependent, slow inactivation. This current is blocked by TEA but not by 4-AP. This slowly inactivating K^+ current may greatly modulate the spike repolarization and control firing frequency at the beginning of the phasic burst in the MNCs (Cobbett *et al.*, 1989). In addition, a time- and voltage-dependent slow, outwardly rectifying K^+ current is expressed in OT-MNCs but not in VP-MNCs (Stern & Armstrong, 1995). This K^+ current is sensitive to TEA and Ba^{2+} but insensitive to Cs^+, 4-AP, iberiotoxin, and apamin (Stern & Armstrong, 1997). It appears to be Ca^{2+}-dependent because it is sensitive to block by Cd^{2+} (Stern & Armstrong, 1997). The properties of this current suggest that it may regulate firing rate and pattern in the OT-MNCs (Stern & Armstrong, 1995). In addition, the MNCs also express some background K^+ currents such as TASK (TWIK-related acid-sensing K^+ currents), TREK (TWIK-related K^+ currents), and TRAAK (TWIK-related arachidonic acid activated K^+ currents), some of which are sensitive to mechanical factors or extracellular pH but not to voltage (Talley *et al.*, 2001; Han *et al.*, 2003). However, the physiological significance of these channels in the MNCs is not known. The MNCs of the PVN express a slowly inactivating K^+ current mediated possibly by a K_V1 K^+ channel and this current rapidly repolarizes the cell membrane after a strong depolarization (Bains *et al.*, 2001).

A voltage-gated cation current, which activates slowly at voltages more positive than -60 mV and shows slow deactivation, has recently been identified in the MNCs in our laboratory

(Liu *et al.*, 2005). This cation current is insensitive to TEA and 4-AP but sensitive to Ba^{2+} and could be conducted by a subtype of K^+ channels (Liu *et al.*, 2005). This current displays osmosensitivity in about 60% of the MNCs, and was therefore referred as the osmosensitive current (OC). Although both the mechanisms underlying the OC and its physiological roles are incompletely understood, it may, if it is a K^+ current, contribute to a slowing or termination of firing by a hyperpolarizing effect during the discharge of action potentials in the MNCs and thus contribute to the transition between fast continuous and phasic firing.

1.4 Effects of dehydration on MNCs

MNCs respond to osmotic stimuli not only by changing their firing rate and pattern but also by displaying other functional changes. The cell membrane of the MNCs, like other biological membranes, is semipermeable, and the MNCs can undergo changes in cell volume in response to changes in osmolality of the ECF (Zhang & Bourque, 2003). The cell, through the modulation of intracellular signal transduction, alters cellular functions such as cell excitation, gene expression, hormone secretion, cell proliferation, and so on (Lang *et al.*, 1998a). However, the MNCs show different changes in cell volume in response to osmotic stimuli between acute hypertonic stimuli in isolated cells (Zhang & Bourque, 2003) and chronic dehydration in live rats (Hatton, 1997). While most cells display regulatory volume decrease (RVD) or increase (RVI) upon swelling or shrinking (Lang *et al.*, 1998a), respectively, the MNCs acutely isolated from the rat SON show significant sustained decrease or increase upon changes in external osmolality (Zhang & Bourque, 2003). These sustained changes are not accompanied by a change in cell membrane capacitance (Zhang & Bourque, 2003), which is partly dependent on total membrane surface area and is obtained from electrophysiological method. These suggest that the MNCs do

not change their total membrane surface area but rather change size (which is obtained from the cell images) by folding or unfolding reserved membrane (Zhang & Bourque, 2003). Such changes in response to osmotic stimuli involve the interaction of the cell membrane with actin filaments (Zhang *et al.*, 2007b). Thus, osmosensitive channels can continuously respond to alteration in osmolality of the ECF (Zhang & Bourque, 2003). However, sustained dehydration in live rats causes hypertrophy of the MNCs in both somata and dendrite such that the space in the perivascular basal lamina are extensively filled by the MNCs (Hatton, 1997). Sustained dehydration induced by 7 days of substitution of tap water with 2% saline leads to an increase in volume of MNC region in the SON by 54% (Salm & Hawrylaka, 2004). In addition to the change in morphology of the MNCs, other structural changes are also detected in both the SON and in the neurohypophysis during sustained dehydration (Hatton, 1997). Glial cells around the SON also undergo a long-lasting structural change in response to sustained dehydration such that their coverage of the MNCs significantly decreases. Moreover, the MNCs also experience functional plasticity, which includes increases in excitatory glutamatergic and noradrenergic inputs, decreases in GABAergic and taurine input, and an increase in inhibitory kappa opioid receptors due to their translocation to the MNC plasma membrane (Shuster *et al.*, 1999; Di & Tasker, 2004; Bourque, 2008). Furthermore, cellular processes such as signal transduction and gene expression are also modulated in the MNCs during sustained dehydration, leading to an increase in VP gene expression (Zingg *et al.*, 1986; Ding *et al.*, 1994; Hurbin *et al.*, 2002) and in c-Fos gene expression in the OVLT, PVN, and SON (Oldfield *et al.*, 1994; Gottlieb *et al.*, 2006). Therefore, sustained osmotic stimuli can cause long-term changes in brain function and such plastic changes take several days or even months to reverse following rehydration (Zingg *et al.*,

1986; Miyata *et al.*, 1994; Yagita *et al.*, 1994; Hatton, 1997; Hawrylak *et al.*, 1998). The mechanisms of these changes are incompletely understood.

1.5 Modulation of MNC firing by neuroactive agents

Since the MNCs are targeted by the neurons from the OVLT, MnPO, and SFO (Bourque, 1998), electrical activity of the MNCs can be modulated by neuroactive agents released from presynaptic terminals (Figure 1.1). These neuroactive agents include some excitatory agents such as acetylcholine, glutamate, histamine, angiotensin, and substance P, and some inhibitory agents such as γ-aminobutyric acid, taurine, and opioid peptides. Their physiological roles in modulating MNC firing and hormone release have been reviewed in detail (Renaud & Bourque, 1991; Sladek & Kapoor, 2001; Sladek, 2004). In addition, the MNCs not only release VP and OT from their axon terminals in the neural lobe of neurohypophysis but also release the hormones from their dendrites (Mason *et al.*, 1986; Di Scala-Guenot *et al.*, 1987; Landgraf & Ludwig, 1991; Ludwig *et al.*, 1995). Dendritic release can optimize subsequent hormone release from the terminals by generating autocrine and/or retrograde feedback control (Wotjak *et al.*, 1994; Gouzenes *et al.*, 1998; Hirasawa *et al.*, 2004). In addition, neuropeptides such as dynorphin and apelin (a peptide originally extracted from bovine stomach) are co-stored and co-released with VP from the dendrites of the MNCs and thus generate a direct autocrine feedback regulation by exerting inhibitory effects on MNC firing (Brown *et al.*, 1999; Brown & Bourque, 2004; De Mota *et al.*, 2004; Brown *et al.*, 2006). Unlike dendritically released VP, which causes inhibitory effects during the whole periods of a phasic burst not only in the original neuron but also on neighbouring neurons (Brown & Bourque, 2004; Brown *et al.*, 2007), dendritically released dynorphin has an inhibitory effect only on the MNC releasing it (Brown *et*

al., 2007). Phasic bursts may be terminated by a dynorphin-induced postspike inhibitory effect on the DAP and plateau potentials, and therefore dynorphin may contribute to phasic firing in the MNCs to facilitate VP release (Brown & Bourque, 2004; Brown *et al.*, 2006).

1.6 M-Currents

MNCs change their electrical behaviour in response to small changes in external osmolality through the regulation of ion channels (Bourque, 2008), and ion channels, especially osmosensitive ion channels, therefore play key roles in the modulation of MNC firing and hormone release. In addition to the SIC (Bourque *et al.*, 2002) and the activity dependent currents (Roper *et al.*, 2003), other currents may play roles in regulating firing rate and pattern of the MNCs. M-currents, which are voltage-gated K^+ currents (Robbins, 2001), play key roles in regulating cellular excitability and mutations of the proteins mediating the KCNQ/M currents can lead to cellular dysfunction and many diseases (Jentsch, 2000). However, it is not known whether M-currents are expressed in MNCs and whether they play roles in the modulation of MNC firing and hormone release.

1.6.1 Subtypes, structure, and biophysical properties of KCNQ/M channels

M-currents, which are inhibited by the activation of muscarinic receptors (Figure 1.9), were first identified in frog sympathetic neurons (Brown & Adams, 1980). The M-currents are mediated by channels composed of members of a family of time- and voltage-gated K^+ channels termed KCNQ channels or K_V7 channels (Robbins, 2001). Like other subtypes of voltage-

dependent K^+ channels, the KCNQ/M channel is composed of four α subunits and one β subunit (Robbins, 2001), and each of the α subunits contains six transmembrane domains from S1 to S6. The α subunits contribute to the generation of a single P-loop forming a K^+-selective pore. The S4 segment with positively charged residues is a voltage sensor. Five members of the KCNQ channel gene family encoding the α subunits have been identified (KCNQ1-5; Robbins, 2001). Each of the channel subunits can be homomultimers of any of the five types such as KCNQ1, KCNQ2, and so on, or of some combinations of heteromultimers such as KCNQ2/3, KCNQ3/4, and KCNQ3/5 (Table 1.3; Robbins, 2001). Only KCNQ3 subunits can co-assemble with KCNQ2, KCNQ4, and KCNQ5 subunits to form heteromultimers (Robbins, 2001). The classic M-current is mediated by KCNQ2/3 heteromultimers (Wang *et al.*, 1998). However, homomultimers of KCNQ1, KCNQ2, KCNQ3, KCNQ4, and KCNQ5 (Robbins, 2001) have many properties similar to those of the classic M-current (Wang *et al.*, 1998; Lerche *et al.*, 2000; Selyanko *et al.*, 2000). Heteromultimers of KCNQ3/4 and KCNQ3/5 can also mediate M-currents in expression systems (Kubisch *et al.*, 1999; Lerche *et al.*, 2000), and coexpression of the KCNQ4 and the KCNQ5 with the KCNQ3 can lead the channel-mediated currents to increase in amplitude by about 2 fold or 4-5 fold, respectively. Although there is evidence for the existence of KCNQ3/5 (Yus-nájera *et al.*, 2003), native KCNQ3/4-mediated M-current has not been reported in any cells. The KCNQ/M channels are low threshold-activated K^+ channels and M-currents activate slowly at potentials more positive than -60 mV (Robbins, 2001). One important feature of the M-currents is that they display little and no inactivation (Robbins, 2001). Although recent studies have shown that the KCNQ4 and the KCNQ5 display some intrinsic inactivating properties (Jensen *et al.*, 2007), the small inactivation of the KCNQ4 channel completely disappears after the application of the KCNQ/M channel openers (Jensen *et al.*, 2007; see below).

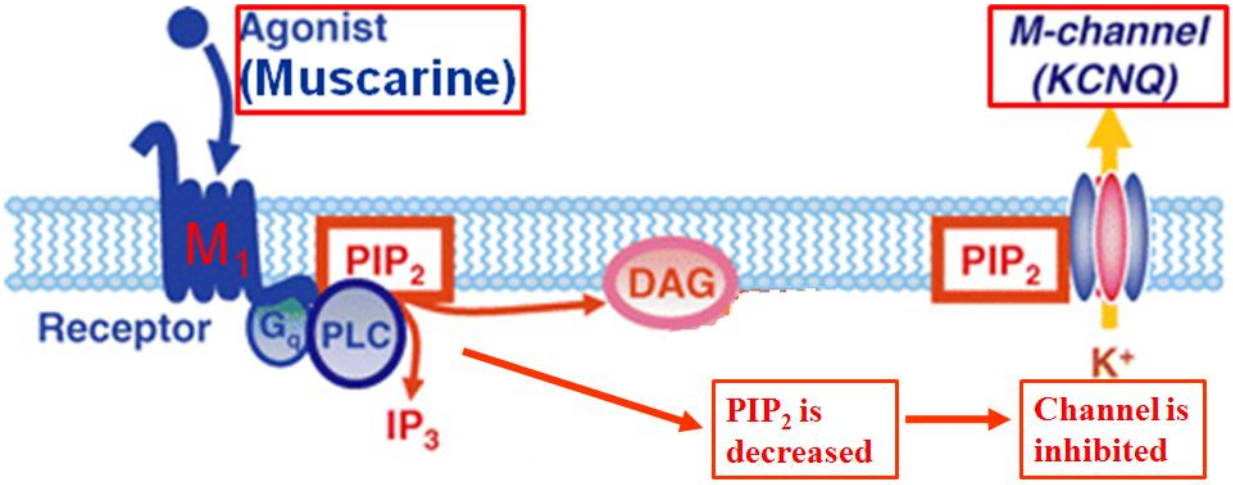


Figure 1.9 Signal pathways of M-currents. DAG, diacylglycerol; ER, endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; PIP_2, Phosphatidylinositol (4,5)-bisphosphate; PKC, protein kinase C; PLC, Phospholipase C. (Suh & Hille, 2007b)

1.6.2 Pharmacology of KCNQ/M channels

After M-currents were identified, several known K^+ channel regulators were used to try to modulate the channels. The pharmacology of the KCNQ/M channels has recently been reviewed in detail (Table 1.3; Robbins, 2001; Miceli *et al.*, 2008; Xiong *et al.*, 2008). Unlike voltage-dependent A-type K^+ channels, the KCNQ/M channels are insensitive to 4-AP (Robbins, 2001). The KCNQ/M channels display different sensitivity to TEA (Hadley *et al.*, 2000; Lerche *et al.*, 2000), and this is dependent on the subtypes of the channels. Extracellular TEA blocks KCNQ1, KCNQ2, KCNQ3, KCNQ4, and KCNQ5 with IC_{50}s (in mM) of 5.0, 0.3, >30, 3, and >30, respectively. Thus, KCNQ1, KCNQ2, and KCNQ4 are very sensitive to extracellular TEA, as is KCNQ2/3 which has an IC_{50} of 3.5 mM (Wang *et al.*, 1998). However, KCNQ3 and KCNQ5 are much less sensitive to the extracellular TEA. Both KCNQ3 and KCNQ5 channel subunits contain a threonine residue within the pore loop of the channels and this makes them have a low sensitivity to extracellular TEA (Wang *et al.*, 1998; Lerche *et al.*, 2000). There is a tyrosine residue instead of the threonine residue in the pore loop of the KCNQ2 channel subunits (Wang *et al.*, 1998), which makes the channel have a high sensitivity to extracellular TEA. In addition, internal TEA accelerates activation of and retards deactivation of KCNQ/M channel (Suh & Hille, 2007a). Furthermore, internal TEA as an open channel blocker causes an inactivation of the KCNQ/M channels (Suh & Hille, 2007a), as does it in other K^+ channels (Armstrong, 1966). Ba^{2+}, a non-selective blocker of K^+ channels, can also display a blocking effect on KCNQ/M channels (Robbins, 2001).

The identification of KCNQ/M currents was difficult until the discovery of the selective channel blockers (Costa & Brown, 1997; Wang *et al.*, 1998), linopirdine and its analogue, XE991 [10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone]. These agents have been

proposed as cognitive enhancers because they increase the release of acetylcholine (Saletu *et al.*, 1989) and other neurotransmitters (Tam *et al.*, 1991). Linopirdine directly blocks the KCNQ/M channels from the extracellular side of the channels (Costa & Brown, 1997). The IC_{50} of linopirdine is dependent on the subtype of the channel (Robbins, 2001). KCNQ1, KCNQ2, KCNQ3, and KCNQ2/3 are much more sensitive to linopirdine with IC_{50} of less than 10 μM; however, KCNQ4 and KCNQ3/4 are much less sensitive to linopirdine with IC_{50}s of more than 200 μM and less than 200 μM, respectively (Kubisch *et al.*, 1999). XE991 is more powerful not only in increasing release of the neurotransmitters (Zaczek *et al.*, 1998) but also in blocking KCNQ/M channels (Wang *et al.*, 1998). Thus, the KCNQ/M channels are more sensitive to block by XE991 (Wang *et al.*, 1998).

Table 1.3 Pharmacology of KCNQ/M channels. (Robbins, 2001; Søgaard *et al.*, 2001; Tatulian *et al.*, 2001)

Subtype	KCNQ1	KCNQ2	KCNQ3	KCNQ4	KCNQ5	KCNQ2/3	KCNQ3/4	KCNQ3/5
Ba^{2+} 1mM		50%			60%	67%		
5 mM				80%				
TEA IC_{50} mM	5.0	0.1~0.3	30~224	3.0	30~71	3.8~30		200
Linopirdine IC_{50} μM	8.9	3.7~4.8	4.8	> 200	16~51	0.6~10	< 200	7.7~15
XE991 IC_{50} μM	0.8	0.7	< 50	5.5	65	0.6		
Retigabine EC_{50} μM	100.1	2.5	0.6	5.2		1.9		1.4

Several KCNQ/M channel openers have recently been identified (Wu & Dworetzky, 2005; Xiong *et al.*, 2008). A well known opener of the KCNQ/M channels is retigabine (Main *et al.*, 2000), which can selectively open the KCNQ/M channels (Tatulian *et al.*, 2001), especially at a low concentration (less than 10 μM). Retigabine directly binds to a tryptophan residue within the S5 segment of the KCNQ/M channels to cause activation of the channels (Schenzer *et al.*, 2005). The S5 segments of KCNQ2, KCNQ3, KCNQ4, and KCNQ5 subunits contain such a tryptophan residue while the KCNQ1 subunit does not (Schenzer *et al.*, 2005). Thus, KCNQ2, KCNQ3, and KCNQ4, and KCNQ5, but not KCNQ1, are sensitive to retigabine (Tatulian *et al.*, 2001; Wickenden *et al.*, 2001). Retigabine increases the KCNQ/M currents by increasing open probability of the channels (Tatulian & Brown, 2003), speeding activation of the KCNQ/M currents (Tatulian *et al.*, 2001), slowing deactivation of the currents (Tatulian *et al.*, 2001), shifting voltage sensitivity of the channels to more negative voltages (Tatulian *et al.*, 2001), and canceling inactivation of the channels (Jensen *et al.*, 2007). Flupirtine, an analog of retigabine, and BMS-204352 are also used as openers of the KCNQ/M channels (Wu & Dworetzky, 2005).

1.6.3 Signal pathways of M-currents

M-currents, which are inhibited by the activation of muscarinic receptors (Brown & Adams, 1980), are mediated by voltage-gated KCNQ channels (Wang *et al.*, 1998). Thus, two interesting and important issues appear. What subtype of the muscarinic receptors is involved in the activation? What messengers contribute to the signal transduction pathway from activation of the receptors to closure of the KCNQ/M channels? Oxotremorine-M, an agonist of the muscarinic receptors, displays no inhibitory effects on native M-currents in sympathetic ganglion

neurons of mice with mutation of M1 muscarinic receptors (Hamilton *et al.*, 1997). Another recent study also showed that the activation of M1 muscarinic receptors stimulated by 10 μM oxotremorine-M causes inhibition of the M-currents mediated by expressed KCNQ2/3 channels (Selyanko *et al.*, 2000). These studies suggest that M1 muscarinic receptors couple to the KCNQ/M channel. The activation of M1 receptors by oxotremorine-M can reduce the native M-currents by about 60-90% (Hamilton *et al.*, 1997; Haley *et al.*, 1998; Winks *et al.*, 2005), and this inhibitory effect is not significantly different from that oxotremorine-M does on the M-currents mediated by expressed KCNQ2/3 channels (Selyanko *et al.*, 2000).

In addition, the knockdown of the α subunit of G_q-proteins in rat superior cervical ganglion neurons leads to a significant decrease in the inhibitory effect of oxotremorine-M on M-currents (Haley *et al.*, 1998), demonstrating that G_q-proteins are involved in the suppression of M-currents by oxotremorine-M. Intracellular Ca^{2+} as a second messenger is not involved in the suppression of the M-currents by oxotremorine-M (Shapiro *et al.*, 2000), but the KCNQ/M currents display a certain sensitivity to intracellular Ca^{2+} (Delmas & Brown, 2005), possibly because the C-terminus of the KCNQ/M channels contains a binding site for calmodulin, which serves as a Ca^{2+} sensor (Bal *et al.*, 2008; Haitin & Attali, 2008). In addition, recent studies have shown that plasma membrane-associated phospholipase C (PLC) is involved in the receptor-channel signal transduction pathways of the M-currents (Suh & Hille, 2002). U73122, a PLC inhibitor, prevents the inhibitory effect of muscarine on M-currents. Thus, the membrane lipid phosphatidylinositol (4,5)-bisphosphate (PIP_2), which is derived from the cleavage of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and is also cleaved by PLC into diacylglycerol (DAG) and IP_3 (Oude Weernink *et al.*, 2004), may be involved in the receptor-channel transduction pathways (Suh & Hille, 2002).

PIP_2 is one of eight common phosphoinositides (PI), which are distinguished by phosphorylation of the inositol 3-, 4-, and 5-hydroxyl positions and include PI, three isomers of phosphatidylinositol phosphate (PIP), three isomers of phosphatidylinositol bisphosphate and PIP_3 (Suh & Hille, 2005). PIP_2 has, in recent years, been identified as a crucial second messenger that regulates a number of cellular processes including regulation of ion channels, the actin cytoskeleton, vesicle trafficking, gene expression, and cell survival (McLaughlin *et al.*, 2002; Doughman *et al.*, 2003; Suh & Hille, 2005). PIP_2 is synthesized by PIP kinase type I using PI(4)P as substrate and type II using PI(5)P as substrate (Doughman *et al.*, 2003). In addition, type III PIP kinases may contribute to the synthesis of PIP_2 by using PI to synthesize PI(5)P (Sbrissa *et al.*, 1999). Phenylarsine oxide (Wiedemann *et al.*, 1996) and wortmannin (Nakanishi *et al.*, 1995), both of which can inhibit PI (4) kinase to block the synthesis of the PI(4)P and thus block the replenishment of PIP_2, can prevent recovery of M-currents from the suppression of the muscarinic receptor activation and can also directly inhibit M-currents (Suh & Hille, 2002). Oxotremorine-M can cause a decrease in PIP_2 levels in the plasma membrane and a subsequent increase in the cytoplasmic IP_3 level (Suh *et al.*, 2004). In addition, dioctanoyl PIP_2 (diC8-PIP_2), a synthetic form of PIP_2, increases KCNQ/M currents by increasing open probability of the channels (Li *et al.*, 2005). These data suggest that PIP_2 is required for the activation of the KCNQ/M channels.

Thus, muscarine, by acting on the M1 muscarinic receptors to activate the G_q-proteins, activates PLC to deplete the PIP_2 and thus suppresses the KCNQ/M-currents (Figure 1.9; Suh *et al.*, 2004). Intracellular ATP is involved in PIP_2 metabolism and is necessary for the synthesis of the PIP_2, and it can therefore increase recovery of the M-currents from muscarinic suppression (Suh & Hille, 2002). Intracellular Mg^{2+} is involved in G-protein cycling and is necessary for

activation of the proteins (Suh *et al.*, 2004). It can therefore enhance the inhibition of M-currents by muscarine and decrease KCNQ/M currents (Suh & Hille, 2007a).

1.6.4 KCNQ/M channels and cell volume

Osmotic stimuli can cause cell swelling or shrinking, and the cells will display RVD or RVI upon the swelling or shrinking (Lang *et al.*, 1998a), respectively. Changes in cell volume play an important role in the modulation of intracellular signaling pathways and cellular functions (Lang *et al.*, 1998a), which include changes in intracellular ion composition and subsequently in cell membrane potential because the alterations of activity of ion transporters and channels including K^+ channels. Several types of K^+ channels, including K_{Ca} channels (Fernandez-Fernandez *et al.*, 2002; Roman *et al.*, 2002), KCNQ/M channels (Grunnet *et al.*, 2003; Hougaard *et al.*, 2004; Jensen *et al.*, 2005b), voltage-gated K_V1 (Deutsch & Chen, 1993; Felipe *et al.*, 1993) and K_V4 channels (Wang *et al.*, 2005), and inwardly rectifying K^+ channels (Wurm *et al.*, 2006), can alter their activity in response to the changes in cell volume. Several subtypes of KCNQ/M channels have been demonstrated to display a sensitivity to cell volume. Expressed KCNQ1 channels in cultured COS-7 cells were the first of the KCNQ/M channel family to be found to sense small changes in cell volume (Kubota *et al.*, 2002). In addition, native KCNQ1 channel-mediated currents also sense changes in cell volume in primary neonatal rat cardiomyocytes and thus play an important role in regulating cardiomyocyte volume during injury (Calloe *et al.*, 2007). Cell swelling enhances the KCNQ1 channel-mediated current, whereas cell shrinkage decreases the current. The osmosensitivity of the KCNQ1 channel disappears after the cells are treated by cytochalasin D, which has no any effects on the KCNQ1

channel-mediated current under normal osmolality (Grunnet *et al.*, 2003; Calloe *et al.*, 2007). Furthermore, phalloidin, a compound promoting actin polymerization, can abolish the effect of cytochalasin D on the KCNQ1 channel (Grunnet *et al.*, 2003). These data suggest that the KCNQ1 channel underlies osmosensivity due to the involvement of the actin cytoskeleton, as does the SIC (Zhang *et al.*, 2007b). Besides the KCNQ1 channel, KCNQ4 (Hougaard *et al.*, 2004) and KCNQ5 (Jensen *et al.*, 2005b) channels have also been found to be modulated by changes in cell volume.

1.6.5 Distribution, physiology, and pathophysiology of the KCNQ/M channels

KCNQ/M channels are broadly expressed in various tissues (Robbins, 2001) and play key roles in regulating cellular excitability. Mutations of the proteins mediating the KCNQ/M currents can lead to cellular dysfunction and many diseases (Table 1.4; Jentsch, 2000). KCNQ1 is primarily expressed in cardiac tissues, intestine, kidney, and inner ear, and contributes to a slow delayed rectifier K^+ (I_{Ks}) current to repolarize cardiac action potentials, Cl^- homeostasis by regulating the balance of ionic currents, regulation of cell volume, and K^+ recycling (Robbins, 2001), respectively. Dysfunction of the KCNQ1 channel or mutations of the proteins mediating the channel will cause long QT syndrome in the heart (Barhanin *et al.*, 1996). Four of the KCNQ/M channels, KCNQ2, KCNQ3, KCNQ4, and KCNQ5, are expressed in the brain (Robbins, 2001) and play crucial roles in regulating neuronal excitability. The KCNQ2/3 heteromultimer forms the classic M-current with a low threshold (Wang *et al.*, 1998), and regulates the subthreshold excitability in various neurons (Delmas & Brown, 2005). In addition, the voltage-gated KCNQ2/3-mediated M-current can be modulated by several neurotransmitters

and intracellular signal pathways (Jentsch, 2000; Delmas & Brown, 2005). This enables them to control firing frequency and excitability in multiple ways in various central and peripheral neurons (Delmas & Brown, 2005). Mutations of the proteins mediating the classic M-current can disrupt neuronal firing pattern and ultimately lead to a genetic form of epilepsy and other forms of neuronal dysfunction (Jentsch, 2000). Epilepsy is a very common neurological disease and over 0.5-0.8% of the population in the world is affected by this disease (Schroeder *et al.*, 1998; Hirtz *et al.*, 2007). Mutations in the KCNQ/M-type K^+ currents are responsible for benign familial neonatal convulsions (BFNC; Yang *et al.*, 1998), an autosomal dominant epilepsy in infancy. A decrease in the M-currents by 25% can lead to BFNC (Schroeder *et al.*, 1998). Homozygous mouse pups in which KCNQ2 channel gene has been knocked out only survive for a few hours after birth and neurons in mice expressing fewer KCNQ2 channels are hyperexcitable (Watanabe *et al.*, 2000). KCNQ4 is expressed primarily in the cochlea, but also in some brain regions (primarily in the central auditory pathway and in other nuclei of the brain stem), heart and skeletal muscle (Kubisch *et al.*, 1999; Kharkovets *et al.*, 2000), and contributes to a large voltage-gated K^+ current ($I_{K,L}$) in type I hair cells and an $I_{K,n}$ K^+ current activated at negative potentials (Housley & Ashmore, 1992) in outer hair cells (Kharkovets *et al.*, 2000). Therefore, the KCNQ4 channel plays a key role in hearing, and mutations of the proteins mediating the KCNQ4 channel gene will lead to nonsyndromic autosomal dominant progressive hearing loss (DFNA2; Kharkovets *et al.*, 2000). KCNQ5 is broadly expressed in the brain, skeletal muscle (Schroeder *et al.*, 2000), and other tissues (Robbins, 2001). KCNQ5 homomultimers and KCNQ3/5 heteromultimers can also mediate native M-currents (Shah *et al.*, 2002; Yus-nájera *et al.*, 2003; Brueggemann *et al.*, 2007). There are no studies demonstrating that mutations of the proteins mediating the KCNQ5 channel gene are related to any diseases.

However, it is possible that the KCNQ5 channel-mediated M-current is involved in the formation of BNFC because the expression of the KCNQ5 channel is altered during formation of the disease (Yus-nájera *et al.*, 2003). In addition, expression of the KCNQ5 channel in skeletal muscle increases during myocyte proliferation and differentiation, and the KCNQ5 channel, therefore, may play a role in myoblast proliferation (Roura-Ferrer *et al.*, 2008).

Table 1.4 Expression and role in diseases of KCNQ/M channels. * Indicates that this protein is known to associate with the subunit in the tissue that is affected in the disease, and that its mutation causes the same disease (for example, KCNE1 mutations also cause the RWS and the JLNS). BFNC, benign familial neonatal convulsions; JLNS, Jervell and Lange-Nielsen syndrome; LQTS, long-QT syndrome; RWS, Romano–Ward syndrome. (?: unknown) (Jentsch, 2000) (Reproduced with permission from Copyright Clearance Center)

Table 1 | **KCNQ channels: expression data and role in disease**

Gene	Locus	Expression	Partner	Disesase	Inheritance	Remaining function
KCNQ1	11p15.5	Heart, cochlea, intestine, kidney	KCNE1*, KCNE3	LQTS, (dominant RWS)	Dominant	50% > *x* > 0% Dominant negative on one allele Often 0% function in homomers
				LQTS, (recessive RWS)	Recessive	50% > *x* > 0% Residual function of mutated homomers, or mutant1/mutant2 heteromeric channels
				LQTS + congenital deafness (JLNS)	Recessive	~0% Loss of function of both alleles
KCNQ2 *KNCQ3*	20q13.3 8q24	Brain Brain	KCNQ3* KCNQ2*, KCNQ5	BFNC	Dominant	~75% (in heteromer) Loss of function of one allele, haploinsufficiency
KCNQ4	1p34	Inner ear, brainstem nuclei	KCNQ3	DFNA2 (dominant progressive hearing loss)	Dominant	~10–50% Dominant negative on one allele or haploinsufficiency
KCNQ5	6q14	Brain, muscle	KCNQ3	?	–	–

Retigabine can cause the activation of KCNQ2, KCNQ3, and KCNQ4, and KCNQ5 (Tatulian *et al.*, 2001; Wickenden *et al.*, 2001), which are expressed in neurons, but not the activation of KCNQ1, which is primarily expressed in cardiac tissues (Robbins, 2001). Such a property of retigabine may allow it to have a clinical application in the treatment of the diseases involving neuronal hyperexcitability, such as epilepsy and neuropathic pain (Blackburn-Munro *et al.*, 2005).

2. RATIONALE, HYPOTHESIS, AND OBJECTIVES

MNCs change their electrical behaviour in response to small changes in external osmolality through the regulation of ion channels (Bourque, 2008) and the rate and pattern of action potentials generated in the MNC somata determine the release of hormone (Bicknell, 1988). Ion channels, especially osmosensitive ion channels, therefore play key roles in the modulation of MNC firing and hormone release. My studies will explore the relationship between osmoregulation and ion channels involved in the modulation of firing rate and pattern in MNCs.

2.1 Voltage-dependent Ca^{2+} channels play many important roles not only in the regulation of cell excitability but also in intracellular signal transduction (Fisher & Bourque, 2001). Several types of the Ca^{2+} channels, including N-, P/Q-, and L-types, have been identified in the MNC somata and terminals (Fisher & Bourque, 1996). Although total Ca^{2+} currents in the MNCs are not affected by an acute change in osmolality of the extracellular solution (Liu *et al.*, 2005), it is not known whether there is a change in total Ca^{2+} currents during sustained dehydration. It is possible that one or more types of Ca^{2+} currents may be affected during sustained dehydration.

2.2 The OC recently identified in our laboratory is an osmosensitive cation current in the MNCs and its properties suggest that it might play physiological roles in the MNCs (Liu *et al.*, 2005). However, the biophysical properties of the OC such as its ion selectivity, the molecular nature of the channel mediated the OC, and its physiological role, are still unclear, and these suggest that further investigation of the OC is necessary.

Based on these observations and the data provided by the previous studies, I therefore tested the following hypotheses.

1). Sustained dehydration causes an increase in one or more types of Ca^{2+} currents expressed in the MNCs.

2). The MNCs express a K^{+} current mediated by KCNQ/M subunits, and this current is activated by increases in the osmolality of the extracellular solution and plays a role in osmosensitivity of MNC electrical behaviour.

The objectives of the first project will investigate whether L-type Ca^{2+} currents, which initiate local signaling events that activate gene expression by phosphorylation of the CREB proteins and thus cause long-term changes in brain function (see section 1.2.2.1.3), are upregulated during sustained dehydration, which induces an adaptation including ultrastructural changes and gene expression in the SON (see section 1.4). The objectives of the second project will investigate the following properties and roles of the OC.

1). Ion selectivity of the channels mediating the OC.

2). Molecular nature of the channels mediating the OC.

3). Physiological roles of the OC in regulating MNC firing pattern.

3. MATERIALS AND METHODS

3.1 Chemicals

XE991 was from Biotrend USA and was dissolved in DMSO. Retigabine was kindly provided by Valeant Research and Development (USA) and dissolved in DMSO. Flupirtine was from Sigma (St. Louis, USA) and dissolved in DMSO. E4031 was from BIOMOL International LP, USA and dissolved in water. Linopirdine and nifedipine were from Sigma (St. Louis, USA) and dissolved in ethanol and DMSO, respectively. All other chemicals were also from Sigma (St. Louis, USA). The final concentration of ethanol was not over 0.15% and ethanol at this concentration did not affect the electrophysiological properties of the OC. The final concentration of DMSO was not over 0.1% in most of these studies. (The DMSO concentration was slightly higher when XE991 at 20 and 30 μM were used and when XE991 was used with retigabine or flupirtine). DMSO at these concentrations had no significant effect on the amplitude of evoked Ca^{2+} currents and OC. The drugs were delivered to the extracellular recording solutions by a pipette.

3.2 Animal and cell preparation

Male Long Evans rats (Charles River, QC, Canada) weighing 250-300g, were anaesthetized by halothane inhalation and killed by decapitation in accordance with the guidelines of the Canadian Council on Animal Care and our University Ethics Committee. For the studies on L-type Ca^{2+} current, rats deprived of water for 16-24 h prior to killing were used for the dehydrated condition. Plasma osmolality can be increased to 330-340 mosmol kg^{-1} by such dehydration, which can also cause structure changes in the SON (Wakerley *et al.*, 1978;

Hatton, 1997). Osmolality of the solutions for dissection and recording were maintained at 325 ± 3 mosmol kg^{-1} for the dehydrated rats. MNCs were prepared as previously described in Oliet *et al.* (Oliet & Bourque, 1992). Briefly, after the decapitation, the rat head was quickly put in cool physiological saline. The skull was carefully opened and the brain was quickly taken away from the cranial vault after optic nerves were cut. The inferior surface of the brain was exposed and the brain was moistened by ice-cold PIPES [piperazine-1, 4-bis (2-ethanesulfonic acid)] solution composited of (in mM, pH 7.1): PIPES 20, NaCl 110, KCl 5, $MgCl_2$ 1, glucose 25, and $CaCl_2$ 1. The PIPES solution had an osmolality of 295 mosmol kg^{-1}. A brain slice with a thickness of about 1.0 mm containing most of SON was obtained. The slice was put in a dish containing ice-cold PIPES solution and then fixed with pins. Two tissue blocks containing most of SON were obtained and then placed in a 15 ml glass tube containing 10 ml PIPES solution with trypsin (Type XI, 0.6 mg/ml). The solution was continuously bubbled with 100% O_2 in a water bath at 34°C for 90 min, and the tissues were then transferred to a trypsin-free PIPES solution, which was also continuously bubbled with 100% O_2 at room temperature for 30 min. Finally, a series of fire-polished Pasteur glass pipettes with decreasing diameters were used to triturate the two blocks of tissue. Thus, MNCs acutely dispersed were obtained and the cells were plated on culture dishes with glass bottoms. The dishes were covered to prevent evaporation and the cells were allowed to settle and attach for about one hour at room temperature before recording.

3.3 Whole-cell patch-clamp recording

Whole-cell patch-clamp recordings were performed in the cells with a cross-sectional diameter greater than 15 μm at room temperature, and over 96% of the cells with this size are MNCs (Oliet & Bourque, 1992). The recordings were performed using an EPC-9 amplifier

(HEKA, Germany) operated with Pulse software (HEKA, Germany). Electrodes were pulled from borosilicate capillary glass (1.2 × 0.68 mm; A-M Systems Inc, USA) on a P-97 horizontal pipette puller (Sutter Instrument Company, USA) and polished using a microforge (Narashige). For the isolation of Ca^{2+} currents, a protocol similar to that described in Liu *et al.* (Liu et al., 2005), was used and NMDG (N-methyl-D-glucamine)/Ca^{2+} recording solution was used for Ca^{2+} current recording. The extracellular solution contains (in mM): NMDG 90, $CaCl_2$ 2, glucose 10, Hepes 10, TTX 0.0002, TEA 20, 4-AP 4, and pH 7.35. A VAPRO pressure osmometer (WESCOR, Logan, UT, USA) was used to measure the osmolality of the recording solution before each experiment, and mannitol was added for adjustment of the osmolality (295 or 325 mosmol kg^{-1}) as required. The recording electrodes (2.5–6 MΩ) were filled with a solution that contains (in mM): Tris 110, $MgCl_2$ 1, EGTA 1.6, TEA 40, Na_2ATP 2, phosphocreatine 14, and pH 7.2. The internal recording solution had an osmolality of 290 mosmol kg^{-1}. For the further investigation of the properties of the OC previously identified as a tail current (the remaining current during the closing period of an opening channel after voltage steps), the external recording solution contains (in mM, pH 7.35): NaCl 120, KCl 3, glucose 10, $CaCl_2$ 2, HEPES 10, TEA 20, 4-AP 4, and TTX 0.0002. The recording electrodes (2-4 MΩ) were filled with a solution that was composed of (in mM; pH 7.2): KCl 125, $MgCl_2$ 5, Tris 5, EGTA 1, TEA 15, Na_2ATP 1, GTP 0.1, phosphocreatine 7, and pH 7.2. We call these recording solutions (for the isolation of the OC) the TEA/physiological solutions.

3.4 Extracellular recording

Single unit extracellular recording was performed in a hypothalamic explant as previously described in Bourque and Renaud (Bourque & Renaud, 1983). Briefly, after the brain

was obtained from male Long-Evans rats, it was placed in a bubbled (with a 95% O_2 and 5% CO_2 gas mixture) artificial cerebrospinal fluid (ACSF; pH 7.4) containing (in mM): KCl 4, NaH_2PO_4 1.23, NaCl 112, $NaCO_3$ 25.95, glucose 10, $MgCl_2$ 1, and $CaCl_2$ 1. The osmolality of the ACSF solution was adjusted to 295-300 mosmol kg^{-1} using mannitol. The brain with the inferior surface exposed was glued to a lid of a culture dish with LOCTITE quick set 404 industrial adhesive. A block of explant (about 8 mm × 8 mm × 2 mm) containing the hypothalamus was cut with a razor blade and was fixed to a small block of Sylgard with pins in a recording chamber. The ACSF was bubbled with a 95% O_2 and 5% CO_2 gas mixture and preheated to 34°C using a single channel heater controller (Warner Instruments, Canada) and flowed at about 1 ml/min onto the surface of the explant from a perfusion pipette. Finally, a small opening in the arachnoid membrane of the explant was made with fine forceps, and then the explant was allowed to equilibrate in bubbled (with a 95% O_2 and 5% CO_2 gas mixture) ACSF for at least 90 min. Single unit extracellular recording was performed from SON neurons, located in a region along the edge of the optic chiasm to the junction of the middle cerebral arteries and anterior cerebral arteries. Recordings were made with electrodes made with borosilicate capillary glass (1.2 × 0.68 mm; A-M Systems Inc, USA) pulled on a vertical pipette puller (Narashige). Electrodes (15-30 MΩ) filled with 1 M NaCl were inserted to the explant to a depth less than 200 µm to detect firing of individual MNCs. An Axoclamp 2A amplifier (Axon Instruments) and a Digi-data 1200B interface (Axon Instruments) were used for recording, and data analysis was performed with pClamp 9.0 software (Axon Instruments).

3.5 Immunocytochemistry

Immunocytochemistry studies were performed in acutely isolated MNCs following patch-clamp recording to identify VP- and OT-MNCs as previously described (Fisher *et al.*, 1998). Following recordings, mild positive pressure was applied to allow dissociation of the patch pipette from the recorded cell. A round mark around the recorded cell was then made on the bottom of the dish using ink, and an image of the cell and the spots around the cell was obtained using a cooled CCD camera attached to a Zeiss Axiovert 200 microscope with a 40 x objective or was sketched in a notebook. About 80% of these cells were found the next day. The dish containing the marked cell was rinsed using phosphate-buffered saline (PBS, pH 7.4) three times, and the cell was then fixed in PBS with 4% paraformaldehyde, 0.05% sodium periodate, and 0.34% lysine at room temperature for 20 min. The cell was thoroughly washed using PBS (8 times) to remove paraformaldehyde and then incubated with blocking solution composed of PBS with 4% normal donkey serum (Jackson Immunoresearch) and 0.02% Triton X-100 at room temperature for 30-60 min. After blocking, the cell was incubated with primary antibody overnight at 4°C. For the identification of VP- and OT-MNCs, goat primary antibodies (Santa Cruz Biotechnology, USA), anti-neurophysin (NP) I (1:100; which recognizes OT-releasing MNCs) and anti-NP II (1:100; which recognizes VP-releasing MNCs) were used. For the identification of KCNQ/M channel subunits in MNCs, rabbit antibodies recognizing the channel proteins of KCNQ2, KCNQ3, KCNQ4, and KCNQ5 from Santa Cruz Biotechnology were used (1:100). Other rabbit antibodies recognizing KCNQ2 (1:500) and KCNQ3 (1:500), gifts from Dr. K. Mackie of Indiana University, USA, and KCNQ4 (1:800), a gift from Dr. B. Kachar of NIDCD/NIH, USA, were also used and yielded similar results. One dish without the primary antibody was used as a negative control during each experiment. After the incubation with

primary antibodies, the cells were rinsed using the blocking solution 8 times, and then treated with secondary antibodies, either donkey anti-goat (Cy3; Jackson Immunoresearch, 1:500) which was used for the identification of VP- and OT-MNCs, or donkey anti-rabbit (FITC; Jackson Immunoresearch, 1:500) which was used for the identification of KCNQ/M channel subunits. The dishes were covered with a box to avoid light, and incubated with the secondary antibodies for 30 min at room temperature. The cells were then rinsed first with blocking solution 4 times and subsequently with PBS 4 times. Finally, following addition of mounting solution (Citifluor, Marico Inc.), the cells were covered with a glass coverslip. The cells were viewed with the Zeiss inverted Axiovert-200 microscope under two conditions, differential contrast interference (DIC) and fluorescence imaging. A cooled CCD camera was used to obtain images.

3.6 Data analysis

Currents including total Ca^{2+} currents, L-type Ca^{2+} currents and the OC, unless stated otherwise, were measured from the baseline to the peak. In addition, current density was generated by division of the current amplitude by cell membrane capacitance, which was displayed automatically by the Pulse software. Data were entered in a Prism worksheet (GraphPad software), and results were shown as mean ± SEM with "*n*" (number of cells). Statistical analysis was estimated by Student's *t* test or one-way ANOVA, as appropriate. The difference was regarded to be significant when $P < 0.05$.

4. RESULTS

4.1 Total Ca^{2+} currents and L-type Ca^{2+} current in cells from normal and dehydrated rats

VDCCs play many important roles in the regulation of cell excitability and cellular signal transduction (Fisher & Bourque, 2001). Although total Ca^{2+} currents in the MNCs are not affected by an acute change in osmolality of the extracellular solution (Liu *et al.*, 2005), it is not known whether they are affected by sustained dehydration. It is possible that one or more types of Ca^{2+} currents may be affected by sustained dehydration. We first investigated the amplitude of the total Ca^{2+} current and the L-type Ca^{2+} current in normal and dehydrated rats.

4.1.1 Isolation of total Ca^{2+} currents and L-type Ca^{2+} current

Two groups of rats were involved in this study. One group was normally hydrated (the control rats) while the other was deprived of water for 16-24 h (the dehydrated rats). MNCs were acutely isolated from the SON of two groups of rats and whole-cell patch-clamp recording was performed in NMDG/Ca^{2+} solution with osmolalities of 295 mosmol kg^{-1} for control MNCs and 325 mosmol kg^{-1} for dehydrated MNCs. The recording solutions did not contain K^+. The solutions contained TTX to block Na^+ channels, and TEA and 4-AP to block K^+ channels. The cells were held at -100 mV or -80 mV, depolarized to -10 mV or 0 mV for 400 ms, respectively, and then returned to the holding potentials. A large inward Ca^{2+} current, after the long depolarizing step, was observed (Figure 4.1A). When the Ca^{2+} current become steady, a selective blocker of L-type Ca^{2+} channels, nifedipine (10 μM), was applied to the extracellular recording solution. The L-type Ca^{2+} current was then measured by subtracting currents evoked with nifedipine from those without nifedipine. Nifedipine was dissolved in DMSO and the final

concentration of DMSO was not over 0.1%. DMSO at this concentration had no significant effect on Ca^{2+} currents. Traces in Figure 4.1B display L-type Ca^{2+} currents after the subtraction in control and dehydrated MNCs. The traces suggest that amplitude of the L-type Ca^{2+} current appears to be much larger in cells from dehydrated rats than that in control rats. Since there was no significant difference in total and nifedipine-sensitive Ca^{2+} currents evoked by the two depolarizing steps mentioned above, the data from the experiments performed using the two different depolarizing protocols were pooled together for analysis.

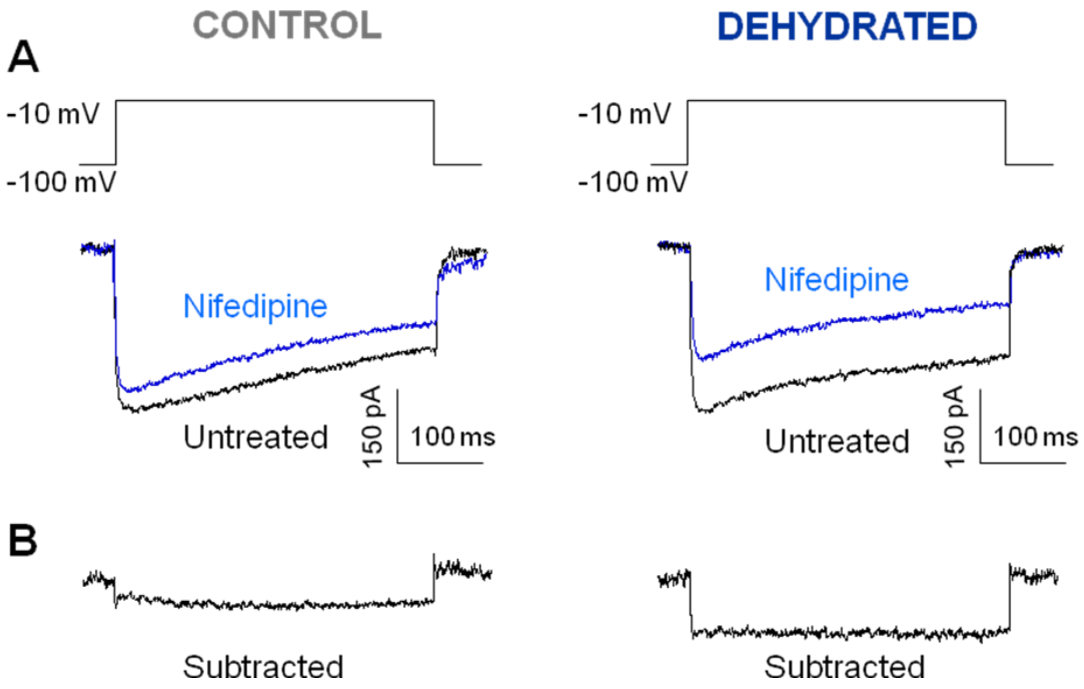


Figure 4.1 Total Ca^{2+} currents and L-type Ca^{2+} current in cells from normal and dehydrated rats. A, left, Currents were evoked by steps from -100 mV to -10 mV for 400 ms before and after the addition of 10 μM nifedipine in MNSs from normal rats. **A, right,** Currents were evoked by steps from -100 mV to -10 mV for 400 ms before and after the addition of 10 μM nifedipine in MNCs from dehydrated rats. **B, left,** The subtracted current showing the nifedipine-sensitive current in normal rats. **B, right,** The subtracted current showing the nifedipine-sensitive current in dehydrated rats.

4.1.2 Sustained dehydration significantly increases the amplitude and density of L-type Ca^{2+} current but not total Ca^{2+} currents

Figure 4.2A shows that the amplitude of total Ca^{2+} currents were slightly but not significantly increased from -214 ± 16 pA to -237 ± 17 pA during sustained dehydration (n = 36 and 42 for cells from control and dehydrated rats, respectively). However, there was a significant difference in amplitude of the L-type Ca^{2+} current between control (-55.5 ± 6.2 pA) and dehydrated rats (-99.1 ± 10.0 pA; Figure 4.2B), demonstrating that sustained dehydration causes a significant increase in amplitude of the L-type Ca^{2+} current (n = 36 and 42 for control and dehydrated rats, respectively; $P < 0.001$). Thus, the L-type Ca^{2+} currents expressed as a percentage of total Ca^{2+} currents, following 16-24 h dehydration, was increased from 26.0% to 41.8%.

OT-MNCs undergo hypertrophy and an increase in cell membrane capacitance during lactation (Teruyama & Armstrong, 2005), and both types of MNCs undergo hypertrophy during sustained dehydration (Lin *et al.*, 1996). Our data also showed that there was a significant difference in cell membrane capacitance between control (13.4 ± 0.5 pF) and dehydrated rats (16.7 ± 0.5 pF), demonstrating that sustained dehydration causes a significant increase in the cell membrane capacitance (n = 36 and 42 for cells from control and dehydrated rats, respectively; $P < 0.001$; Figure 4.3A). The L-type Ca^{2+} current was then divided by the cell membrane capacitance to obtain current density. Figure 4.3B shows that there was also a significant difference in the L-type Ca^{2+} current density between control (-4.5 ± 0.6 pA/pF) and dehydrated rats (-6.4 ± 0.6 pA/pF), demonstrating that sustained dehydration also causes a significant increase in the L-type Ca^{2+} current density (n = 36 and 42 for cells from control and dehydrated

rats, respectively; $P < 0.05$). These data demonstrate that the L-type Ca^{2+} current was increased by 78.6% in amplitude and by 42% in density during sustained dehydration.

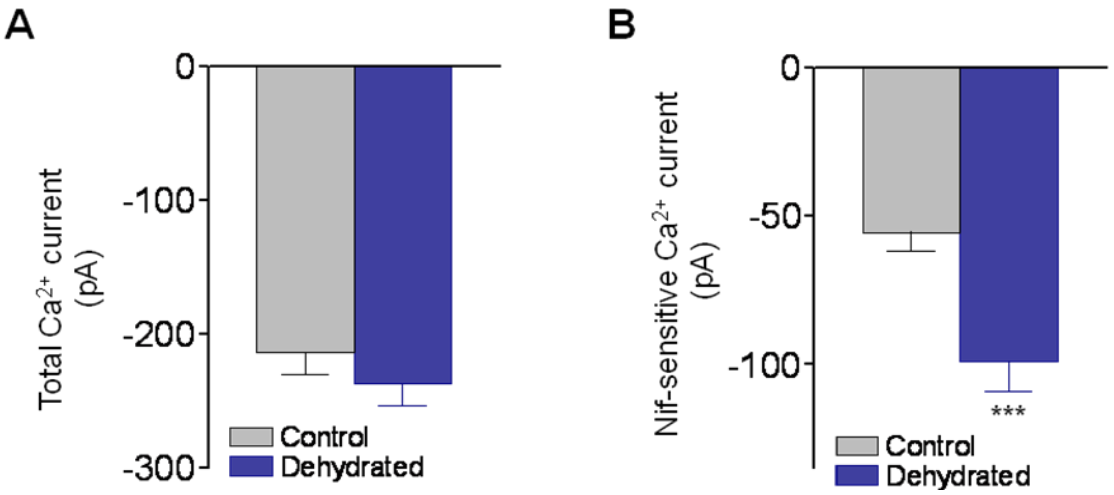


Figure 4.2 Sustained dehydration causes a significant increase in amplitude of L-type Ca^{2+} current but not total Ca^{2+} currents. A, A bar graph showing the amplitude of total Ca^{2+} currents in normal and dehydrated rats (n = 36 and 42 for control and dehydrated rats, respectively; $P > 0.05$). **B,** A bar graph showing the amplitude of L-type Ca^{2+} currents in normal and dehydrated rats (n = 36 and 42 for control and dehydrated rats, respectively; $^{***}P < 0.001$).

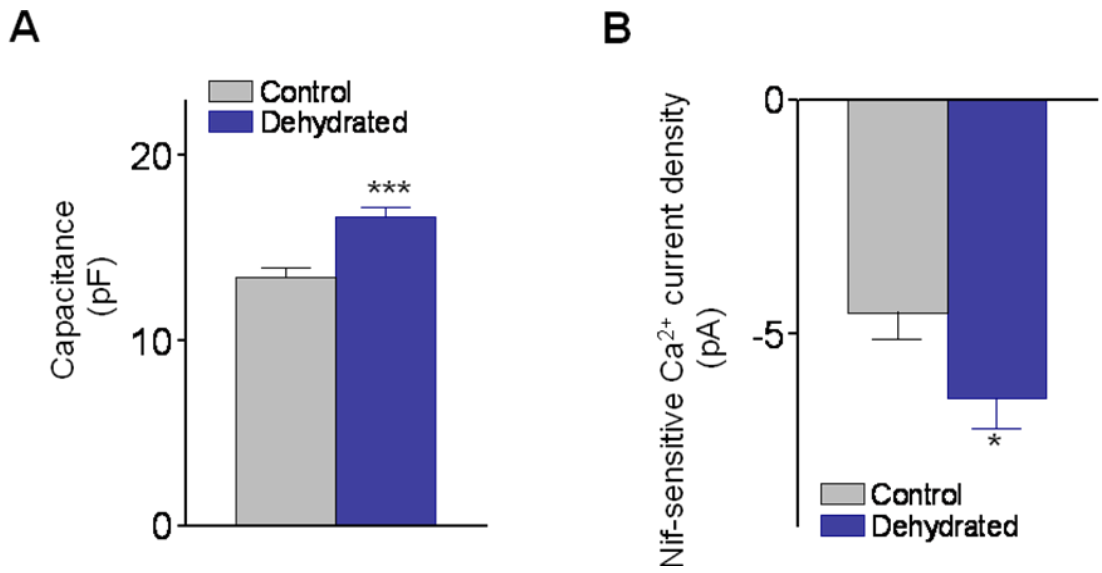


Figure 4.3 Sustained dehydration increases cell capacitance and density of L-type Ca^{2+} current. A, A bar graph showing cell capacitance in cells from normal and dehydrated rats (n = 36 and 42 for cells from control and dehydrated rats, respectively; $^{***}P < 0.001$). **B,** A bar graph showing the density of L-type Ca^{2+} current in normal and dehydrated rats (n = 36 and 42 for control and dehydrated rats, respectively; $^{*}P < 0.05$).

We also used acutely isolated MNCs from control rats to determine whether a 1.5 h treatment in hyperosmolar solution could cause increases in the cell capacitance or L-type Ca^{2+} currents. The cell capacitance in the MNCs cultured in 320 mosmol kg^{-1} (15.0 ± 1.4 pF, *n*=9) physiological PIPES solution for about 90 min was not significantly larger than that in the MNCs maintained in 290 mosmol kg^{-1} (13.8 ± 1.1 pF, *n*=10) physiological PIPES solution. The amplitude of L-type Ca^{2+} currents in the MNCs cultured in 320 mosmol kg^{-1} (-76.9 ± 15.0 pA, *n*=9) physiological PIPES solution for about 90 min was not significantly larger than that in the MNCs maintained in 290 mosmol kg^{-1} (-65.0 ± 9.5 pA, *n*=10) physiological PIPES solution.

4.1.3 Current-voltage relationship of total Ca^{2+} current and L-type Ca^{2+} current in cells from normal and dehydrated rats

The cell was depolarized from a holding potential of -80 mV to a series of potentials from -70 mV to +20 mV (in 10 mV increments every 1.5 s) for 400 ms and then returned to -80 mV. After the Ca^{2+} currents were stable, 10 μM nifedipine was added to the extracellular solution to block L-type Ca^{2+} current. The amplitude of L-type currents were then determined by subtracting currents evoked with nifedipine from those without nifedipine. The current-voltage relationship of the total Ca^{2+} currents in control and dehydrated MNCs is displayed in Figure 4.4A, which shows that peak Ca^{2+} current was obtained at -10 mV in the MNCs. The results indicate that sustained dehydration induced a significant increase in amplitude of the total Ca^{2+} currents only at 0 mV (-6.4 ± 0.6 *versus* -4.5 ± 0.6 pA/pF, n = 27 and 31 for cells from control and dehydrated rats, respectively; $P < 0.01$), with no significant difference at other voltages. Peak current of the L-type Ca^{2+} current was also obtained at -10 mV in the MNCs (Figure 4.4B).

In addition, Figure 4.4B also shows that there were significant differences in amplitudes of the L-type Ca^{2+} current between control and dehydrated rats at voltages between -20 and +10 mV (*n* = 27 and 31 for cells from control and dehydrated rats, respectively), demonstrating that sustained dehydration causes a significant increase in amplitude of the L-type Ca^{2+} current. This is consistent with the data described above. It is interesting to note, as is shown in Figure 4.4B, that the L-type Ca^{2+} current activated at -50 mV, confirming that a low threshold-activated L-type Ca^{2+} current is present in the MNCs. This is consistent with a previous study demonstrating that MNCs express a non-inactivating nifedipine-sensitive Ca^{2+} current that displays activation at -50 mV (Fisher & Bourque, 1995).

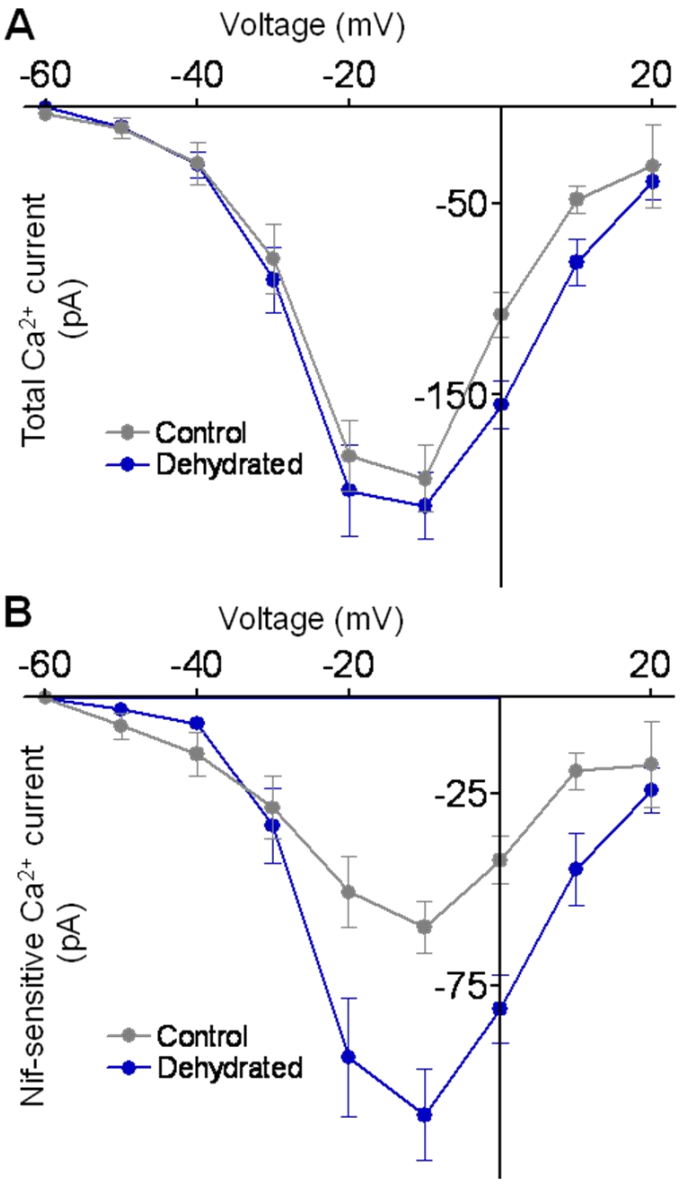


Figure 4.4 Current-voltage relationship of total Ca^{2+} current and L-type Ca^{2+} current in cells from normal and dehydrated rats. The cell was depolarized from a holding potential of -80 mV to a series of potentials from -70 mV to +20 mV (in 10 mV increments every 10 s) for 400 ms. **A,** The current-voltage relationship of total Ca^{2+} currents evoked by a series of potentials in control and dehydrated rats (*n* = 27 and 31 for cells from control and dehydrated rats, respectively). **B,** The current-voltage relationship

of L-type Ca^{2+} currents evoked by a series of potentials in control and dehydrated rats (*n* = 27 and 31 for cells from control and dehydrated rats, respectively).

4.1.4 Sustained dehydration increases cell membrane capacitance and L-type Ca^{2+} current in both VP- and oxytocin (OT)-MNCs

Since SON contains two types of neurons, VP-MNCs and OT-MNCs, and VP- and OT-MNCs can display differences in firing patterns (see section 1.3.1) during osmotic stimulation (Poulain *et al.*, 1977; Wakerley *et al.*, 1978), we tested whether there were significant differences in the increase in the L-type Ca^{2+} current between VP-MNCs and OT-MNCs in control and dehydrated rats. We used immunocytochemical techniques to identify the cells after whole-cell patch-clamp recording. Cells were identified as VP-MNCs if they were immunopositive for NP II or immunonegative for NP I or as OT-MNCs if they were immunopositive for NP I or immunonegative for NP II. One dish without the primary antibody was used as a negative control during each experiment. Immunocytochemical identification, after whole-cell patch-clamp recording, was successfully performed in 31 MNCs, including 8 VP-MNCs and 5 OT-MNCs in control rats, and 14 VP-MNCs and 4 OT-MNCs in dehydrated rats. Figure 4.5A, left, shows a cell that was immunoreactive for anti-NP II following recording, suggesting that it was a VP-MNC. Figure 4.5A, right, shows a recorded cell that was immunoreactive to anti-NP I, suggesting that it was an OT-MNC. After the types of the recorded cells were determined using post-recording immunocytochemical technique, we investigated whether there was a difference in cell membrane capacitance between VP-MNCs and OT-MNCs in control and dehydrated rats. Figure 4.5B, shows that there was an increase in the cell

membrane capacitance in both VP- and OT-MNCs during sustained dehydration. The cell membrane capacitance of the VP-MNCs was 14.5 ± 1.1 pF in control rats and 16.5 ± 0.8 pF in dehydrated rats (n = 8 and 14, respectively), but this increase was not significant (probably due to the relatively small number of cells sampled). The cell membrane capacitance of OT-MNCs was 12.4 ± 1.3 pF in control rats and 16.2 ± 0.9 pF in dehydrated rats (n = 5 and 4, respectively), and the difference was significant ($P < 0.05$). The amplitude of L-type Ca^{2+} current of VP-MNCs was -57.4 ± 15.0 pA in control rats and -109.1 ± 16.9 pA in dehydrated rats (n = 8 and 14, respectively; Figure 4.5C), and this difference was significant ($P < 0.05$). In addition, Figure 4.5C also shows that the amplitude of L-type Ca^{2+} current in OT-MNCs was also much larger in dehydrated rats than that in control rats (-122.0 ± 28.8 pA *versus* -59.0 ± 16.1 pA; n = 4 and 5, respectively; $P < 0.05$). These data demonstrate that sustained dehydration increases the L-type Ca^{2+} current in both VP- and OT-MNCs.

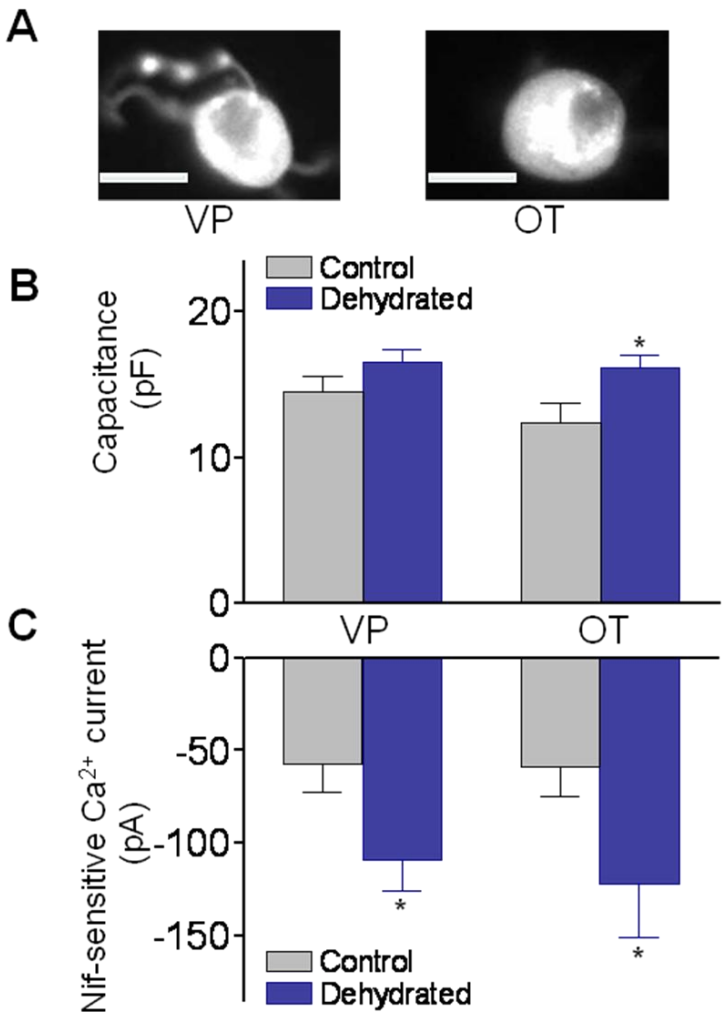


Figure 4.5 Sustained dehydration increases cell capacitance and L-type Ca^{2+} current in both VP- and OT-MNCs. A, Immunofluorescence images following post-recording showed immunoreactivity to anti-NP II (left) and anti-NP I (right). The scale bar is 15 µM. **B,** A bar graph showing cell capacitance in control and dehydrated rats that were immunoreactive for VP (n = 8 and 14, respectively) and OT (n = 5 and 4, respectively; $^{*}P < 0.05$). **C,** A bar graph showing the amplitude of L-type Ca^{2+} current in control and dehydrated rats that were immunoreactive for VP (n = 8 and 14, respectively; $^{*}P < 0.05$) and OT (n = 5 and 4, respectively; $^{*}P < 0.05$).

4.1.5 Summary and discussion: sustained dehydration increases L-type Ca^{2+} current

Our data from whole-cell patch-clamp recording studies indicate that although the total VDCC currents only displayed a small increase (which was significant at only one voltage), the L-type Ca^{2+} current was increased by 78.6% in current amplitude and by 42% in current density following 16-24 h dehydration. This is consistent with the results of radioligand binding studies in our laboratory (Star, 2005). In those studies, the density of L-type Ca^{2+} channels was determined by binding of ^{3}H-isradipine, which is a DHP and can selectively bind to L-type Ca^{2+} channels, in the SON and neurohypophysial tissues. Specific binding was estimated by subtracting non-specific binding of the mean of triplicate assays from total binding and the B_{MAX} (maximal number of binding) and K_D (dissociation constant) were determined by GraphPad software. The radioligand binding studies show that the numbers of L-type Ca^{2+} channels were significantly increased (by 32.2%) in the SON, but not in the neurohypophysis, during sustained dehydration (Star, 2005).

Our data from the whole-cell patch-clamp recording also showed that a low threshold-activated L-type Ca^{2+} current was detected in the MNCs (Figure 4.4B). This current may be mediated by the $Ca_V1.3$ channel, which can activate at a low voltage (Koschak *et al.*, 2001). This finding is consistent with a previous report indicating that the MNCs express a non-inactivating L-type Ca^{2+} current that displays activation at -50 mV (Fisher & Bourque, 1995). A study using the single-cell RT-PCR technique in our laboratory also indicated that the MNCs express both the $Ca_V1.2$ and the $Ca_V1.3$ channel subunits (Zhang *et al.*, 2007a). This is consistent with a previous immunocytochemical study, which demonstrated that both the $Ca_V1.2$ and the $Ca_V1.3$ channel subunits are expressed in somata and proximal dendrites of the MNCs (Joux *et al.*, 2001).

Our results from post-recording immunocytochemistry demonstrate that the increase in the L-type Ca^{2+} current during sustained dehydration occurred in both VP-MNCs and OT-MNCs. Although VP-MNCs release VP to regulate plasma osmolality, OT, in some cases, can also play a role in osmoregulation by natriuresis, and plasma levels of both vary with changes in plasma osmolality (Bourque & Oliet, 1997). Furthermore, hypertrophy can be caused in both VP-MNCs and OT-MNCs during sustained dehydration (Lin *et al.*, 1996). Thus, it is reasonable that sustained dehydration causes the increase in the L-type Ca^{2+} current in both the VP-MNCs and the OT-MNCs (also see 5.1.1).

In addition, MNC membrane capacitance was also increased by 24.6% following sustained dehydration, which is consistent with previous studies demonstrating that sustained dehydration causes hypertrophy of the MNCs in live rats (Hatton, 1997). This leads to an increase in volume of MNC region in the SON by 54% following sustained dehydration induced by 7 days of substitution of tap water with 2% saline (Salm & Hawrylaka, 2004), despite a decrease in glial coverage (Hatton, 1997). Although our data indicate that the increase in cell capacitance in VP-MNCs was not significant, it may be due to the relatively small number of cells compared.

In summary, our data demonstrate that sustained dehydration causes increases in cell membrane capacitance and in the amplitude and density of L-type Ca^{2+} currents in MNCs, and that increases in L-type Ca^{2+} currents occur in both VP-MNCs and OT-MNCs.

4.2 The osmosensitive current (OC) is increased by acute hypertonic stimulation

MNCs change their electrical behaviour in response to small changes in external osmolality through the regulation of ion channels (Bourque, 2008), and ion channels, especially osmosensitive ion channels, therefore play key roles in the modulation of MNC firing and hormone release. Our data show that L-type Ca^{2+} currents are increased in MNCs following sustained dehydration. However, MNCs may express other currents, which display osmosensitivity and regulate MNC firing rate and pattern. An osmosensitive current (the OC) has been identified in acutely isolated MNCs in our laboratory (Liu *et al.*, 2005). The biophysical properties of the OC such as its ion selectivity, the molecular nature of the channel mediated the OC, and its physiological role, are still unclear, and we will investigate its properties and physiological role.

4.2.1 Isolation of the OC

The OC is a cationic current and was isolated as a tail current (the remaining current during the closing period of an opening channel after voltage steps) in external and internal recording solutions without K^+ (I_{Ca} medium; Liu *et al.*, 2005). The OC is voltage-dependent and activated at potentials more positive than -60 mV. The OC was observed in the presence of 20 mM TEA and 4 mM 4-AP in the external recording solution and 40 mM TEA in the internal recording solution, but was blocked by 0.25 mM Ba^{2+}. We performed experiments to investigate the biophysical properties, molecular nature, and physiological role of the OC. Since the OC, which was isolated in I_{Ca} medium in the absence of K^+, might be mediated by a subtype of K^+ channels (Liu *et al.*, 2005), we evoked the OC in the presence of 3 mM K^+ in the external

solution and 125 mM K^+ in the internal solution. TEA (20 mM) and 4-AP (4 mM) in the external solution, and TEA (15 mM) in the internal solution were included to block most K^+ currents. TTX (0.2 µM) was used to block inward Na^+ current. These recording solutions are called the TEA/physiological solutions. The reason why the inward Ca^{2+} current was not blocked will be discussed in the following section.

Cells were depolarized from a holding potential of -80 mV to 0 mV for 400 ms, and then returned to -80 mV in TEA/physiological solution with an osmolality of 295 mosmol kg^{-1}. During the depolarization, inward Ca^{2+} current and outward currents were obtained (Figure 4.6A, left). The stimulation was repeated until the currents became stable. The cell was then perfused with TEA/physiological solution with an osmolality of 325 mosmol kg^{-1}, and the depolarizing stimulation was performed until the currents became stable again, typically 2-5 minutes. The sizes of both the inward currents and the outward currents were then measured. Figure 4.6B, left, shows the inward Ca^{2+} currents evoked in a MNC in NMDG/Ca^{2+} solutions with osmolality of 295 mosmol kg^{-1} and then with osmolality of 325 mosmol kg^{-1}. Ca^{2+} current density was -8.0 ± 1.4 pA/pF and -7.9 ± 1.3 pA/pF in 295 mosmol kg^{-1} and 325 mosmol kg^{-1} solutions (n=6, P > 0.05), respectively. This suggests that there were no significant differences in the Ca^{2+} currents between the two conditions (Figure 4.6B, right). Figure 4.6A, left, shows that the amplitude of the net outward current was increased after the cell was switched to 325 mosmol kg^{-1} solution. This current, which can display an increase with increases in osmolality, is called the osmosensitive current, or OC. The subtracted outward current was obtained by subtracting currents evoked in 295 mosmol kg^{-1} solution from those in 325 mosmol kg^{-1} solution, and is displayed in Figure 4.6A, left (inset). The net outward current was increased in 40 of 61 MNCs (66%) tested, which is similar to the percentage of MNCs that displayed an increase (59%) in the

density of the OC isolated as a tail current in I_{Ca} medium (Liu *et al.*, 2005). Figure 4.6A, right, shows that the density of the net outward current was significantly increased from 12.3 ± 1.3 pA/pF in 295 mosmol kg^{-1} solution to 21.4 ± 1.8 pA/pF in 325 mosmol kg^{-1} solution (n=40, P < 0.0001). Figure 4.6A, inset, also shows that the OC was slowly activating and displayed slow inactivation. The OC inactivated by 35.7% ± 4.6% by the end of the 400 ms depolarizing step.

Since osmotic pressure is normally maintained within a very narrow range in mammals, we tested whether the OC was sensitive to small changes in external osmolality. Our data show that the OC displays a significant increase from 14.0 ± 2.3 pA/pF to 19.6 ± 3.3 pA/pF (n=6, P < 0.05) after external osmolality was increased from 295 to 305 mosmol kg^{-1}.

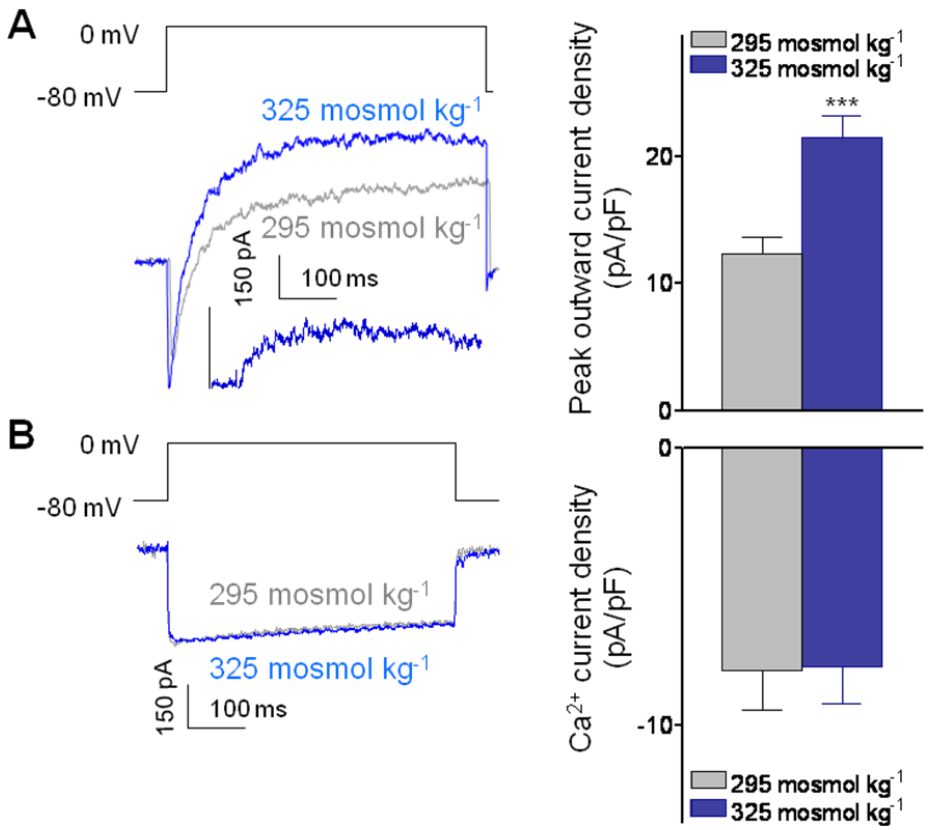


Figure 4.6 A slow outward current sensitive to increases in external osmolality. A, left, Currents evoked by steps from -80 mV to 0 mV for 400 ms in 295 mosmol kg^{-1} and then 325 mosmol kg^{-1} TEA/physiological external solutions. **Inset,** The difference between the two currents. **A, right**, The density of evoked outward currents in 40 responding cells in 295 mosmol kg^{-1} and 325 mosmol kg^{-1} TEA/physiological external solutions ($^{***}P < 0.0001$). **B, left,** Ca^{2+} currents were evoked by a step from a holding potential of -80 mV to 0 mV for 400 ms in 295 mosmol kg^{-1} and then 325 mosmol kg^{-1} NMDG/Ca^{2+} external solutions. **B, right**, The density of evoked Ca^{2+} currents in 5 cells in 295 mosmol kg^{-1} and 325 mosmol kg^{-1} NMDG/Ca^{2+} external solutions (n=6, $P > 0.05$).

4.2.2 Voltage-dependence of the OC

In order to determine which ion channel mediates the OC in MNCs, we investigated the biophysical and pharmacological properties of the OC. Since voltage sensitivity is an important feature of many types of ion channels, we tested whether the OC was voltage-dependent. The cell was depolarized from a holding potential of -80 mV to a series of potentials from -70 mV to 0 mV (in 10 mV increments every 10 s) for 400 ms and then returned to -80 mV in 295 mosmol kg^{-1} solution and then in 325 mosmol kg^{-1} solution. Figure 4.7A, left, shows the subtracted currents at a series of potentials from -60 mV to 0 mV. The plot in Figure 4.7A, right, shows that the OC is activated at potentials more positive than -60 mV, and the current-voltage relationship of the OC demonstrates that it is mediated by a voltage-dependent ion channel.

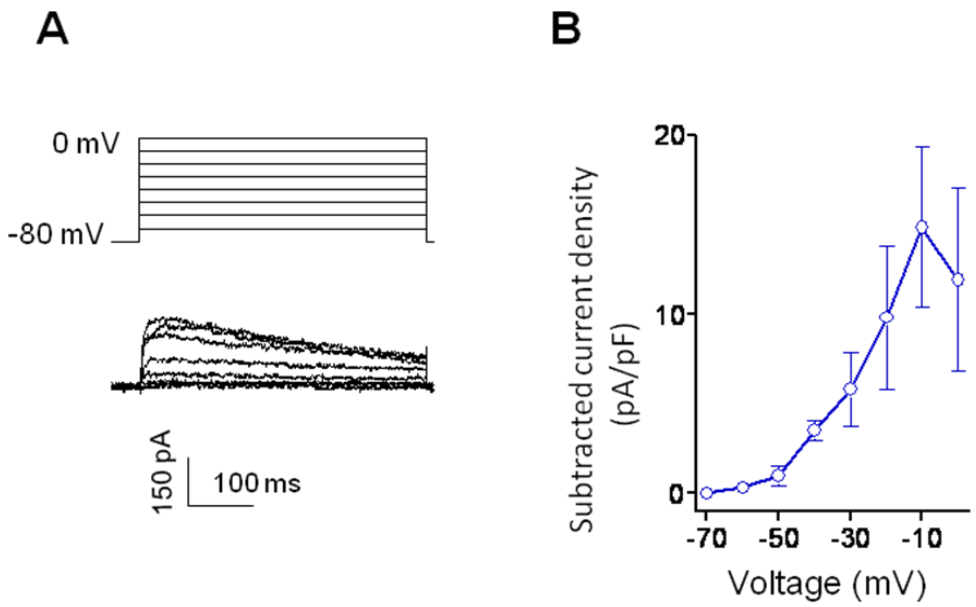


Figure 4.7 The OC is voltage-dependent. A, Cells were stepped from a holding potential of -80 mV to a series of potentials between -70 mV and 0 mV for 400 ms (in 10 mV increments every 10s) in 295 mosmol kg^{-1} and then 325 mosmol kg^{-1} external solutions. The traces show the difference between the two conditions. **B,** A plot of the density of the subtracted outward currents evoked from -80 mV to a series of potentials between -70 mV and 0 mV for 400 ms (*n*=3).

4.2.3 Ca^{2+}-dependence of the OC

Ca^{2+}-sensitivity is an important feature of several types of ion channels. Previous studies from our laboratory showed that 16 μM Cd^{2+} blocked the OC as a tail current in I_{Ca} medium by about 40%, but the application of BAPTA (a Ca^{2+} chelator) in the pipette solution, did not block the increase of the OC when the cells were switched from 295 to 325 mosmol kg^{-1} external solutions (Liu, 2004). These data suggest that although the OC is not primarily Ca^{2+} dependent, the OC could be partly Ca^{2+}-dependent or sensitive to Ca^{2+} entry through a subtype of Ca^{2+} channels, and this is the reason why we did not block the inward Ca^{2+} current. We therefore further investigated the Ca^{2+}-dependence of the OC. Nifedipine at 10 μM significantly decreased the OC as a tail current in I_{Ca} medium with 325 mosmol kg^{-1}, and the density of the tail current was -9.2 ± 1.8 pA/pF and -3.6 ± 0.6 pA/pF before and after the addition of nifedipine (n=4, P < 0.05). In addition, nifedipine also decreased the outward current in TEA/physiological solution with 295 mosmol kg^{-1}. Figure 4.8A shows the outward current in 9 MNCs in 295 mosmol kg^{-1} solution. Nifedipine decreased the density of the outward current from 11.9 ± 1.9 pA/pF to 6.7 ± 1.5 pA/pF (n=9, P < 0.05; Figure 4.8B). Figure 4.8C shows the outward current was increased from 6.3 ± 1.9 pA/pF to 10.2 ± 2.3 pA/pF in 6 out of 9 MNCs when the cells were switched from 295 to 325 mosmol kg^{-1} external solutions in the presence of 10 μM nifedipine (n=6, P < 0.05). These results are consistent with the previous studies, which suggest that the increase of the OC does not require Ca^{2+} (Liu, 2004). These data demonstrate that the OC could be sensitive to Ca^{2+} entry through L-type Ca^{2+} channels and that the increase of the OC does not require Ca^{2+}.

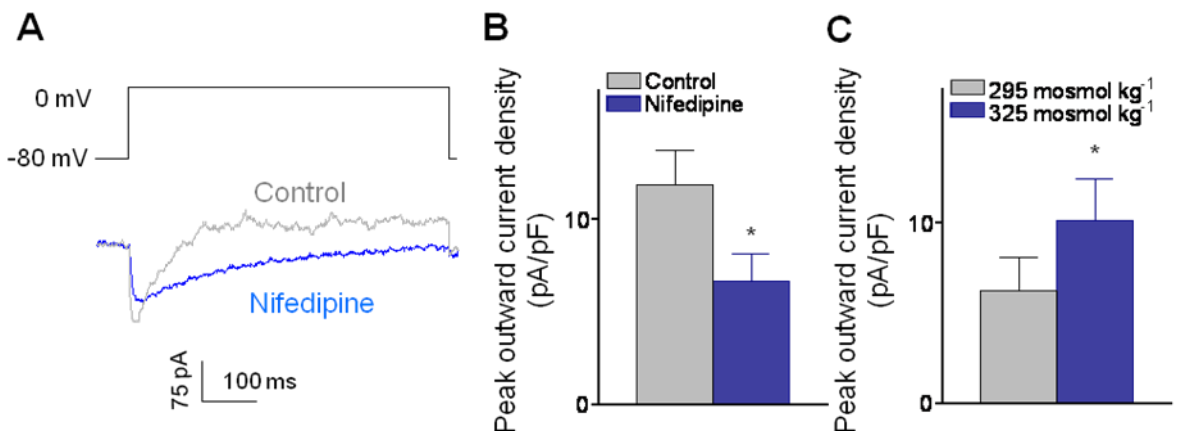


Figure 4.8 Ca^{2+}-dependence of the OC. Currents were evoked by steps from -80 mV to 0 mV for 400 ms. **A,** Currents evoked in 295 mosmol kg^{-1} solution before and after the addition of 10 µM nifedipine. **B,** A bar graph showing the density of the peak outward currents in 295 mosmol kg^{-1} solution before and after addition of 10 µM nifedipine (n=9, $^{*}P < 0.05$). **C,** A bar graph of the density of the outward currents in 295 and 325 mosmol kg^{-1} external solutions in the presence of 10 µM nifedipine (n=6, $^{*}P < 0.05$).

4.2.4 Reversal potential of the OC

Since the reversal potential of an ion current reflects the selectivity of the channel type mediating the current, we determined the reversal potential of the OC. To do this, we obtained the density and direction of subtracted tail currents at a series of different potentials following activation of the current by a depolarizing voltage step. The cell in Figure 4.9A was depolarized from a holding potential of -80 mV to 0 mV for 250 ms and then returned to different potentials from -120 mV to -50 mV (in 10 mV increments every 10 s) in 295 mosmol kg^{-1} solution and then in 325 mosmol kg^{-1} solution. The trace in Figure 4.9A, upper, shows subtracted tail currents at a series of potentials from -120 mV to -50 mV. These experiments were performed in an external solution with 3 mM K^+. The reversal potential can be determined from the subtracted tail currents (Figure 4.9A, upper). The direction of the subtracted tail currents in this cell changed at about -100 mV. The plot in Figure 4.9A, bottom, shows the mean current-voltage results for 5 cells and demonstrates that the reversal potential of the OC was between -100 mV and -90 mV. This was near E_K (predicted using the Nernst equation to be at -94 mV with 3 mM K^+ in the external solution and 125 mM K^+ in the internal solution), suggesting that the OC could be mediated by a K^+-selective channel. If this is the case, it would be predicted that the reversal potential would shift with changes in the K^+ concentration in the external solution. We therefore repeated the experiments in an external solution that contained 30 mM K^+ (and 27 mM less Na^+). The cell in Figure 4.9B was depolarized from a holding potential of -80 mV to 0 mV and then returned to different potentials from -80 mV to -10 mV (in 10 mV increments every 10 s) for 250 ms in 295 mosmol kg^{-1} solution and then in 325 mosmol kg^{-1} solution. The trace in Figure 4.9B, upper, shows the subtracted tail currents at a series of potentials from -80 mV to -10 mV. The direction of the subtracted tail currents in this cell changed at about -40 mV (Figure 4.9B, upper).

The plot in Figure 4.9B, bottom, shows the mean current-voltage results for 5 cells and demonstrates that the reversal potential of the OC was between -40 mV and -30 mV, which again was near E_K (predicted to be at -36 mV with 30 mM K^+ in external solution and 125 mM K^+ in internal solution). These data demonstrate that the reversal potential of the OC is near the E_K and shifts with changes of the K^+ concentration in the external solution, suggesting that the OC is a voltage-sensitive K^+-selective current.

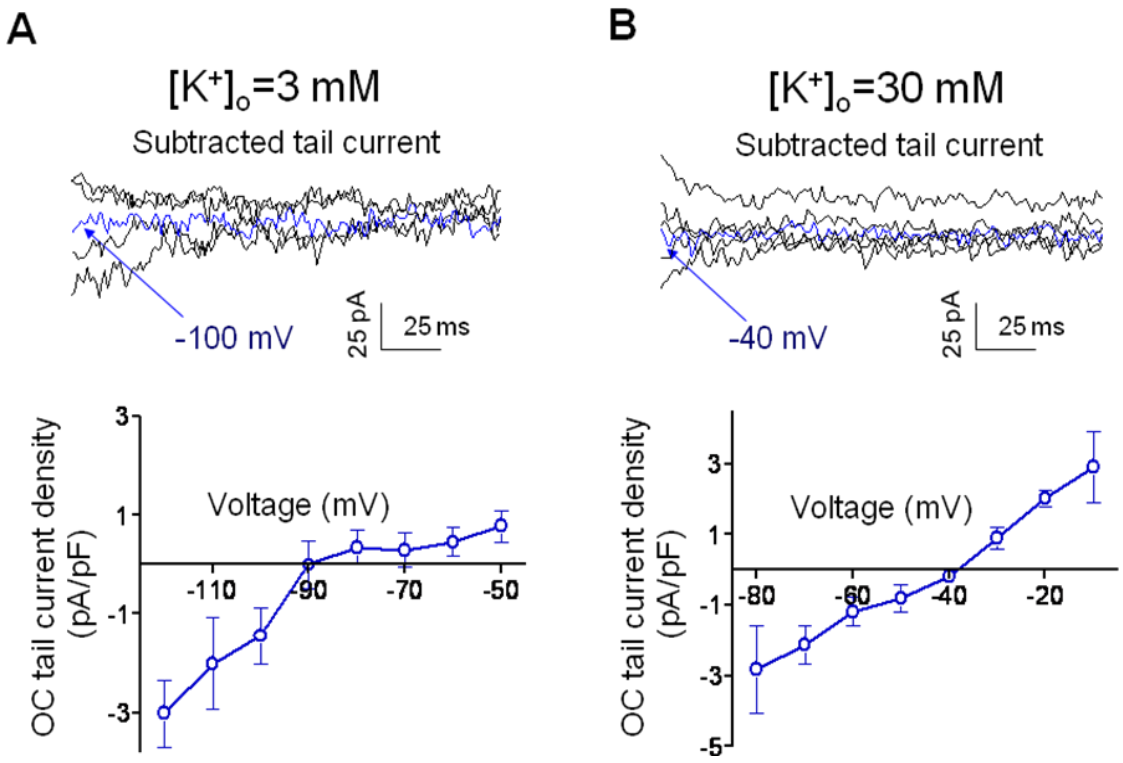


Figure 4.9 The OC is a K^+-selective current. A, above, Traces of OC tail currents in 3 mM external K^+. Currents were evoked by stepping from -80 mV to 0 mV for 250 ms and then to a series of potentials between -120 mV and -50 mV. Subtraction of currents evoked in 295 mosmol kg^{-1} from those evoked in 325 mosmol kg^{-1} led to the OC tail currents. **A, below,** A plot of the density of the subtracted tail currents in 3 mM external K^+ (*n*=5). **B, above,** Traces of OC tail currents in 30 mM external K^+. Currents were evoked by stepping from -80 mV to 0 mV for 250 ms and then to a series of potentials between -80 mV and -10 mV. **B, below,** A plot of the density of the subtracted tail currents in 30 mM external K^+ (*n*=5).

4.2.5 Pharmacology of the OC

4.2.5.1 Ba^{2+} blocks the OC

Since the OC is a voltage-gated K^+ current, we tested the effect of Ba^{2+}, a non-selective blocker of K^+ channels. When the outward currents were increased in MNCs switched from 295 mosmol kg^{-1} to 325 mosmol kg^{-1} solution, 0.3 mM Ba^{2+} was applied to the external solution. The trace in Figure 4.10A, left, shows that the outward current in 325 mosmol kg^{-1} solution was decreased by 0.3 mM Ba^{2+}. Ba^{2+} (0.3 mM) decreased the density of the outward currents from 17.4 ± 4.0 pA/pF to 10.5 ± 3.3 pA/pF (n=7, P < 0.05; Figure 4.10B). The outward currents evoked under these conditions may contain ionic currents other than the OC. Therefore, to confirm that Ba^{2+} blocks the OC, we compared the effects of 0.3 mM Ba^{2+} added at 295 mosmol kg^{-1} and then at 325 mosmol kg^{-1} solution in MNCs expressing the OC. Figure 4.10C shows that the density of Ba^{2+}-sensitive currents was increased from 5.6 ± 1.4 pA/pF to 9.8 ± 2.0 pA/pF (n=4, P< 0.05) after the cell was switched to 325 mosmol kg^{-1} solution, suggesting that the OC is sensitive to Ba^{2+}. This is consistent with previous studies on the pharmacology of the OC isolated as a tail current in I_{Ca} medium (Liu *et al.*, 2005).

Evoked currents were unaffected by stromatoxin, which selectively blocks $K_V4.2$ channels and members of the K_V2 family of channels with IC_{50} of less than 3 nM and 25 nM, respectively (Escoubas *et al.*, 2002). The density of the outward current in 295 mosmol kg^{-1} solution was 16.9 ± 5.5 pA/pF and 17.5 ± 5.5 pA/pF (n=4, P > 0.05) before and after the addition of 30 nM stromatoxin (Figure 4.11).

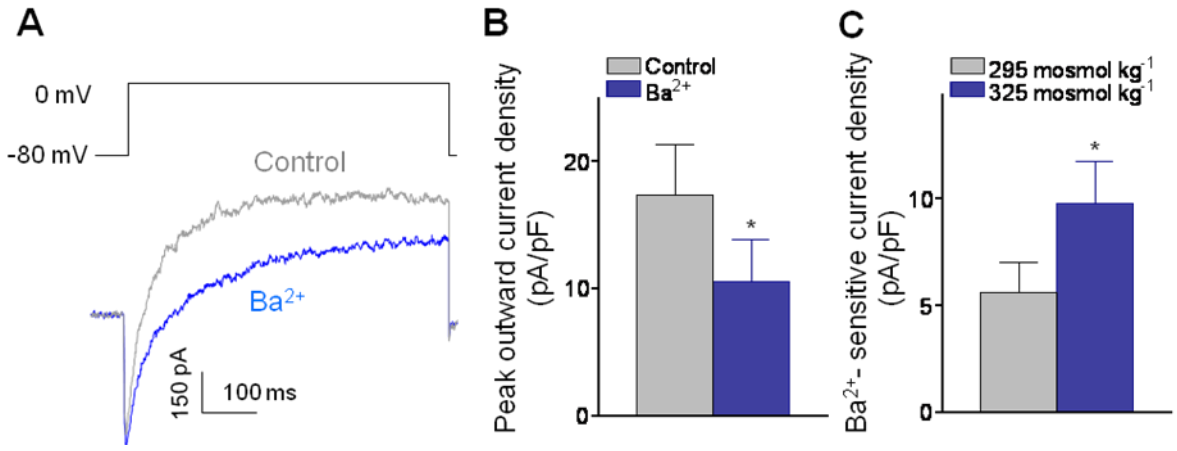


Figure 4.10 The OC is sensitive to Ba^{2+}. Currents were evoked by steps from -80 mV to 0 mV for 400 ms. **A,** Currents evoked in 325 mosmol kg^{-1} solution before and after the addition of 0.3 mM Ba^{2+}. **B,** A bar graph showing the density of the peak outward currents in 325 mosmol kg^{-1} solution before and after addition of 0.3 mM Ba^{2+} (n=7, $^{*}P < 0.05$). **C,** A bar graph of the density of the currents blocked by 0.3 mM Ba^{2+} in 295 and 325 mosmol kg^{-1} solutions (n=4, $^{*}P < 0.05$).

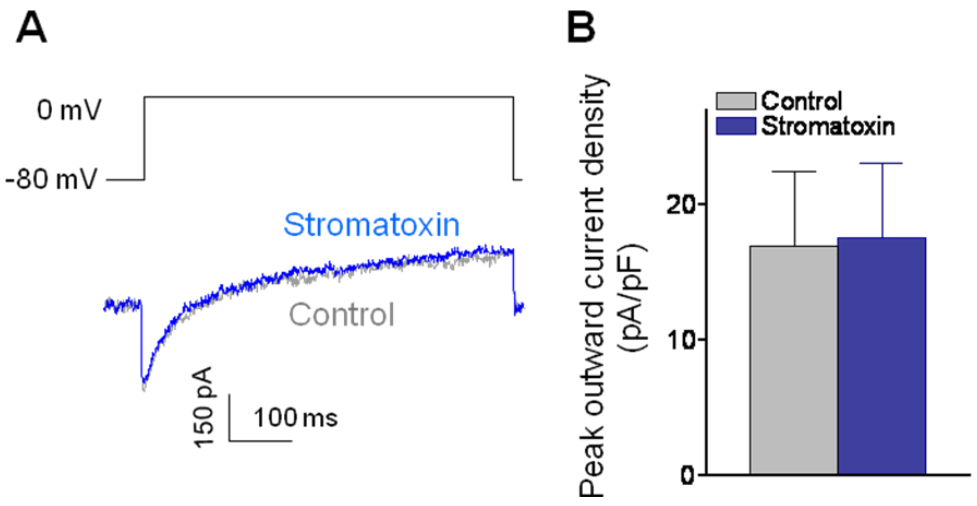


Figure 4.11 The outward current is insensitive to stromatoxin. Currents were evoked by steps from -80 mV to 0 mV for 400 ms. **A,** Currents evoked in 295 mosmol kg^{-1} solution before and after the addition of 30 nM stromatoxin. **B,** A bar graph showing the density of the peak outward currents in 295 mosmol kg^{-1} solution before and after addition of 30 nM stromatoxin (n=4, $P > 0.05$).

4.2.5.2 The OC is insensitive to apamin.

Since the OC was partly Ca^{2+} dependent, we tested whether the OC was mediated by K_{Ca} channels. Since BK channels are very sensitive to extracellular TEA (which was included in our external recording solutions) and IK channels are not voltage dependent (Faber & Sah, 2003), the OC could be not mediated by them. We tested the effect of apamin, a selective blocker of SK channels, on the OC. When the outward currents were increased in MNCs switched from 295 mosmol kg^{-1} to 325 mosmol kg^{-1} solution, 100 nM apamin was applied to the external solutions. The trace in Figure 4.12A shows that the outward current in 325 mosmol kg^{-1} solution before and after the addition of 100 nM apamin. The density of the outward currents was 23.9 ± 5.4 pA/pF to 23.3 ± 5.7 pA/pF before and after the addition of 100 nM apamin (n=5, P > 0.05; Figure 4.12B). This result indicates that the OC is not mediated by SK channels.

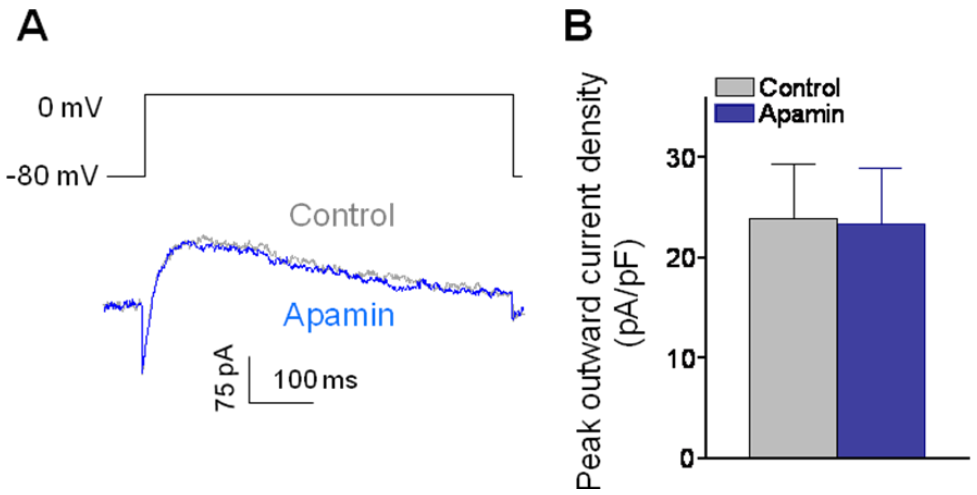


Figure 4.12 The OC is insensitive to apamin. Currents were evoked by steps from -80 mV to 0 mV for 400 ms. **A,** Currents evoked in 325 mosmol kg^{-1} solution before and after the addition of 100 nM apamin. **B,** A bar graph showing the density of the peak outward currents in 325 mosmol kg^{-1} solution before and after addition of 100 nM apamin (n=5, P > 0.05).

4.2.5.3 The OC is sensitive to block of linopirdine and XE991

Since voltage-dependent KCNQ/M currents activate slowly at potentials more positive than -60 mV and display little and no inactivation (Robbins, 2001), the slowly activating OC could be mediated by a subtype of the KCNQ/M channels. Therefore, we tested effect of linopirdine, a selective blocker of KCNQ/M channels (Wang *et al.*, 1998). The IC_{50} of linopirdine on KCNQ/M channels is dependent on the subtype of the channel (Robbins, 2001). The trace in Figure 4.13A shows that outward currents were decreased by 150 μM linopirdine in 325 mosmol kg^{-1} solution. Figure 4.13B shows that the mean density of outward currents was decreased from 23.5 ± 3.6 pA/pF to 8.8 ± 2.1 pA/pF (n=8, $P < 0.001$) following addition of 150 μM linopirdine in 325 mosmol kg^{-1} solution. Figure 4.13C shows that the density of linopirdine-sensitive current was increased from 6.0 ± 1.8 pA/pF to 14.0 ± 2.0 pA/pF (n=9, $P < 0.01$) after the cell was switched to 325 mosmol kg^{-1} solution, suggesting that the OC is sensitive to 150 μM linopirdine. We also tested the effect of 10 μM linopirdine in MNCs, and linopirdine at 10 μM did not display a significant inhibitory effect (Figure 4.13D). The density of the OC in 325 mosmol kg^{-1} solution was 25.8 ± 6.2 pA/pF and 24.0 ± 6.1 pA/pF (n=4, $P > 0.05$) before and after the addition of 10 μM linopirdine (Figure 4.13E), suggesting that the OC was significantly blocked only by high doses of linopirdine. We did not test the effect of linopirdine at concentrations higher than 150 μM because of the limited solubility of linopirdine.

Since the OC was also sensitive to Ba^{2+}, we tested whether the current sensitive to Ba^{2+} was the same as that blocked by linopirdine. After the outward currents were blocked by 150 μM linopirdine in 325 mosmol kg^{-1} solution, 0.3 mM Ba^{2+} was subsequently added to the solution (Figure 4.14A). The density of the outward currents in 4 cells was 25.8 ± 6.2 pA/pF, 11.7 ± 3.3 pA/pF, and 10.2 ±2.5 pA/pF before and after addition of 150 μM linopirdine and after addition

of 0.3 mM Ba^{2+} in 325 mosmol kg^{-1} solution (Figure 4.14B), respectively. This result indicated that subsequent addition of 0.3 mM Ba^{2+} did not further significantly block the currents, suggesting that the current blocked by Ba^{2+} is the same as that sensitive to linopirdine.

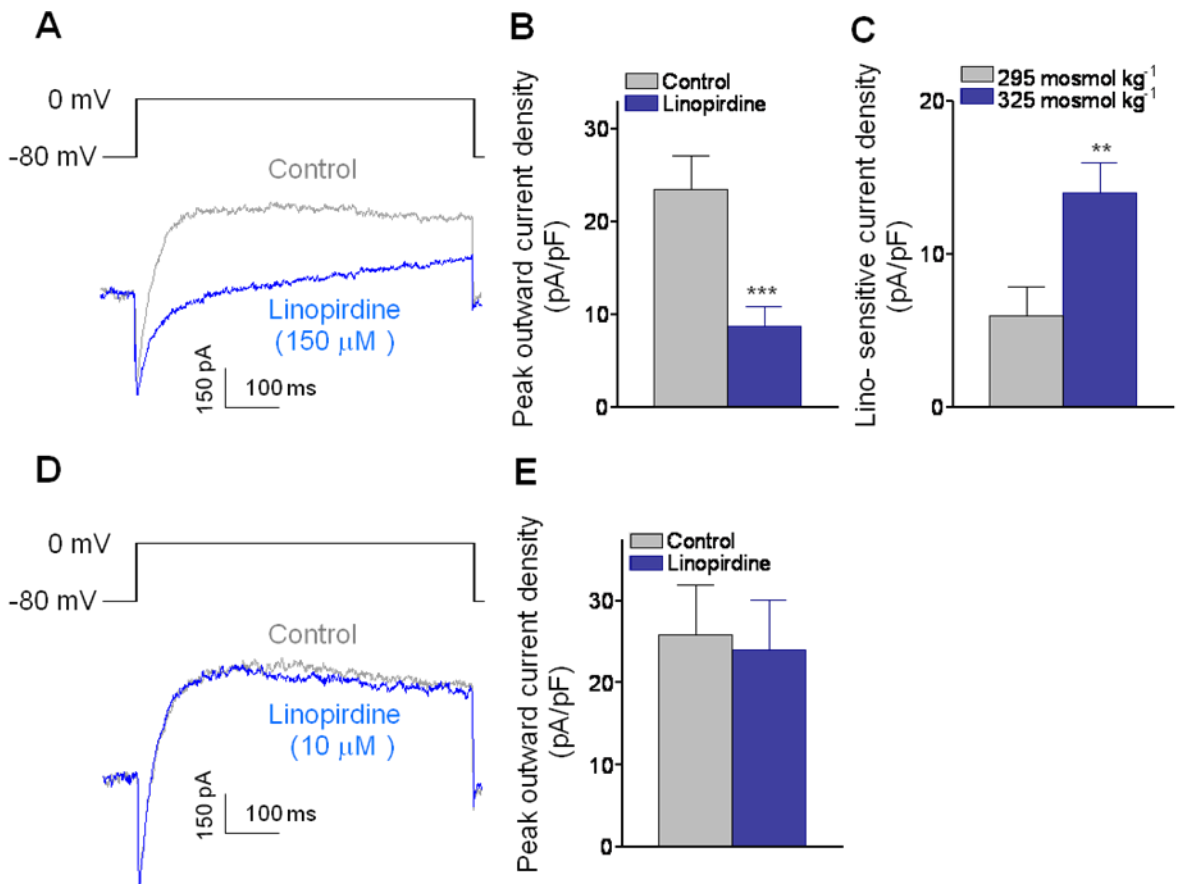


Figure 4.13 The OC is sensitive to linopirdine at a high dose but not at a low dose. Currents were evoked by steps from -80 mV to 0 mV for 400 ms. **A,** Currents evoked in 325 mosmol kg^{-1} solution before and after the addition of 150 µM linopirdine. **B,** A bar graph showing the density of the peak outward currents in 325 mosmol kg^{-1} solution before and after addition of 150 µM linopirdine (n=8, $^{***}P < 0.001$). **C,** A bar graph of the density of the currents sensitive to linopirdine in 295 and 325 mosmol kg^{-1} solutions (n=9, $^{**}P < 0.01$). **D,** Currents evoked in 325 mosmol kg^{-1} solution before and after the addition of 10 µM linopirdine. **E,** A bar graph showing the density of the peak outward currents in 325 mosmol kg^{-1} solution before and after addition of 10 µM linopirdine (n=4, $P > 0.05$).

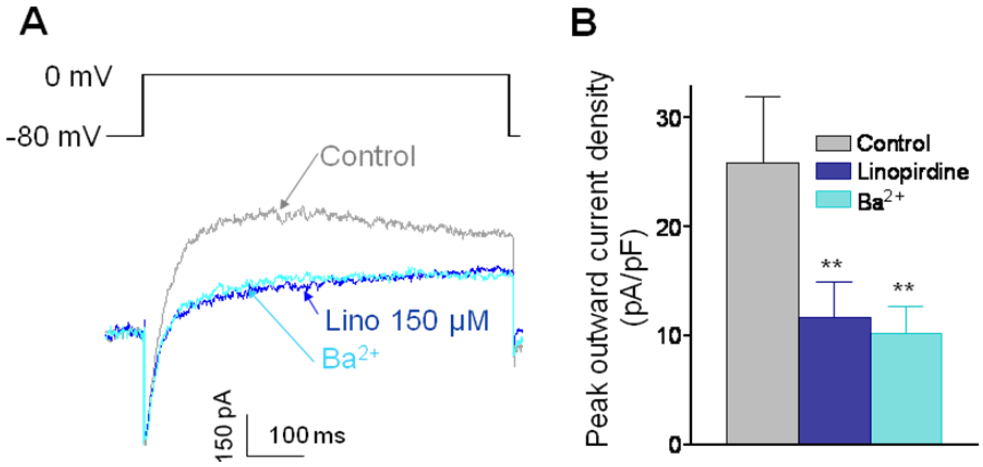


Figure 4.14 The currents sensitive to Ba^{2+} and linopirdine are the same. Currents were evoked by steps from -80 mV to 0 mV for 400 ms. **A,** Currents evoked in 325 mosmol kg^{-1} solution before and after addition of 150 µM linopirdine and 0.3 mM Ba^{2+}. **B,** A bar graph of the density of peak outward currents in 325 mosmol kg^{-1} solution before and after addition of 150 µM linopirdine and 0.3 mM Ba^{2+} (n=4, $^{**}P < 0.01$).

Linopirdine is a selective blocker of KCNQ/M channels (Wang *et al.*, 1998), but at 150 μM (we tested its effect on the OC at this concentration) may block other types of K^+ channels. Therefore, we tested the effect of XE991, a more powerful blocker of KCNQ/M channels (Wang *et al.*, 1998). The trace in Figure 4.15A shows that the outward currents were blocked by 5 μM XE991 in 325 mosmol kg^{-1} solution. Figure 4.15B shows that the density of the outward current was decreased from 25.8 ± 3.6 pA/pF to 10.6 ± 3.3 pA/pF (n=8, $P < 0.01$). Figure 4.15C shows that the density of XE991-sensitive current was increased from 8.7 ± 1.5 pA/pF to 16.0 ± 4.2 pA/pF (n=7, $P < 0.05$) after the cell was switched to 325 mosmol kg^{-1} solution, suggesting that the OC is sensitive to 5 μM XE991. We further tested the effect of XE991 by applying a series of concentrations (0.3, 1, 3, 10, 20, and 30 μM) in a 295 mosmol kg^{-1} solution to obtain the dose-dependence of the inhibitory effect of XE991 on the outward currents (Figure 4.16A). The data are shown in Figure 4.16B (n=7). The IC_{50} of XE991 was 3.9 μM, suggesting that the OC is very sensitive to XE991 and could be a KCNQ/M current.

Figure 4.17A shows that adding 0.3 mM Ba^{2+} after the outward currents were blocked by 5 μM XE991 in 325 mosmol kg^{-1} solution had little or no effect. The density of the outward currents in 6 cells was 24.4 ± 3.6 pA/pF, 7.0 ± 2.7 pA/pF, and 5.7 ± 2.3 pA/pF before and after addition of 5 μM XE991 and after addition of 0.3 mM Ba^{2+} in 325 mosmol kg^{-1} solution (Figure 4.17B), respectively. This suggests that the current blocked by Ba^{2+} is the same as that sensitive to XE991.

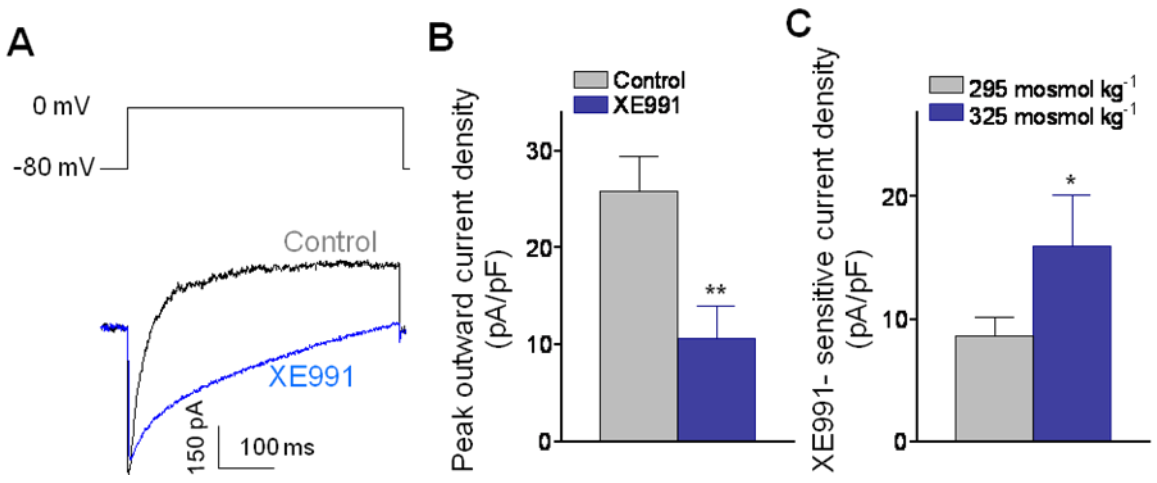


Figure 4.15 The OC is sensitive to XE991. Currents were evoked by steps from -80 mV to 0 mV for 400 ms. **A,** Currents evoked before and after the addition of 5 µM XE991. **B,** A bar graph showing the density of the peak outward currents in 325 mosmol kg^{-1} solution before and after addition of 5 µM XE991 (n=8, $^{**}P < 0.01$). **C,** A bar graph of the density of the currents sensitive to XE991 in 295 and 325 mosmol kg^{-1} solutions (n=7, $^{*}P < 0.05$).

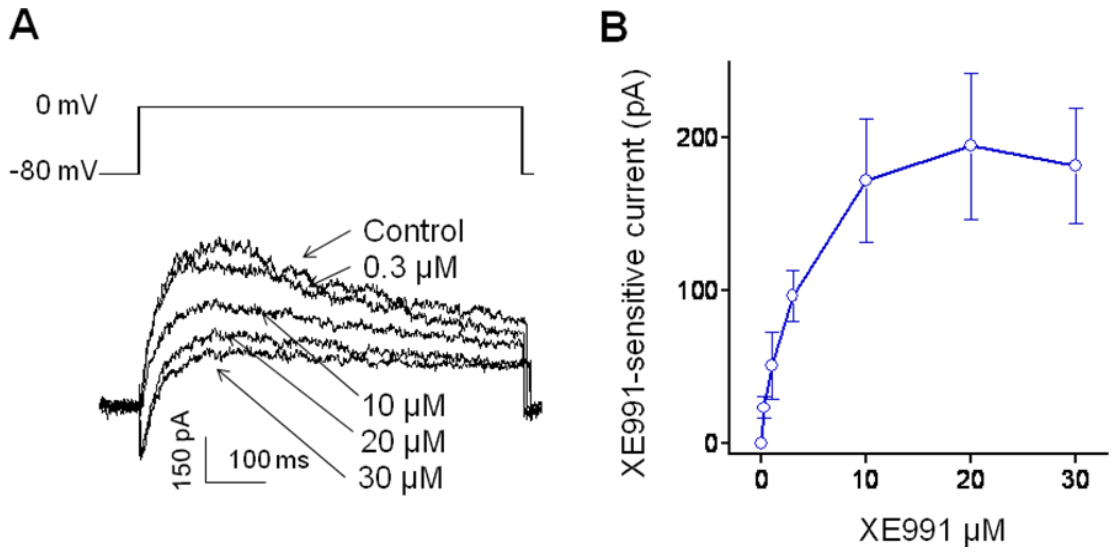


Figure 4.16 XE991 blocks the outward current with a low IC_{50}. A, left, Currents evoked by steps from -80 mV to 0 mV in 295 mosmol kg^{-1} solution before and after the addition of 0.3, 1, 3, 10, 20, and 30 µM XE991. **A, right,** A bar graph of the density of peak outward currents in 295 mosmol kg^{-1} solution before and after addition of 0.3, 1, 3, 10, 20, and 30 µM XE991 (*n*=7).

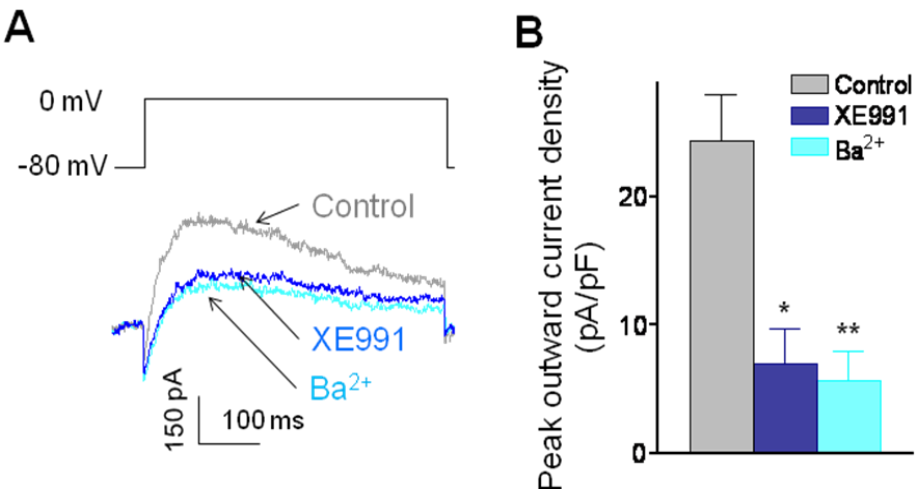


Figure 4.17 The current sensitive to Ba^{2+} is also sensitive to XE991. Currents were evoked by steps from -80 mV to 0 mV. **A,** Currents evoked in 325 mosmol kg^{-1} solution before and after addition of 5 µM XE991 and 0.3 mM Ba^{2+}. **B,** A bar graph of the density of peak outward currents in 325 mosmol kg^{-1} solution before and after addition of 5 µM XE991 and 0.3 mM Ba^{2+} (n=6, $^{*}P < 0.05$, $^{**}P < 0.01$).

4.2.5.4 Both flupirtine and retigabine increase the OC

Since blockers of the KCNQ/M channels can decrease the OC in MNCs, we tested whether openers of KCNQ/M channels can increase the current. We first tested the effect of retigabine. The cells were depolarized from a holding potential of -100 mV to -40 mV for 400 ms and returned to -100 mV in 295 mosmol kg^{-1} solution. When the current became stable, 10 μM retigabine was added to the external solution. The current was measured at the end of a 400 ms depolarizing step. Figure 4.18A shows that the outward currents evoked in 295 mosmol kg^{-1} solution were increased by 10 μM retigabine. The density of the outward current was significantly increased from 6.5 ± 0.7 pA/pF to 11.1 ± 1.6 pA/pF (n=6, $P < 0.05$) by adding retigabine in 295 mosmol kg^{-1} solution (Figure 4.18B).

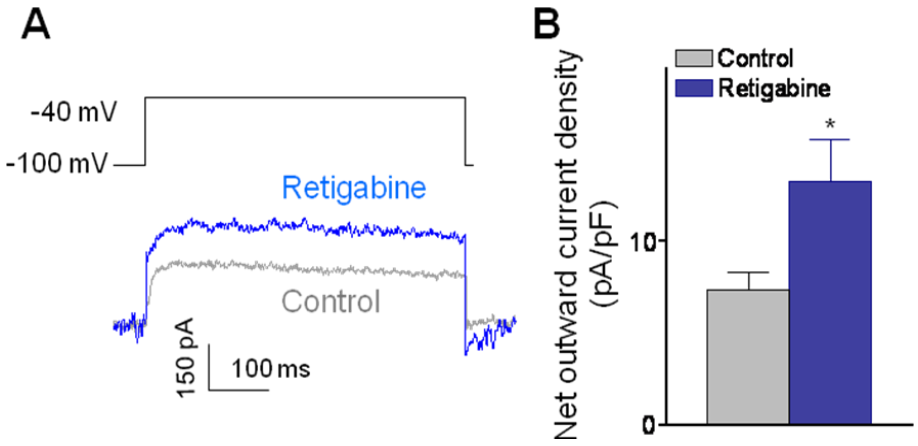


Figure 4.18 Retigabine increases the outward current. A, Currents evoked by steps from -100 mV to -40 mV in 295 mosmol kg^{-1} solution before and after the addition of 10 μM retigabine. **B,** A bar graph of the density of the outward currents measured at the end of 400 ms depolarizing step in 295 mosmol kg^{-1} solution before and after addition of retigabine (n=6, $^{*}P < 0.05$).

The effect of flupirtine, which is an analog of retigabine and can also increase KCNQ/M currents (Wu & Dworetzky, 2005), was also tested. The addition of 10 µM flupirtine caused an increase in outward currents evoked by a depolarizing step from the -80 mV to 0 mV for 400 ms in 295 mosmol kg^{-1} solution (Figure 4.19A). The current was measured at the end of the 400 ms depolarizing step. Figure 4.19B shows that the density of the outward currents was increased from 7.4 ± 2.3 pA/pF to 17.9 ± 3.3 pA/pF (n=7, $P < 0.01$) by 10 µM flupirtine. The holding currents required to hold the cells at -80 mV were increased after the addition of retigabine (75.6 ± 3.9 pA *versus* 189 ± 31.0 pA, n=6; $P < 0.05$) or flupirtine. This increase can be significantly blocked by 10 µM XE991, suggesting that the openers may activate the OC at -80 mV. Figure 4.19C shows that the holding current, before and after the addition 10 µM flupirtine and after the addition of 10 µM XE991, were -91.0 ± 9.5pA, -352.5 ± 45.7 pA, and -158.5 ± 17.7pA (n=4). Thus, the openers of the KCNQ/M channels may shift the threshold voltage of the OC to more negative potentials. Therefore, we tested the effect of retigabine on the current-voltage relationship of the OC.

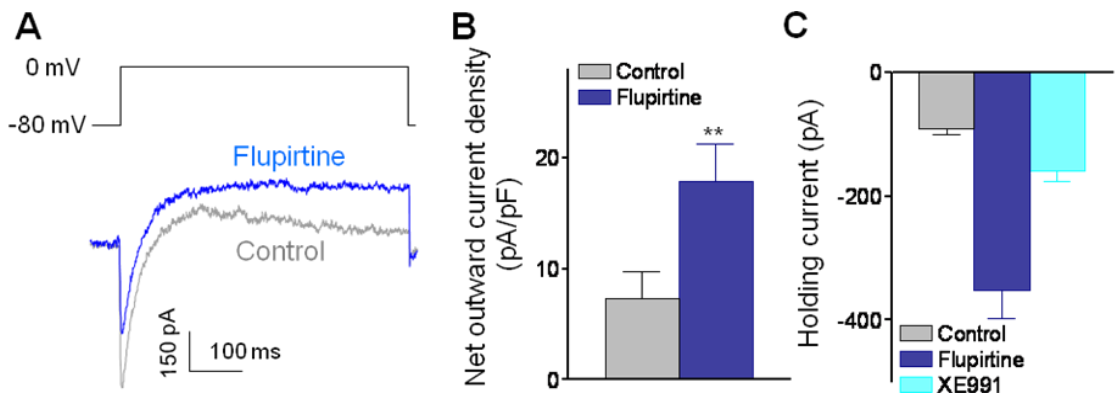


Figure 4.19 Flupirtine increases the OC. A, Currents evoked by steps from -80 mV to 0 mV before and after addition of 10 µM flupirtine in 295 mosmol kg^{-1} solution**. B,** A bar graph of the density of outward current measured at the end of 400 ms depolarizing step before and after addition of flupirtine in 295 mosmol kg^{-1} solution (n=7, $^{**}P < 0.01$). **C,** A bar graph showing the holding currents before and after the addition of flupirtine and XE991 (n=4).

The cell was depolarized from a holding potential of -100 mV to a series of potentials from -90 mV to -20 mV (in 10 mV increments every 10 s) for 400 ms and returned to -100 mV in 295 mosmol kg^{-1} solution. After the currents became stable, 10-20 μM retigabine was applied to increase the currents and then 10 μM XE991 was added to block the currents. Thus, XE991-sensitive current in the absence of retigabine was obtained by subtracting currents evoked in the presence of XE991 from those before addition of retigabine. In addition, XE991-sensitive current in the presence of 10-20 μM retigabine was obtained by subtracting currents evoked with XE991 from those after addition of retigabine. Figure 4.20A, shows XE991-sensitive currents evoked by depolarizing steps to a series of potentials between -90 mV and -20 mV in 295 mosmol kg^{-1} solution in the presence of 10 μM retigabine. Data of the effect of retigabine on the current-voltage relationship of the OC from 4 cells were plotted (Figure 4.20B), and retigabine shifted the potential for the channel activation from about -40 mV to -80 mV. The results demonstrate that retigabine shifts activation curve of the OC to more hyperpolarized potentials. In the presence of retigabine, the XE991-sensitive currents activated at potentials equal to or more positive than -80 mV, which is consistent with the results described above showing that the holding current at -80 mV was increased by retigabine.

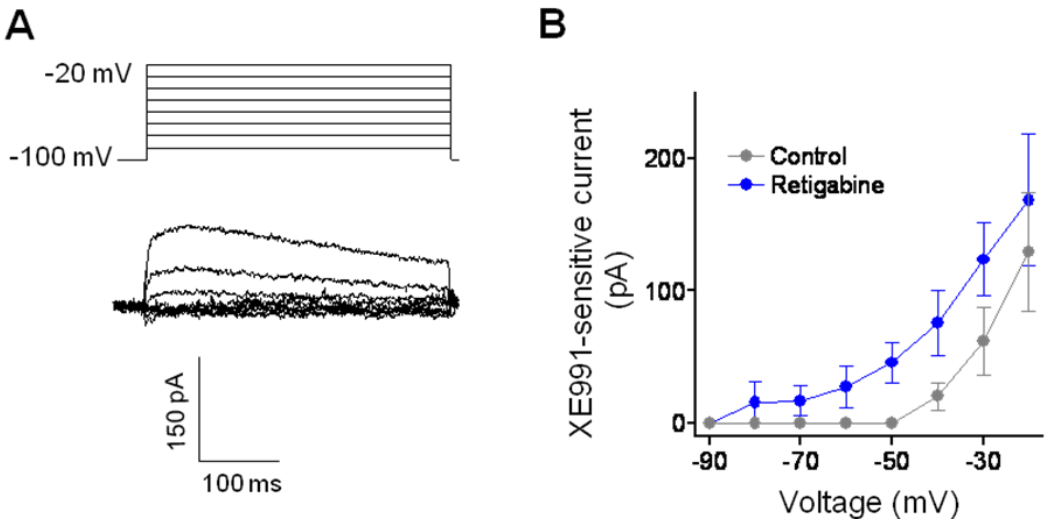


Figure 4.20 Retigabine shifts the activation curve of the OC in 295 mosmol kg^{-1} solution. Cells were stepped from a holding potential of -100 mV to a series of potentials between -90 mV and -20 mV for 400 ms (in 10 mV increments every 10s) in 295 mosmol kg^{-1} solution. **A.** Traces showing XE991-sensitive currents in the presence of 10 µM retigabine. **B,** A plot of XE991-sensitive currents in the absence and presence of retigabine in 295 mosmol kg^{-1} solution (n=4). Retigabine shifted the potential for the channel activation from about -40 mV to -80 mV.

4.2.5.5 Muscarine inhibits the OC

Since the OC is affected by selective modulators of the KCNQ/M channels, the OC could be mediated by a subtype of KCNQ/M channels. If so, muscarine should be able to inhibit the OC. Therefore, we tested the effect of muscarine on the OC. Currents were evoked by a depolarizing step from the -80 mV to 0 mV for 400 ms in 295 mosmol kg^{-1} solution before and after the addition of 30 μM muscarine. Muscarine inhibited the outward currents in 6 out of 9 MNCs. An example of this is shown in Figure 4.21A. The density of the outward currents in 6 cells responding to muscarine was significantly decreased from 13.4 ± 2.8 pA/pF to 10.7 ± 3.1 pA/pF (n=6, P < 0.01; Figure 4.21B). Thus, the outward currents were also sensitive to muscarine. Next, we tested whether the current sensitive to muscarine was the same as that blocked by XE991 and linopirdine.

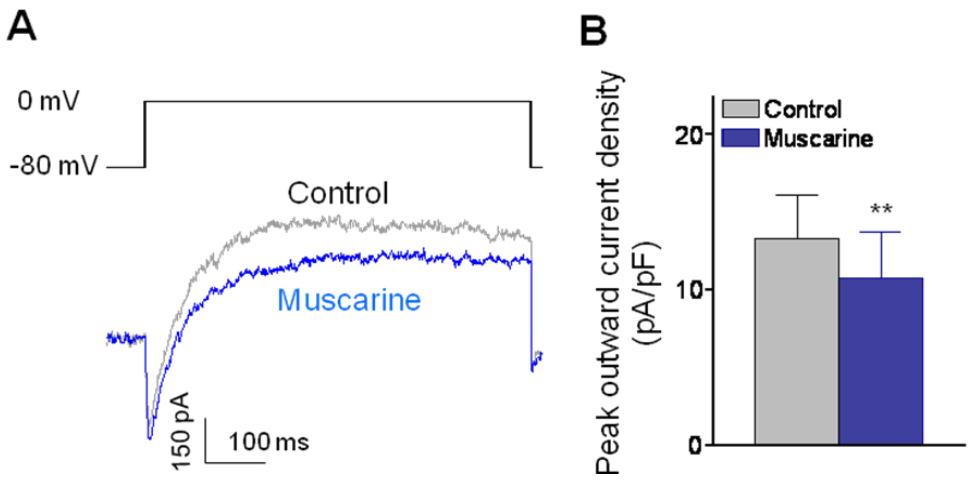


Figure 4.21 Muscarine inhibits the outward current. Currents evoked by steps from -80 mV to 0 mV in 295 mosmol kg^{-1} solution**. A,** Currents evoked before and after the addition of 30 µM muscarine. **B,** A bar graph of the density of peak outward currents in 295 mosmol kg^{-1} solution before and after addition of muscarine (n=6, $^{**}P < 0.01$).

After outward currents were blocked by 5 μM XE991 in 295 mosmol kg^{-1} solution, 30 μM muscarine was subsequently added to the solution (Figure 4.22A, left). The density of the outward currents in 295 mosmol kg^{-1} solution in 5 cells was 15.2 ± 1.3 pA/pF, 5.2 ± 1.5 pA/pF, and 5.5 ± 1.6 pA/pF before and after the addition of 5 μM XE991 and after the addition of 30 μM muscarine (Figure 4.22A, right), respectively. Subsequent addition of muscarine did not further significantly inhibit the currents, suggesting that the current inhibited by muscarine is also sensitive to XE991. A similar study was performed with linopirdine, and Figure 4.22B, left, shows that 30 μM muscarine, after the outward currents were blocked by 150 μM linopirdine in a MNC in 295 mosmol kg^{-1} solution, did not effect the outward current. The density of the outward currents in 295 mosmol kg^{-1} solution in 6 cells was 12.7 ± 2.3 pA/pF, 5.2 ± 1.4 pA/pF, and 4.0 ± 1.0 pA/pF before and after the addition of 150 μM linopirdine and after the addition of 30 μM muscarine (Figure 4.22B, right), respectively. This result indicated that subsequent addition of muscarine did not further significantly inhibit the currents, either, suggesting that the current inhibited by muscarine is sensitive to linopirdine. These data suggest the OC is sensitive to muscarine.

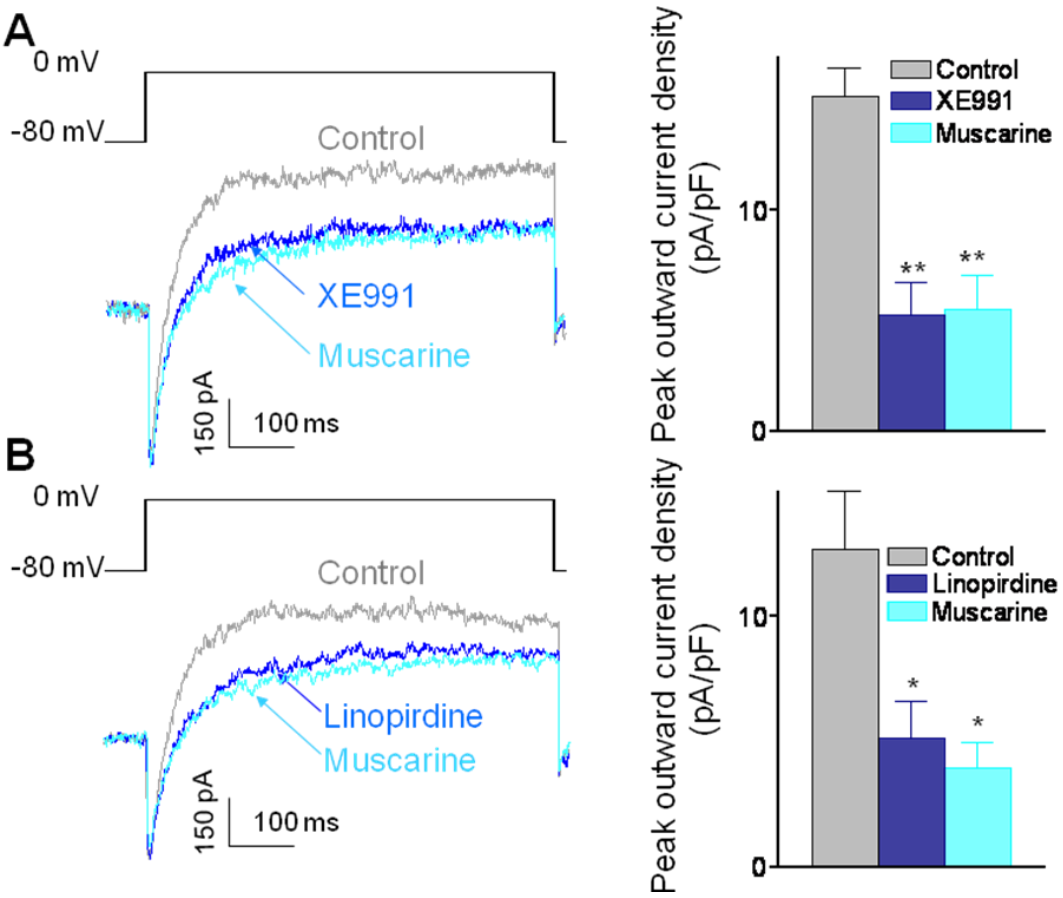


Figure 4.22 The current sensitive to muscarine is also sensitive to XE991 and linopirdine. Currents evoked by steps from -80 mV to 0 mV in 295 mosmol kg^{-1} solution**.** After the outward current was blocked by XE991 or linopirdine, and muscarine was subsequent added to block the current again. **A, left,** Currents evoked before and after addition of 5 µM XE991 and 30 µM muscarine. **A, right,** A bar graph of the density of peak outward currents before and after addition of XE991 and muscarine in 295 mosmol kg^{-1} solution (n=5, $^{**}P < 0.01$). **B, left,** Currents evoked before and after addition of 150 µM linopirdine and 30 µM muscarine. **B, right,** A bar graph of the density of peak outward currents in 295 mosmol kg^{-1} solution before and after addition of linopirdine and muscarine (n=6, $^{*}P < 0.05$).

4.2.5.6 The OC is insensitive to E4031

Therefore, many properties of the OC suggest that it is conducted by a subtype of KCNQ/M channels. KCNQ/M-currents were originally identified in sympathetic neurons (Brown & Adams, 1980), and can be blocked by selective blockers of the KCNQ/M channels, linopirdine and XE991 (Wang *et al.*, 1998), but with different IC_{50}s (Robbins, 2001). In addition to currents mediated by KCNQ/M channels, there are other K^+ currents that have similar biophysical and pharmacological properties and are therefore called KCNQ/M-like currents (Selyanko *et al.*, 1999). One such type of voltage-gated K^+ channels, the *ether-a-go-go*-related gene (ERG) K^+ channel, mediates KCNQ/M-like currents in neurons (Selyanko *et al.*, 1999). The ERG K^+ channel consists of three subtypes: ERG1, ERG2, and ERG3 (Schwarz & Bauer, 2004). The ERG K^+ channel is expressed in heart, brain, and other tissues and plays important physiological roles in these tissues (Schwarz & Bauer, 2004). The ERG K^+ channel-mediated current shows many similarities to those mediated by KCNQ/M channels (Selyanko *et al.*, 1999; Hirdes *et al.*, 2004). Furthermore, the ERG K^+ channel-mediated current is also sensitive to the changes in cell volume (Rees *et al.*, 1995; Lees-Miller *et al.*, 1997). Therefore, we tested the effect of E4031, a selective blocker of ERG K^+ channels with an IC_{50} less than 200 nM (Shi *et al.*, 1997), on the OC.

After the outward current was evoked by a depolarizing step from holding potential of -80 mV to 0 mV for 400 ms in 325 mosmol kg^{-1} solution, 0.5 μM E4031 was added to the solution (Figure 4.23A). The density of the outward currents before and after the addition of 0.5 μM E4031 was 21.4 ± 3.4 pA/pF and 19.4 ± 2.4 pA/pF (Figure 4.23B), respectively, and there was no significant difference (n=7, P > 0.05), suggesting that the OC is not mediated by a subtype of the ERG K^+ channels.

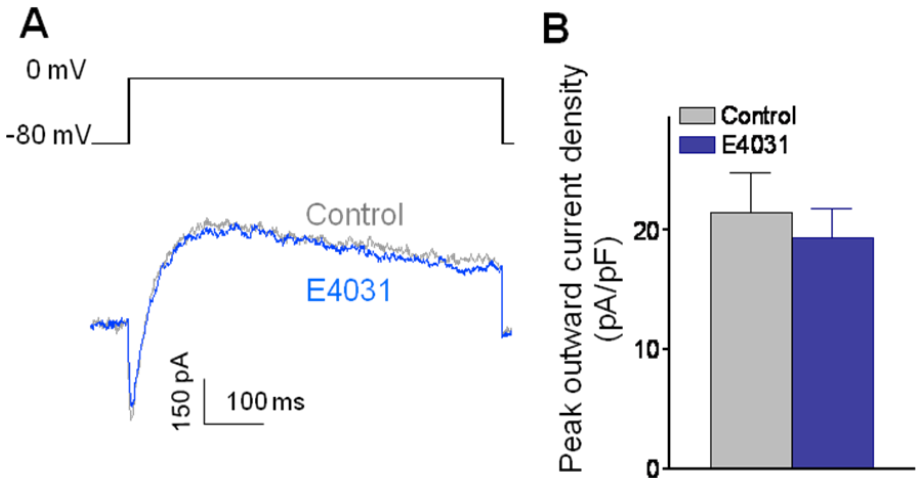


Figure 4.23 The OC is insensitive to E4031. A, Currents evoked in 325 mosmol kg^{-1} solution before and after addition of 0.5 µM E4031. **B,** A bar graph of the density of peak outward currents in 325 mosmol kg^{-1} solution before and after addition of 0.5 µM E4031 (n=7, $P > 0.05$).

4.2.6 Expression of KCNQ/M channel subunits in MNCs

Since the biophysical and pharmacological properties of the OC suggest that it could be mediated by a subtype of KCNQ/M current in acutely isolated MNCs, we tested whether the MNCs express subunits of the KCNQ/M channels. RT-PCR (reverse transcription-polymerase chain reaction) studies using whole SON tissue, which were performed by Dr. WRA Kosala JS Rajapaksha, a former post-doctoral fellow in Dr. Fisher's laboratory, indicated that the tissue expresses KCNQ2, KCNQ3, KCNQ4, and KCNQ5, but not KCNQ1 (Zhang *et al.*, 2008). Since the SON also consists of other types of cells that could also express subunits of the KCNQ/M channels, a study of single-cell RT-PCR was performed by Dr. Rajapaksha in 25 MNCs (Zhang *et al.*, 2008), which indicated that 6, 18, 3, and 7 cells expressed KCNQ2, KCNQ3, KCNQ4, and KCNQ5 channel subunits, respectively.

Immunocytochemical studies using antibodies directed against KCNQ/M channels specifically labeled MNCs. Rabbit antibodies recognizing the channel proteins of KCNQ2, KCNQ3, KCNQ4, and KCNQ5 from Santa Cruz Biotechnology were purified. One dish without the primary antibody was used as a negative control during each experiment. These studies indicated that MNCs express KCNQ2, KCNQ3, KCNQ4, and KCNQ5 channel subunits (Figure 4.24). In Figure 4.24, KCNQ4 primary antibody was from Dr. B. Kachar of NIDCD/NIH, USA. Double staining studies suggested that these channel subunits were expressed in both VP- and OT-MNCs (Figure 4.24), which is consistent with a previous study showing that the OC isolated in I_{Ca} medium as a tail current was expressed in both types of MNCs (Liu, 2004). These data suggest that subunits of KCNQ/M channels are expressed in MNCs and that the MNCs are able to express the KCNQ/M currents.

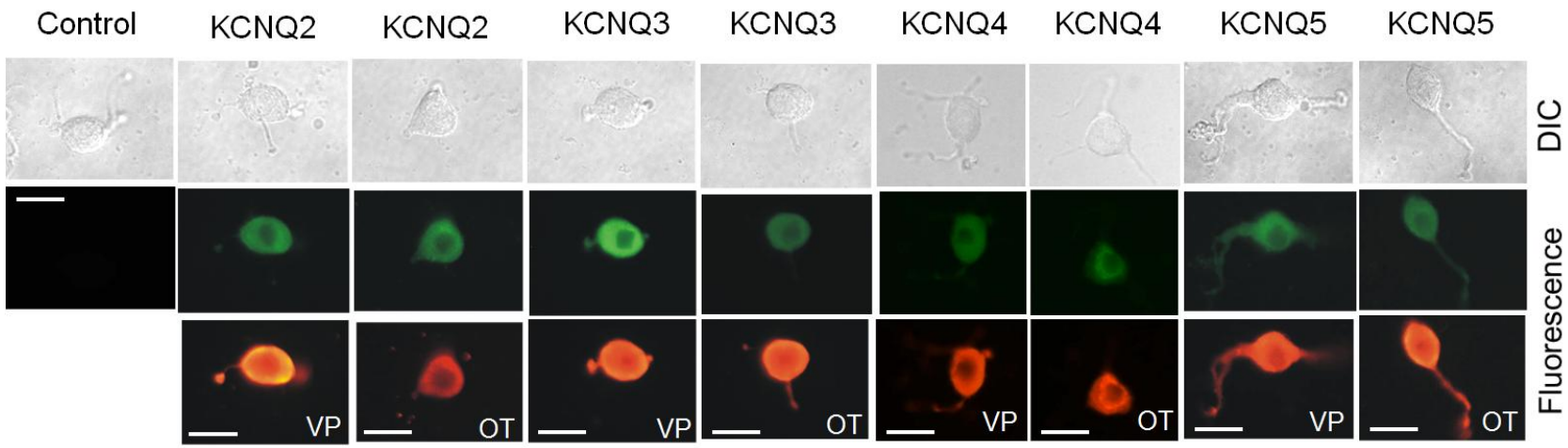


Figure 4.24 Immunocytochemical studies suggest that MNCs express KCNQ2, KCNQ3, KCNQ4, and KCNQ5. A, Images show the DIC (upper) and immunofluorescence images (middle and bottom) of both VP- and OT-MNCs that showed immunoreactivity to anti- KCNQ2, KCNQ3, KCNQ4, and KCNQ5. The negative control without primary antibodies recognizing the KCNQ channel proteins was also displayed. The scale bar is 20 µM.

4.2.7 The role of the OC in regulating MNC firing patterns

The physiological role of the OC in regulating MNC firing is not known. Our studies suggest that the OC could be a KCNQ/M current, and the physiological roles of KCNQ/M currents have not been investigated in the MNCs. However, studies from other labs have shown that the MNCs express a muscarine-sensitive sAHP, which is insensitive to apamin, and that muscarine increases the firing rate and the duration of spontaneous phasic bursts in rat SON (Ghamari-Langroudi & Bourque, 2004). The muscarinic modulation in MNC firing pattern could be due to involvement of KCNQ/M channels. Since the OC is sensitive to 5 µM XE991 and 10 µM retigabine (and these drugs are selective for KCNQ/M channels at such concentrations; Wang *et al.*, 1998; Xiong *et al.*, 2008), we tested the effects of these modulators on MNC firing patterns to investigate whether the KCNQ/M currents regulate MNC firing.

Single unit extracellular recording was performed in hypothalamic explants as previously described in Bourque and Renaud (Bourque & Renaud, 1983). Such recording makes it possible to study a more intact preparation in which MNCs fire in patterns similar to those showed in *vivo*. XE991 and retigabine were delivered to the cells through a perfusion system. Figure 4.25A shows that 10 µM XE991 increased the firing rate in MNCs showing fast continuous firing (n=2). XE991 (10 µM) also initiated firing in silent MNCs (Figure 4.25B, n=4) and increased the duration of spontaneous phasic bursts (Figure 4.25C, n=1). Furthermore, Figure 4.25D shows that 20 µM retigabine decreased the firing rate or caused a cessation of firing in 4 out of 6 MNCs showing fast continuous firing. These results suggest that a XE991- and retigabine-sensitive current plays a role in the regulation of MNC firing. This also supports the hypothesis that modulation of the OC by changes in osmolality might affect the MNC firing pattern.

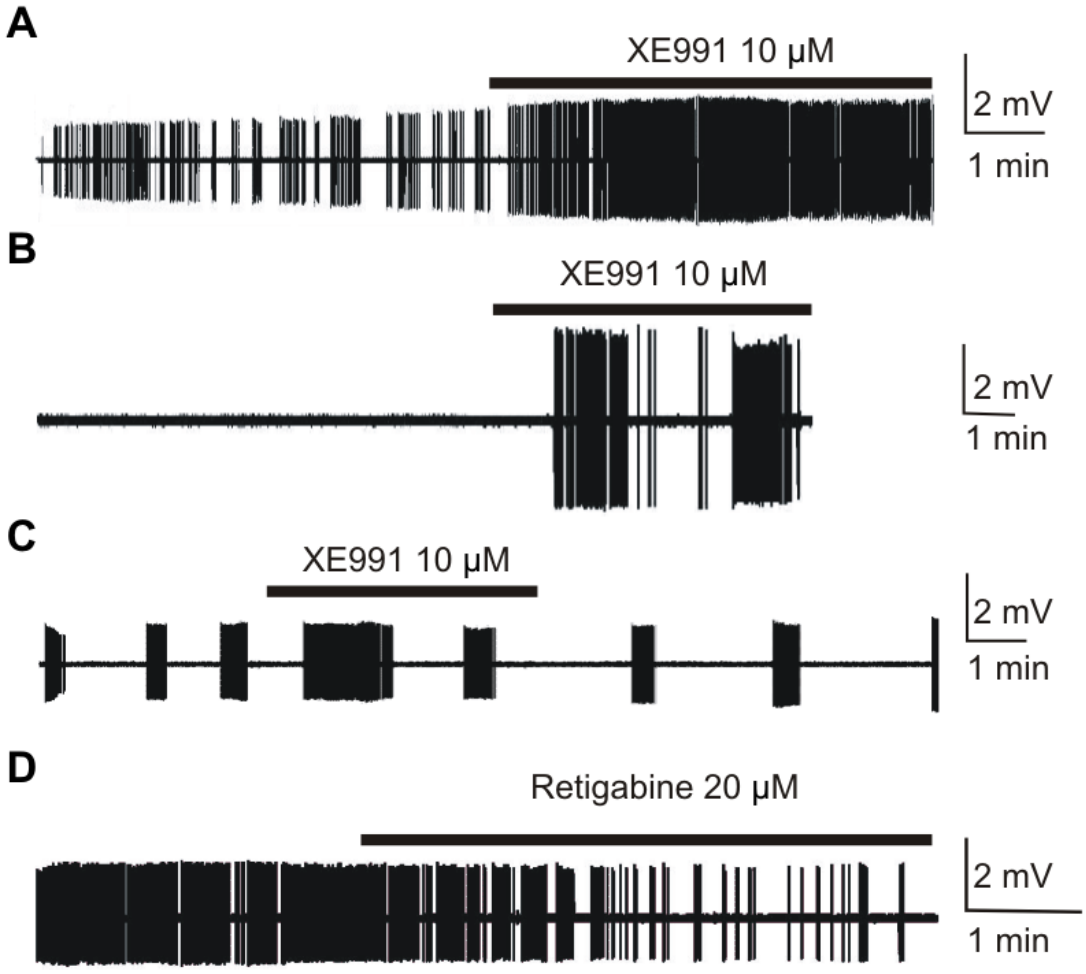


Figure 4.25 XE991 and retigabine modulate MNC firing patterns. A, XE991 increased firing rate in MNCs with fast continuous firing (*n*=2). **B,** Effect of XE991 in silent MNCs (*n*=4). **C,** Effect of XE991 in a MNC with phasic firing (*n*=1). **D,** Retigabine decreased firing rate in MNCs (*n*=4).

4.2.8 Summary and discussion: the OC is increased by acute hypertonic stimulation and could be a KCNQ/M-like current

The MNCs can regulate plasma osmolality by releasing VP to control water excretion from the kidney. Since the release of VP is dependent on the rate and pattern of MNC firing (Bicknell, 1988; Bourque, 2008), ion channels, especially osmosensitive ion channels, contribute to the changes in their electrical activity and thus regulate the release of hormone. The mechanisms underlying the transition of the electrical behaviour are not completely understood. The SIC contributes to increases in MNC firing during elevations in osmolality (Bourque *et al.*, 2002). We here report that the MNCs express an osmosensitive inhibitory K^+ current (the OC). The properties of the OC, although it also senses changes in osmolality of the ECF, are different from those of the SIC (Oliet & Bourque, 1993) and those of a stretch-sensitive background K^+ current (Han *et al.*, 2003). Neither of these channels are voltage-dependent, while the OC is. The SIC is mediated by a non-selective cation (K^+, Na^+, and Ca^{2+}) channel (Zhang & Bourque, 2006), while the OC is conducted by a K^+-selective channel. The SIC is mediated by TRPV1 (Sharif-Naeini *et al.*, 2006), whereas we propose that the OC is a KCNQ/M-like current. Our evidence for this assertion is summarized below.

Firstly, the OC is voltage-dependent and activates at the voltages more positive than -60 mV, and these biophysical properties are similar to those of the KCNQ/M channels. Although the inactivation of the OC is greater than that reported for KCNQ/M channels (Jensen *et al.*, 2007), the OC under our conditions was isolated in internal recording solutions containing 15 mM TEA. TEA from the internal side of the plasma membrane displays open-channel blocking effects and changes kinetics of K^+ channels, including KCNQ/M channels (Suh & Hille, 2007a), and makes the channels show an inactivating effect (Armstrong, 1966). Thus, the presence of 15

mM TEA in the internal recording solution in our study may change the kinetics of the OC and contribute to an increase in its inactivation.

Secondly, the OC is regulated by selective modulators of the KCNQ/M channels such as linopirdine (Wang *et al.*, 1998), XE991 (Wang *et al.*, 1998), and retigabine (Xiong *et al.*, 2008), but not by E4031, a specific blocker of the ERG K^+ channels (Shi *et al.*, 1997). Although our data show that the OC is significantly blocked only by 150 μM linopirdine but not by 10 μM linopirdine and linopirdine at 150 μM can blocks other K^+ channels, the KCNQ/M channels are blocked by linopirdine with different doses (Robbins, 2001). KCNQ1, KCNQ2, and KCNQ3 are much more sensitive to linopirdine, with IC_{50}s of less than 10 μM, but KCNQ4, KCNQ3/4, and KCNQ5 are much less sensitive to linopirdine (especially KCNQ4 and KCNQ3/4 with IC_{50} of more than 200 μM and less than 200 μM, respectively; Kubisch *et al.*, 1999). The OC is blocked by XE991 with an IC_{50} of less than 5 μM, and XE991 at this concentration selectively blocks KCMQ/M channels (Wang *et al.*, 1998). Furthermore, retigabine, which is a selective opener of the KCNQ/M channels, increases the OC amplitude and shifts its voltage sensitivity to more negative potentials, and this is consistent with its effects on KCNQ/M channels (Tatulian *et al.*, 2001).

Thirdly, the OC is significantly inhibited by muscarine, and this is also consistent with the characteristics of KCNQ/M channels (Schroeder *et al.*, 2000; Selyanko *et al.*, 2000). Muscarine can inhibit about 80 % of the KCNQ/M currents by acting on M1 receptors coupled to G-proteins to deplete PIP_2 (Haley *et al.*, 1998; Selyanko *et al.*, 2000; Suh & Hille, 2002); however, the inhibitory effect of muscarine on the OC is much smaller. This may be due to the fact that there is 20 mM TEA in our external recording solution, since TEA can specifically bind to the M1 receptors (Tiger *et al.*, 1989; Balduini *et al.*, 1990). The specific binding of TEA to the

M1 receptors will directly interfere with binding of muscarine to the receptors in the MNCs. Thus, the inhibitory effect of muscarine on the OC may be greatly underestimated in the MNCs due to the presence of TEA in the external solution.

Fourthly, RT-PCR and immunocytochemical studies suggest that the MNCs express the subunits of the KCNQ/M channels, KCNQ2, KCNQ3, KCNQ4, and KCNQ5, suggesting that the MNCs are able to express KCNQ/M channels.

Finally, modulators of KCNQ/M channels regulate firing rates and patterns in the MNCs. Since the OC is sensitive to linopirdine at a high concentration and linopirdine at 150 μM can block other K^+ currents, we did not test whether linopirdine can regulate firing rates and patterns in the MNCs. XE991 (10 μM) increased the firing rate, whereas 10 μM retigabine decreased the firing rate or caused a cessation of firing in rat supraoptic neurons. These data suggest that the modulators at such concentrations may modulate the MNC firing by regulating the KCNQ/M channels. We cannot conclude that the modulators regulate the MNC firing by regulating the OC because the modulators could modulate the firing by regulating other subtypes of the KCNQ/M channels in the MNCs. These data suggest that the MNCs could express one or more subtypes of the KCNQ/M currents and that the OC may regulate MNC firing rate and pattern.

In summary, an osmosensitive current, the OC, has been identified in acutely isolated MNCs. We propose that the OC is a KCNQ/M-like current because most properties of the OC are similar to those of KCNQ/M channels. However, this has not yet been confirmed by the study using mice with knockout of the KCNQ/M channel genes. In addition, some properties (such as sensitivity to TEA and linopirdine, and inactivating property) of the OC are inconsistent with those of the classic KCNQ/M current. Since XE991 and retigabine as selective modulators

of KCNQ/M channels and they can modulate the OC and MNC firing, the OC may play an important role in regulating the rate and pattern of MNC firing.

5. GENERAL DISCUSSION

5.1 Sustained dehydration causes an increase in L-type Ca^{2+} current

5.1.1 Possible mechanisms underlying the increase in L-type Ca^{2+} current

Our results from whole-cell patch-clamp recordings demonstrate that 16-24 h of water deprivation significantly increases the L-type Ca^{2+} currents by 78.6% in amplitude and by 42% in density. This increase occurs in both VP- and OT-releasing MNCs. This is consistent with radioligand binding studies of tissue from the SON, which show that the density of binding sites for the L-type Ca^{2+} channel ligand is increased by 32.2% following sustained dehydration (Star, 2005). These radioligand binding assays showed an increase in L-type Ca^{2+} channels that was smaller than that suggested by the whole-cell patch-clamp recordings, which is possibly due to the different experimental approaches used for the two studies. In the whole-cell patch-clamp recording, isolated MNCs were used and the L-type Ca^{2+} current was obtained from single MNCs. However, whole SON tissues, which contain other types of cells, were used for the radioligand binding study, and other types of cells may also express L-type Ca^{2+} channels, which in these cells could be insensitive to sustained dehydration. This would lead to an underestimation of the increase in channels in MNCs. The L-type Ca^{2+} channel is expressed not only in somata of the MNCs but also in their dendrites (Joux *et al.*, 2001), and the increase in the L-type Ca^{2+} channels during sustained dehydration could occur in both locations. Since most of the MNC dendrites were lost during the isolation procedure, the recording primarily reflected the L-type Ca^{2+} currents in the somata of the MNCs and could underestimate or overestimate the overall change depending on where the change in current primarily occurs.

Sustained dehydration does not cause significant changes in the levels of mRNA coding for Ca^{2+} channel α_1 subunits (Zhang *et al.*, 2007a). The mechanisms underlying the increase in L-type Ca^{2+} current are unknown in rat MNCs. However, there are three possibilities, including increases in the functional activity of pre-existing channels, increases in the number of channels on the plasma membrane due to an increase in synthesis of the L-type Ca^{2+} channel, or translocation of the channels from intracellular stores to the plasma membrane.

One possibility is an increase in the functional activity of the pre-existing channels, which could be caused by changes in ionic strength or in a second messenger system, or by mechanical force. The L-type Ca^{2+} channel can be modulated by second messengers such as Mg^{2+} and cAMP (Yamaoka & Kameyama, 2003), which are altered by osmolality (Okada, 1997). Sustained dehydration could change the functional activity of the pre-existing L-type Ca^{2+} channels in the plasma membrane by modulating a second messenger system. In addition, the L-type Ca^{2+} channel is also mechanosensitive (Gannier *et al.*, 1994; Matsuda *et al.*, 1996). L-type Ca^{2+} channels can be activated by membrane stretch caused by carbon fibres (Gannier *et al.*, 1994) or by applying a positive pressure into the cell via patch pipette (Matsuda *et al.*, 1996), which leads to a rapid increase in $[Ca^{2+}]_i$ in isolated ventricular myocardial cells. This phenomenon can be blocked by streptomycin, a blocker of mechanosensitive channels (Gannier *et al.*, 1994). In addition, the L-type Ca^{2+} current displays sensitivity to acute changes in osmolality of the extracellular recording solution. The L-type Ca^{2+} current in guinea-pig gastric smooth muscle was increased by 35% by an acute decrease in osmolality of the extracellular solution from 290 mosmol kg^{-1} to 202 mosmol kg^{-1} and decreased by 50% by an increase in osmolality of the extracellular solution from 290 mosmol kg^{-1} to 350 mosmol kg^{-1} due to an actin cytoskeleton-mediated change in membrane tension (Xu *et al.*, 1996; Xu *et al.*, 1997). This is

consistent with another study demonstrating that the L-type Ca^{2+} current in the human gastric smooth muscle cells was increased by 57% by a rapid decrease in osmolality of extracellular solution from 290 mosmol kg^{-1} to 222 mosmol kg^{-1} (Kim *et al.*, 2000). The L-type Ca^{2+} current in rat anterior pituitary cells was decreased by 50% by a 63% increase in the osmolality of extracellular solution, with no change in cell capacitance (Ben-Tabou De-Leon *et al.*, 2006). The decrease, which is independent of the actin cytoskeleton, is attributed to a decrease in channel activity (which is primarily dependent on single channel conductance, open possibility of the channel, and ionic-strength), but single channel conductance of the channel does not change (Ben-Tabou De-Leon *et al.*, 2006). The changes in channel activity could be due to alteration in membrane tension at the phospholipid bilayer and/or in ionic-strength underneath the plasma membrane (in which case changes in cytoplasmic salt levels cause activation of ion channels or transporters; Cannon *et al.*, 1998), as suggested by Ben-Tabou De-Leon *et al*. (Ben-Tabou De-Leon *et al.*, 2006). These data, which showed that L-type Ca^{2+} current is increased by cell swelling, are opposite to those in our study in which L-type Ca^{2+} current is increased following sustained dehydration. Although this difference could be due to different molecular bases of osmosensory transduction in different cell types (Lang *et al.*, 1998b) or to different mechanisms underlying the osmosensitivity under different experimental conditions, this does not explain why there is an increase in DHP binding sites following sustained dehydration in our studies (Star, 2005). In addition, an increase in channel activity due to mechanical force would be expected to occur quickly, whereas the Ca^{2+} current in the MNCs does not significantly change with acute change in extracellular osmolality (Liu *et al.*, 2005). Furthermore, the density of L-type Ca^{2+} current in acutely isolated MNCs maintained in 320 mosmol kg^{-1} physiological PIPES solution for about 90 min was not significant larger than that in the MNCs maintained in 290

mosmol kg^{-1} physiological PIPES solution. These data suggest that the increase in the L-type Ca^{2+} current in our studies was not caused by a change in the activity of pre-existing channels.

A second possibility is that the synthesis of the L-type Ca^{2+} channels in the MNCs increases. Stimulus-activated increases in the synthesis of the L-type Ca^{2+} channel in the plasma membrane have been reported in other types of cells. Isoprenaline-induced cardiac hypertrophy causes 26% increase in L-type Ca^{2+} current amplitude (although with no increase in current density) possibly because of an increase in protein synthesis of the channel (Meszaros *et al.*, 1997). Other studies have shown that the numbers of cardiac L-type Ca^{2+} channels are increased due to an increase in protein synthesis of the channel by a sustained decrease in estrogen levels (Johnson *et al.*, 1997), blockers of L-type Ca^{2+} channels (Schroder *et al.*, 2007), nerve growth factors (Tanaka & Koike, 1995), and β-adrenergic stimulators (Akuzawa-Tateyama *et al.*, 2006). Although we cannot rule this possibility out, we (Zhang *et al.*, 2007a) and other researchers (Hindmarch *et al.*, 2006) have seen no evidence for increases in mRNA levels for any type of Ca^{2+} channel following sustained dehydration.

A third possibility is the translocation of the L-type Ca^{2+} channel from intracellular stores to the plasma membrane. Translocation of Ca^{2+} channels from intracellular stores to the plasma membrane has been reported in *Aplysia* bag cell neurons (Strong *et al.*, 1987; White *et al.*, 1998). The bag cell neurons express two types of Ca^{2+} channel α1 subunits. One mediating a basal Ca^{2+} current with a conductance of 12 pS is expressed on the plasma membrane. The other, which mediates a Ca^{2+} current with a conductance of 24 pS, is only localized on vesicles of the neurons under normal condition. The bag cell neurons can therefore express a basal Ca^{2+} current conducted by a 12 pS channel under normal condition. After activation of protein kinase C, the bag cell neurons, however, express another type of Ca^{2+} current with a conductance of 24 pS.

This study suggests that the Ca^{2+} channel with a conductance of 24 pS is translocated from the vesicles to the plasma membrane. Other studies have reported the translocation of L-type Ca^{2+} channels in other types of cells. In HEK 293 cells, trafficking to or retention of the L-type Ca^{2+} channel on the plasma membrane during chronic hypoxia causes an increase in $Ca_V1.3$ L-type Ca^{2+} currents (Green & Peers, 2001; Scragg *et al.*, 2004). Chronic hypoxia increases levels of amyloid β peptides, which selectively cause insertion into the plasma membrane of $Ca_V1.3$ L-type Ca^{2+} channels by interacting with the channels. In pancreatic β-cells, an increase in intracellular Ca^{2+} level decreases expression of $Ca_V1.3$ L-type Ca^{2+} channel subunits in the plasma membrane and increases the cytoplasmic distribution of the channel subunits, with no changes in the channel expression in the total cellular membranes (Huang *et al.*, 2004), suggesting that the L-type Ca^{2+} channel is translocated from the intracellular stores to the plasma membrane. In addition, the α1 subunits of the L-type Ca^{2+} channel distribute not only in the plasma membrane of the MNCs but also in an intracellular subplasmalemmal pool (Fisher *et al.*, 2000; Joux *et al.*, 2001), possibly in the internal membrane of vesicles. This observation suggests the possibility that the L-type Ca^{2+} channel can translocate from the intracellular stores to the plasma membrane in the MNCs and that such translocation can cause an increase in the channel-mediated current.

These observations suggest that the L-type Ca^{2+} current could be functionally changed by multiple mechanisms, and there are some studies that could be able to determine the mechanisms underlying the increase in the L-type Ca^{2+} current following sustained dehydration. Immunocytochemical studies at the electron microscopic level using antibodies against Ca^{2+} channel subtypes might be able to show whether there is an increase in L-type Ca^{2+} channel density in the MNC plasma membrane in normal and dehydrated rats. If it is possible to find

conditions in which the increase in L-type Ca^{2+} current can be activated in cultured isolated MNCs by treating with high osmolality and/or potential modulators such as VP (which causes the translocation of AQP2 water channels in the epithelial cells of the kidney collecting duct; Fenton *et al.*, 2008), it will be helpful for studies of the mechanisms underlying the increase in the L-type Ca^{2+} current following sustained dehydration. Under these conditions, it would be possible to perform experiments about membrane recycling using activity dependent dye FM1-43 to see whether changes in osmolality cause membrane recycling. Since Ca^{2+} channels could translocate from cytoplasmic vesicles to the plasma membrane, such an approach would make it possible to observe the uptake of fluorescently labeled DHPs which can selectively bind to the L-type Ca^{2+} channel and thus determine whether the channel will be translocated to the plasma membrane with increases in osmolality. Furthermore, such an approach would also make it possible to observe the translocation of the L-type Ca^{2+} channel following increases in osmolality using transfection with a plasmid coding for the channels tagged with green florescent proteins. In addition, it would also be possible to block electrical activity in MNCs with drugs, the translocation with toxins such as tetanus toxin to block exocytotic fusion (Verderio *et al.*, 1999), or protein synthesis, and thus confirm whether the L-type Ca^{2+} channels are translocated to the plasma membrane with increases in osmolality. These experiments could help to elucidate the mechanisms underlying the increase in the L-type Ca^{2+} current in MNCs following sustained dehydration.

5.1.2 Physiological roles of the increased L-type Ca^{2+} current during sustained dehydration

The L-type Ca^{2+} channel plays many crucial physiological roles in many brain areas, including the SON (Fisher & Bourque, 1996; Moosmang *et al.*, 2005) and activation of L-type Ca^{2+} current can mediate long-lasting changes in brain function (Hetzenauer *et al.*, 2006; Striessnig *et al.*, 2006). An increase in the L-type Ca^{2+} current in the MNCs during sustained dehydration might lead to the modulation of cell excitability, hormone release, gene expression, and structural adaptation.

Firstly, an increase in the L-type Ca^{2+} current will further depolarize the MNCs to cause activation of voltage-dependent channels, and cause an increase in $[Ca^{2+}]_i$ which would lead to the activation of Ca^{2+}-dependent potentials such as the DAP, the HAP, and the AHP. These Ca^{2+}-dependent potentials modulate MNC firing rate and pattern. Although it is not known whether the DAP is modulated by influx of Ca^{2+} through a specific Ca^{2+} channel, L-type Ca^{2+} channels play important roles in the modulation of a slow after-depolarizing potential, which plays a role in the generation of bursts, and a hyperpolarization-activated, postspike depolarizing afterpotential, which plays a role in modulating spike frequency, in neuroendocrine Dahlgren cells of teleost *Platichthys flesus*. The blockade of the L-type Ca^{2+} current in these cells reduces the amplitude of the after-depolarizing potential by 95% and the hyperpolarization-activated, postspike depolarizing afterpotential by 76% (Brierley *et al.*, 2004). Thus, an increase in the L-type Ca^{2+} current during sustained dehydration might modulate the DAP and associated plateau potentials in the MNCs and thus regulate MNC firing pattern.

In addition, an increase in the L-type Ca^{2+} current could also modulate the Ca^{2+}-dependent HAP and/or AHP. There is evidence from other cell types to suggest that an increase

in L-type Ca^{2+} current could cause selective increases in K_{Ca} currents. In rat CA1 hippocampal pyramidal neurons, the BK channel contributing to the fAHP is selectively activated by the N-type Ca^{2+} channel, whereas the SK channel contributing to mAHP is selectively activated by the L-type Ca^{2+} channel (Marrion & Tavalin, 1998). Furthermore, another study suggests that the BK channel is selectively activated by the L-type Ca^{2+} channel due to their co-localization in the brain (Grunnet & Kaufmann, 2004). Mechanisms underlying such coupling between the L-type Ca^{2+} channel and the K_{Ca} channel are unknown. However, an α-actinin2 cytoskeletal protein may play an important role in the formation of the coupling between the $Ca_V1.3$ L-type Ca^{2+} channel and the SK2 channel (Lu *et al.*, 2007). In addition, Ca^{2+} entry through the L-type Ca^{2+} channel also contributes to the activation of the sAHP (Shah & Haylett, 2000), which may play a role in regulating firing in neurons (Faber & Sah, 2003) including the MNCs (Ghamari-Langroudi & Bourque, 2004). A specific co-localization between the $Ca_V1.3$ L-type Ca^{2+} channel and the SK1 channel, which may contribute to sAHP generation (Kohler *et al.*, 1996), has been found in rat CA1 hippocampal neurons (Bowden *et al.*, 2001). In CA pyramidal neurons, the blockade of the L-type Ca^{2+} current reduces the sAHP by 24% (Pineda *et al.*, 1999).

It is not known whether AHPs are increased in the MNCs during sustained dehydration, but increases do occur during lactation (Teruyama & Armstrong, 2005). During lactation, the amplitudes of both an apamin-sensitive mAHP and an apamin-insensitive sAHP are significantly increased in OT-MNCs, but the density of only mAHP is significantly increased. In addition, there is a significant increase in cell capacitance in OT-MNCs but not in VP-MNCs during lactation. Total Ca^{2+} currents are also significantly increased in amplitude (but not in density) only in OT-MNCs during lactation. However, it is not known whether there is an increase in the L-type Ca^{2+} current and whether the increase in the L-type Ca^{2+} current is responsible for the

increases in the sAHP and/or mAHP during lactation. These data, however, suggest that an increase in the L-type Ca^{2+} current during sustained dehydration could contribute to the modulation of MNC firing rate and pattern by regulating the DAP, the HAP, and the AHP. Future studies attempting to determine whether water deprivation leads to increases in Ca^{2+}-dependent potentials using patch-clamp recording in the MNCs would be very helpful for the understanding of roles of the increased L-type Ca^{2+} current. In addition, studies investigating the roles of DHPs on MNC firing would be also helpful.

Secondly, an increase in the L-type Ca^{2+} current could facilitate the release of VP/OT (Mason *et al.*, 1986) and co-localized neuropeptides such as dynorphin (Brown & Bourque, 2004) and apelin (De Mota *et al.*, 2004) from the MNC somatodendritic region. Dendritic release can optimize subsequent hormone release from the terminals by generating autocrine and/or retrograde feedback control (Wotjak *et al.*, 1994; Gouzenes *et al.*, 1998; Hirasawa *et al.*, 2004). Unlike N-and P/Q-type Ca^{2+} channels, which play a key role in neurotransmission, the L-type Ca^{2+} channel is responsible for endocrine release and exocytotic release from neuronal somata (Fisher & Bourque, 2001). The L-type Ca^{2+} current mediates dynorphin release from the dendrites, but not from axons, in hippocampal granule cells (Simmons *et al.*, 1995). In addition, the L-type Ca^{2+} channel may play an important role in VP release from the somatodendritic region in the SON (Shibuya *et al.*, 1998). In a rat SON slice preparation, pituitary adenylate cyclase activating polypeptides can cause VP release from the somatodendritic region by evoking Ca^{2+} entry. Blockers of the L- but not N-type Ca^{2+} channel selectively block the Ca^{2+} entry and the VP release, suggesting a role of the L-type Ca^{2+} current in MNC dendritic release. An increase in the L-type Ca^{2+} current during sustained dehydration might modulate MNC firing rate and patterns by contributing to the somatodendritic release of VP (Mason *et al.*, 1986;

Wotjak *et al.*, 1994) and co-localized dynorphin (Brown & Bourque, 2004) and apelin (De Mota *et al.*, 2004), which are able to generate autocrine feedback control.

Finally, an increase in the L-type Ca^{2+} current may contribute to regulation of increases in gene expression and other long-lasting changes in function of the SON during sustained dehydration. The L-type Ca^{2+} channel-mediated Ca^{2+} signals, specifically in the central nervous system, initiate intracellular signal transduction events that activate long-lasting changes in brain function and behaviour (Hetzenauer *et al.*, 2006; Striessnig *et al.*, 2006) because Ca^{2+} influx from the L-type Ca^{2+} channels of the $Ca_V1.2$ and $Ca_V1.3$ subfamily in the brain can preferentially activate transcription factors such as CREB proteins (Hardingham *et al.*, 1999; Weick *et al.*, 2003; Zhang *et al.*, 2006) and c-Fos (Hetzenauer *et al.*, 2006; Zhao *et al.*, 2007). Formation of LTP and various forms of memory are tightly associated with activity of the CREB proteins (Frank & Greenberg, 1994; Yin & Tully, 1996; Silva *et al.*, 1998). Furthermore, signal transduction and gene expression are also modulated in the MNCs following sustained dehydration, leading to increases in VP gene expression (Zingg *et al.*, 1986; Ding *et al.*, 1994; Hurbin *et al.*, 2002) and in c-Fos gene expression in the OVLT, PVN, and SON (Oldfield *et al.*, 1994; Gottlieb *et al.*, 2006). An increase in L-type Ca^{2+} current during sustained dehydration may increase the rate at which the CREB proteins are phosphorylated in the MNCs, and such an increase could be important in the regulation of gene expression that occurs during water deprivation. In addition, studies showed that although separate mechanisms might be involved in the hypertrophy of the MNCs and in cardiomyocytes, the L-type Ca^{2+} channel, in part, is responsible for cardiomyocyte hypertrophy induced by serum stimulation (Lubic *et al.*, 1995), and calcineurin activated by Ca^{2+} entry from the L-type Ca^{2+} channel may contribute to such mechanical stretch-induced hypertrophy (Fiedler *et al.*, 2002; Zou *et al.*, 2002; Zobel *et al.*,

2007). Thus, an increase in the L-type Ca^{2+} current may contribute to the structural adaptation that occurs in the SON during sustained dehydration.

5.2 The OC increased by acute hypertonic stimulation could be a KCNQ/M-like current

5.2.1 Physiological roles of the OC

We have identified a slowly activating and inactivating, osmosensitive current (the OC) in acutely isolated MNCs. This current increases with increases in extracellular osmolality in about 66% of the MNCs. The reversible potential of the OC demonstrates that the OC is mediated by a type of voltage-dependent K^+ channels. What could be the physiological role of a slowly activating and inactivating voltage-dependent K^+ current that increases as osmolality is increased? It appears paradoxical that a K^+ current would be activated by hypertonic stimulation that increases the output from these cells (i.e. the release of VP). However, the OC might play a role in mediating the transition between fast continuous and burst firing in MNCs. Activation of the OC might greatly decrease the firing rate and eventually lead to cessation of firing. The slowly activating and inactivating OC might activate during a burst of action potentials and might contribute to a slowing or termination of firing in MNCs. VP-MNCs respond to osmotic stimuli by adopting a phasic pattern of firing, and a phasic burst lasts for about 20 seconds (Poulain & Wakerley, 1982; Bicknell, 1988). Firing rate rapidly increases during the initial phase of the bursts to 30 Hz, and then decreases to about 10 Hz, leading to formation of a constant firing phase (Roper *et al.*, 2004). The constant firing lasts over 10 s and abruptly ceases, leading to the termination of the bursts (Roper *et al.*, 2004). The voltage-dependent, slowly activating OC could progressively activate during the burst, and thus gradually decrease firing rate. The

time for complete activation of the OC is unknown in MNCs since internal TEA in our experiments could affect biophysical properties of the OC (Armstrong, 1966). Our current data suggest that the OC could terminate MNC firing and cause formation of a shorter burst, which occurs in MNCs during sustained dehydration (Dyball & Pountney, 1973). Thus, the OC could be important in mediating the MNC firing in shorter bursts (Figure 5.1).

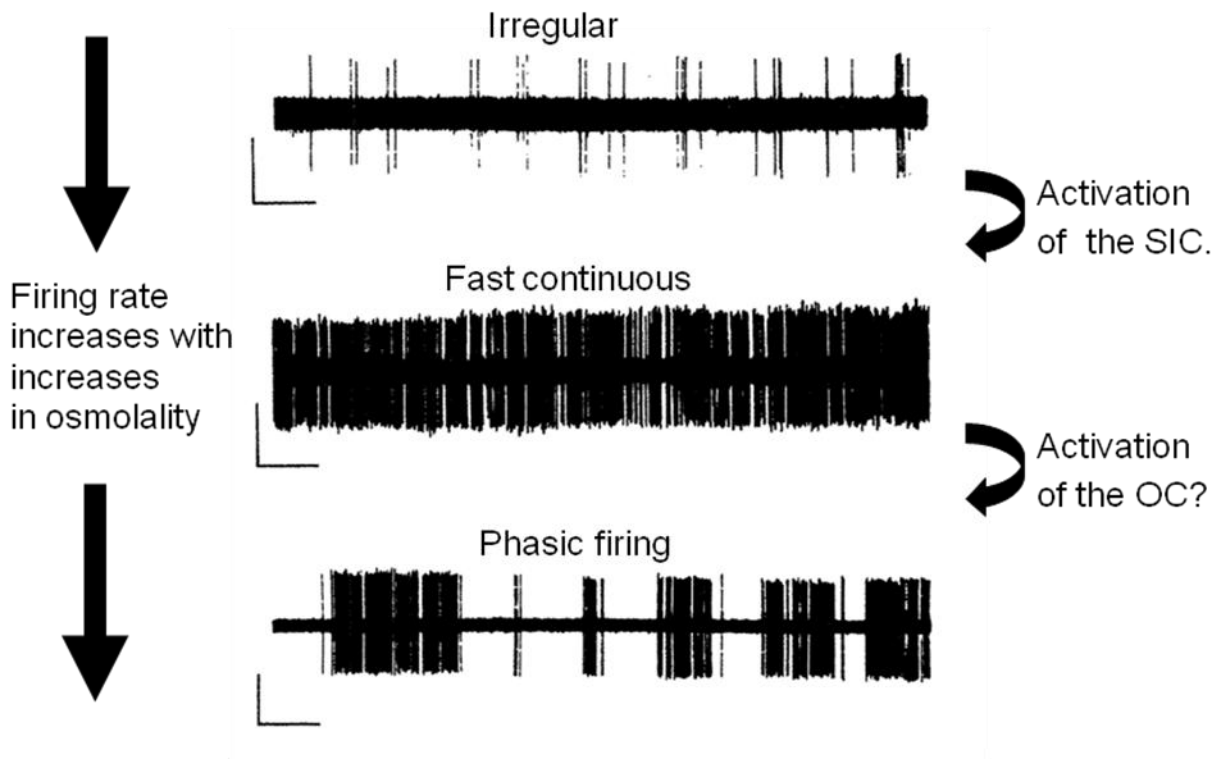


Figure 5.1 Hypothesized physiological role of the OC in regulating MNC firing rate and pattern. SIC play an important role in the increase in MNC firing during elevations in osmolality (Bourque *et al.*, 2002). However, the properties of the OC suggest that it might slowly activate during a burst of action potentials and might contribute to a slowing or termination of firing in MNCs. Thus, the OC might contribute to the transition between fast continuous and phasic firing in MNCs. Scale mark is 200 μV and 10 s. The traces of this figure are adapted from Poulain *et al*. (Poulain *et al.*, 1977).

The molecular nature of the OC is not confirmed. However, pharmacological studies of the OC demonstrated that the OC is insensitive to extracellular TEA and is blocked by XE991 with an IC_{50} of 3.9 μM. XE991 at this concentration selectively blocks KCMQ/M channels (Wang *et al.*, 1998). In addition, retigabine, which is a selective opener of the KCNQ/M channels, increases the OC in amplitude and shifts the voltage sensitivity of the OC to more negative potentials, and this is consistent with its properties on the KCNQ/M channels (Tatulian *et al.*, 2001). Thus, the pharmacological properties of the OC suggest that it could be mediated by a subtype of KCNQ/M channels. If the OC is mediated by a subtype of KCNQ/M channels, this is the first demonstration that the KCNQ/M current can regulate cell firing in the MNCs. If the OC is mediated by a subtype of KCNQ/M channels, which are located at the axon initial segments and nodes of Ranvier (Lai & Jan, 2006) and also in somatodendrites and neuronal terminals (Delmas & Brown, 2005) and play key roles in regulating neuronal firing frequency and excitability by multiple mechanisms (Delmas & Brown, 2005; Vervaeke *et al.*, 2006), it could modulate MNC firing by the following possible mechanisms.

The KCNQ/M channels contribute to the regulation of the resting membrane potential and modulate the critical threshold of action potentials in hippocampal pyramidal neurons (Shah *et al.*, 2008), in rat visceral sensory neurons (Wladyka & Kunze, 2006), and in isolated mouse inner hair cells (Oliver *et al.*, 2003). It is unknown whether the OC, if it is KCNQ/M current, regulates the resting membrane potential and critical threshold of action potentials in MNCs and thus modulate their excitability. This is possible because a recent study has shown that the resting membrane potential is depolarized by 1.6-6.3 mV by applying about 50 μM muscarine in all MNCs (Ghamari-Langroudi & Bourque, 2004). However, it is not known whether muscarine, as a non-selective agonist of muscarinic receptors (Garnier *et al.*, 1998), changes the resting

membrane potential in the MNCs by acting on M1 receptors (which couple to KCNQ/M channels) to inhibit the KCNQ/M currents.

In CA1 pyramidal cells, an inhibition of the KCNQ/M channels enhances a slow afterdepolarization, which generates a burst, and thus controls neuronal activity (Chen & Yaari, 2008). This is consistent with a previous study demonstrating that the spike afterdepolarization and burst generation in hippocampal neurons were regulated by the KCNQ/M channels (Yue & Yaari, 2004). MNCs can also intrinsically generate a DAP, which follows action potentials and can summate into plateau potentials, and thus evoke a phasic burst (Andrew & Dudek, 1983). The DAP in the MNCs cannot be reduced by muscarine (Ghamari-Langroudi & Bourque, 2004), and this suggests that KCNQ/M channels may not display any effects on generation of the DAP. However, besides this Cs^+-sensitive DAP in the MNCs (Ghamari-Langroudi & Bourque, 1998), a Cs^+-insensitive fast DAP was recently identified in the MNCs that could also play a role in regulation of MNC firing (Teruyama & Armstrong, 2007). It is not known whether the OC can reduce this Cs^+-insensitive voltage-dependent DAP by hyperpolarizing the cells and thus regulate the rate and pattern of MNC firing.

Interestingly, a recent study using sharp electrode, whole-cell and perforated patch-clamp recordings has shown that the mAHP is not mediated by SK channels but by a subtype of KCNQ/M channels in hippocampal CA1 pyramidal neurons (Gu *et al.*, 2005). Moreover, dominant negative expression of the KCNQ2 subunits in CA1 pyramidal neurons leads to a decrease in spike frequency adaptation and in mAHP, and causes an increase in neuronal excitability (Peters *et al.*, 2005). KCNQ/M channels mediate both a fast and a slow component of the AHP in dopamine neurons of ventral tegmental area and thus regulate the interspike intervals but not spike parameters such as amplitude, duration, and threshold (Koyama & Appel, 2006). In

addition, the KCNQ/M channels play a role in the modulation of the subthreshold accommodation and spike frequency adaptation by contributing to a slowly activating and deactivating K^+ current (I_{Ks}) in nodes of Ranvier and thus control firing frequency in the sciatic nerve (Schwarz *et al.*, 2006). A muscarine-sensitive sAHP has been identified in MNCs (Ghamari-Langroudi & Bourque, 2004). This sAHP does not contribute to modulation of spike parameters such as amplitude, duration, and broadening either, but muscarine increases firing rate and duration of the spontaneous phasic bursts in the MNCs (Ghamari-Langroudi & Bourque, 2004). However, it is not known whether this sAHP is mediated by a subtype of the KCNQ/M channels and whether the OC can mediate an AHP and thus regulate MNC firing.

These observations suggest that the OC, if it is a KCNQ/M-like current, can modulate the rate and pattern of MNC firing possibly by multiple mechanisms. Activation of the OC could slowly turn down the firing or behave as a 'brake' on neuronal firing, as do the KCNQ/M currents in various types of neurons (Delmas & Brown, 2005). Future experiments using patch-clamp techniques in the MNCs will investigate whether the OC affects the voltage-dependent DAP by hyperpolarizing the cells and whether the OC contributes to an AHP. In addition, since the OC is very sensitive to XE991 and retigabine, these drugs could be used to see whether the OC contributes to the transition between fast continuous and burst firing. These modulators are used to modulate MNC firing at different doses. For example, a high dose of retigabine could cause complete activation of the OC and thus terminate MNC firing. However, a smaller dose of retigabine could mimic the enhancement of the OC induced by an increase in extracellular osmolality and thus mimic the effects of the OC at different osmolalities.

5.2.2 The OC and somatodendritic release

If the OC is a KCNQ/M current, which can be modulated by many neurotransmitters such as acetylcholine, and neuropeptides such as luteinizing hormone releasing hormone and angiotensin (Delmas & Brown, 2005), it could be a cellular target through the synaptic transmission and thus contribute to the modulation of osmosensory transduction. Furthermore, the MNCs can also release VP and other potential autocrine regulators from their dendrites (Mason *et al.*, 1986; Di Scala-Guenot *et al.*, 1987; Landgraf & Ludwig, 1991; Ludwig *et al.*, 1995). Dendritic release of VP can optimize subsequent hormone release in the terminals by generating feedback control (Wotjak *et al.*, 1994; Gouzenes *et al.*, 1998; Hirasawa *et al.*, 2004). VP can display an excitatory effect to increase burst duration and/or decrease the duration of silence in faintly active neurons (periods of activity of < 10 sec) and thus contribute to the transition to phasic firing (Gouzenes *et al.*, 1998). VP can generate excitatory effects in excitable cells possibly by enhancing Ca^{2+} entry and/or by inhibiting K^+ currents. VP can increase Ca^{2+} entry in vascular smooth muscle by causing the activation of T-type Ca^{2+} channels (Brueggemann *et al.*, 2005), L-type Ca^{2+} channels (Katori *et al.*, 2001), receptor-operated Ca^{2+} channels (Katori *et al.*, 2001), Ca^{2+} release channels of the sarcoplasmic reticulum (Katori *et al.*, 2001), store-operated Ca^{2+} channels (Byron & Taylor, 1995), and TRPC channels (Maruyama *et al.*, 2006). However, VP can also rapidly and greatly inhibit the L-type Ca^{2+} channels in L6 cells, but this inhibition slowly recovers due to the activation of protein kinase C (Hantash *et al.*, 2006). VP can suppress the currents mediated by KCNQ/M channels in A7r5 rat aortic smooth muscle cells (Brueggemann *et al.*, 2007) and in rat mesenteric arteries (Mackie *et al.*, 2008), and by ATP-dependent K^+ channels in insulin-secreting cells and vascular smooth muscle cells (Martin *et al.*, 1989; Kawano *et al.*, 2007; Shi *et al.*, 2007). Thus, the OC, if it is a KCNQ/M

current, could be one of targets of VP released from the dendrites and this could contribute to the VP-induced feedback control.

5.2.3 The OC and hormone release

Many properties of the OC suggest that it could be a KCNQ/M current. KCNQ/M channels play a role in regulating the secretory release of neurotransmitters such as γ-aminobutyric acid, norepinephrine, aspartate, dopamine and serotonin, and acetylcholine, and this has been reviewed in detail (Hansen *et al.*, 2008). In addition, KCNQ/M channels also play a role in exocytotic secretion in pancreatic neuroendocrine cells (Thevenod, 2002; Lee *et al.*, 2008). Our data have demonstrated that the OC can modulate MNC firing. Since the release of hormone is determined primarily by the firing rate and pattern in the somata of the MNCs (Bicknell, 1988), the OC could play an important role in the control of the release of VP by regulating the rate and pattern of MNC firing. Muscarine (Bisset & Fairhall, 1996) and acetylcholine (Sladek & Kapoor, 2001) can increase the release of VP and OT, but it is not known whether modulation of KCNQ/M channels contributes to this effect. These studies suggest that the OC could be able to modulate the release of hormone in the MNC.

5.2.4 Possible subtypes of the KCNQ/M channels mediating the OC

The molecular nature of the OC remains unknown. If the OC is mediated by a subtype of KCNQ/M channels, the pharmacological data on the OC may help to determine which subunits of the KCNQ/M channels could be involved. Since different subunits of the KCNQ/M channels are blocked by TEA, linopirdine, and XE991 with different IC_{50}s, (Robbins, 2001), our data have implications concerning which subtypes mediate the OC in the MNCs. The OC is

insensitive to TEA. Since the classic M-current expressed in many brain neurons is mediated by KCNQ2/3 heteromultimers and displays high sensitivity to extracellular TEA (Wang *et al.*, 1998), it is unlikely that the OC is mediated by these heteromultimers. The channel mediating the OC could therefore contain the TEA-insensitive KCNQ3 and/or KCNQ5 subunits (Hadley *et al.*, 2000; Lerche *et al.*, 2000). Since the OC was significantly blocked by 150 μM but not by 10 μM linopirdine, the channel mediating the OC could contain the much less linopirdine-sensitive KCNQ4 subunits (Kubisch *et al.*, 1999). In addition, the channels composed of the KCNQ5 subunits are blocked by linopirdine at a high concentration (more than 10 μM; Robbins, 2001). Only the KCNQ3 subunit can co-assemble with KCNQ2, KCNQ4, and KCNQ5 subunits to form heteromultimers (Robbins, 2001). Thus, it appears that the most likely composition of the channel mediating the OC is KCNQ3/4, KCNQ3/5, or KCNQ5 subunits. Both the KCNQ4 channel (Hougaard *et al.*, 2004) and KCNQ5 channel (Jensen *et al.*, 2005a) are sensitive to changes in cell volume, and this suggests that such composition of the channel mediating the OC (if it is a KCNQ/M current) is possible.

Co-immunoprecipitation studies on KCNQ/M channels have been used to determine the existence of KCNQ2/3 and KCNQ3/5 heteromultimers in the central neurons (Cooper *et al.*, 2000; Yus-nájera *et al.*, 2003). Such techniques in the future studies on the OC will be helpful for demonstrating which subtypes of the KCNQ/M channels could mediate the current. In addition, the knock down of KCNQ current expression using antisense and/or siRNA techniques could decrease the OC, if it is a KCNQ/M current. Such experiments may demonstrate the subtype(s) mediating the OC. If the knockout mice of some KCNQ/M channel genes are able to be obtained and lack the OC, the molecular nature of the OC may be confirmed. Knockout mice of some KCNQ/M channel genes, which are identified to be expressed in SON, will be involved

this study in the future. The OC should be completely eliminated in these knockout mice if it is mediated by the channels whose genes are knocked out. Studies have used the knockout mice of KCNQ4 channel gene to indicate that mutation of KCNQ4 subunits causes the hearing loss in DFNA2 (Kharkovets *et al.*, 2006).

5.2.5 Osmosensitivity of the OC

The mechanisms underlying the osmosensitivity of the OC are unknown. There are three most likely possibilities for the channel displaying the osmosensitivity. One possibility is that the channel is directly gated by mechanical force. Mechanical force can modulate the activity of ion channels by an interaction between the channel proteins and mechanical force inducer (Hamill & Martinac, 2001) such as the lipid bilayer, as with the TREK-TRAAK family (Kim, 2003), and/or cytoskeleton, as with the SIC in MNCs (Zhang *et al.*, 2007b). The cells change their volume as an important physiological function in response to osmotic stimuli, and changes in volume, therefore, lead to alterations in the membrane tension, which determines the function of some ion channels and transporters (Lang *et al.*, 1998a; Lang, 2007). A second possibility is that the channel is activated by a change in ionic strength (in which case changes in cytoplasmic salt levels cause activation of ion channels or transporters) due to osmotic stimuli, as with the volume-regulated chloride channel (Nilius *et al.*, 1998; Voets *et al.*, 1999; Wittels *et al.*, 2000). A third possibility is that the channel is modulated by second messengers such as Ca^{2+}, Mg^{2+}, cAMP, and/or by phosphorylation reactions of kinases such as protein kinase C (Okada, 1997; Stutzin & Hoffmann, 2006), which are altered in function by osmolality. Many cellular signaling events such as second messenger cascades, phosphorylation of proteins, and gene expression may be osmosensitive (Okada, 1997; Lang *et al.*, 1998a).

Several subtypes of the KCNQ/M channels are sensitive to cell volume, and the current amplitudes are increased with decreases in extracellular osmolality (Grunnet *et al.*, 2003; Hougaard *et al.*, 2004; Jensen *et al.*, 2005b). However, the OC is different, and it displays an increase with an increase in osmolality of extracellular recording solution (Figure 4.6). If the OC is mediated by a KCNQ/M channel, this difference could be due to different molecular mechanisms of osmosensory transduction in different cell types (Lang *et al.*, 1998b). SIC, a splice variant of TRPV1 with a truncated N-terminus and a C-terminus of TRPV4, can functionally decrease or increase in MNCs in response to hypotonic or hypertonic stimuli, respectively (Sharif-Naeini *et al.*, 2006). However, TRPV1 does not display any osmosensitivity (Liedtke *et al.*, 2000). TRPV4 is activated by cell swelling, and inhibited in HEK293 cells (Strotmann *et al.*, 2000) or unaffected in CHO cells (Liedtke *et al.*, 2000) by cell shrinking. The mechanisms underlying the osmosensitivity of the OC (if it is a KCNQ/M current) in the MNCs may be different from that of the KCNQ/M channels mentioned above (in which case osmosensitivity of the channel is attributed to an interaction between the cellular cytoskeleton and the channel proteins; Grunnet *et al.*, 2003). However, a second messenger cascade or intracellular signal pathway that regulates the OC in the MNCs might respond to osmolality of the extracellular solution in the MNCs. Many intracellular signaling pathways change with alterations in cell volume and thus play an important role in the modulation of cellular functions (Wehner *et al.*, 2003; Stutzin & Hoffmann, 2006). One example is that the KCNQ/M channels can be modulated by PIP_2 and protein kinase C (Delmas & Brown, 2005). PIP_2 can directly regulate the KCNQ/M channels (Suh & Hille, 2002) possibly by binding to the channels to increase their open probability (Li *et al.*, 2005). KCNQ/M channels can also indirectly be modulated by PIP_2 by the activation of the protein kinase C pathway and subsequent cytoskeletal

reorganization due to PIP_2 hydrolysis (Lan *et al.*, 2006). Phosphoinositide signalling (Wehner *et al.*, 2003) is altered in response to osmotic stimuli due to the changes in activity of many kinases and enzymes. PLC, which can cleave cellular PIP_2, can be activated by membrane stretch (Brophy *et al.*, 1993; Ruwhof *et al.*, 2001; Barfod *et al.*, 2005; Nam *et al.*, 2007), and such activation can cause the hydrolysis of PIP_2 and subsequent activation of large conductance background K^+ channels in B lymphocytes (Nam *et al.*, 2007), and K^+ and Cl^- channels in HTC rat hepatoma cells (Barfod *et al.*, 2005). Such stretch-induced activation in B lymphocytes is not dependent on the cellular cytoskeleton (Nam *et al.*, 2007). Furthermore, the level of PIP_2 in the plasma membrane is significantly increased by hypertonic stimuli in guinea pig cardiac myocytes, M1, COS, and HEK-293 cells (Nasuhoglu *et al.*, 2002), in Ehrlich Lettre ascites cells (Nielsen *et al.*, 2007; Rasmussen *et al.*, 2008), and in HeLa cells (Nasuhoglu *et al.*, 2002; Yamamoto *et al.*, 2006). This increase could be attributed to the activation of type I PIP kinases in response to hypertonic stimuli (Yamamoto *et al.*, 2006). Thus, the level of membrane PIP_2 can display changes with alterations in osmolality of the extracellular solution (Nasuhoglu *et al.*, 2002; Yamamoto *et al.*, 2006; Nam *et al.*, 2007; Nielsen *et al.*, 2007; Rasmussen *et al.*, 2008). Osmotic modulation of the KCNQ/M currents might occur through the PIP_2 system, and PIP_2 metabolism might play an important role in modulating the osmosensitivity of the OC. The OC, if it is a KCNQ/M current, might respond to a different intracellular signaling pathway that has been known for osmosensory transduction from other KCNQ/M channels reported to sense changes in cell volume.

5.3 Does the OC as a KCNQ/M-like current couple to the L-type Ca^{2+} current?

Although the increase in the L-type Ca^{2+} current, which is identified in dehydrated rat, and the increase in the OC, which is found in isolated MNCs in response to acute hypertonic stimulation and could be a KCNQ/M current, are recognized in the MNCs under different experimental conditions, there might be a relationship between the two currents because the two channels can couple to many other plasma membrane proteins. The L-type Ca^{2+} channel can couple to a number of plasma membrane proteins including K_{Ca} channels (Marrion & Tavalin, 1998; Grunnet & Kaufmann, 2004), ryanodine receptor-mediated Ca^{2+} release channels (Catterall, 1991), Na^{+}/Ca^{2+} exchangers (Pott *et al.*, 2007), TRPC6-like cation channels (Poteser *et al.*, 2003), angiotensin AT1A receptors (Oz *et al.*, 1998), and β1-adrenoreceptors (Yatani *et al.*, 1995). In addition, KCNQ/M channels can couple to several plasma membrane proteins including M1 muscarinic receptors (Schroeder *et al.*, 2000; Selyanko *et al.*, 2000), dopamine D_2 receptors (Ljungstrom *et al.*, 2003), and β3-adrenoreceptors (Kathofer *et al.*, 2000). Interestingly, a recent study showed a relationship between the L-type Ca^{2+} channel and a KCNQ/M channel in hippocampal CA1 pyramidal neurons (Wu *et al.*, 2008). This study indicated that the L-type Ca^{2+} channel opener Bay K 8644 increases the KCNQ/M current, and that the selective L-type Ca^{2+} channel blocker, nimodipine, decreases the KCNQ/M current (Wu *et al.*, 2008). These data strongly suggest a functional coupling between the L-type Ca^{2+} channel and the KCNQ/M channel. Thus, an interesting question about the sensitivity of the KCNQ/M channels to Ca^{2+} appears. The KCNQ/M channels show some sensitivity to intracellular Ca^{2+} (Delmas & Brown, 2005) possibly because of a binding site of calmodulin operating as a Ca^{2+} sensor in the C-terminus of the channels (Bal *et al.*, 2008; Haitin & Attali, 2008). The effects of intracellular Ca^{2+} on the modulation of the KCNQ/M channels, however, are discrepant. Intracellular Ca^{2+} has

been reported to both increase and decrease the KCNQ/M channel-mediated current; its effect is dependent on $[Ca^{2+}]_i$ and the current is enhanced with an increase in $[Ca^{2+}]_i$ when $[Ca^{2+}]_i$ is less than 200 nM (Tokimasa, 1985; Marrion *et al.*, 1991; Yu *et al.*, 1994; Marrion, 1996; Selyanko & Brown, 1996; Cruzblanca *et al.*, 1998; Gamper & Shapiro, 2003; Gamper *et al.*, 2005). Our data also showed that the OC isolated as a tail current in I_{Ca} medium was sensitive to the Ca^{2+} channel blocker, Cd^{2+} (Liu, 2004), and to the selective L-type Ca^{2+} channel blocker, nifedipine. Furthermore, the outward current was also blocked by nifedipine in TEA/physiological solution. These studies suggest that there might be a possibility that the OC couples to the L-type Ca^{2+} channel in MNCs. However, an increase in the OC in response to acute hypertonic stimulation may be not attributed to an increase in the L-type Ca^{2+} current because the OC still displays an increase in the presence of nifedipine when the cells are switched from 295 to 325 mosmol kg^{-1} external solutions (Figure 4.8). In addition, an increase in the L-type Ca^{2+} current was small and not significant after the MNCs isolated from normal rat were cultured in 320 mosmol kg^{-1} physiological PIPES solution for about 90 min.

5.4 Significance of these studies

The osmotic pressure of the ECF in mammals has to be set at an ideal point, and VP is the hormone primarily responsible for its maintenance (Bourque, 1998). MNCs sense small changes in external osmolality to change their electrical activity and thus determine the release of VP (Bicknell, 1988; Bourque & Oliet, 1997). The uncontrolled synthesis and release of VP leads to a number of physiological perturbations and diseases (Bourque & Oliet, 1997). The MNCs change their electrical behaviour through the regulation of ion channels (Bourque, 2008), especially osmosensitive ion channels. Our studies indicate that sustained dehydration causes an

increase in the L-type Ca^{2+} current in the MNCs, and such an increase may contribute to the modulation of MNC firing, hormone release, and adaptation during sustained dehydration. In addition, our studies also demonstrate that a KCNQ/M-like current is increased by acute hypertonic stimulation and that this current may be able to regulate the rate and pattern of MNC firing and hormone release. The identification of this current may lead to a better understanding of the mechanisms of the transition between fast continuous to burst firing in MNCs. Therefore, our studies will be beneficial to understand the mechanisms that control VP and OT in response to acute changes in osmolality and also the mechanisms underlying MNC adaptation during sustained dehydration, and may be helpful for the treatment of these related diseases and the improvement of health.

5.5 Future directions

For L-type Ca^{2+} channel project, future studies will investigate whether an increase in the L-type Ca^{2+} current causes the modulation of Ca^{2+}-dependent potentials, somatodendritic release, gene expression, and MNC hypertrophy. In addition, future studies will also investigate and whether the increase is attributed to a reversible translocation of the channels from internal storage sites to the plasma membrane and/or an enhancement of the channel activity.

For the OC project, future studies will investigate whether an increase of the OC in MNCs displaying fast continuous firing can cause the transition between fast continuous to burst firing or the generation of phasic firing. In addition, future studies will also investigate whether the OC affects the DAP and the associated plateau potential by hyperpolarizing the cells, and whether the OC contributes to an AHP. The molecular nature of the OC remains unknown. Co-

immunoprecipitation studies on KCNQ/M channels and knock down of KCNQ current using antisense and/or siRNA techniques will help to determine whether the OC is mediated by a KCNQ/M channel and which subtype of the KCNQ/M channels mediates the OC if it is a KCNQ/M current. If the knockout mice of some KCNQ/M channel genes are able to be obtained, the molecular nature of the OC may be confirmed in the MNCs. In addition, the mechanisms of the OC underlying osmosensitivity need to be explored. Mechanical force, or changes in cellular signaling events or ionic strength due to osmotic stimuli, may be responsible for the osmosensitivity of the OC.

6. CONCLUSIONS

Our data indicate that there is a selective increase in the L-type Ca^{2+} current in MNCs during sustained dehydration. Such an increase may be attributed to an increase in functional channel numbers of plasma membrane and/or channel activity and may contribute to plastic changes in the SON during sustained dehydration.

In addition, we have also identified a slowly activating osmosensitive current in acutely isolated MNCs from rat SON, and many properties of this current suggest that it could be mediated by a subtype of the KCNQ/M channels. Slow activation of this current during MNC firing might suppresses activity by hyperpolarizing the cell and this contributes to the transition between fast continuous and burst firing. Thus, this osmosensitive current might contribute to promotion of phasic firing in the MNCs and thus regulate the release of hormone.

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