THE ACTIVATION OF NOVEL CALCIUM-DEPENDENT PATHWAYS DOWNSTREAM OF N-METHYL-D-ASPARTATE RECEPTORS

by

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Calcium (Ca$^{2+}$) influx through N-methyl-D-aspartate receptors (NMDARs) is widely held to be the requisite step initiating delayed neuronal death following ischemic stroke. However, blocking NMDARs fails to prevent the accumulation of intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_i$) and subsequent neurotoxicity. This suggests that alternate, as yet uncharacterized Ca$^{2+}$-influx pathways exist in neurons. Transient receptor melastatin 2 (TRPM2) is a Ca$^{2+}$-permeable member of the transient receptor potential melastatin family of cation channels whose activation by reactive oxygen/nitrogen species (ROS/RNS) and ADP-ribose (ADPR) is linked to cell death. While these channels are broadly expressed in the central nervous system (CNS), the presence of TRPM2 in neurons remains controversial and more specifically, whether they are expressed in neurons of the hippocampus is an open question. Here, I employ a combination of molecular,
biochemical and electrophysiological approaches to demonstrate that functional TRPM2 channels are expressed in pyramidal neurons of the hippocampus. Unlike in heterologous expression systems, the ADPR-dependent activation of TRPM2 in neurons required a concomitant rise in $[\text{Ca}^{2+}]_i$ via either voltage-dependent $\text{Ca}^{2+}$ channels or NMDARs. While short, repeated NMDA applications activated a TRPM2-like current in the absence of exogenous ADPR, sustained NMDA application to hippocampal neurons resulted in the activation of a pannexin1 (Px1) hemichannel. Px1 hemichannels are large conductance, nonjunctional gap junction channels that can be activated following periods of oxygen-glucose deprivation (OGD) in neurons. Activation of Px1 required the influx of $\text{Ca}^{2+}$ through NMDARs. Supplementing the intracellular milieu with adenosine triphosphate (ATP) prevented Px1 activation, suggesting that hemichannels may be activated during periods of mitochondrial dysfunction and metabolic failure. Our findings have potential implications for the treatment of diseases such as cerebral ischemia and Alzheimer’s disease (AD) as they implicate two novel ion channels in the excitotoxic signaling cascade activated downstream of NMDARs.
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Who did what:

All electrophysiological experiments described in this thesis in cultured and acutely isolated hippocampal neurons were designed, carried out, and analyzed by myself with critical input from Dr. Mike Jackson and my supervisor, Dr. John F. MacDonald. Dr. Mike Jackson carried out some recordings in cultured hippocampal neurons in parallel with me and his data contribute to the $n$ values presented in these cases. This was essential in order to make full use of batches of cultured cells that demonstrated TRPM2-like currents. Recordings in slice were carried out by Drs. Yael Perez and Hong Bin Li.

The molecular and biochemical results presented were designed, carried out and analyzed by me (Westerns, TRPM2 immunostaining), Dr. S. Kiyonaka (in situ hybridization) and Dr. Hong Sun (TRPM2 shRNA immunostaining).
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LIST OF ABBREVIATIONS

ACA - N-(p-amylcinnamoyl)anthranilic acid
ACSF - artificial cerebrospinal fluid
AD – Alzheimer’s disease
ADPR - adenosine diphosphate (ribose)
AMPA - a-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ATP - adenosine triphosphate
BAPTA - 1,2-bis(o-aminophenoxy)ethane-N,N,N,N-tetraacetic acid
CA - cornu ammonis (region of hippocampus)
CaM - calmodulin
CLT - clotrimazole
CNS - central nervous system
CREB - cyclic AMP response element binding
Cx - connexin
DG - dentate gyrus
DIV - days in vitro
EC - entorhinal cortex
ECS/ICS - extracellular solution/intracellular solution
EGTA - ethylene glycol tetraacetic acid
FFA - flufenamic acid
GFP - green fluorescent protein
GPCR - G-protein coupled receptor
HEK - human embryonic kidney
I_{pe} – post-exposure current

I_{2nd} – NMDA secondary current

MEM - minimal essential medium

mGluR - metabotropic glutamate receptor

mPTP - mitochondrial permeability transition pore

NAADP - nicotinic acid-adenine dinucleotide phosphate

NAD - nicotinamide adenine dinucleotide

NOS - nitric oxide synthase

NU Dix/ NUDT9 - nucleotide diphosphate linked to X/ NUDIX type motif 9

OGD - oxygen glucose deprivation

P2X - purine receptor, class 2, ligand gated ion channel

PARG/PARP - poly-(ADP-ribose) glycohydrolase/polymerase

PSD - postsynaptic density

Px - pannexin

RNAi - RNA interference

ROS/RNS - reactive oxygen/nitrogen species

RT-PCR - real time polymerase chain reaction

shRNA - short hairpin RNA

siRNA - small interfering RNA

TNF - tumor necrosis factor

TRP(C,M,V) - transient receptor potential (canonical, melastatin, vanilloid)

VDCC - voltage dependent calcium channel
Introduction

1.1. The excitotoxic model of cell death

N-methyl-D-aspartate receptors (NMDARs) form a structurally and pharmacologically defined class of ionotropic glutamate receptors that generate essential physiological signals required for synaptic development and plasticity (Dingledine et al., 1999). Over-activation of these receptors, however, has been implicated in a variety of pathological states including cerebral ischemia (stroke) (Arundine & Tymianski, 2004), epilepsy (Meldrum, 1993) and Alzheimer's disease (Doraiswamy, 2003). This process was termed excitotoxicity by Olney in the late 1960s and describes the mechanism whereby excessive release of glutamate overexcites neurons to their eventual demise (Olney, 1969). While over-activation of NMDARs also induces Na+ influx and consequent cell swelling, loading of neurons with Ca2+ through these receptors is widely believed to be the requisite step leading to the delayed cell death and damage seen in pathological states such as stroke (Choi et al., 1987). However, in spite of promising results derived from animal studies (Simon et al., 1984; Ozyurt et al., 1988), clinical trials for drugs that block NMDARs have failed to benefit stroke patients, as they are effective only when administered before or during a short therapeutic window following the ischemic attack (Ikonomidou & Turski, 2002). While it is clear that novel therapeutic means of minimizing the debilitating effects of stroke must be found, targeting potential mediators of neuronal death that lie downstream of NMDAR activation appears a more viable option.
1.1.1. Changes in synaptic transmission during ischemia

The amino acid L-glutamate is the major excitatory neurotransmitter in the brain and spinal cord, where it is released from pre-synaptic terminals (Biscoe & Straughan, 1966; Curtis et al., 1972; Teichberg et al., 1981; Dolphin et al., 1982; Walker et al., 1995). Chemical synaptic transmission is initiated at the pre-synaptic terminal upon the Ca$^{2+}$-dependent release of glutamate. Glutamate then diffuses across the synaptic cleft and binds to its receptors on the post-synaptic membrane. Na$^+$-influx through the ensuing activation of $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors is the predominant contributor to depolarization of the post-synaptic membrane and the subsequent post-synaptic response. Under physiological conditions, the glutamate-mediated gating of AMPA receptors causes sufficient depolarization of the post-synaptic membrane to partially relieve the voltage-dependent Mg$^{2+}$-block of NMDA receptors (Mayer et al., 1984; Nowak et al., 1984). Consequently, NMDA receptor-mediated currents contribute to the late phase of the excitatory post-synaptic potential. However, the number of glutamate molecules released by the pre-synaptic terminal is insufficient to depolarize the membrane to an extent where there is complete relief of Mg$^{2+}$ block (Hausser et al., 2000). This functions to keep NMDAR-mediated signaling in check and works to prevent the inappropriate activation of pathological signaling cascades.

During ischemia, abnormalities in glutamatergic signaling occur and these are associated with the delayed death of neuronal cells. In 1984, Diemer’s group was the first to demonstrate that ischemia caused a rise in the level of extracellular glutamate in the rat hippocampus (Benveniste et al., 1984), a finding that has since been corroborated by several groups (Erecinska et al., 1984; Korf et al., 1988; Torp et al., 1993). Given the complexity of
glutamate-dependent signaling events, it is not surprising that changes in several processes likely lead to this increase. While exocytotic release was widely believed to be the predominant source of increased glutamate, only the early phase of ischemia-induced glutamate increase was prevented in the absence of Ca$^{2+}$ (Wahl et al., 1994). More likely of greater consequence, the loss of transmembrane ionic gradients and intracellular acidosis that occur during ischemia impair and possibly even reverse the function of the astrocytic glutamate/aspartate transporter (Rossi et al., 2000). Under physiological conditions, this Na+-dependent transporter clears glutamate from both the synaptic cleft, in particular, and extracellular space, in general, and serves to attenuate glutamatergic signaling (Schousboe et al., 1977). Its failure during ischemia likely contributes significantly to the rise in extracellular glutamate. Regardless of its source, high extracellular glutamate concentrations during ischemia lead to the inappropriate activation of post-synaptic glutamate receptors, including NMDARs.

1.2. Calcium and neurotoxicity

Calcium ions play important roles as intracellular messengers and are implicated in the regulation of diverse physiological processes such as cell growth, membrane excitability, exocytosis and neuronal activity. In large part due to its ubiquitous role as a signaling molecule, cellular Ca$^{2+}$ homeostasis is tightly regulated (Miller, 1991; Zhou & Neher, 1993; Sattler & Tymianski, 2000). At rest, the concentration of free intracellular Ca$^{2+}$ ([Ca$^{2+}$]) is buffered to approximately 100 nM (Gorman et al., 1984), 100 times lower than in the extracellular space. This low concentration is essential for enhancing the signal to noise ratio within the cytoplasm and establishes an environment in which small or localized increases in
[Ca\(^{2+}\)]_i are sufficient to initiate Ca\(^{2+}\)-dependent events (Sattler & Tymianski, 2000). Both the regulation of [Ca\(^{2+}\)]_i and the location of these ions within cells is accomplished by the complex interplay between Ca\(^{2+}\) influx, efflux, buffering and internal Ca\(^{2+}\) storage. In this way, local rises in [Ca\(^{2+}\)]_i can effectively and appropriately activate nearby enzymes and ion channels.

Neurons are the sites of varied Ca\(^{2+}\)-dependent events, many of which are regulated independently and via distinct signaling pathways. Under physiological conditions, these processes function in harmony within the cell’s Ca\(^{2+}\)-buffering capacity. However, excessive Ca\(^{2+}\) entry via either influx from the extracellular space or release from intracellular stores likely overwhelms the cell’s intrinsic regulatory mechanisms and results in the inappropriate activation of neurotoxic, Ca\(^{2+}\)-dependent pathways (Schanne et al., 1979). While early work suggested that the amount of neuronal death was directly related to the degree of Ca\(^{2+}\) accumulation (Manev et al., 1989; Marcoux et al., 1990), a large body of evidence now indicates that the site of Ca\(^{2+}\) entry into neurons is a better predictor of the extent of neurotoxicity. For example, depolarizing neurons with high extracellular potassium or non-NMDA-type glutamate receptor agonists resulted in less neuronal death than was predicted based only on the net rise of [Ca\(^{2+}\)]_i (Hartley et al., 1993). These findings were extended using free Ca\(^{2+}\) indicators, which measure relative changes in Ca\(^{2+}\) and demonstrated that, in cultured embryonic spinal neurons, Ca\(^{2+}\) loading through NMDARs was toxic to neurons while loading of similar amounts of Ca\(^{2+}\) through voltage-dependent Ca\(^{2+}\) channels (VDCCs) was not (Tymianski et al., 1993b). Later work in which Ca\(^{2+}\)-influx pathways were pharmacologically isolated and the total amount of Ca\(^{2+}\) loading measured supported the idea that the site of Ca\(^{2+}\) influx determines the extent of neurotoxicity (Sattler et al., 1998).
1.2.1. **A secondary rise in intracellular Ca\(^{2+}\) following excitotoxic stimulation**

Despite strong evidence for a relationship between Ca\(^{2+}\)-overload and neuronal death, the precise mechanism whereby Ca\(^{2+}\)-influx through NMDARs results in neurotoxicity remains elusive. It is clear, however, that the primary rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) during NMDA exposure is followed by a secondary, post-exposure rise in [Ca\(^{2+}\)]\(_i\) that is not inhibited by application of NMDAR antagonists. Moreover, evidence from correlative studies implicated this secondary increase in [Ca\(^{2+}\)]\(_i\) as the causal factor in delayed neuronal death (Randall & Thayer, 1992; Tymianski *et al.*, 1993a). Experiments by Chen *et al.* (1997) demonstrated that transient application of NMDA to acutely isolated hippocampal neurons induced a continuously increasing inward current after removal of the NMDA stimulus. The current, termed the postexposure current (I\(_{pe}\)), increased in amplitude until cell death occurred and its suppression prevented neuronal death even after NMDAR activation. Furthermore, I\(_{pe}\) was associated with an influx of Na\(^+\) and Ca\(^{2+}\) into neurons and exhibited a linear current-voltage relationship that reversed near 0 mV, consistent with the notion that I\(_{pe}\) is a non-selective cation conductance (Chen *et al.*, 1997). When NMDA was applied in the absence of extracellular Ca\(^{2+}\), I\(_{pe}\) was not activated and the ensuing neuronal death prevented. These data provide a mechanistic link between activation of NMDARs and cell toxicity, whereby an NMDAR-mediated rise in [Ca\(^{2+}\)]\(_i\) activates a cation conductance that is itself permeable to Ca\(^{2+}\).
1.2.2. The “Ca\textsuperscript{2+} paradox” is analogous to ischemia-reperfusion injury after stroke

Over-activation of NMDARs during anoxia can lead to a dramatic fall in extracellular Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]e) (Silver & Erecinska, 1990). The decrease in [Ca\textsuperscript{2+}]e during the ischemic period is followed by a rapid rise in [Ca\textsuperscript{2+}]e once over-stimulation of NMDARs by glutamate has ceased. This situation is at least in part analogous to the changes in [Ca\textsuperscript{2+}]e that occur following reperfusion of tissue made ischemic during stroke (Hearse et al., 1978). Tissue damage caused by ischemia-reperfusion injury is not limited to the brain and also occurs notably in the gut, liver, kidney and heart (Ferrari et al., 1993; Cuzzocrea & Wang, 2005). In fact, studies carried out in the 1960s on isolated rat heart revealed that perfusion of the heart with Ca\textsuperscript{2+}-free solution prior to the reintroduction of Ca\textsuperscript{2+} caused a rapid and irreversible contracture of the myocardium followed by cell death (Zimmerman & Hulsmann, 1966). This phenomenon, whereby removing extracellular Ca\textsuperscript{2+} results in Ca\textsuperscript{2+}-overloading of cells upon its reintroduction, is termed the “calcium paradox.” More recently, the complexity of the “calcium paradox” has increased. Studies in hippocampal neurons have demonstrated that after simply decreasing extracellular Ca\textsuperscript{2+}, and not eliminating it entirely, the influx of Ca\textsuperscript{2+} into cells was enhanced (Xiong et al., 1997). This “Ca\textsuperscript{2+}-sensing” current responded to transient decreases in extracellular divalents, was outwardly rectifying in the presence of extracellular divalent cations and was inhibited by intracellular Mg\textsuperscript{2+} (Xiong et al., 1997; Wei et al., 2007). Our previous results indicate that one member of the melastatin family of transient receptor potential channels, TRPM7, mediates the response to low [Ca\textsuperscript{2+}]e in neurons (Wei et al., 2007). Taken together, these results suggest a role for extracellular Ca\textsuperscript{2+} in regulating the influx of Ca\textsuperscript{2+} into cells.
1.3. **A role for TRPM2 activation in mediating delayed neuronal death after stroke**

Unlike TRPM7, which conducts only small inward currents in the presence of extracellular divalent cations, the activation of another member of this family of channels, TRPM2, requires extracellular Ca\(^{2+}\). Consequently, its activation may underlie the second, “reperfusion” phase of the “calcium paradox” and delayed cell death in hippocampal neurons. Although significant characterization of TRPM2 channels has been carried out in cell lines and immune cells, their function in hippocampal neurons remains unknown (Perraud *et al.*, 2001; Heiner *et al.*, 2005). Initially in fact, the existence of functional TRPM2 in neurons was not widely accepted (Perraud *et al.*, 2003). TRPM2 is highly expressed in immune cells and brain, where the majority of the signal is attributed to expression of TRPM2 in microglia. These channels exhibit a linear current-voltage relationship, are Ca\(^{2+}\)-permeable and are believed to respond to changes in oxidative stress (Perraud *et al.*, 2001; Sano *et al.*, 2001; Hara *et al.*, 2002; Perraud *et al.*, 2003). They are gated by the binding of intracellular ADP-ribose to the channel’s enzymatic Nudix domain (Perraud *et al.*, 2001). The sensitivity of this activation is enhanced by elevated [Ca\(^{2+}\)]\(_i\), (McHugh *et al.*, 2003), which can occur following overactivation of NMDARs in ischemia. Furthermore, endogenous ADP-ribose likely rises during ischemia due to resulting metabolic disturbances and consequent activation of DNA repair enzymes, leading to activation of TRPM2 (Fonfria *et al.*, 2004). Interestingly, extracellular application of the oxidant hydrogen peroxide has also been demonstrated to activate TRPM2, although the gating-mechanism appears distinct from that of ADP-ribose-mediated gating (Wehage *et al.*, 2002). Regardless of the activator, a sustained TRPM2-mediated increase in [Ca\(^{2+}\)]\(_i\) is observed upon channel activation and cell death ensues (Hara *et al.*, 2002). Furthermore, Ca\(^{2+}\) influx
through NMDARs may play a role in mediating delayed neuronal death following stroke by providing a continuous driving force for ADP-ribose production and activation of TRPM2.

1.4. The hippocampus and why we study it

A part of the limbic system and therefore intimately linked with human emotion (at least historically) (Hayman et al., 1998), attention (Kaada et al., 1949; Holmes & Adey, 1960) and memory (Orbach et al., 1960), the hippocampal formation lies deep within the medial temporal lobe of the brain. This formation consists of a group of brain structures that includes the dentate gyrus (DG), hippocampus, subiculum, presubiculum, parasubiculum and entorhinal cortex (EC). Although the terms “hippocampal formation” and “hippocampus” are often used interchangeably, the hippocampus proper typically refers to the region that comprises the CA fields, CA3, CA2 and CA1. Historically, the regions encompassing the hippocampal formation were believed to be linked in a predominantly unidirectional excitatory circuitry termed the trisynaptic circuit (synapse 1: EC to DG, synapse 2: DG to CA3, synapse 3: CA3 to CA1) (Andersen et al., 1969). While the notion of a unidirectional, trisynaptic circuit simplifies the study of many hippocampal-dependent processes, the finding that CA1 neurons project back to the subiculum (Amaral et al., 1991) and EC (Naber et al., 2001) must be taken into account when considering hippocampal function as a whole.

The hippocampus is organized in a clear, laminar pattern, with a tightly-packed pyramidal (principal) cell layer in the CA1 region. More loosely packed in the CA2 and CA3 regions, this layer contains the pyramidal cell bodies. The basal dendrites sit in the largely cell-free stratum oriens, located deep to the principle layer, while the apical dendrites extend through the stratum radiatum and the stratum lacunosum-moleculare. Interestingly,
Unlike their CA3 counterparts (Ishizuka et al., 1990), the dendritic trees of CA1 pyramidal neurons are relatively similar from cell to cell (Pyapali et al., 1998). However, this finding does not necessarily correlate with functional homology as the lateral EC projects to the distal portion of CA1 while the medial EC projects to the proximal region of CA1 (Dolorfo & Amaral, 1998).

Despite these differences, CA1 pyramidal cells and the hippocampus are commonly studied due, in part, to several unique features. The hippocampal’s laminar structural arrangement allows for the visual identification of different cell types. This permits the study of distinct neuronal subtypes and circuitry in slice preparations. Furthermore, the fact that the hippocampus is readily distinguished from the cortex and other brain structures facilitates its dissection from the intact brain. In this way, we can be confident that cultured hippocampal preparations contain the cells of interest and not those of surrounding regions. Finally, the particular sensitivity of the hippocampus to ischemic insult (Schmidt-Kastner & Freund, 1991) makes it a useful preparation to study the activation of novel Ca\(^{2+}\)-dependent currents downstream of over-activated NMDARs.
While we hypothesize that TRPM2 channels are activated downstream of NMDARs, the ubiquity of Ca\(^{2+}\)-signaling strongly suggests that multiple pathways are activated in tandem. The following section consists of a discussion of glutamate receptors in the hippocampus and three potential sources of Ca\(^{2+}\)-entry into neurons following stimulation of NMDARs: TRPM2, TRPM7 and pannexin hemichannels, which are activated following OGD of hippocampal neurons (Thompson et al., 2006).

1.5. **Glutamate receptors**

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) and exerts its effects by binding to two major classes of receptors: the ionotropic and metabotropic glutamate receptors. These two classes are distinguished on the basis of their pharmacological, biophysical and molecular profiles (Dingledine et al., 1999). The eight metabotropic glutamate receptors (mGluR1-8) are dimeric, seven transmembrane domain G-protein-coupled receptors (GPCRs). Although binding of glutamate stabilizes them in the active conformation (Kunishima et al., 2000), it is the G proteins themselves that are coupled to downstream signaling pathways and therefore exert the majority of mGluR-mediated effects (Pin & Duvoisin, 1995). The ionotropic glutamate receptors are ligand-gated receptors that are subdivided into AMPA (\(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), kainate and NMDA (\(N\)-methyl-\(D\)-aspartic acid) receptor classes. While these classes are named after the relatively selective compounds used to activate the corresponding receptor, AMPA, NMDA and kainate do not occur naturally within the brain (Gasic & Hollmann, 1992; McBain & Mayer, 1994; Nakanishi et al., 1998).
In the hippocampus, most excitatory neurotransmission is mediated by post-synaptic AMPA and NMDA receptors. These two classes of receptors have different kinetic, permeation and pharmacological profiles. Although kainate receptors share many characteristics with AMPA receptors, their role in synaptic transmission is, by comparison, poorly understood. Since the focus of this thesis is on the activation of ion channels downstream of NMDA receptors, the remainder of the discussion on glutamate receptors will focus on this class of ionotropic receptors.

1.5.1. **NMDA receptors**

1.5.1.1. **Structure of NMDA receptors**

NMDA receptors are widely expressed throughout the CNS and generate important excitatory signals required for synaptic development and plasticity. This class of ligand-gated ionotropic glutamate receptors is encoded by three gene families that give rise to the NR1, NR2 and NR3 subunits. Eight different NR1 subunit splice variants exist as a result of alternative splicing of the extracellular N- and intracellular C-termini. In addition, four distinct genes encoding the NR2 subunit have been identified. Accordingly, the NR2 subunit exists in one of four forms, NR2A through NR2D (Dingledine *et al.*, 1999). Of note, the NR3 subunit does not seem to play an important role in the hippocampus. The subunit stoichiometry of NMDARs is a subject of some controversy. Based on the size of chemically cross-linked receptors, NMDARs were initially believed to be pentamers (Brose *et al.*, 1993). However, experiments by (Behe *et al.*, 1995), in which various subunits were coexpressed in oocytes, identified single channel conductance patterns consistent with each NMDAR being
composed of two NR1 and two NR2 subunits. More recent evidence supports this tetrameric model of the NMDAR (Dingledine et al., 1999; Furukawa et al., 2005). Irrespective of the existence of a pentameric or tetrameric subunit stoichiometry for NMDARs, coexpression studies in non-neuronal cell lines indicated that each receptor must consist of at least one glycine-binding NR1 subunit and at least one agonist-binding NR2 subunit in order to form functional receptors (Monyer et al., 1992).

1.5.1.2. Temporal and spatial variability in NMDAR subunit expression

NMDAR subunits exhibit variable expression patterns depending on both the stage of development and subcellular localization. Evidence for the changing subunit composition of NMDARs throughout development first emerged from Lily Jan’s laboratory in 1994. While the group initially demonstrated that mRNA and protein levels of NR1, NR2A and NR2B subunits were developmentally regulated, coimmunoprecipitation assays directly confirmed that the subunit composition of native NMDARs changed following a similar pattern. The NR2B subunit was coimmunoprecipitated with NR1 from postnatal day 1 (P1) cortical membranes through the end of the testing period (P53). Conversely, no detectable NR2A was coimmunoprecipitated with NR1 at P1. However, the amount of NR2A coimmunoprecipitated with NR1 increased gradually and reached adult levels by P21. Interestingly after P7, NR2A and NR2B were coimmunoprecipitated by antibodies against either subunit, pointing to the existence of NR1/NR2A/NR2B heterotrimers (Sheng et al., 1994).

Synaptic NMDARs in both adult hippocampal and cortical neurons were found to contain the NR2A subunit (Stocca & Vicini, 1998; Tovar & Westbrook, 1999) and NR2A-
containing NMDARs are widely believed to be the most prevalent NMDAR at the synapse. Conversely, several studies point to the presence of a high proportion of NR2B-containing receptors at extrasynaptic versus synaptic sites. In both hippocampal pyramidal neurons and cerebellar Golgi cells, high frequency stimulation recruited a large NR2B-mediated component that was not observed when glutamate spillover was restricted with a single stimulus (Brickley et al., 2003; Lozovaya et al., 2004). Furthermore, glutamate from both (1) spillover from neighbouring neuronal synapses and (2) astrocytic sources activated NMDA responses, which were blocked by NR2B antagonists (Fellin et al., 2004; Scimemi et al., 2004). However, the relegation of NR2B subunits to extrasynaptic sites and NR2A subunits to synaptic ones is not absolute (Li et al., 1998; Thomas et al., 2006). Distinct signaling pathways are linked to synaptic versus extrasynaptic NMDARs and this, rather than their subunit contribution exclusively, may contribute to their different properties. This occurs in large part via the coupling of synaptic NMDARs to PSD-95 in the post-synaptic density and may explain the finding that synaptic NMDARs are more susceptible to Ca\(^{2+}\)-dependent “run-down” (Li et al., 2003). Interestingly, the activation of synaptic NMDARs has been linked to neuronal survival via a CREB-dependent mechanism. Conversely, stimulation of extrasynaptic NMDARs inhibited CREB function and lead to the activation of cell death pathways (Hardingham et al., 2002). Although it is tempting to consider NR2B/extrasynaptic receptors as mediators of cell death and NR2A/synaptic receptors as pro-survival channels, the relationship between the subcellular localization of NMDARs, the subtype of receptor expressed and associated downstream signaling pathways is complex. As further evidence in support of this, the activity of synaptic NMDARs has been implicated
in neuronal death, as uncoupling PSD-95 from these receptors was found to be neuroprotective (Sattler et al., 2000).

1.6. TRP channels

Transient receptor potential (TRP) proteins constitute a large and functionally diverse family of ion channels that are related on the basis of primary amino acid sequence and cation permeability. TRP proteins are putative six-transmembrane domain (S1-S6) subunits that assemble to form non-selective cation channels, with most members exhibiting at least some permeability to Ca$^{2+}$. Although their primary structure contains seven hydrophobic regions, comparisons with other ion channels such as the voltage-gated K$^+$ channels and mutagenesis experiments strongly suggest that TRP channels possess six membrane-spanning segments. In fact, most of what is understood about TRP channel structure results from analogy with the comparatively well-studied voltage-gated K$^+$ (Kv) class of ion channels. Like Kv channel subunits, TRP subunits likely assemble to form homo- or heterotetrameric cation channels with a pore-forming loop between S5 and S6 and intracellular amino- and carboxy-termini (Clapham et al., 2001).

TRP channels were first identified in Drosophila photoreceptors, where a spontaneous mutant lacking the TRP protein exhibited transient receptor potentials in response to continuous light stimulation (Cosens & Manning, 1969; Minke, 1977). Mammalian homologues of Drosophila TRP channels have now been identified and classified into six protein families based on sequence identity, the largest of which are the classical (TRPC), vanilloid (TRPV) and melastatin (TRPM) families. Although known functions of mammalian TRP channels are diverse, the notion that TRP channels sense and respond to
changes in their extracellular environments may act as a unifying functional theme (Clapham, 2003).

1.6.1. **TRPM channels**

TRPM channels remain the most poorly understood members of the TRP channel family, in large part due to their complexity and diversity of function. In 1998, the founding TRPM member, TRPM1, was cloned and named melastatin for its putative tumor suppressor function in melanoma cell lines (Duncan et al., 1998). Since then, seven other members of the TRPM family of channels have been identified, each with unique gating mechanisms and ion permeability profiles. Although our understanding of the physiological and pathological functions of TRPM channels is relatively limited, their diverse structural and biophysical profiles correlate strongly with the broad array of functional properties linked to TRPM channel activation. For example, TRPM8 activity underlies cold-temperature sensations (Peier et al., 2002), TRPM3 regulates osmolarity (Grimm et al., 2003) and TRPM5 is involved in producing sweet, umami and bitter taste perception (Zhang et al., 2003b).

1.6.1.1. **TRPM channel structure**

Structurally, TRPM channels are united by an approximately 700 amino acid N-terminus “TRPM-homology region,” which is found in all eight members of the TRPM family but not in any other molecule identified to date (Perraud et al., 2004). The evolutionary function of this region of homology remains unknown. However, splice variants of either TRPM1 or TRPM2 channels containing the N-terminal domain or the N-
terminal domain plus the transmembrane-spanning region, respectively, act as dominant negatives to suppress channel function (Xu et al., 2001; Zhang et al., 2003a). Furthermore, the N-terminal region appears to be involved in assembly and/or trafficking of TRPM channels, as deletion of the first 110 amino acids resulted in the loss of TRPM2 channels at the cell membrane, despite high levels of protein (Perraud et al., 2003). This result likely underlies the failure to observe TRPM2 currents in two variants missing short segments of the N-terminal domain (Hara et al., 2002; Wehage et al., 2002).

Given the limited sequence homology within the TRPM family itself, it is not surprising that TRPM channels share little homology with their TRPV and TRPC channel brethren. A small, approximately 250 amino acid region that constitutes the six TRP transmembrane-spanning domains is the sole region of homology with other TRP family members. Moreover, despite the presence of large intracellular termini, TRPM channels have even fewer similarities to other known ion channels. The high level of diversity of the large C-terminus suggests that this region may play a role in the unique functions of each TRPM channel (Perraud et al., 2004). Of interest, select members of the TRPM family are particularly unusual as they consist of a TRP channel linked to a carboxy-terminal enzyme domain. These ion channel/enzyme fusions are termed chanzymes. TRPM2 is linked to an ADP-ribosepyrophosphatase (Perraud et al., 2001) while the closely related TRPM7 and TRPM6 proteins contain a fused, atypical protein kinase domain at their C-terminal ends (Runnels et al., 2001; Schlingmann et al., 2002; Ryazanova et al., 2004). To date, these three members of the TRPM family are the only known ion channels with this unique, chzyme distinction (Montell, 2003; Scharenberg, 2005).
1.6.2. TRPM7 channels

TRPM7 activity was first identified as a requirement for cell viability due to its Mg\(^{2+}\)-permeability and consequent role in regulating the intracellular Mg\(^{2+}\) concentration (Nadler et al., 2001). Paradoxically, overexpression of TRPM7 channels in HEK293 cells was also linked to cell death in this and other studies (Nadler et al., 2001; Monteilh-Zoller et al., 2003; Schmitz et al., 2003). TRPM7 is highly permeable to both Mg\(^{2+}\) and Ca\(^{2+}\), and exhibits an outwardly-rectifying current-voltage relationship that is modified by the presence of extracellular divalents (Runnels et al., 2002; Wei et al., 2007). The permeation of divalent cations through TRPM7 channels obstructs the inward flow of monovalent ions (Nadler et al., 2001). However, during ischemia, the extracellular concentration of Ca\(^{2+}\) falls, suggesting that TRPM7 may be a mediator of Na\(^+\)- and, to a lesser extent, Ca\(^{2+}\)-loading of neurons.

Previous studies in our lab and our collaborator’s unveiled a novel pathway for Ca\(^{2+}\) influx into neurons in an in vitro model of stroke. Blocking NMDARs during anoxia revealed the presence of a sustained depolarizing current (I\(_{\text{anoxia}}\)) with properties matching those of the TRPM7 current. I\(_{\text{anoxia}}\) was activated by the production of reactive oxygen/nitrogen species (ROS), which occurs during ischemia following a rise in [Ca\(^{2+}\)]\(_i\) via overactivated NMDARs. Inhibiting TRPM7 expression permitted survival of neurons that would otherwise have died due to prolonged anoxia, indicating that I\(_{\text{anoxia}}\) was likely mediated by TRPM7 channels (Aarts et al., 2003). However, two properties of this channel make it is unlikely that TRPM7 is the final mediator Ca\(^{2+}\)-overload and delayed cell death in neurons: (1) the inward current contributed by TRPM7 is minimal in the presence of extracellular divalents and (2) if TRPM7 carries a substantial inward current under conditions
of low extracellular Ca\(^{2+}\), the major component of this current is likely Na\(^+\). Taken together, this reasoning suggests that TRPM7’s ability to directly mediate Ca\(^{2+}\)-loading of neurons is marginal. However, Na\(^+\) (and Ca\(^{2+}\), to a lesser extent) influx through TRPM7 channels may play a role in mediating delayed neuronal death following an ischemic insult by providing a continuous driving force for ADP-ribose production and activation of TRPM2.

1.6.3. **TRPM2 channels**

1.6.3.1. **TRPM2 expression and structure**

The TRPM2 gene is located on a region of chromosome 21 that is associated with a wide range of human diseases including non-syndromic hereditary deafness (Veske et al., 1996) and bipolar disorder (Straub et al., 1994). In an attempt to search for candidate genes underlying these disorders, TRPM2 was isolated and cloned due to its sequence homology with *C. elegans* genes and both Drosophila and human *trp* genes. Analysis of the deduced amino acid sequence indicated that TRPM2 likely functions as a Ca\(^{2+}\)-permeable ion channel. Furthermore, Northern blot assays revealed that TRPM2 was highly expressed in the brain, with comparatively weaker expression in the heart, placenta and lungs (Nagamine et al., 1998). Later RT-PCR studies extended these results by demonstrating that TRPM2 is expressed in a wide variety of other tissues including bone marrow, spleen and liver (Sano et al., 2001).

TRPM2 contains a NUDIX domain in its C-terminal end with significant sequence homology to NudT9, a mitochondrial ADP-ribose-hydrolase (ADPRase) (Perraud et al., 2001; Sano et al., 2001; Hara et al., 2002). NUDIX domain-containing enzymes form a
family of over 300 proteins believed to function as housekeeping enzymes by breaking-down toxic compounds in cells (Bessman et al., 1996). While the TRPM2 NUDIX domain possesses ADPRase activity, differences at several sites in the consensus sequence result in drastically decreased enzymatic activity when compared to that of NudT9 (Perraud et al., 2001). Introduction of these sequence alterations into NudT9 reduced its intrinsic enzymatic activity to a level comparable to that of the TRPM2 NUDIX domain, indicating that differences at these sites in the NUDIX domain account for TRPM2’s weak ADPRase activity (Shen et al., 2003).
**Figure 1** – Schematic model of the structural domains of a TRPM2 channel subunit embedded in the lipid bilayer. Salient features include 6 transmembrane domains (S1-S6) with a pore-forming loop between S5 and S6, intracellular N- and C- termini, an N-terminal TRPM homology region (HR) and a C-terminal NUDIX box (NUDT9-H) possessing ADP-ribase activity.
1.6.3.2. **Biophysical properties of TRPM2**

The presence of the C-terminal NUDIX domain in TRPM2 prompted studies by two independent groups to determine if TRPM2 were gated by ADPR or a related nucleotide. Both groups demonstrated that, in HEK 293 cells over-expressing recombinant TRPM2, inclusion of micromolar concentrations of ADPR in the patch-pipette activated a current with large inward and outward components, a linear current-voltage (IV) relationship and reversal potential (E\text{rev}) equal to 0 mV. Single channel recordings from inside-out excised membrane patches demonstrated that TRPM2 has a single channel slope conductance of 60 pS (Perraud et al., 2001) and that it was directly activated by ADPR, most likely by ADPR binding to the channel’s NUDIX homology domain (Perraud et al., 2001; Sano et al., 2001). Importantly, the sensitivity of this activation was enhanced by elevated [Ca$^{2+}$], (McHugh et al., 2003). Furthermore, ion substitution experiments indicated that the channel was equally permeable to the monovalent cations Na$^+$, K$^+$ and Cs$^+$ and the divalent cation Ca$^{2+}$ (Perraud et al., 2001; Sano et al., 2001). Taken together, these results indicate that TRPM2 is a Ca$^{2+}$-permeable, non-selective cation channel gated by ADPR.

1.6.3.3. **TRPM2 gating**

Although the role of TRPM2 as a non-selective, Ca$^{2+}$-permeable cation channel is well established, some debate exists surrounding its mechanism of gating. Sano et al. (2001) describe the delayed gating of TRPM2 by millimolar nicotinamide adenine dinucleotide
(NAD), a precursor of ADPR, while Perraud et al. (2001) found that NAD failed to activate TRPM2. Since NAD is a precursor of ADPR, the delayed activation of TRPM2 might in fact be mediated by ADPR generated from the breakdown of NAD. However, single channel analysis from inside-out patches indicated that application of NAD could rapidly activate TRPM2 current (Sano et al., 2001; Hara et al., 2002). The comparatively high concentration of NAD versus ADPR required to gate TRPM2 suggests that the NAD solution contained sufficiently high levels of contaminating ADPR due to the hydrolysis of NAD. More recently, information derived from structural modeling of the NudT9 domain indicated that the breakdown of NAD to ADPR is necessary for gating of TRPM2 (Shen et al., 2003).

In addition to gating by adenine nucleotides, a large body of evidence strongly implicates changes in oxidative stress in the gating of TRPM2 channels. Hara et al. (2002) first reported that extracellular application of micromolar hydrogen peroxide (H2O2) elicited a significant rise in [Ca$^{2+}$], in HEK 293 cells transiently expressing TRPM2. This increase in [Ca$^{2+}$], was sustained and resulted in cell death. These results were extended to endogenously expressed TRPM2 channels as siRNA-mediated suppression of TRPM2 in rat insulinoma RIN-5F cell also prevented the H2O2-induced rise in [Ca$^{2+}$], and ensuing cell death (Hara et al., 2002). Interestingly, whole-cell voltage clamp recordings demonstrated that a TRPM2 mutant lacking a 34 amino acid sequence in its C-terminal domain was insensitive to gating by intracellular ADPR yet responded to 5 mM H2O2 (Wehage et al., 2002). These results indicate that oxidant-induced gating of TRPM2 acts via a distinct mechanism versus that mediated by ADPR and suggests the existence of a dual activation pathway. While the above evidence strongly suggests that, like ADPR, H2O2 can directly activate TRPM2 channels, an alternate model exists whereby oxidative stress results in the
production of endogenous ADPR and consequent TRPM2 activation. Both mechanisms may act in parallel to activate TRPM2 channels in vivo.

1.6.3.4. Sources of ADPR production

Under conditions of oxidative stress, H2O2 and other reactive species induce oxidative DNA damage and subsequent activation of poly(ADP-ribose) polymerase 1 (PARP-1), the most abundant isoform of the nuclear PARP family of enzymes. This damage can also be induced by nitrosative stress, which causes comparatively more DNA damage (Szabo & Ohshima, 1997) and likely activates PARP-1 to a greater extent. PARP-1 is a highly conserved DNA-binding protein that catalyzes the post-translational modification of proteins via poly-ADP-ribosylation. PARP-1 is activated upon binding to breakage sites on damaged DNA and employs NAD as a substrate to polymerize ADPR onto nuclear proteins, a critical process for DNA repair (de Murcia et al., 1994). However, the overactivation of PARP-1 leads to the depletion of NAD stores, energy failure due to a decrease of ATP and consequent cell death (Zhang et al., 1994; Berger et al., 1996; Eliasson et al., 1997; Endres et al., 1997).

Neurons contain high levels of PARP-1 activity after periods of oxidative stress (Yu et al., 2003) and this may, in part, explain their susceptibility to PARP-1-dependent, NMDA-mediated cell death (Mandir et al., 2000). Interestingly, the poly-ADP-ribsylation of nuclear proteins by PARP-1 is short-lived, in large part due to the activity of the antagonist enzyme PARG (Lu et al., 2003). The resulting free ADPR may then exit the nucleus and directly gate TRPM2 channels on the plasma membrane. In fact, Fonfría et al., 2004 demonstrated that three, structurally distinct PARP inhibitors prevented the H2O2-induced
activation of TRPM2 currents in both recombinant and endogenously expressing cell systems. Furthermore, these inhibitors had no effect on ADPR-activated TRPM2, indicating that the effects of PARP inhibitors occurred upstream of channel gating by ADPR.

In addition to the generation of ADPR in the nucleus, mitochondria offer an alternative source of free ADPR in cells. Mitochondria contain millimolar levels of NAD and have been demonstrated to serve as sites of NAD hydrolysis in response to oxidative stress in vitro (Richter et al., 1990; Schweizer et al., 1993). Under these conditions, the hydrolysis of NAD has been associated with the opening of the mitochondrial permeability transition pore (mPTP) (Di Lisa et al., 2001), which allows the passage of molecules up to 1500 Da from the mitochondria into the cytoplasm (Massari & Azzone, 1972). In isolated brain mitochondria, NAD release was prevented in the presence of the mPTP inhibitor 2-aminoethoxy-diphenyl borate (Chinopoulos et al., 2003). This NAD is then hydrolyzed to ADPR by the outer mitochondrial membrane enzyme NAD+ glycohydrolase (Boyer et al., 1993). The consequent rise in cytosolic ADPR could lead to TRPM2 activation. However, pharmacological suppression of mitochondrial permeability transition with cyclosporine A, a nonspecific mPTP inhibitor, did not prevent oxidant-induced TRPM2 (Perraud et al., 2005). While this result suggests that the ADPR generated via NAD hydrolysis is not responsible for gating TRPM2, these experiments were carried out in HEK293 cells transiently expressing recombinant TRPM2. Such systems express proteins at higher levels than their endogenous counterparts. This is evidenced by the 10 nA TRPM2-mediated currents recorded in this study, which are enormous by any standard. Since several pathways likely work in parallel to activate TRPM2 following oxidative stress, it remains possible that blocking the ADPR generated via NAD hydrolysis alone is insufficient to observe changes in the amplitude of
TRPM2 currents. Perhaps even more compelling support for the hypothesis that
mitochondria-dependent NAD hydrolysis generates free cytosolic ADPR that gates TRPM2
is the unexpected finding that HEK293 cells express neuron-specific proteins (Shaw et al.,
2002). The failure of cyclosporine A to block mPTP opening in neurons suggests that it may
also be ineffective at blocking mitochondrial permeability transition in HEK293 cells
(Chinopoulos et al., 2003; Bambrick et al., 2006).

While it remains unknown whether the release of mitochondrial NAD leads to the
activation of TRPM2 currents in endogenous systems, ADPR itself may be released from the
mitochondria, providing an alternate source of mitochondrial-derived ADPR. Support for
this idea comes from a clever set of experiments whereby expression of a NUDT9 isoform
with mitochondrial-restricted expression substantially suppressed H2O2-induced Ca^{2+} influx
in TRPM2-expressing HEK293 cells (Perraud et al., 2005). This result indicates that the
mitochondria can serve as a source of ADPR, which is likely released through the mPTP
during conditions of oxidative stress. Evidence of intra-mitochondrial poly(ADP-
ribosylation) and the finding that both PARP and PARG are expressed within the
mitochondria support a role for these enzymes in the generation of mitochondria-derived
ADPR (Burzio et al., 1981; Masmoudi et al., 1988; Mosgoeller et al., 1996; Du et al., 2003).

1.6.3.5. **Functional relevance of ADPR-mediated gating of TRPM2**

Despite findings that exogenous application of extracellular oxidants or intracellular
ADPR activates TRPM2 channels, the functional relevance of ADPR-mediated gating in vivo
depends absolutely on the endogenous concentration of intracellular ADPR. Data from
HPLC analysis indicated that, at least in lymphatic cells, the resting concentration of
intracellular ADPR ranged from 33 to 84 µM, depending on the specific cell line (Gasser & Guse, 2005). These values fall within the lower range of ADPR concentrations required for gating TRPM2 (EC50 = 90 µM in T cells) (Perraud et al., 2001; Sano et al., 2001). However, these findings must be considered within the environment in which TRPM2 is activated. TRPM2 activation not only requires gating by intracellular ADPR but also depends absolutely on synergistic gating by Ca\(^{2+}\) (McHugh et al., 2003). In neutrophils, only small TRPM2 currents were activated upon inclusion of 5-100 µM ADPR in the patch pipette when [Ca\(^{2+}\)]\(_{i}\) was buffered to low (<10 nM) levels. Conversely, in the presence of 1 µM of intracellular Ca\(^{2+}\), these same concentrations of ADPR were permissive for near maximal activation of TRPM2 currents (Heiner et al., 2006). These results indicate that ADPR can act as a physiologically relevant activator of TRPM2, even at relatively low intracellular concentrations. Furthermore, maintaining the concentration of ADPR at levels near the threshold of channel activation is consistent with a model whereby rapid gating of TRPM2 is required in response to appropriate stimuli. In this way, only a small increase of intracellular ADPR, coupled with a rise in [Ca\(^{2+}\)]\(_{i}\), is required for TRPM2 activation (Kuhn et al., 2005).

1.7. **Hemichannels: More than just half gap junctions**

Gap junctions are specialized connections that form direct communication conduits between adjoining cells. These points of contact allow the passage of both ions and small molecules up to ~1 kD in size. In neurons, gap junctions act as low-pass filters, which preferentially allow the passage of sub-threshold potentials and act to synchronize groups of
cells (Sohl et al., 2005). This electrical coupling in neurons was first observed in the giant motor synapse of the crayfish (Furshpan & Potter, 1957) and later recorded in mammalian systems in the mesencephalic nucleus of cranial nerve V (Hinrichsen, 1970), the lateral vestibular nucleus (Wylie, 1973) and the inferior olivary nucleus (Llinas et al., 1974).

Today, it is well established that gap junctions form the molecular counterparts of electrical synapses (Bennett & Zukin, 2004). However, although electrical coupling between cells was the first function attributed to gap junctions, the flux of nutrients, metabolites and signaling molecules through these structures is also of significant physiological importance (Evans & Martin, 2002).

In vertebrates, connexins were the first identified family of gap junction-forming proteins. These proteins are expressed in a wide variety of cell types and over 20 known mammalian genes contribute to their overlapping yet unique distribution and functions (Sohl & Willecke, 2003). Six connexin molecules oligomerize to form heximers, which are termed ‘connexons’ or ‘hemichannels.’ In turn, apposing hemichannels on adjacent cell membranes dock to form complete gap junction channels. Individual gap-junction channels can then form aggregates of hexagonal groups called gap junction plaques.

Until recently, a significant body of literature existed in support of the notion that connexins were the only gap junction proteins in vertebrates. However, a search of the human genome for innexin orthologs revealed a novel family of three putative gap-junction forming proteins (Panchin et al., 2000). These were named pannexins on the basis of their broad physiological expression and shared sequence homology with the invertebrate gap junction proteins, innexins (Baranova et al., 2004). However, despite significant predicted structural similarities, pannexins share little sequence homology with connexins.
Hemichannels are integral transmembrane proteins that are permeable to ions and small molecules up to 1 kD in size. Each channel consists of either six connexin or pannexin molecules, which assemble to form a connexon or pannexon, respectively. These non-selective channels form the functional subunits of gap junction channels by pairing with their counterparts on apposing cell membranes (Sohl et al., 2005). While hemichannels may remain unpaired in the nonjunctional membrane, the formation of intercellular connections was initially believed to be a requisite step for their activation. However, recent years have seen the emergence of strong biochemical and electrophysiological evidence for functional extra-junctional connexin (Musil & Goodenough, 1991; Paul et al., 1991; DeVries & Schwartz, 1992) and pannexin hemichannels (Bruzzone et al., 2003; Thompson et al., 2006).

The open probability of most connexin hemichannels is significantly reduced by hyperpolarized membrane potentials and physiological concentrations of extracellular Ca\(^{2+}\) (DeVries & Schwartz, 1992; Pfahnl & Dahl, 1999). Despite this, connexin hemichannels may function in pathological conditions, as expression of Cx46 in Xenopus oocytes resulted in cell swelling and death. Extracellular Ca\(^{2+}\) levels have been shown to fall during both ischemic and seizure-like activity (Harris & Symon, 1984) and bathing the oocytes in high Ca\(^{2+}\) solution prevented both deleterious consequences of Cx46 hemichannel activation (Paul et al., 1991). Conversely, Cx26 hemichannels could be activated in a voltage-independent manner and are less sensitive to extracellular counterparts than other family members. Since their activation in oocytes did not lead to cell swelling and death, these hemichannels may play a physiological role in cellular signaling (Ripps et al., 2004).
1.7.1. Pannexin hemicannels

Like connexins, the three pannexin proteins (Px1, Px2 and Px3) are predicted to contain four transmembrane domains, separated by two extracellular and one intracellular loop, and cytoplasmic N and C termini. The presence of conserved cysteine residues in the extracellular loop further supported a gap junctional role for these proteins (Baranova et al., 2004). While classical dye-coupling experiments in *Xenopus* oocytes demonstrated that pannexins could form functional gap junction channels (Bruzzone et al., 2003), the slow dynamics of cell coupling suggest that many pannexin hemicannels remain undocked in the membrane (Bruzzone et al., 2005; Shestopalov & Panchin, 2008).

In *Xenopus* oocytes, overexpression of Px1 or Px1/2 but not Px2 alone resulted in the formation of functional hemicannels. The homomeric and heteromeric channels differed from each other on the basis of gating kinetics and current amplitude. Homomeric Px1 channels had a very large single channel conductance of approximately 550 pS in symmetrical KCl (Bruzzone et al., 2003), over 200 pS larger than that of any connexin hemicannel reported to date (Thompson & Macvicar, 2008). Importantly, unlike connexins, Px1 and Px1/2 were insensitive to increased extracellular Ca$^{2+}$ (Bruzzone et al., 2005) and exhibited little voltage dependence (Bruzzone et al., 2003), suggesting that they may play a more varied role in physiological and pathological processes than their connexin counterparts.

Px1 and Px2 are widely expressed in the brain in such regions as the hippocampus, cerebellum, thalamus, hypothalamus and neocortex (Ray et al., 2005). Of particular relevance to our studies, Px1 and Px2 may be the only gap junction protein expressed in the pyramidal cells of the hippocampus (Sohl et al., 2005). Evidence of functional pannexin
hemichannel activity in hippocampal neurons has been reported in response to oxygen-glucose deprivation (OGD) (Thompson et al., 2006). The OGD-activated current had a single channel conductance similar to that of homomeric Px1 hemichannels when expressed in oocytes and was inhibited by CBX as well as La$^{3+}$, a non-specific blocker of connexin hemichannels. Furthermore, Px1 hemichannel activation has been linked to cell death in macrophages as ATP-mediated activation of P2X7 receptors activated a dye uptake pathway that was inhibited by suppressing Px1 either pharmacologically or by RNAi (Pelegrin & Surprenant, 2006). Taken together, this evidence suggests that the large conductance pathway mediated by pannexin hemichannels may result in the ionic dysregulation and ensuing death of hippocampal neurons.
1.8. **Summary of objectives and hypotheses**

1.8.1. **Overall hypothesis**

TRPM2 channels generate excitatory signals that have important roles in the physiology and pathophysiology of hippocampal neurons. The activation of these channels following inappropriate NMDAR activation represents a novel route of Ca\(^{2+}\) entry into these cells.

1.8.2. **Summary of objectives (1)**

1) To examine the molecular and biochemical characteristics of neuronal TRPM2
2) To identify and characterize TRPM2-mediated currents in hippocampal neurons
3) To identify a functional link between NMDARs and TRPM2 channels

1.8.3. **Sub-hypotheses (1)**

1) TRPM2 channels are expressed in CA1 hippocampal neurons and are in close proximity to NMDARs.
2) Hippocampal neurons possess functional TRPM2 channels that, when activated, provide a novel pathway for Ca\(^{2+}\)- influx into these cells.
3) TRPM2 channels are activated downstream of NMDAR activation via a Ca\(^{2+}\)- dependent pathway.
The results from this section demonstrated that short, repeated NMDA applications activated TRPM2-mediated currents. However, the current activated following sustained NMDA application did not respond to pharmacological antagonists of TRPM2 channels. We therefore sought to characterize this secondary current.

1.8.4. **Summary of objectives (2)**

1) To characterize and identify the secondary current activated following sustained NMDA applications

2) To determine a mechanistic link between sustained NMDAR stimulation and the activation of this secondary current

1.8.5. **Sub-hypotheses (2)**

1) Pannexin hemichannels are activated following sustained NMDA application in hippocampal neurons

2) The activation of the secondary current requires $\text{Ca}^{2+}$-influx through NMDARs
Materials and Methods

1.9. Preparation of primary cultured murine hippocampal neurons

All primary cultures of mouse hippocampal neurons were prepared by Lidia Brandes (University of Toronto) using the following protocol: Fetuses were rapidly removed from time-pregnant (E17-18) Swiss mice under anesthesia (isoflurane). Hippocampal tissue was microdissected from each fetus and placed in cold Hanks’ solution. The neurons were then mechanically dissociated by trituration using a Pasteur pipette. The cells were plated at a density of less than 1 x 10⁶ cells/ml on either 35-mm collagen-coated culture dishes (used for electrophysiological recordings 18-30 days in vitro (DIV)) or poly-D-lysine-coated 25-mm glass coverslips (used for immunofluorescence staining experiments 14 DIV). The mixed cultures were incubated at 37 C in 5% carbon dioxide for 1 week in minimal essential medium (MEM) (Gibco, Grand Island, NY) that was supplemented with fetal bovine and maintained in serum. All experiments were carried out according to guidelines stipulated by the University of Toronto Animal Care Committee.

1.10. Criteria for the selection of neurons

Recordings were made only from those neurons that were pyramidal in shape, had long, well-defined basal dendrites and that were bright field in appearance under phase contrast optics. Since recordings were made in neurons 18-30 DIV (vs. the standard 14-21 DIV), cells were often granular in appearance and this correlated highly with the ability to activate TRPM2 currents. This additional criterion proved especially important in order to
record TRPM2 currents downstream of NMDA receptors in the absence of exogenous ADPR in the patch pipette.

For neurons within a single dissection, it was not uncommon for intracellular ADPR plus either VDCC or NMDAR stimulation to fail to activate a TRPM2-like current in every cell recorded from. So as to attempt to control for this variability, two additional criteria were implemented for recordings: (1) On any given day, a baseline control recording was carried out using both standard ICS and ECS to ascertain if a TRPM2-like current could be activated. Currents were evoked with ADPR in the patch pipette and either voltage-ramps or NMDA applications. If a current developed, Ca\(^{2+}\)-free ECS or clotrimazole was applied to help confirm that it was, in fact, mediated by TRPM2 and not merely a non-specific “leak” conductance and (2) each experiment was carried out using neurons from an average of 3 to 5 distinct dissections. This latter criterion proved particularly important for validating negative results. Furthermore, since not all neurons within a single dissection demonstrated TRPM2-like currents, negative results were also confirmed in an average of 5 cells from a single dissection.

1.11. **Recording electrodes**

Whole-cell patch electrodes were constructed using a Narishige two-stage puller (PP-83; Narishige, Greenvale, NY) from thin-walled borosilicate glass (TW150-F3; WPI, Sarasota, FL). Electrodes had a final resistance of 3 to 5 M\(\Omega\) when filled with intracellular solution (ICS) containing (in mM): 140 CsGluconate, 10 HEPES, 2 MgCl\(_2\), 0-3 ADPR, pH 7.3 (adjusted with CsOH) and an osmolarity of between 295 and 300 mOsm as measured
with a vapor pressure-based osmometer (model 5500, Wescor Inc.). Calcium buffers were omitted from the ICS in order to promote the activation of TRPM2-mediated currents.

1.12. **Whole-cell recordings from primary cultured hippocampal neurons**

Culture dishes were rinsed twice with standard extracellular solution (ECS) at room temperature to remove the culture media and mounted in the holding chamber of a phase-contrast, inverted microscope (Zeiss, Germany). The standard ECS was composed of (in mM): 140 NaCl, 5.4 KCl, 25 HEPES, 33 glucose, 2 CaCl$_2$, 1 MgCl$_2$, 0.2 µM TTX, pH of 7.4 (adjusted with NaOH) and had an osmolarity between 305 and 315 mOsm. The selection of neurons appropriate for high-quality patch-clamp recordings in these experiments was made on the basis of several important criteria (discussed below). After selecting an appropriate neuron, a patch electrode was filled with standard ICS and mounted onto the head stage. Positive pressure was applied with a syringe to prevent the collection of debris around the electrode tip as it was advanced towards the cell body. This pressure was removed once the electrode tip was positioned over the cell. The junction potential was nullified with a correction potential from either a Multiclamp 700A or Axopatch-1D amplifier (Molecular Devices, Sunnyvale, CA) and a seal test (10 mV voltage step) applied to the membrane. Electrodes with a final resistance that fell outside the range of 3 to 5 MΩ were discarded, as this suggested the tip of the electrode was too large or small or that it was occluded by debris. The following steps were carried out to form a high resistance seal between the electrode tip and cell membrane: Negative pressure was applied, again by way of a syringe, once the electrode made contact with the cell, as indicated by an increase in pipette resistance. Once a megaohm seal was achieved, a command potential of -60 mV was applied to the membrane.
This served to facilitate the formation of a gigaohm seal. The patch of membrane within the electrode tip was ruptured with additional negative pressure. This was typically sufficient to achieve the whole-cell configuration. The development of large capacitive transients indicated good electrical access to the cell’s interior. To ensure that “re-sealing” of the membrane patch did not occur, input resistance was monitored by applying a -10 mV voltage step. When series resistance varied by >10%, the cell was excluded from further analysis.

Electrical access was also qualitatively monitored on an oscilloscope periodically throughout each experiment. Recordings in which “resealing” occurred were discarded. All recordings were performed at room temperature (20–22 °C). Neurons were voltage-clamped at -60 mV and a stream of ECS applied using a computer-controlled, multi-barreled perfusion system (SF-77B; Warner Institute, Hamden, CT, USA) to achieve rapid exchange of solutions (τ of exchange ≈ 2 ms). All data were filtered at 2 kHz, digitized, and acquired using pClamp (Molecular Devices).

Peroxynitrite (ONOO-, Calbiochem) evoked currents were recorded using solutions prepared under the following conditions: ONOO- was thawed rapidly and dispensed into aliquots. Tubes were purged with 100% nitrogen gas and frozen and stored at < -70°C. Aliquots were thawed immediately prior to use and added to ECS that had been vacuum-evacuated of air to enhance ONOO- solubility and reduce its oxygen-dependent breakdown. ONOO- containing ECS was prepared immediately prior to each recording. All ONOO- containing solutions were protected from light. ADPR-dependent currents were induced by the application of a voltage ramp (± 100 mV, 500 ms) to the membrane every 10 sec. Current-voltage relationships were either constructed from this data or from steady-state voltage steps.
recorded using the following protocol: 0.3 mM ADPR were included in the patch pipette and voltage steps (-80 to +40 mV, 1 sec) applied to the membrane every 60 sec immediately following both breakthrough and activation of the NMDA-mediated current.

1.13. Hippocampal slice recordings

6-8 week-old male Sprague Dawley rats were anesthetized with isoflurane, decapitated and their brains rapidly excised. Transverse hippocampal slices were prepared and placed in a holding chamber for at least 1 hr prior to recording. In one set of experiments, a single slice was pretreated with 100 µM NMDA for 5 min and then transferred to a recording chamber and superfused with artificial cerebrospinal fluid (ACSF, 2 ml/min) composed of (in mM): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 1.3 MgCl2, 2.6 CaCl2, 26 NaHCO3, 10 glucose and 0.01 bicuculline methiodide, saturated with 95% O2- 5%CO2 at 28-30°C. The patch pipette (4–6 MΩ) solution contained (in mM) 132.5 K-glucuronate, 17.5 KCl, 10 HEPES, 0.2 EGTA, 5 QX 314, and 1 ADPR (pH 7.25, 290 mOsm). Patch recordings were done using the “blind” patch method. Cells were voltage clamped at -20 mV, and experiments started only after the access resistance had stabilized. To activate the ADPR-current, voltage ramps (±100 mV, 500 ms) were applied continuously every 10 sec throughout the recording. In a second set of experiments, slices were transferred to the recording chamber and continuously perfused with ACSF (solution as above) at 8ml/min without pretreatment in NMDA. Visually guided patch recordings were obtained from CA1 pyramidal cells and visually identified interneurons in stratum radiatum. Patch pipettes contained a solution of (in mM) 150 Cs-gluconate, 10 HEPES, 10 MgCl2, 4 Mg-ATP and 1 ADPR (4-6 MΩ resistance). Cells were voltage-clamped at -60 mV. ADPR currents were
activated by a 2 µL puff of NMDA (100 mM) directly injected into the recording chamber in a stream of ACSF in close proximity to the slice, in combination with 500 msec long voltage steps to +60mV applied every 10 sec continuously throughout the recordings. Considering the 2 ml volume of the chamber and the flow rate of 8 ml/min, this technique allowed the rapid application and washout of high concentrations of NMDA locally (minimal estimated final concentration of NMDA of 100 µM). Data were amplified using an Axopatch 1-D or MultiClamp 700A, sampled at 5-10 KHz, and analyzed with Pclamp6 software (Axon Instruments, Foster City, CA). Series resistance ranged from 10 to 20 MΩ, as estimated from series resistance compensation of current responses to voltage steps of -10 mV. Cells were discarded if the resistance changed by more than 25%.

1.14. **Statistics for electrophysiological recordings**

Statistical analysis of electrophysiological data was carried out using GraphPad Prism software. All data were reported as mean ± SEM. Data were analyzed by either ANOVA or Student’s t-test.

1.15. **In situ hybridization**

Brains were dissected from anesthetized 8-week-old B6 mice after perfusion with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, post-fixed overnight at 4°C in the same fixative and subsequently dehydrated, embedded in paraffin, sliced at 8 µm, and mounted onto silicon-coated glass slides. The recombinant pBluescript SK(+) plasmid, pBSII-TRPC7-1B, containing the TRPM2 cDNA fragment (1,072-2,980) was amplified from a full-
length mouse cDNA library (Hara et al., 2002), linearized by digesting the EcoRI or NotI site (on vector) and transcribed using T3 or T7 MAXIscript RNA polymerase (Ambion, Austin, TX) with DIG RNA Labeling Mix (Roche Molecular Biochemicals) for synthesis of the sense or antisense digoxigenin (DIG)-labeled RNA probes. Sections were partially digested by 0.8% pepsin in 0.2 N HCl and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) after de-paraffinization and rehydration. Hybridization was performed for 12-16 h at 50°C with 400 ng/ml antisense or sense probe in 10 mM Tris-HCl (pH 7.6), 600 mM NaCl, 0.25% SDS, 1 mM EDTA (pH 8.0), 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, 0.02% bovine serum albumin, 0.2 mg/ml yeast tRNA, 10% dextran sulfate, and 50% formamide. The sections were washed in 2× SSC with 50% formamide at 50°C for 30 min, treated with 10 µg/ml RNase A in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0) and 500 mM NaCl to remove free probes and washed at increasingly high stringencies up to a final condition of 0.2× SSC at 50°C for 20 min.

Washed slides were incubated in 1.5% blocking reagent (Roche Molecular Biochemicals) in 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl (DIG buffer) at RT for 1 h. Immunochemical detection of the hybridized probes was performed using alkaline phosphatase-conjugated anti-DIG antibody (Roche Molecular Biochemicals) at 500 times dilution in DIG buffer for 60 min. The alkaline phosphatase activity was visualized by 12-16 h incubation with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate alkaline phosphatase detection system (Roche Molecular Biochemicals) in 100 mM Tris-HCl (pH 9.5), 50 mM MgCl₂, and 100 mM NaCl under light protection.
1.16. **RT-PCR**

After aspirating the cell into the tip of the micropipette, the tip was broken off and the contents expelled into an Eppendorf tube containing lysis buffer (0.5 ml, Stratagene) supplemented with β-mercaptoethanol (as per manufacturer’s instructions). The contents from 10 cells were pooled and single-stranded cDNA was generated from the resulting sample by reverse transcription SuperScript II RNase H-reverse transcriptase (Invitrogen) with oligo(dT) primer (Invitrogen) following the manufacturer's protocol. The PCR was carried out by using 5 ml of cDNA template, 10’ PCR buffer (5 ml), MgCl2 (3 ml; 25 mM), dNTPs (1 ml; 10 mM), primers (1 ml each; 25 mM), AmpliTaq polymerase (0.5 ml; 5 units/ml; Applied Biosystems), and DEPC-treated water for a final volume of 50 ml. 35 cycles were run at the optimal annealing temperature for each primer set (57°C for TRPM2), and then the annealing temperature was decreased by 0.5°C each cycle for a total of 45 cycles. Primer sequences for TRPM2 were as follows for primer set 1: Fw, 5′-AAGGAACACAGACAATGCCTG, and Rv, 5′-AGGATGGTCTTGTGGTTCGC; for primer set 2: Fw, 5′-ACTGGATCGTGACAACCCTG and Rv, 5′-AGAGCCACATCCTTCTCAGC

1.17. **Membrane preparation and western blotting**

Crude membrane fractions were prepared from cultured hippocampal neurons using a protocol modified from (Rogers *et al.*, 1991). Cells were washed in ice-cold solubilization buffer (250 mM sucrose, 1 mM EDTA and MINI cocktail (Roche, Mannheim, Germany)) and Dounce homogenized. The homogenate was centrifuged at 800 x g for 15 min at 4 °C.
The supernatant was retained, centrifuged at 50,000 x g for 90 min and the resulting pellet (crude membrane fraction) was dissolved in buffer containing 2% SDS and 100 mM DTT. Samples were heated to 70 °C for 5 min, loaded onto 7.5% SDS-PAGE gels and separated by electrophoresis. Proteins were transferred to nitrocellulose membranes, blocked with 5% non-fat dry milk in Tris-buffered saline for 1 hr at RT and incubated with anti-mouse TRPM2 rabbit antiserum for 1 hr at RT (gift from Dr. Y. Mori, Japan). Membranes were washed three times in Tris-buffered saline with 0.1% Tween-20 and incubated with HRP-conjugated secondary antibody for 1 hr. Following a final wash, bound antibodies were visualized via enhanced chemiluminescence using a Kodak Image Processing Station. Quantification of Px1 Western Blot data was made using Kodak Image Processing software based on pixel density. Values were normalized to uninfected control.

1.18. Immunofluorescence localization

Cultured hippocampal neurons were washed with PBS and fixed in 4% paraformaldehyde + 4% sucrose in PBS at room temperature (RT) for 20 min. Neurons were washed three times with 0.1% Triton-X-100 in PBS (PBST) and blocked in PBST containing 10% normal goat serum (NGS) for 1 hr at RT. Immunostaining was carried out in PBST + 1% NGS and neurons were double-labeled as follows: rabbit anti-TRPM2-N-terminal (1:500, overnight at 4 °C, #A300-414A, Bethyl Laboratories, Montgomery, TX), wash with PBST, goat anti-rabbit IgG (1:800, 1 hr at RT, #A11011, Alexa 568, Molecular Probes), mouse monoclonal anti-PSD-95 (1:500, 1 hr at RT, ab2723, Abcam, Cambridge, MA), wash with PBST, goat anti-mouse IgG (1:800, 1 hr at RT, #A11019, Alexa 488, Molecular Probes. To visualize nuclei, neurons were stained with Hoechst Stain (1:2000, 20 min, Sigma) and
coverslips mounted with Prolong Gold antifade solution (Invitrogen, Carlsbad, USA). Fluorescent images were visualized using an Olympus IX81 microscope.

1.19. RNA interference

1.19.1. shRNA preparation and neuronal infection

An shRNA sequence uniquely targeting TRPM2 (5’-
\[\text{tAACCTTAGCTCATGGATTCCC} \text{caagagGGGAATCCATGAGCTAAGGTTttttttc}\]
corresponding to coding regions 169-189 relative to the first nucleotide of the start codon of murine TRPM2 (GenBank NM_138301)), Px1 (5’- GCATCAAATCAGGATCCT -3’
corresponding to coding regions 1125-1143 relative to the first nucleotide of the start codon of human Px1 and a scrambled (control) sequence (5’-
\[\text{tAATTCTCCGAACGTGTCACGT} \text{caagagACGTGACACGTTCGGAGAATTttttttc}\]
were cloned into a pLB lentiviral vector (Addgene plasmid 11619), which expresses shRNA under control of a U6 promoter and green fluorescent protein (GFP) under control of a CMV promoter. Lentiviruses were generated by transiently transfecting the resulting pLB vectors along with packaging vectors pMDLg/pRRE, pRSV-Rev and pMD2.G into HEK293T cells using Lipofectamine 2000. The medium containing viruses was harvested 40 hours following the start of transfection and concentrated using Millipore Centricon Plus-20 filters. The resulting concentrate (50-100 µl) was used to infect cultured hippocampal neurons at 7 DIV. Infected cultures were used for recordings at 21-24 DIV.
1.19.2. **Immunostaining**

Mouse cultured hippocampal neurons on cover slips treated with lentivirus shRNA\textsubscript{TRPM2} and shRNA\textsubscript{control} were first washed in phosphate-buffered saline (PBS). The culture neurons were then fixed and cryo-protected in 2.5% sucrose with 4% paraformaldehyde in PBS solution at room temperature (RT) for 20 min. Immunostaining was performed as described previously (Sun et al., 2006). Neurons were blocked using a 1% BSA, 3% goat serum, 0.3% Triton-X-100 in PBS solution for 90 min at RT; and double labelled with rabbit anti-TRPM2 antibody (#ab11168, 1:50; Abcam, Cambridge, MA., USA), and mouse anti-NeuN antibody (1:100; Chemicon, Temecula, CA., USA) overnight at 4°C (gently rocking). Neurons were subsequently washed with PBS and blocked briefly with the blocking solution. They were incubated with goat anti-rabbit Alexa568 (1:100, Molecular Probes), and goat anti-mouse Alexa350 (1:100; Molecular Probes) for 1 hour at room temperature. After washing, the cover slips were then mounted using ProLong Gold antifade reagent (Invitrogen/Molecular Probes) and viewed with a confocal laser-scanning microscope.

1.19.3. **Confocal Microscopy**

Cultured neurons were double-stained with anti-TRPM2 and NeuN antibodies. Triple fluorescence images were visualized with a laser scanning confocal microscope (Zeiss LSM 510 META) using a 63x microscope lens (Zeiss). Optical stacks of 5-10 confocal images taken at 0.5 μm intervals were used to generate figures as described previously (Sun et al., 2006).
(green represents GFP; red with anti-TRPM2; and blue with anti-NeuN stain)

1.19.4. Quantification and Statistics

Uninfected neurons (WT, no GFP) from the same fields from the TRPM2 staining images were used as controls - 4 groups of the cells to compare the fluorescence signals (WT and LV scramble RNA, and WT and LV TRPM2 siRNA groups). The fluorescence intensities from neuronal cell bodies labelled with TRPM2 and NeuN antibodies were measured using NIH Image J and expressed as arbitrated units (A.U.). Data are presented as mean ± SEM. Group data were compared using one-way ANOVA and multiple comparison test, Fisher LSD test (SigmaStat3.0 of SPSS, Chicago, IL, USA). For all tests, P<0.05 was considered significant.
Ca$^{2+}$-induced ADPR-dependent currents in hippocampal neurons

The majority of results presented in the following sections of this thesis have previously been published in the Journal of Physiology (Olah et al., 2009).

1.20. Background and rationale

TRPM2 forms non-selective cation channels that are stimulated by reactive oxygen/nitrogen species (ROS/RNS) (Hara et al., 2002; Wehage et al., 2002) through the generation of intracellular ADPR (Perraud et al., 2005). ADPR gates TRPM2 channels by binding to the NUDT9H domain of its C-terminus (Perraud et al., 2001). In addition to being expressed in peripheral cell types including immunocytes, neutrophils and rat islet cells (Sano et al., 2001; Togashi et al., 2006; Eisfeld & Luckhoff, 2007), northern blotting and quantitative PCR techniques indicate that TRPM2 is broadly expressed in the CNS (Nagamine et al., 1998; Hara et al., 2002; Fonfria et al., 2006b). However, as this evidence was derived from homogenized tissue samples, it does not allow expression in neurons to be distinguished from that in glia. The importance of making such a distinction is highlighted by a recent study that failed to identify TRPM2 transcripts or functional channels in cerebellar granule cells or astrocytes (Kraft et al., 2004). Rather, TRPM2 was detected in microglial cells, leading to the suggestion that the CNS distribution of TRPM2 corresponds to its expression in non-neuronal cell types (Perraud et al., 2003; Kraft & Harteneck, 2005).

Attempts at functionally identifying TRPM2 channels in neurons are hindered by the absence of selective antagonists or blockers. While the blockers characterized to date (e.g.
clotrimazole and flufenamic acid) have proven useful in characterizing TRPM2 currents in non-excitable cells, their use in neurons proves more problematic as these same pharmacological agents also block numerous voltage- and ligand-gated conductances. Despite these limitations and contrary to the previous report failing to observe TRPM2 in cerebellar granule cells, several lines of evidence indicate that TRPM2 channels are likely expressed in neurons, at least within discrete neuronal populations. For example, TRPM2-like currents have been identified in cultured striatal neurons (Smith et al., 2003; Hill et al., 2006) as well as in acutely isolated cortical neurons (Kaneko et al., 2006). Thus, the issue of whether TRPM2 channels are functionally expressed in central neurons remains controversial and whether or not they are expressed in the principal neurons of the hippocampus remains an open question.

Given the identification of TRPM2 channels as potential mediators of cell death and the selective vulnerability of CA1 pyramidal neurons to ischemia-induced cell damage, we hypothesize that functional TRPM2 channels are expressed in hippocampal neurons.

1.2.1. Results

1.2.1.1. TRPM2 mRNA and protein are expressed in hippocampal neurons

To determine if TRPM2 is expressed in hippocampal pyramidal neurons, we first employed in situ hybridization to examine the distribution of TRPM2 transcripts in mouse sagittal brain sections (Fig 2A and B). TRPM2 mRNA was broadly distributed with high signal intensities detected within both hippocampal pyramidal and granule neuronal cell layers, a distribution profile suggestive of neuronal expression. To obtain more definitive
evidence of specific neuronal expression, patch pipettes were used to aspirate the cytoplasm from visually identified cultured hippocampal neurons. These neurons were selected from a subset of recordings presented below. The resulting samples were pooled and subjected to RT-PCR analysis. Using 2 distinct primer sets targeted to non-overlapping regions of TRPM2 mRNA, we identified amplified products of the expected size (Fig. 3A). In addition, the identity of one of the products was confirmed by DNA sequencing (not shown).

Next, we examined TRPM2 protein expression in a membrane-enriched preparation of cultured hippocampal neurons. Western blot analysis revealed the presence of 2 distinct bands in samples prepared from cultured hippocampal neurons. Consistent with the predicted size of TRPM2, the anti-TRPM2 antibody detected a ~170 kD band as well as a ~95 kD band (Fig 2B), which may correspond to a previously reported short TRPM2 variant (Zhang et al., 2003; Bari et al., 2009). One caveat to this result is that neuronal enriched cultures may also contain other cell types (e.g. fibroblasts and glia), which could contribute to the signal detected on Westerns. To confirm the specific expression of TRPM2 protein in neurons and to begin to examine its subcellular distribution, we performed immunostaining using an antibody recognizing the N-terminus of TRPM2 (Zhang et al., 2003a). TRPM2-immunoreactivity was widely distributed throughout both cell bodies and processes of visually identified pyramidal neurons. TRPM2 staining was diffuse and failed to co-localize preferentially with the synaptic marker, PSD-95 (Fig. 4A-C). Although we cannot exclude the possibility that TRPM2 is present in excitatory synapses, these data suggest that TRPM2 is not uniquely found in these regions.
Figure 2 – Expression and localization of TRPM2 in mouse brain. **A** Immunochemical *in situ* hybridization for TRPM2 in sagittal, adult brain slice. **B** Expanded region illustrates presence of hybridization in hippocampal cell layers.
**Figure 3** - TRPM2 mRNA and protein are expressed in mouse hippocampal neurons.  

**A,** RT-PCR of TRPM2 using distinct primer sets (1 and 2) amplifying non-overlapping regions of mRNA.  

**B,** Western blot analysis of TRPM2 expression in crude membrane fraction demonstrating the presence of 2 distinct bands detected by anti-TRPM2. The ∼170 kDa band is consistent with the predicted size of TRPM2, while the ∼95 kDa one may correspond to a previously reported, short TRPM2 splice variant consistent of the N-terminus and the first 2 transmembrane domains.
Figure 4 - TRPM2 does not preferentially colocalize with the synaptic marker PSD-95. Subcellular localization of TRPM2 by immunofluorescence staining in cultured hippocampal neurons: A, anti-TRPM2 (red); B, anti-PSD-95 (green); C, overlay of A and B including visualization of Hoechst nuclear stain (blue).
1.21.2. ROS/RNS activate TRPM2-like currents in cultured hippocampal neurons

To begin to examine whether hippocampal neurons possess functional TRPM2 channels, we applied H$_2$O$_2$ to voltage-clamped neurons (V$_h$ = -60 mV) and monitored changes in the holding current. H$_2$O$_2$ (1 mM) induced the development of a large inward current that reached a steady-state amplitude (-198.7 ± 40.23 pA, n = 8) within several minutes. The current was then maintained at this level for the remainder of the application period (Fig. 5A). The H$_2$O$_2$-evoked current was inhibited by application of the TRPM2 antagonist clotrimazole (10 µM) (Hill et al., 2004) (-38.3 ± 22.75 pA, n = 9) and was abolished by the removal of extracellular Ca$^{2+}$ (24.77 ± 38.59 pA, n = 8), which has been previously shown to cause rapid inactivation of TRPM2-mediated currents (Kraft & Harteneck, 2005; Scharenberg, 2005; Eisfeld & Luckhoff, 2007) (Fig. 5C).

In addition to ROS, TRPM2 can be activated by select RNS-generating agents (Hara et al., 2002). Among nitrogen species generated during oxygen-glucose deprivation of cultured neurons, peroxynitrite (ONOO$^-$), generated through NMDAR-dependent stimulation of nitric oxide synthase, is especially toxic to neurons. Consequently, it was of interest to examine whether ONOO$^-$ could also induce TRPM2-like currents in neurons. Application of ONOO$^-$ (0.1 mM) to cultured hippocampal neurons activated inward currents (-88.8 ± 15.7 pA, n = 7), which were inhibited by removal of extracellular Ca$^{2+}$ (10.2 ± 24.1 pA, n = 6) (Fig. 5B and C).
Figure 5 - Reactive species activate a TRPM2-like current in cultured hippocampal neurons.

A, representative whole-cell recording demonstrating activation of a TRPM2-like current upon extracellular H2O2 (1 mM) application and block by 10 µM CLT. Bars indicate periods of H2O2 or CLT application, as indicated on figure. Additionally, current development was monitored by assessing changes in the IV relationship, as measured by applying voltage-ramps (±100 mV) before and after H2O2 application (appear as vertical lines on current trace). B, inward current recorded from a single neuron in response to extracellular ONOO- (0.1 mM) application. The current was reduced in the absence of extracellular Ca$^{2+}$. Bars indicate duration of ONOO and 0 Ca$^{2+}$ ECS applications. C, summary bar graph displaying the mean normalized amplitude of the H2O2 and ONOO-current remaining after removal of extracellular Ca$^{2+}$ (H2O2, n = 8; ONOO-, n = 6 or 7) or addition of CLT (10 µM) to the ECF (H2O2, n = 9).
1.21.3. **ADPR alone is insufficient to activate TRPM2 in neurons**

Though the sensitivity of hippocampal neurons to ROS/RNS is suggestive, the hallmark of TRPM2 channel expression is the activation of large inward currents by intracellularly applied ADPR (Perraud et al., 2001; Sano et al., 2001). We therefore examined the effects of including ADPR in our patch pipette solution when recording from cultured hippocampal neurons. Intracellular calcium buffering was not used in these recordings. Under these conditions, little or no inward current was observed with intracellular application of ADPR (300 µM or supramaximal concentrations of 1 mM) (data not shown). This is in contrast to reports of recombinant TRPM2 channel activation in HEK293 cells where the threshold and saturating concentrations of ADPR were 60-100 and 300 µM respectively (Perraud et al., 2001). However, we noted that when we applied a series of voltage-ramps to monitor changes in the I-V relationship, robust inward currents developed in recordings with added ADPR (300 µM, t = 10 min: -1221.8 ± 153.7 pA, n = 12) but not in those without (-227.7 ± 33.2 pA, n = 11) (Fig. 6A and B). Under the conditions employed in our recordings, the I-V curves generated by applied voltage-ramps were contributed by the sum total of voltage-dependent and resting conductances. At the start of recordings (Fig. 6C, trace 1), I-V curves showed moderate outward rectification and reversed at negative potentials, reflecting a predominant contribution by resting K+ conductances. The development of the ADPR-dependent current (Fig. 6C, trace 2) was associated with an increase in membrane current (both inward and outward), a pronounced depolarizing shift in the reversal potential of the I-V curve (reversal after TRPM2 activation: -21.2 ± 1.7 mV, n = 11) and reduction of outward rectification. Subtraction of the ramp generated at the start of recording from that recorded after 10 minutes of recording (Fig. 6C,
trace 2-1) revealed the current component recruited by ADPR. The resulting I-V curve was linear and reversed at 0 mV, consistent with the reported properties of TRPM2. Since TRPM2 currents are stable and long lasting, we also determined the reversal potential of I-V curves after TRPM2 activation using voltage steps instead of ramps. The reversal potential under these conditions did not differ significantly (-24.1 ± 2.6 mV, n = 5).

To explore the relation between applied voltage-ramps and the development of the ADPR-dependent current further, we delayed the start of applied voltage-ramps until 10 minutes after the beginning of recordings with or without added ADPR. In these recordings, the generation of ADPR-dependent inward currents was delayed until after the start of applied voltage-ramps ( t = 10 min: -369.1 ± 34.2 pA, n = 12; t = 15 min: -822.7 ± 178.3 pA, n = 12) (Fig. 7A). These experiments demonstrate that the application of voltage-ramps provides neurons with an additional stimulus that greatly increases the reliability of generating ADPR-evoked currents in hippocampal neurons.
Figure 6 – ADPR-dependent currents in cultured hippocampal neurons. A, averaged data illustrates the time-course for the development of inward currents in recordings with ADPR added to the patch pipette (n = 12). In contrast, the holding current necessary to maintain neurons at -60 mV was unchanged in recordings without added ADPR (control, no ADPR, n = 11). In both cases, I-V relationships were monitored via application of voltage ramps from -100 to +100 mV every 10 sec to the membrane. B, holding current at start of recordings and after 10 min is shown for each individual recording from each of the recording groups. C, I-V curves from a recording in which ADPR was added to the patch pipette. Traces were taken from voltage-ramps applied at the start of recording (trace 1) and after 10 min (trace 2). The specific contribution from the ADPR-dependent current was derived by subtracting the I-V curve generated at the start from that which was recorded at 10 min trace (2-1).
Figure 7 – Applied voltage-ramps are necessary for the induction of ADPR-dependent currents. Average whole-cell currents recorded from neurons with or without added ADPR and in which voltage-ramps were applied continuously or delayed for 10 min.
1.21.4. Functional expression of ADPR-dependent currents in CA1 pyramidal neurons

To confirm the specific expression of ADPR-dependent currents in CA1 pyramidal neurons, we recorded from the CA1 region of acutely prepared hippocampal slices. When voltage-ramps were applied to the membrane as previously described, inclusion of 1 mM ADPR in the patch pipette resulted in the generation of an inward current (Fig. 8A). This was associated with the development of a linear I-V relationship and a depolarizing shift in the reversal potential to 0 mV, again consistent with the properties of TRPM2. Inclusion of 10 µM clotrimazole in the ECS reduced the current amplitude and left-shifted the reversal potential towards resting values (Fig. 8B and C). In an additional series of recordings from visually identified neurons in hippocampal slices, we compared the amplitude of the ADPR-dependent current in pyramidal neurons and stratum radiatum interneurons of the CA1 region. In this instance, the ADPR-dependent current was induced by a brief application of NMDA to the bath in combination with depolarizing steps to +60 mV every 10 sec. In recordings in which 1 mM ADPR was included in the patch pipette, the brief pulse of NMDA provoked the development of a large inward current in pyramidal neurons that was abolished by the removal of extracellular Ca$^{2+}$ (in pA ± SEM, 607 ± 142 vs 111 ± 28). This current was absent in interneurons (143 ± 30 vs 169 ± 34; Fig. 8D).
**Figure 8** – Activation of the ADPR-dependent current in hippocampal slices in CA1 hippocampal pyramidal neurons but not in stratum radiatum interneurons.  
A, representative whole-cell recording from a CA1 hippocampal pyramidal neuron in slice demonstrating activation of an ADPR-dependent current upon inclusion of 1 mM ADPR in the patch pipette. The amplitude of the current was reduced upon extracellular application of 10 µM CLT as shown. Ramps were blanked for clarity.  
B, I-V curves from the same cell as above. Traces were taken from voltage-ramps applied at the start of recording (trace 1), after 30 min (trace 2) and following perfusion of the slice for 20 min with ACSF containing 10 µM CLT (trace 3).  
C, summary bar graph demonstrating inhibition of ADPR-dependent current by 10 µM CLT (n = 4).  
D, summary bar graph of ADPR-dependent currents induced by application of NMDA and abolished by removal of extracellular Ca$^{2+}$ in CA1 pyramidal cells (n=6 and 8) and stratum radiatum interneurons (n=7 and 6).
1.21.5. **TRPM2 channel pharmacology**

We next explored the pharmacology of the ADPR-dependent currents evoked by voltage-ramps to confirm that these are in fact mediated by TRPM2. Recombinant TRPM2 currents are blocked by a series of compounds including clotrimazole, ACA and flufenamic acid (Harteneck et al., 2007). In addition, TRPM2 currents can be inactivated by the removal of extracellular Ca\(^{2+}\) (McHugh et al., 2003). In contrast, TRPM2 currents are either insensitive or enhanced by extracellular application of La\(^{3+}\) (Kraft et al., 2004), an effective blocker of many non-selective cation channels including several other members of the TRP family of ion channels (Kraft & Harteneck, 2005). In the absence of TRPM2 selective antagonists, we therefore tested each of these blockers on the ADPR-dependent current in hippocampal neurons. As illustrated by the series of representative I-V curves, removal of extracellular Ca\(^{2+}\) (Fig. 9A and B) as well as application of either clotrimazole (Fig. 9A and C), flufenamic acid (Fig. 9A and D) or ACA (Fig. 9A and E) depressed the ADPR-induced current and shifted the reversal potential of I-V curves back towards their initial, more negative value. In contrast, application of La\(^{3+}\) enhanced the ADPR-dependent current (Fig. 9A and F). The resulting pharmacological profile is entirely consistent with the activation of TRPM2 channels in hippocampal culture neurons.
Figure 9 – The pharmacological profile of the ADPR-dependent current in cultured hippocampal neurons matches that of TRPM2. Summary data (A) and representative voltage-ramps (B-F) of effect of removing extracellular Ca$^{2+}$ (A and B) or application of either clotrimazole (10 µM) (A and C), flufenamic acid (100 µM) (A and D), ACA (20 µM) (A and E) or La$^{3+}$ (100 µM) (A and F) on the maximal ADPR-dependent current.
1.21.6. **Ca\(^{2+}\)**-dependence of the ADPR-activated current

While recombinant TRPM2 channels are sensitive to the removal of extracellular Ca\(^{2+}\), this effect is ultimately mediated by the action of Ca\(^{2+}\) at an intracellular site located on either the channel or a closely associated protein (McHugh et al., 2003). We therefore examined whether the voltage-ramp mediated activation of ADPR-dependent currents in hippocampal neurons required an elevation of intracellular Ca\(^{2+}\). The intracellular buffering capacity in our recordings was increased by adding 20 mM BAPTA to our ADPR-containing ICS. Using the same ramp protocol as in previous experiments, buffering intracellular Ca\(^{2+}\) suppressed the induction of TRPM2-like currents in cultured hippocampal neurons (ADPR alone: \(-477.1 \pm 97.6\) pA, \(n = 13\); ADPR + BAPTA: \(-265.0 \pm 27.9\) pA, \(n = 10\)) (Fig. 10).

We therefore suspected that the observed dependence of TRPM2-like currents on voltage-ramps was due to Ca\(^{2+}\) entry via recruitment of VDCCs. To examine the involvement of VDCCs, we tested the effects of co-applied Cd\(^{2+}\) (100 µM) and Ni\(^{2+}\) (100 µM), two broad spectrum VDCCs blockers (Fox et al., 1987), on the ability of voltage-ramps to promote the generation of ADPR-dependent current (300 µM). Blocking VDCCs completely eliminated the induction of the ADPR-dependent current by voltage ramps in cultured hippocampal neurons \((-85.0 \pm 16.0\) pA, \(n = 6\)) (Fig. 10).
Figure 10 – Applied voltage-ramps are necessary for the induction of ADPR-dependent currents. Summary bar graph illustrating the effects of the various indicated treatments on the amplitude of the ADPR-dependent current measured 10 min after the start of recordings for ramp-induced and 16 min for NMDA-induced. No ADPR (n = 5); ramps: ADPR (n = 13), ADPR + Ni²⁺/Cd²⁺ (n = 6), ADPR + BAPTA (n = 10); NMDA: ADPR (n = 16).
TRPM2 channel activation downstream of NMDA receptors

1.22. Background and Rationale

The results thus far demonstrate that, in addition to ADPR, the gating of TRPM2-like currents in cultured hippocampal neurons requires a rise in intracellular Ca\(^{2+}\), as resting levels are insufficient to support channel activation. This is in contrast to findings in recombinant cell systems where the intracellular application of ADPR alone is sufficient to activate TRPM2 (Perraud et al., 2001). We have demonstrated that the influx of extracellular Ca\(^{2+}\) through activated VDCCs accounts for one source of increased \([\text{Ca}^{2+}]_i\). However, whether the requisite rise in \([\text{Ca}^{2+}]_i\) for TRPM2 activation depends absolutely on Ca\(^{2+}\) influx through VDCCs remains unknown. Alternatively, Ca\(^{2+}\) entry via other sources may also act with ADPR to synergistically activate TRPM2. We therefore examined whether TRPM2-like channel activation specifically required VDCC or whether other routes of Ca\(^{2+}\) entry could activate these channels. Since NMDARs serve as a major Ca\(^{2+}\) influx pathway in hippocampal neurons and their over-activation leads to excitotoxic cell death, we tested whether stimulation of NMDARs could activate the ADPR-dependent current. Furthermore, since over-activation of NMDARs may lead to the generation of ADPR, we hypothesize that TRPM2-like currents can be activated downstream of NMDARs in the absence of exogenously applied ADPR.
1.23. Results

1.23.1. NMDAR inhibition by clotrimazole

Pre-treatment of cultured hippocampal or granule cells with clotrimazole has previously been demonstrated to reduce cell death following glutamate challenge. These effects were, at least in part, due to the inhibition of NMDARs by clotrimazole, as application of 50 µM clotrimazole significantly depressed the steady-state of the NMDA-evoked response (Isaev et al., 2002). Despite its lack of specificity, clotrimazole is commonly employed as the hallmark TRPM2 antagonist. We worried that any reduction observed in the NMDA-induced, ADPR-dependent current in the presence of clotrimazole might in fact be due to clotrimazole block of NMDAR-mediated currents (INMDA). We therefore constructed a concentration-inhibition curve fitted using the logistic equation with the goal of finding a concentration of clotrimazole that inhibited TRPM2-mediated currents but not those mediated by NMDARs. In cultured hippocampal neurons, extracellular application of clotrimazole resulted in the concentration-dependent (0.3-100 µM) inhibition of INMDA (50 µM NMDA, 3 µM glycine, n = 6) (Fig. 11A). The IC$_{50}$ equaled 18.9 µM for the steady-state current and >100 µM for the peak I$_{NMDA}$ (Fig. 11B), indicating that clotrimazole preferentially inhibits the steady-state versus peak of I$_{NMDA}$. Since 10 µM clotrimazole produced a greater than 90% block of TRPM2 currents and the time to half-block was approximately 1 minute (Hill et al., 2004), we selected this concentration of clotrimazole for our experiments as it had no significant effect on either the peak or steady-state I$_{NMDA}$. 
Figure 11 – Clotrimazole inhibits NMDA-evoked currents at concentrations that do not affect TRPM2. A, a representative trace recorded from a cultured hippocampal neuron voltage-clamped at – 60 mV. When added to the extracellular fluid, clotrimazole (0.3 – 100 µM) caused a concentration-dependent inhibition of NMDA-evoked currents. B, summary concentration-inhibition curve fit using the logistic equation. Currents were normalized to the maximum response. IC$_{50}$ = 18.9 µM ($I_{\text{NMDA(steady-state)}}$) and >100 µM ($I_{\text{NMDA(peak)}}$)
1.23.2. Activation of the ADPR-dependent current downstream of NMDA receptors

To minimize activation of VDCCs during these recordings, changes in the IV relationship were monitored by applying voltage ramps (±100 mV) once every minute instead of once every 10 seconds. No significant effects of VDCC activation were observed when this recording protocol was employed. As anticipated, in the absence of NMDA application, no increase in holding current was observed when 300 µM ADPR was included in the patch pipette. However, brief, repeated NMDA applications (100 µM for 5 sec every 2 min) induced a large ADPR-dependent current (-1221.0 ± 132.0 pA, n = 16, Fig. 10). Like TRPM2-mediated currents, this current was sensitive to low extracellular Ca\(^{2+}\) and blocked by 10 µM clotrimazole. Taken together with our previous findings, this result suggests that multiple pathways of Ca\(^{2+}\)-influx are permissive for activation of the ADPR-dependent current in hippocampal neurons, at least with respect to VDCC and NMDA receptors.

1.23.3. Requirement of calmodulin activity for activation of the ADPR-mediated current

Tong et al. (2006) reported that a direct association between the Ca\(^{2+}\)-sensing protein calmodulin (CaM) and TRPM2 was required for TRPM2 activation in HEK293 cells following either H\(_2\)O\(_2\) or TNF-alpha stimulation. This was evidenced by the finding that cotransfection of TRPM2-expressing HEK293 cells with a Ca\(^{2+}\)-insensitive CaM mutant significantly inhibited the increase in [Ca\(^{2+}\)], following TRPM2 activation. Furthermore, immunoprecipitation experiments indicated that CaM directly associated with TRPM2 and this physiological association was enhanced following treatment of cells with H\(_2\)O\(_2\) (Tong et
Since one mechanism of CaM activation is via $\text{Ca}^{2+}$-influx through NMDARs (Keller et al., 2008), we sought to determine if inhibiting CaM activity prevented the activation of the ADPR-dependent current. As previously described, short, repeated NMDA applications (100 µM for 5 sec every 2 min) induced a large ADPR-dependent current when 300 µM ADPR was included in the ICS. We next tested the effects of including a CaM inhibitory peptide based on the calmodulin-binding domain of myosin light chain kinase in the patch pipette on the ADPR-dependent current. Again, NMDA applications were employed to induce the ADPR-dependent current. While current activation was not suppressed in the presence of the CaM inhibitory peptide, the amplitude of the current 20 minutes following establishment of the whole-cell configuration was significantly reduced compared with control at the end of 20 minutes of recording (ADPR alone, $466 \pm 136$ pA, $n = 6$; ADPR + NMDA, $1230 \pm 183$ pA, $n = 14$; peptide, $963 \pm 178$ pA, $n = 6$; p < 0.05 by ANOVA) (Fig. 12). This suggests that, while not essential for activation of the ADPR-dependent current, CaM activity in hippocampal neurons plays a modulatory role in its development.
Figure 12 – Calmodulin activity modulates the amplitude of the ADPR-dependent current.

Summary data demonstrating the development of the NMDA-induced, ADPR-dependent current in the absence of ADPR (■), with 300 μM ADPR (●) and with 300 μM ADPR and the addition of the CaM inhibitory peptide (○). NMDA currents were evoked every minute for 5 seconds (▽).
1.23.4. shRNA knockdown of TRPM2

In order to further substantiate the participation of TRPM2 channels in the ADPR-dependent current, we used short hairpin RNA (shRNA) mediated gene silencing to reduce the expression levels of TRPM2 in our cultured neurons. Several sequences were first screened in HEK 293 cells expressing TRPM2. We identified a specific sequence (shRNA_{TRPM2}) that could substantially reduce ADPR-evoked current amplitudes in these cells (not shown). A scrambled shRNA sequence was used as control (shRNA_{scrambled}).

Given the difficulty of achieving high efficiency transfection of post-mitotic cells such as neurons, shRNA_{TRPM2} and shRNA_{scrambled} were cloned into a lentiviral vector that also allowed for expression of GFP. Visual inspection of GFP expression confirmed that high infection rates (>80%) were achieved in our cultured hippocampal neurons with both shRNA_{TRPM2} and shRNA_{control} lentiviruses. We first confirmed the ability of shRNA_{TRPM2} to reduce the expression of TRPM2 in infected cultures by quantifying the intensity of TRPM2 immunostaining (Fig. 13). This staining was compared both between infected (GFP positive) and non-infected (GFP negative, wild type (WT)) neurons within each culture dish and between cultures expressing either shRNA_{TRPM2} or shRNA_{control}. As an internal negative control, we also compared the intensity of NeuN (Fig. 13D) under identical conditions. In neurons expressing shRNA_{TRPM2}, TRPM2 expression was reduced by ~45% compared to neurons expressing shRNA_{control} (shRNA_{TRPM2} = 125.3 ± 9; shRNA_{control} = 222.8 ± 5.7) whereas no difference was observed between shRNA_{control} expressing neurons and uninfected WT neurons (Fig. 13A, B and C). In contrast, no difference was observed in NeuN staining between the various treatment groups (Fig. 13A, B and D). We also observed no difference in
the relative GFP intensity between shRNA\textsubscript{control} and shRNA\textsubscript{TRPM2} expressing neurons (shRNA\textsubscript{TRPM2} = 227.9 ± 1.0; shRNA\textsubscript{control} = 234.0 ± 0.9, not shown).

Having confirmed the effectiveness of shRNA\textsubscript{TRPM2} at reducing the expression of TRPM2, we attempted to record ADPR-dependent currents in visually identified GFP positive neurons from cultures infected with either shRNA\textsubscript{TRPM2} or shRNA\textsubscript{scrambled} lentiviruses. In these experiments, NMDA applications were used to activate the ADPR-dependent current. In this way, the amplitude of NMDAR-mediated currents could be used as an additional control for potential off-target effects of shRNA\textsubscript{TRPM2}. ShRNA\textsubscript{TRPM2} reduced the amplitude of ADPR-dependent currents (Fig. 13E) whilst NMDAR-mediated currents were unchanged (NMDAR-mediated peak amplitudes: shRNA\textsubscript{TRPM2} = -4655.2 ± 490.9 pA; shRNA\textsubscript{control} = -3894.1 ± 445.0 pA, difference was not significant).
Figure 13 – shRNA knockdown of TRPM2 prevents activation of the ADPR-dependent current. A and B, confocal images from cultures treated as indicated. High magnification (X63 objective) image of the TRPM2 immunosignal (red) (A and B, top left) and GFP fluorescence (green) (A and B, lower right). Anti-NeuN staining (blue) of GFP positive cells (A and B lower left). Scale bar = 20 µm. C, comparison of fluorescence intensities from cell bodies of WT (uninfected) and neurons infected with shRNAcontrol and shRNA_{TRPM2} (n = 8 / group). The TRPM2 fluorescence intensity from cell bodies of the shRNA_{TRPM2} treated group is statistically significant lower than that of the shRNAcontrol treated group (also from that of WT cells (GFP negative) from both treatment groups) as indicated by “*” (difference, \( P < 0.001, \) ANOVA; \( P < 0.001, \) multiple comparison test (Fisher’s least significant difference test)). D, As a control, the NeuN fluorescence intensity was also quantified and used as reference in all 4 groups for the comparison (n = 8 / groups). No difference in the NeuN expression of these neurons was observed. E, Comparison of the ADPR-dependent current induced by NMDA application in neurons treated with shRNA_{TRPM2} (n = 9) or shRNAcontrol (n = 7). The amplitude of the current (normalized to cell capacitance) was determined after 10 min of recordings. The ADPR-dependent current recorded from shRNA_{TRPM2} treated neurons was significantly smaller than in shRNAcontrol treated neurons (**, \( P < 0.05, \) Student’s \( t \) test)
A LentiVirus shRNA_{control}  
\[ \text{TRPM2} \quad \text{merged} \]
\[ \text{NeuN} \quad \text{GFP} \]

B LentiVirus shRNA_{TRPM2}  
\[ \text{TRPM2} \quad \text{merged} \]
\[ \text{NeuN} \quad \text{GFP} \]

C comparison of TRPM2 expression  
\[ \text{WT} \quad \text{shRNA}_{\text{control}} \quad \text{WT} \quad \text{shRNA}_{\text{TRPM2}} \]

D comparison of NeuN expression  
\[ \text{WT} \quad \text{WT} \quad \text{shRNA}_{\text{TRPM2}} \]

E comparison of ADPR-evoked current  
\[ \text{shRNA}_{\text{control}} \quad \text{shRNA}_{\text{TRPM2}} \]

Bar graphs show normalized intensity (A.U.) for TRPM2 and NeuN expression, as well as current density (pA/PF) for ADPR-evoked current.
1.23.5. **Activation of TRPM2-like currents in the absence of exogenous ADPR**

In addition to their high Ca\(^{2+}\) permeability, several lines of evidence exist to suggest that TRPM2 channels are activated downstream of NMDARs in the absence of exogenous ADPR. Firstly, TRPM2 channels are directly gated by intracellular ADPR, although channel activation by ADPR is strictly dependent on the presence of intracellular Ca\(^{2+}\) (Perraud et al., 2001; McHugh et al., 2003). Mitochondria are an important cellular store of NAD\(^+\) (Richter et al., 1990), the precursor of ADPR, and hydrolysis of this NAD\(^+\) may play an essential role in the activation of TRPM2. Ca\(^{2+}\) loading of mitochondria causes collapse of the mitochondrial transmembrane potential, resulting in opening of the permeability transition pore (mPTP) (Di Lisa et al., 2001; Baines, 2009). Opening of the mPTP may allow NAD\(^+\) or ADPR itself to leave the mitochondria, thus leading to the activation of TRPM2, as it is permeable to molecules up to 1.5 kDa. Interestingly, Ca\(^{2+}\) influx through NMDARs has “preferential” access to the mitochondria and sustained Ca\(^{2+}\) influx via over-activated NMDARs lead to an increase in intracellular Ca\(^{2+}\), transmembrane potential collapse and opening of the mPTP (Peng & Greenamyre, 1998). This suggests that excessive activation of NMDARs may lead to the activation of TRPM2 channels. Additionally, the generation of ROS following over-stimulation of NMDARs could lead to TRPM2 activation as extracellular application of the oxidant hydrogen peroxide has been demonstrated to gate open TRPM2 (Wehage et al., 2002). Furthermore, a recent report by Miller’s group indicated that TRPM2 activation depended absolutely on calmodulin binding to the N-terminal IQ-like motif of the channel (Tong et al., 2006). The activation of calmodulin by Ca\(^{2+}\)-influx through NMDARs may provide an additional functional link between NMDARs
and TRPM2 channels. Given this evidence we asked whether TRPM2-like currents could be activated in the absence of exogenously applied ADPR.

To determine whether TRPM2-like currents are activated downstream of NMDARs, we again carried out our recordings under conditions that minimized the activation of VDCCs (i.e. I-V relationships were monitored by applying voltage ramps from -100 to +100 mV once every minute instead of once every 10 seconds. As before, no significant effects of VDCC activation were observed when this recording protocol was employed. However, brief, repeated NMDA applications (100 µM for 5 sec every minute) activated an inward current, as assessed by monitoring changes in the holding current at – 60 mV (Fig. 14 A). Like TRPM2, the NMDA-induced current was inhibited by removing extracellular Ca\(^{2+}\) or by including clotrimazole in the ECS (Fig. 14 B). Furthermore, consistent with the activation of TRPM2-mediated current, application of La\(^{3+}\) did not block the NMDA-induced current (Fig. 14 B). The activation of a TRPM2-like current following NMDAR stimulation likely results from the concomitant effects of Ca\(^{2+}\) influx through NMDARs and the subsequent generation of endogenous ADPR.
**Figure 14** – Activation of a TRPM2-like current in the absence of exogenous ADPR. **A,** representative continuous recording from cultured hippocampal neuron voltage-clamped at -60 mV. In the absence of ADPR in the patch pipette, short, repeated NMDA applications (5 sec every 1 min, denoted by arrows) activated an inward current as observed by an increase in the holding current at -60 mV. **B,** summary bar graph of peak NMDA-induced, TRPM2-like currents. Consistent with the activation of TRPM2-like current, the NMDA-induced currents were insensitive to block by La$^{3+}$ and blocked by CLT or perfusion with nominally Ca$^{2+}$-free ECF.
Discussion

1.24. **TRPM2 mRNA and protein are expressed in hippocampal neurons**

TRPM2 is expressed in diverse cell types including beta cells of the pancreas, immune cells and cardiac myocytes. However, despite convincing evidence for high expression in the mammalian brain, much of this signal has been attributed to strong expression in non-neuronal cells. Thus, the existence of functional TRPM2 channels in neurons remains controversial at best. Using cellular, biochemical and molecular approaches we now show that functional TRPM2 channels are highly expressed in pyramidal neurons of the hippocampus, including those of the CA1 region. As even our neuronal-enriched cultures contained fibroblasts, we circumvented the problem of amplifying a non-neuronal TRPM2 signal by employing a patch pipette to harvest individual cultured hippocampal neurons. Moreover, neurons were selected using our standard set of morphological criteria and were in fact selected from a subset of cells used in our electrophysiological recordings. Conversely, we did not find any functional evidence for TRPM2 expression in a subgroup of CA1 interneurons in hippocampal slices. While this suggests that TRPM2 channels are not expressed within this subgroup of cells, it remains possible that the conditions for their activation are simply different than those in CA1 pyramidal neurons.

Our immunofluorescence results indicate that the expression of TRPM2 in hippocampal neurons is diffuse and not preferentially localized at the synapse, as it did not co-localize with the synaptic marker PSD-95. Furthermore, using a commercially available antibody directed against the N-terminus of TRPM2 (Bethyl Labs), we detected a band in both at 170 kDa, consistent with the predicted molecular weight of TRPM2 (Zhang et al.,
However, multiple bands were detected and the predominant band ran below 100 kDa. While it remains possible that the antibody is not specific for TRPM2 in native cell systems, six distinct TRPM2 isoforms of varying molecular weights have been reported in a variety of cell types (Zhang et al., 2003a). It is possible that the multiple bands observed in Western blot experiments correspond to one or more of these TRPM2 isoforms. As several of these lack key regulatory domains and one acts as a dominant negative of TRPM2 function, it will be important to learn which of these TRPM2 isoforms exists in hippocampal neurons.

1.25. \( \text{Ca}^{2+} \) influx is required for TRPM2 gating in hippocampal neurons

Intracellular \( \text{Ca}^{2+} \) homeostasis is controlled in part by three major adenine intracellular second messengers: ADPR, cyclic ADPR (cADPR) and nicotinic acid-adenine dinucleotide phosphate (NAADP) (Harteneck et al., 2007). cADPR activates the ryanodine receptor, NAADP may stimulate a novel \( \text{Ca}^{2+} \) channel (Kraft et al., 2004) and ADPR gates \( \text{Ca}^{2+} \) permeable TRPM2 channels. Interestingly, each of these messengers is metabolically related, suggesting they may make distinct but coordinated contributions to \( \text{Ca}^{2+} \) signaling by modulating the gating of their respective target channels. In recombinant systems, applications of ADPR lead to the direct gating of TRPM2 channels, indicating that ADPR is an intracellular TRPM2 agonist. In addition to ADPR, ion substitution experiments aimed at characterizing the cation permeability of TRPM2 channels revealed a role for extracellular \( \text{Ca}^{2+} \) as a positive regulator of TRPM2 (Hara et al., 2002). Subsequently, McHugh et al. (2003) demonstrated that the gating of TRPM2 by ADPR was critically dependent on intracellular \( \text{Ca}^{2+} \), consistent with earlier suggestive evidence (Perraud et al., 2001). Here,
we demonstrate that ADPR alone is insufficient to gate TRPM2 in hippocampal neurons. Rather, a concomitant influx of Ca\(^{2+}\) through VDCCs or NMDARs is necessary to fully activate TRPM2 channels. This is in contrast to recombinant systems, where the activation of an additional Ca\(^{2+}\)-entry route is not required for TRPM2 channel activation.

In recombinant expression systems, the Ca\(^{2+}\) required for ADPR gating may, at least in part, be derived from entry into cells via TRPM2 channels themselves. In this scenario, initial infrequent openings of even a few channels provide a highly localized influx of Ca\(^{2+}\). However, due to the high channel density that exists in these systems, this small initial Ca\(^{2+}\) influx is sufficient to facilitate the opening of neighbouring channels, leading to rapid and self-sustained positive feedback. However, Ca\(^{2+}\) influx through TRPM2 is insufficient to allow gating by ADPR in endogenous systems, where channel density is comparatively lower. Indeed, minimal gating of TRPM2 channels by ADPR alone occurred in neutrophil granulocytes unless recordings were made using pipette solutions containing high Ca\(^{2+}\), leading to the suggestion that endogenous ADPR “enables” the activation of TRPM2 (Kraft & Harteneck, 2005).

Our recordings from cultured hippocampal neurons were carried out in the absence of exogenous intracellular Ca\(^{2+}\) buffering in order to promote maximal channel activation. However, even under these conditions, application of supramaximal concentrations (1 mM) of ADPR could not reliably evoke TRPM2 currents on its own and an additional stimulus providing an influx of Ca\(^{2+}\) was required. This could be achieved in neurons by applying voltage-ramps to the membrane. The voltage-ramp-mediated induction of these currents was prevented by applying extracellular Ni\(^{2+}\) and Cd\(^{2+}\) or by buffering intracellular Ca\(^{2+}\) with BAPTA. Taken together, these results confirm the role of Ca\(^{2+}\) entry through VDCCs.
Therefore, TRPM2 channels in hippocampal neurons appear to require both “priming” by ADPR as well as “induction” by Ca\(^{2+}\) influx through VDCCs. This requirement may account for the previous report that TRPM2-like currents are exceedingly small in cultured cortical neurons (Kraft et al., 2004).

1.26. **Source-specificity of Ca\(^{2+}\) influx**

We have demonstrated that Ca\(^{2+}\)-influx through either VDCCs or NMDARs is required for activation of the ADPR-dependent current. Regardless of the source of Ca\(^{2+}\) influx, the ADPR-dependent current had a biophysical and pharmacological profile consistent with the activation of TRPM2 currents. Until recently, no evidence that Ca\(^{2+}\) can itself directly gate TRPM2 channels existed. Rather, elevated [Ca\(^{2+}\)]\(_i\) was believed to shift the ADPR concentration-effect relationship, thereby facilitating activation of TRPM2 by ADPR (Perraud et al., 2001; Heiner et al., 2006). However, a recent study by Du et al. (2009) provides evidence to the contrary. In transiently transfected HEK293 cells, intracellular Ca\(^{2+}\) activated TRPM2 currents in the absence of exogenous ADPR. Two lines of evidence support the direct gating of TRPM2 channels by intracellular Ca\(^{2+}\): (1) TRPM2 mutants with disrupted ADPR-binding sites were insensitive to activation by intracellular application of 100 nM Ca\(^{2+}\) and 200 \(\mu\)M ADPR (used to activate wildtype TRPM2) yet were activated by high intracellular Ca\(^{2+}\) alone (100 \(\mu\)M) and (2) application of Ca\(^{2+}\) alone to the cytosolic face of inside-out patches activated wildtype TRPM2 currents. Furthermore, inclusion of 10 \(\mu\)M ADPR shifted the EC\(_{50}\) of Ca\(^{2+}\)-dependent activation from 16.9 to 0.49
µM, suggesting a synergistic effect of Ca\(^{2+}\) and ADPR on wildtype TRPM2 activation (Du \textit{et al.}, 2009).

While these results imply that Ca\(^{2+}\) and not ADPR acts as the key activator of TRPM2 channels, the authors employed 100 mM Ca\(^{2+}\) to directly activate TRPM2 channels, a 1000 fold increase above resting [Ca\(^{2+}\)]. As this concentration likely exceeds the physiological limits of [Ca\(^{2+}\)], whether or not these results hold true for endogenous TRPM2 channels in hippocampal neurons remains the subject of future experiments. Interestingly, however, truncation mutants mimicking endogenous splice isoforms of TRPM2 that are insensitive to gating by ADPR or H2O2 alone and lack portions of either or both of the N- or C- terminals could also be activated by high [Ca\(^{2+}\)] (Du \textit{et al.}, 2009). Since some or all of these isoforms may be expressed in the hippocampus, these findings raise the possibility of novel physiological and/or pathological roles for TRPM2 splice variants in hippocampal neurons.

1.27. \textbf{TRPM2 pharmacology}

The study of many TRP family channel members is made difficult due to the absence of selective blockers. This is especially true when characterizing these channels in neurons, where multiple ligand- and voltage-gated channels can be coincidently activated by, for example, membrane depolarization and oxidative stress. We therefore utilized a number of comparative criteria to establish that the voltage-ramp induced currents were in fact mediated by TRPM2. Firstly, the currents were never observed unless ADPR was included in our intracellular pipette solution. The use of ADPR for the identification of TRPM2 is beneficial in this respect as, to our knowledge, only one other channel can reportedly be activated by
ADPR in mammalian cells. A $K_{Ca}^{2+}$ channel expressed in vascular smooth muscle cells is activated by ADPR and mediates K+ efflux from cells (Li et al., 1997). As these currents are outwardly directed, they did not contribute to our inward, ADPR-dependent currents.

Secondly, our current resembled TRPM2 currents recorded in HEK293 cells in that both: a) demonstrated near linear I-V curves b) had reversal potentials consistent with non-selective cation channels (approximately 0 mV) c) were suppressed by the removal of extracellular Ca$^{2+}$ and d) required intracellular Ca$^{2+}$ for gating of the channels by ADPR. Thirdly, the voltage-ramp activated, ADPR-primed currents in cultured neurons were strongly depressed by appropriate concentrations of antagonists of recombinant TRPM2 channels including clotrimazole, flufenamic acid and ACA. Furthermore, like TRPM2 currents, the ADPR-primed currents were insensitive and, in some cells, even enhanced by applications of La$^{3+}$, a broad-spectrum blocker of numerous non-selective channels including several TRP family members (Zhu et al., 1998; Halaszovich et al., 2000; Inoue et al., 2001; Riccio et al., 2002).

1.28. TRPM2 channels mediate the ADPR-dependent current

Nevertheless, the participation of TRPM2 channels in the ADPR current in hippocampal neurons was further confirmed using a vector-based approach to express an shRNA sequence (shRNA$_{TRPM2}$) that selectively reduced TRPM2 expression. Infection of cultured hippocampal neurons with shRNA$_{TRPM2}$ lentivirus reduced the expression of TRPM2, as revealed by immunostaining, and reduced the amplitude of ADPR currents, which were evoked by NMDA application. Collectively this pharmacological, biophysical and molecular profile provides strong evidence that functional TRPM2 channels are expressed in hippocampal neurons and that TRPM2 currents can be induced by influx of Ca$^{2+}$.
via voltage-dependent Ca\(^{2+}\) channels or NMDARs, provided that intracellular ADPR is made available to prime the channels.

1.29. **TRPM2 activation may explain vulnerability of CA1 neurons to ischemia**

In contrast to our findings, Kraft *et al.* (2004) failed to detect TRPM2 in cultured cerebellar granule neurons suggesting instead that TRPM2 was expressed in microglia. Taken together, their findings and ours indicate that, though broadly distributed in the CNS, the particular cell types expressing TRPM2 vary regionally. This in turn suggests that TRPM2 serves distinct regional functions. Given the selective vulnerability of CA1 pyramidal neurons to ischemic damage (Abe *et al.*, 1995), the functional expression of Ca\(^{2+}\) permeable TRPM2 within this neuronal population is noteworthy. Interestingly, the Ca\(^{2+}\) to monovalent ion permeability ratio of TRPM2 is comparable to that of NMDARs (Jahr & Stevens, 1993; Wollmuth & Sakmann, 1998; Togashi *et al.*, 2008). However unlike NMDARs, TRPM2 channels are not subject to voltage-dependent block by extracellular Mg\(^{2+}\), suggesting that TRPM2 channels could provide much greater Ca\(^{2+}\) entry and correspondingly be more toxic to neurons when active. Indeed, sustained intracellular Ca\(^{2+}\) elevation following TRPM2 activation has been causally linked to cell death in a variety of cell types under conditions promoting oxidative and nitrosative stress (Hara *et al*., 2002). Such conditions are well known to exist during experimentally induced oxygen/glucose deprivation. In cultured cortical neurons, oxygen glucose deprivation leads to the release of glutamate and excessive NMDAR activation (Lipton, 1999). Due to the coupling of NMDARs to NOS via PSD-95, cell death pathways are stimulated in a “source specific” manner by Ca\(^{2+}\)-entry via activated NMDAR (Sattler *et al*., 1999). The resulting activation
of NOS leads to accumulation of ONOO$^-$, which proves especially toxic to neurons (Aarts & Tymianski, 2004). Our demonstration that ONOO$^-$ activated TRPM2 channels in pyramidal neurons is thus important in this respect. Importantly, to our knowledge, there is no evidence to suggest that NO may directly activate TRPM2. Any role for NOS in the activation of TRPM2 would therefore strictly be through its contribution to the production of ONOO$^-$.

Future studies will be needed to determine whether this can in fact occur.

Finally, when recording from cultured hippocampal neurons, the various pyramidal neurons of the hippocampus (CA1-CA4) cannot be readily be distinguished. However, recordings from CA1 pyramidal neurons in slices, including a subset from visually identified neurons, confirm the functional expression of TRPM2 in these neurons, known to be especially vulnerable to ischemia. In contrast, we could not find evidence for the functional expression of TRPM2 in visually identified interneurons; a neuronal population that is reportedly resistant to this type of insult (Ferrer et al., 1995; Inglefield et al., 1997; Sugawara et al., 2002; Frahm et al., 2004). Accordingly, the differential functional expression of TRPM2 channels may partially explain the differential sensitivity of these neuronal populations to ischemic insults. Similarly, although not present in cerebellar granule cells, it remains of interest to determine whether TRPM2 channels are expressed in purkinje cells, which, like CA1 hippocampal neurons, are especially vulnerable to ischemia-induced cell death (Brorson et al., 1995). In addition, although in situ hybridization suggests that TRPM2 transcripts are diffusely expressed throughout hippocampal pyramidal subregions (CA1-CA4), additional studies will be needed in order to determine the extent to which TRPM2 is functionally expressed in each of these neuronal populations.
Pannexin hemichannel activation downstream of NMDA receptors

1.30. Background and rationale

Our results to this point demonstrate that short, repeated applications of NMDA activate TRPM2 currents in hippocampal neurons. This activation protocol may be analogous to either reduced but not complete block of perfusion during an ischemic stroke or to chronic, neurodegenerative diseases such as Alzheimer’s disease. However, we wondered whether sustained NMDAR activation, as might occur when perfusion is completely inhibited during either focal or global cerebral ischemia. Interestingly, we found that, although a secondary current was activated following sustained NMDA application, no significant attenuation of the current was observed in the presence of clotrimazole or nominally Ca\(^{2+}\)-free ECS. Although we cannot exclude the possibility that TRPM2 channels contribute to the current activated following sustained NMDAR activation, they are not the predominant mediators of this secondary current. We therefore sought to determine the molecular correlate of this secondary activated current. Based on its biophysical and pharmacological properties, we hypothesized that the secondary current was mediated by pannexin-containing hemichannels since these channels can be activated in physiological concentrations of extracellular Ca\(^{2+}\) and are activated following OGD in neurons (Thompson et al., 2006).
1.31. Results

1.31.1. Activation of a Ca\(^{2+}\)-dependent current following prolonged NMDAR stimulation

To begin to investigate the mechanism whereby prolonged NMDAR stimulation leads to the activation of a secondary inward current, cultured hippocampal neurons were voltage-clamped at -60 mV in the whole-cell configuration and the baseline holding current recorded for 5 minutes. NMDA (100 µM, 10 µM D-Serine) was then applied continuously for 5 minutes and any change in the holding current monitored. During the NMDA application period, we noted that the cell bodies became round and swollen in appearance. This was likely due to the continuous driving force for Na\(^+\)-influx through activated NMDARs when the membrane is clamped at -60 mV for an extended time period. So as to minimize any potential contaminating effects of Na\(^+\)-loading and subsequent cell swelling, we applied NMDA while clamping the total current at 0 pA and allowing the membrane potential to vary physiologically. Neurons retained their pyramidal morphology throughout this protocol. Following stimulation of NMDARs in the presence of 2 mM extracellular Ca\(^{2+}\) and return to voltage-clamp mode (-60 mV), an inward current, secondary to NMDA exposure (I\(_{2nd}\)) developed (Fig. 15A). I\(_{2nd}\) exhibited a linear current-voltage relationship that reversed near 0 mV, indicating that it is likely a non-selective cation current (Fig. 15B). Activation of I\(_{2nd}\) was prevented when NMDA was applied in nominally Ca\(^{2+}\)-free extracellular solution that was substituted with 2 mM Ba\(^{2+}\) to maintain the concentration of divalent ions (Fig. 15 A and B). This result indicates that Ca\(^{2+}\)-influx through NMDARs is required for I\(_{2nd}\) development. Furthermore, the requirement for Ca\(^{2+}\)-influx suggests that I\(_{2nd}\) was not activated by a coincident, Ca\(^{2+}\)-independent event also activated downstream of NMDARs.
Figure 15 - Activation of a secondary, Ca\textsuperscript{2+}-dependent current following sustained NMDAR stimulation. A, summary data illustrating the time-course and Ca\textsuperscript{2+}-dependence of I\textsubscript{2nd} development. Cultured hippocampal neurons were voltage-clamped at -60 mV in the whole-cell configuration and the baseline holding current recorded for 5 minutes. Following a switch to current-clamp mode, NMDA (100 µM plus 10 µM D-Serine) was applied continuously for 10 minutes in either the absence (○) or presence (■) of extracellular Ca\textsuperscript{2+} (2 mM). B, average I-V relationships before (solid line) and after (broken line) NMDA application in the presence (top) and absence (bottom) of extracellular Ca\textsuperscript{2+}. 
1.31.2. Timecourse of maximal $I_{2nd}$ activation

As sustained NMDAR activation resulted in the development of a large, inward current, one possibility was that we were simply recording a non-specific effect of cell membrane breakdown or seal deterioration. Although the method of activating $I_{2nd}$ was reminiscent of Chen et al.’s (1997) post-exposure current, the absence of an antagonist or known molecular identify made it difficult to directly ascertain if this were the case. We therefore tested the idea that if $I_{2nd}$ is in fact mediated by channel openings downstream of NMDARs, varying the length of NMDAR activation should result in the graded development of this secondary current. Conversely, we reasoned that any non-specific effects such as the loss of a high resistance seal were expected to yield “all or nothing” responses. Accordingly, we varied the length of NMDA application and monitored the amplitude of $I_{2nd}$. The threshold of current activation was 1 minute and its amplitude increased with increasing lengths of NMDAR stimulation until a plateau was reached. Increasing the length of the NMDA application period beyond 5 minutes did not significantly increase the amplitude of $I_{2nd}$ (Fig. 16). These data support the hypothesis that a secondary conductance is activated following NMDAR stimulation.
Figure 16 – The amplitude of $I_{2\text{nd}}$ depends on the duration of NMDA application. Summary data demonstrating that varying the length of the NMDA application in I-clamp mode from 1 to 10 minutes alters the amplitude of NMDAR $I_{2\text{nd}}$. Maximal currents developed following a 5 minutes NMDA application.
1.31.3. **Activation of I\textsubscript{2nd} is mediated by pannexin-1 hemichannels**

Electron microscopical immunohistochemistry has revealed that Px1 is expressed in hippocampal pyramidal neurons and colocalizes with PSD-95 (Zoidl et al., 2007). Furthermore, Px1-containing hemichannels were activated following periods of OGD in hippocampal neurons (Thompson et al., 2006), which is intimately linked to the activation of NMDARs (Aarts et al., 2003). Given that our biophysical and pharmacological evidence is consistent with I\textsubscript{2nd} being mediated by Px1-hemichannels, we employed two distinct approaches to ascertain if this was in fact the case. Firstly, we included the Px1 mimetic inhibitory peptide, \textsuperscript{10}panx1, in the patch pipette (100 µM; WRQAAFVDSY) (Pelegrin & Surprenant, 2006) and monitored any change in the holding current following NMDA application. Consistent with the notion that sustained NMDA application activates a Px1 hemichannel in hippocampal neurons, intracellular dialysis of cells with this peptide reduced the I\textsubscript{2nd} activated following a 5 minute NMDA application (20 minutes post-NMDA application: control, -2361 ± 381.8 pA, n = 11 vs. \textsuperscript{10}panx1, -884.7 ± 202.1 pA, n = 12, p < 0.005) (Fig. 17 A and B). Neither peak nor steady state NMDA currents were altered in the presence of \textsuperscript{10}panx1, indicating that the attenuation of I\textsubscript{2nd} was not due to block of NMDAR-mediated currents.

We next employed an shRNA-mediated gene silencing strategy to confirm any contribution of Px1 to I\textsubscript{2nd}. This was of particular importance as a recent study provided strong evidence that the Px1 inhibitory peptide is not nearly as specific as first reported (Wang et al., 2007). An shRNA sequence previously reported to reduce the expression of Px1 and a scrambled control sequence were cloned into a lentiviral vector. As with the
vector we used to express anti-TRPM2 shRNA, this vector allowed for expression of GFP and permitted the confirmation of expression rates via visual inspection. We first confirmed that the anti-Px1 shRNA reduced Px1 expression. Greater than 80% infection of cultured hippocampal neurons with both GFP and the shRNA vector was observed. Comparatively, the infection of non-neuronal cells was minimal. Western blot analysis using an antibody directed against a C-terminal portion of Px1 revealed that Px1 expression was reduced to 43 ± 10% of control (P < 0.05; ANOVA; n = 4) (Fig. 18). Next, we employed sister cultures to determine if I_{2nd} was reduced following the shRNA-mediated reduction of Px1. Recordings were only carried out from shRNA-expressing neuron, as determined by the intensity of the GFP signal. Following a 5 minute NMDA application, the peak I_{2nd} amplitude was reduced by >70% in anti-Px1 shRNA-expressing neurons versus control (Fig. 19 and 20).
Figure 17 – NMDAR I\textsubscript{2nd} is blocked by the Px1-inhibitory peptide 10\textsuperscript{panx1}. **A**, summary data demonstrating that 10\textsuperscript{panx1} (●) prevented the development of NMDAR I\textsubscript{2nd} as compared with control (○) recordings where the inhibitory peptide was excluded from that patch pipette. Neurons were voltage-clamped at -60 mV and 10\textsuperscript{panx1} (100 µM) allowed to dialyze for 5 minutes. Following a 5 minute application of NMDA in current-clamp mode (I = 0 nA), cells were again voltage-clamped at -60 mV and changes in the holding current monitored. **B**, summary graph illustrating holding current at -60 mV that developed in individual neurons with or without 10\textsuperscript{panx1}.
Figure 18 – Anti-Px1 shRNA reduction of Px1 expression in hippocampal neurons.

Summary Western blot data from 4 distinct experiments indicating that cultured hippocampal neurons infected with GFP and shRNA targeted against Px1 demonstrated significant (P < 0.05, ANOVA) knockdown of the hemichannel protein. Lanes were loaded with samples prepared from neurons that were 1: uninfected, 2: infected with GFP and scrambled shRNA and 3: infected with GFP and anti-Px1 shRNA.
**Figure 19** – NMDAR $I_{2nd}$ is blocked by RNA interference of Px1. Average data demonstrating that the development of $I_{2nd}$ is significantly reduced in neurons infected with anti-Px1 shRNA but not in those infected with a scrambled shRNA. Recordings were made from GFP positive neurons to insure infection with the shRNA.
Figure 20 – Quantification of I_{2nd} (at -60 mV) following NMDA application. All statistical comparisons were made to the current amplitude for a 5 minute exposure to NMDA (ANOVA; P < 0.05). Asterisks indicate a significant difference from the 5 minute NMDA application.
1.31.4. ATP regulation of pannexin1 currents

Sustained Ca\(^{2+}\) influx via over-activated NMDARs leads to an increase in intracellular Ca\(^{2+}\), collapse of the mitochondrial transmembrane potential and consequent opening of the permeability transition pore (mPTP). Prevention of mitochondrial collapse is central in minimizing ischemia/reperfusion-induced cell death. Since I\(_{2\text{nd}}\) activation required Ca\(^{2+}\)-influx through NMDARs, we tested the hypothesis that mitochondrial collapse was necessary for I\(_{2\text{nd}}\) activation. When neurons were dialyzed with our standard patch pipette solution (2 mM Mg\(^{2+}\), no ATP), I\(_{2\text{nd}}\) developed following NMDA application. Conversely, cells dialyzed with intracellular solution containing high Mg\(^{2+}\) and ATP, non-specific inhibitors of the mPTP, failed to develop I\(_{2\text{nd}}\) (no exogenous ATP: -30.2 ± 8.6 pA/pF, n = 3 vs. 1 mM MgATP: -9.4 ± (Fig. 21 A and B). While these data suggest a role for mitochondrial collapse in activating I\(_{\text{pc}}\) downstream of NMDARs, the use of specific inhibitors of mPTP failed to corroborate this data. The recent finding that intracellular ATP inhibits Px1 hemichannels provides a novel interpretation of these results (Qiu & Dahl, 2009).
**Figure 21** – Intracellular MgATP dialysis prevents the activation of I$_{2nd}$. **A,** summary data demonstrating the development of I$_{2nd}$ following a 10 minute NMDA application in the absence of exogenous MgATP (control, ■). Inclusion of 10 mM MgATP (○) in the patch pipette prevented the development of this current. **B,** representative I-V relationships in the presence or absence of exogenous ATP. Solid lines represent I-V relationships at the start of the recording protocol whereas dotted lines represent I-V relationships 15 minutes following NMDAR stimulation. The absence of a linear I-V relationship after 15 minutes in the presence of high MgATP is consistent with the block of I$_{2nd}$. 
Discussion

1.32. Biophysical and pharmacological properties of the NMDA-dependent current

Several groups have demonstrated that sustained NMDA application or oxygen/glucose deprivation (OGD) in hippocampal neurons activates a secondary, post-exposure current that is not mediated by the NMDAR ligand-gated current (Chen et al., 1997). However, despite the passing of over 10 years since its initial characterization, the identity of this current remains unknown. Here, we provide the first evidence that pannexin hemichannels are activated downstream of NMDARs and that they constitute a major component of $I_{2nd}$. Like the OGD-activated current ($I_{OGD}$), $I_{2nd}$ exhibited a linear I-V relationship that reversed near 0 mV, consistent with the activation of a non-selective current. While the I-V relationship of heterologously expressed Px1 in Xenopus oocytes (Bruzzone et al., 2003; Bao et al., 2004) and HEK 293 cells (Pelegrin & Surprenant, 2006) was strongly outwardly rectifying, our results resemble the so-called “large-conductance” mode of P2X7 channels (Virginio et al., 1999), which is mediated by Px1-containing hemichannels (Pelegrin & Surprenant, 2006). Finally, both $I_{2nd}$ and $I_{OGD}$ were inhibited by extracellular application of the gap junction and hemichannel inhibitor, carbenoxolone (Thompson et al., 2006; Thompson et al., 2008).

1.33. Specificity of the pannexin-1 inhibitory peptide

Our finding that a peptide inhibitor of Px1 hemichannels (10panx1) prevented the activation of $I_{2nd}$ implicates Px1 as the molecular correlate of $I_{2nd}$. 10panx1 is a Px1-mimetic
peptide that was developed to examine the contribution of Px1 to P2X7-mediated effects. In macrophages, 10\(^{\text{panx1}}\) potently inhibited P2X7-mediated dye uptake without altering other aspects of P2X7 receptor activation including ATP-evoked currents and cytosolic Ca\(^{2+}\) transients (Pelegrin & Surprenant, 2006). However, its specificity and those of related gap junction channel inhibitors have been called into question. 10\(^{\text{panx1}}\) is part of a group of gap junction mimetic peptides designed to bind to the extracellular docking gates of these channels in a sequence-specific manner. In a comprehensive study, Wang et al. (2007) provided strong evidence that the mechanism of inhibition is, in fact, not sequence-specific but rather, that these peptides sterically hinder the passage of large molecules and ions through the channel pores. Of particular importance to our studies, application of 10\(^{\text{panx1}}\) to oocytes expressing the lens-specific gap junction protein Cx46, which forms patent hemichannels in these cells, inhibited Cx46-mediated currents. The extent of inhibition was in the same range as that of Px1 currents. Furthermore, Px1 currents could be effectively attenuated in a size-dependent manner by inert polyethyleneglycols, lending further support to the steric model of inhibition. This model explains the seemingly peculiar finding that 10\(^{\text{panx1}}\) inhibited P2X7-mediated dye uptake but not P2X7 currents, as it is reasonable to suppose that small ions but not large dye molecules might pass through a partially occluded channel pore (Wang et al., 2007).

1.34. Pannexin-1 hemichannels are activated downstream of NMDARs

Accordingly, to further substantiate our findings, we employed an shRNA approach to reduce the expression of Px1 in cultured hippocampal neurons infected using a lentivirus delivery system. Although it is notoriously difficult to express exogenous sequences in
neurons, lentivirus delivery permits highly efficient and comparatively long-lasting gene transfer in these cells (Blomer et al., 1997). In this vein, infection of our cultured neurons with GFP and either scrambled or Px1 shRNA was achieved at greater than 80% efficiency. While we observed a knockdown efficiency of only 43% by means of Western blot analysis and a greater than 70% reduction in I_{2nd}, this difference can be attributed to the fact that neurons selected for recordings expressed high levels of GFP (as observed by the very bright GFP signal) while those used for Western blot analysis had variable rates of infection. The residual current observed is likely due, at least in part, to incomplete shRNA-knockdown of Px1. However, we expect that TRPM2 currents are also activated following sustained NMDA application and cannot exclude the possibility that they contribute to this residual current. In fact, the small residual current that remained following either carbenoxolone or La^{3+} block of I_{OGD} was consistent with a TRP family current. Taken together with the previously discussed biophysical and pharmacological evidence, the finding that I_{OGD} had a single channel conductance matching that of Px1 (Thompson et al., 2006) suggests that both I_{OGD} and I_{2nd} are mediated by Px1-containing hemichannels.

1.35. Role of Ca^{2+} in pannexin-1 activation

Unlike their connexin counterparts, pannexin hemichannels are not blocked by physiological concentrations of extracellular Ca^{2+} and may therefore be activated during the reperfusion of ischemic tissue. The ensuing ionic dysregulation may mediate the delayed neuronal death associated with this phase of injury. Our results suggest that Ca^{2+}-influx through activated NMDARs is required for the activation of Px1 hemichannels, as NMDA application in the absence of extracellular Ca^{2+} prevented the development of I_{2nd}. If the
Ca$^{2+}$ load hypothesis is to be taken at face value, the most simple explanation linking NMDARs and Px1 hemichannels is via a rise in \([\text{Ca}^{2+}]_i\). That is, \(\text{Ca}^{2+}\)-influx through continuously activated NMDARs triggers the activation of hemichannels either directly or through a \(\text{Ca}^{2+}\)-dependent process. Surprisingly, however, our collaborators found that increasing the \(\text{Ca}^{2+}\)-buffering capacity of the intracellular solution (strong: 10 mM BAPTA vs. weak: 0.1-1 mM EGTA) had no significant effect, as it did not prevent hemichannel opening (Thompson et al., 2008). The reconciliation of these two findings is not immediately obvious but may be attributed to the difference between removing extracellular \(\text{Ca}^{2+}\) and buffering it intracellular. Interestingly, in oocytes heterologously expressing Px1, application of micromolar \(\text{Ca}^{2+}\) to the cytoplasmic face of excised inside-out patches was sufficient to activate Px1-mediated currents. This single-channel activity was attenuated upon washout of the \(\text{Ca}^{2+}\)-containing solution, suggesting that \(\text{Ca}^{2+}\) activates Px1 via a direct mechanism and not through a \(\text{Ca}^{2+}\)-dependent intermediate (Locovei et al., 2006).

Despite this discrepancy, the depletion of intracellular ATP levels may link NMDARs to Px1 activation via both \(\text{Ca}^{2+}\)-dependant and independent mechanisms. In addition to \(\text{Ca}^{2+}\)-influx, sustained NMDAR activation results in Na$^+$-loading of neurons, which is then extruded from cells via the Na$^+$/K$^+$ ATPase. The Na$^+$/K$^+$ ATPase is a major consumer of neuronal ATP and enhanced metabolic demand following sustained Na$^+$ extrusion leads to ATP depletion (Attwell & Laughlin, 2001). In fact, a recent study suggested that the depletion of ATP may precede delayed \(\text{Ca}^{2+}\) overloading of neurons and any ensuing \(\text{Ca}^{2+}\)-dependent cell death (Vander Jagt et al., 2008). Additionally, over-activation of NMDARs leads to \(\text{Ca}^{2+}\)-dependent mitochondrial dysfunction and collapse of the ATP gradient. Our results demonstrating that perfusion of hippocampal neurons with MgATP prevented
activation of $I_{\text{2nd}}$ are consistent with the hypothesis that Pxx1 is activated following NMDAR-mediated ATP depletion. While further experiments are necessary, it is likely that both $\text{Ca}^{2+}$-dependent and independent ATP depletion pathways work in parallel and result in the activation of Pxx1 hemichannels. Interestingly, although Pxx1-containing hemichannels are permeable to ATP, ATP itself prevents channel opening (Qiu & Dahl, 2009). The activation of Pxx1 during metabolic distress, which is associated with a drop in cytosolic ATP, supports a role for this hemichannel in mediating neuronal death.
General Discussion

1.36. Reconciling the NMDA-dependent activation of both TRPM2 and pannexin-1

The main findings of this study are 1) functional TRPM2 channels and Px1 hemichannels are found in hippocampal neurons and 2) both can be activated following NMDAR activation. Our results demonstrate that short, repeated NMDA applications activate TRPM2 channels while sustained NMDAR stimulation activates Px1 hemichannels in hippocampal neurons. One important question that arises from these findings is: Under what physiological or pathophysiological conditions does NMDAR activation lead to TRPM2 versus Px1 activation and what are the signaling cascades involved? While further investigation is warranted, one possibility is that the detrimental activation of Px1 occurs under conditions of complete metabolic failure and ATP depletion, such as might be observed during global ischemia or complete focal ischemia where perfusion is blocked. Our finding that supplementing the intracellular milieu with ATP prevents Px1 activation supports this reasoning, as Px1 hemichannels are blocked by ATP (Qiu & Dahl, 2009). Of course, it is likely that TRPM2 channels are also activated under these conditions, since metabolic failure results in the production of free radicals and ADPR. However, the large conductance of Px1 hemichannels coupled with our finding that shRNA-mediated reduction in Px1 expression significantly attenuated the development of NMDAR I_{2nd} provides support for the hypothesis that the predominant current is mediated by Px1 hemichannels, at least under these conditions. In either case, the activation of Ca^{2+}-permeable TRPM2 channels may represent a feed-forward mechanism for the continued activation of Px1 hemichannels.
While the studies presented in this thesis demonstrate that NMDA application can lead to the activation of both TRPM2 channels and Px1 hemichannels, they do not directly address any functional relationship between these channels. In particular, whether Px1 and TRPM2 are activated via distinct or overlapping signaling pathways downstream of NMDARs remains an open question. Furthermore, the time-course of their activation following NMDAR stimulation is unknown and may provide clues to both pathological and physiological roles for these channels in hippocampal neurons.

Coupled with previous demonstrations linking TRPM2 activation to cell death (Hara et al., 2002), our result demonstrating that TRPM2 channels are activated following short, repeated NMDA applications suggests a role for TRPM2 in mediating neurotoxicity. However, no studies exist that examine the contribution of either TRPM2 or Px1 activation to cell death in hippocampal neurons. While TRPM2 activation has been demonstrated to lead to mixed apoptotic and necrotic death in cardiac myocytes (Yang et al., 2006), it will be interesting to determine if this holds true in hippocampal neurons. Finally, it is likely that multiple pathways are activated following inappropriate NMDAR stimulation, of which TRPM2 and Px1 are but two examples. The activation of such NMDAR-independent pathways as OGD-activated TRPM7 may act in parallel with NMDAR-dependent pathways and contribute to hippocampal neuronal death. Determining the contribution of each is not trivial, especially in the absence of highly selective pharmacological tools. However, in combination with shRNA approaches, the recent development of TRPM2 knockout mice will likely facilitate these experiments.
Figure 22 – Proposed model of TRPM2 and Px1 activation following NMDAR activation in hippocampal neurons. Plus signs indicate potential positive feedback mechanisms.
1.37. **What factors confer a physiological vs. pathological role for TRPM2 in neurons?**

Although our knowledge of TRPM2 channels is rapidly expanding, no physiological role for these channels in neurons has been identified to date. The simplest explanation for this is that the role of TRPM2 channels in neurons is restricted to pathological states. While possible, this explanation is unsatisfying and likely oversimplified. Indeed, several neuronal ion channels possess dual physiological and pathophysiological roles, of which the NMDAR and pannexin hemichannels are the most relevant examples (Hardingham *et al.*, 2002; Thompson *et al.*, 2006; Thompson *et al.*, 2008).

Our finding that activation of either VDCCs or NMDARs can synergize with ADPR to gate TRPM2 argues against the “source specificity” hypothesis of Ca\(^{2+}\)-influx and suggests that it is the amount and not location of Ca\(^{2+}\) influx that serves to gate TRPM2. However, all our experiments measured a single end-point, that is, the development of the TRPM2-mediated current itself. Doing so does not permit us to distinguish between potential differences in any signaling cascade activated downstream of TRPM2 channels. One possibility is that VDCC-dependent activation of TRPM2 is coupled to physiological signaling pathways while those coupled to NMDAR-dependent activation are cytotoxic. Support for this concept comes from Tymianski *et al.* (1993b), who demonstrated that Ca\(^{2+}\)-influx through NMDARs is toxic to neurons while that through VDCCs is not. Indeed, our preliminary results suggest that TRPM2 activation is coupled to NR2B-containing NMDARs, which are associated with neurotoxicity. Furthermore, our finding that NMDA applications activate TRPM2-like currents in the absence of exogenous ADPR suggest that NMDAR activation may itself lead to the generation of intracellular ADPR via either
mitochondrial and/or nuclear pathways. Such a feedforward mechanism would be predicted to amplify cytotoxic TRPM2-dependent signaling.

In this vein, the recent finding that ADPR-insensitive splice variants of TRPM2 can be gated by high intracellular Ca\(^{2+}\) alone warrants further investigation in neurons (Du et al., 2009). It is intriguing to think that hippocampal neurons possess multiple TRPM2 isoforms, which are coupled to distinct signaling pathways. Indeed, the presence of a distinct low molecular weight band in our Western blot results suggests the presence of multiple TRPM2 isoforms in hippocampal neurons. Full-length channels, which are synergistically gated by ADPR, may play a role in mediating pathophysiological events while activation of their ADPR-insensitive counterparts may modulate such physiological processes as synaptic plasticity.

1.38. **A potential role for TRPM2 in the Ca\(^{2+}\) paradox and ischemia/reperfusion injury**

Over-activation of NMDARs during anoxia can lead to a dramatic fall of extracellular Ca\(^{2+}\) concentrations (Silver & Erecinska, 1990). The decrease in [Ca\(^{2+}\)]\(_e\) during the ischemic period is followed by a rapid rise in [Ca\(^{2+}\)]\(_e\) once over-stimulation of NMDARs by glutamate has ceased. This situation is in many ways analogous to the changes in [Ca\(^{2+}\)]\(_e\) that occur following reperfusion of tissue made ischemic during stroke and is akin to the so-called “Ca\(^{2+}\) paradox” reported in isolated hearts (Zimmerman & Hulsmann, 1966; Hearse et al., 1978). For clarity, the Ca\(^{2+}\) paradox can be divided into two distinct phases, which are analogous to the two phases of ischemia/reperfusion injury observed *in vivo*. Phase 1 or the Na\(^+\)-loading phase likely occurs during the ischemic event and is associated with low [Ca\(^{2+}\)]\(_e\) as well as probable decreases in the extracellular concentration of Mg\(^{2+}\) and pH. Conversely,
phase 2 encompasses the reperfusion period, during which $[Ca^{2+}]_e$ is restored. This latter phase is associated with cell death. However, detailed studies demonstrating the existence of the $Ca^{2+}$ paradox in neurons have not been carried out and the molecular mechanism underlying this phenomenon remains unclear.

Several properties of TRPM2 indicate that it may play a role in mediating the second, “reperfusion phase” of the $Ca^{2+}$ paradox. Unlike the alternative candidate TRPM7, TRPM2 channels are not blocked by extracellular divalents, including $Ca^{2+}$. Rather, their activity depends on the relative levels of extracellular and intracellular $Ca^{2+}$. For example, if the $[Ca^{2+}]_i$ is low (∼ 30 nM), extracellular $Ca^{2+}$ is absolutely required for full activation of TRPM2 channels. This requirement may be due to a $Ca^{2+}$ binding site within the channel pore that is accessible from either side of the membrane (Starkus et al., 2007). Although over-activation of NMDARs during ischemia promotes $Ca^{2+}$-loading of neurons, $Ca^{2+}$-induced inactivation of NMDARs subsequently depresses currents through these channels (Ehlers et al., 1996), thereby limiting $Ca^{2+}$-entry and subsequent neurodegeneration. However, $Ca^{2+}$-influx through NMDARs is structurally coupled to potentially toxic NO signaling pathways, including production of ONOO-, via PSD-95 (Aarts et al., 2002). ONOO- may then activate TRPM7 channels, which are further activated during the 1st (“ischemic” phase) by the decrease in extracellular divalent cations. Furthermore, ADPR is likely generated downstream of over-activated NMDARs. This likely sets in motion a feed-forward pathway whereby the rise in $[Ca^{2+}]_i$, oxidative stress and ADPR generation prime TRPM2 channels for full activation. Upon reperfusion, the restoration of $[Ca^{2+}]_e$ may be sufficient to fully activate TRPM2 channels, leading to massive $Ca^{2+}$-influx and consequent neuronal death.
In our experiments assessing whether functional TRPM2 channels were present in hippocampal neurons, we noticed that the development of TRPM2-like currents following repetitive NMDA applications was rarely observed in cultured hippocampal neurons less than 18 DIV. However, we could consistently record these currents in neurons between 18 and 30 DIV with exogenous ADPR in the patch pipette. The requirement for “old” neurons became even more stringent when short, repeated NMDA applications were employed to activate TRPM2-like currents in the absence of added ADPR. Under these conditions, TRPM2 currents could not be evoked in neurons younger than 27-28 DIV. A build-up of free radicals in older neurons, which could facilitate TRPM2 activation, might account for these differences. Indeed, ageing is associated with impaired Ca$^{2+}$ homeostasis (Hajieva et al., 2009), depolarization of the mitochondria and increased production of reactive oxygen species (Parihar & Brewer, 2007). Furthermore, in response to glutamate challenge, older neurons were slower to remove excess intracellular Ca$^{2+}$ and return to baseline (Raza et al., 2007), suggesting that they may be more vulnerable to excitotoxicity and oxidative stress than their younger counterparts. An alternative possibility is that the expression of TRPM2 increases throughout development.

Aging is associated with increased vulnerability to stroke as well as neurodegenerative disorders such as Alzheimer’s Disease (AD). In fact, the risk of AD increases dramatically in persons older than 70 years of age. Histopathologically, AD is characterized by senile “plaques,” aggregates of amyloid beta-peptide (Abeta) and “tangles,” aggregates of the microtubule-associated protein tau (Mattson, 2004). The accumulation of
neurotoxic forms of Abeta is believed to be an important mediator of neurodegeneration in AD. Diseased neurons exhibit increased oxidative damage, impaired energy metabolism and altered Ca\textsuperscript{2+} homeostasis. Consequently, one possible mechanism of Abeta-induced neurodegeneration is via the generation of ROS (Mattson, 1997). However, the mechanism linking Abeta and oxidative stress to cell death is poorly understood.

Recently, Abeta-dependent toxicity was found to require the activation of TRPM2 currents in striatal neurons, as blocking TRPM2 prevented both the Abeta-mediated rise in [Ca\textsuperscript{2+}]\textit{i} and ensuing cell death (Fonfria et al., 2005). Preliminary results from our lab corroborated these findings in hippocampal neurons. Interestingly, neurons that express post-synaptic glutamate receptors are particularly vulnerable to neurodegeneration in AD (Mattson, 2004). Our results demonstrate that TRPM2 channels can be activated downstream of the NMDA-subtype of glutamate receptor and provide a potential link between Abeta, oxidative stress and cell death, as NMDA-dependent TRPM2-activation may exacerbate Abeta-mediated toxicity.

Finally, it is interesting to note that oxidative stress was shown to upregulate TRPM2 mRNA expression in a model of middle cerebral artery occlusion (Fonfria et al., 2006a). Although this study was carried out in a microglial cell line, these results imply that, in addition to higher levels of oxidants facilitating TRPM2 activation in older cells, older cells may actually express higher levels of the channel. It will be valuable to determine if these results hold true in neurons, in general, and hippocampal neurons, in particular.
1.40. **Conclusions and significance**

This study provides the first evidence that functional TRPM2 channels are expressed in hippocampal neurons and activated downstream of NMDARs. I demonstrated that, in addition to gating by intracellular ADPR, TRPM2 gating in neurons required a concomitant rise in intracellular Ca\(^{2+}\) through either VDCCs or NMDARs. Furthermore, TRPM2 channels could be activated following short, repeated NMDA applications in the absence of exogenously applied ADPR, suggesting that NMDAR activation itself results in the production of ADPR. Conversely, sustained NMDA applications activated Px1 hemichannels, providing the first evidence that NMDARs and Px1 channels are functional linked. This activation required the influx of Ca\(^{2+}\) through NMDARs and likely occurred via a mechanism dependent on metabolic failure. These results are significant as they expand our knowledge of the roles of TRPM2 channels and Px1 hemichannels in neuronal physiology and pathophysiology and enhance our understanding of excitotoxicity by providing a link between NMDARs, TRPM2 channels and Px1 hemichannels. The identification of these channels downstream of NMDAR activation provides novel pharmacological targets to prevent the neuronal death that occurs following such conditions as cerebral ischemia and AD.
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Appendix
Appendix: Pannexin-like hemichannels are activated by tyrphostin A9 in hippocampal neurons

The following study consists of the biophysical and pharmacological characterization of a novel current activated by a synthetic tyrosine kinase inhibitor, tyrphostin A9. Serendipitously, our results suggest that tyrphostin A9, in fact, activates a pannexin-like current in hippocampal neurons. However, the tyrphostin-activated current is “contaminated” with secondary-activated conductances, reducing any potential utility of tyrphostin A9 as a hemichannel agonist, even in vitro. The salient points to arise from this study are: (1) There are likely many, as yet unknown, ways of activating hemichannels in neurons and (2) activation of hemichannel-like activity must be taken into consideration when interpreting results from experiments employing tyrphostin A9 to inhibit tyrosine kinase activity.

1.41. Introduction

Tyrphostin A9 (TA9), a member of a class of membrane permeable protein tyrosine kinase inhibitors, is a potent PDGFR antagonist commonly employed to assess the role of PDGFR activity in diverse signaling events. While its inhibitory effects are highly specific when compared to its actions on closely related receptor tyrosine kinases (IC$_{50}$ = 500 nM for the PDGFR vs. IC$_{50}$ = 460 mM for the epidermal growth factor receptor), a growing list of kinases inhibited by TA9 has been identified (Aasheim et al., 2005; Fuortes et al., 1999). Moreover, several studies now point to the existence of tyrosine kinase independent effects of TA9 and related compounds, suggesting that tyrphostins are not nearly as specific as first
reported. For example, tyrphostins A51 and A48 inhibit cyclic nucleotide phosphodiesterases (Nichols et al., 2000), A23, A25 and A47 inhibit GTP-dependent enzymes (Wolbring et al., 1994) and A8, A23 and A48 inhibit calcineurin (Martin, 1998). Interestingly, tyrphostin A23 activates a Na\(^+\)-Ca\(^{2+}\) exchanger current but this likely occurs downstream of tyrosine kinase inhibition (Missan et al., 2004). To our knowledge, no studies exist demonstrating that tyrphostins can act as agonists of ionotropic or ligand-gated ion channels, independently of their RTK-inhibitory activity.

Surprisingly, in our experiments employing TA9 to inhibit PDGFR activity in hippocampal neurons, we noted the activation of an inward current (I\(_{TA9}\)) upon extracellular application of TA9 at -60 mV. I\(_{TA9}\) activation was independent of TA9’s tyrosine kinase inhibitory effects as intracellular application via the patch pipette failed to activate the current. We therefore sought to characterize the biophysical and pharmacological properties of this novel current. Here, we demonstrate that tyrphostin A9 activates a Ca\(^{2+}\)-permeable, non-selective current in hippocampal neurons. Furthermore, we provide pharmacological evidence that I\(_{TA9}\) may be mediated by pannexins, a recently identified gap junction hemichannel family of proteins.
1.42. **Methods**

1.42.1. **Cell isolation**

CA1 pyramidal neurons were acutely isolated from hippocampal slices prepared from 15- to 21-day-old Wistar rats using modified methods as previously described (Wang *et al.*, 1995). Briefly, hippocampi from postnatal Wistar rats (15-21 days) were rapidly dissected in ice-cold extracellular solution (ECS) containing in mM: 140 NaCl, 5.4 KCl, 1.3 CaCl₂, 25 HEPES, 33 glucose and 0.0002 tetrodotoxin, with pH adjusted to 7.4 using NaOH and osmolarity between 315 and 325 mosmol, sliced into ~1-mm thick slices. Slices were incubated for 50 min in ECS at room temperature (20-22 °C) before treatment with 2-3 mg/ml crude papain extract for 30 min. Following a 3x wash with ECS, slices were allowed to recover in this solution for at least 2 hrs before use in recordings. All solutions used throughout the dissection were continuously bubbled with 95% O₂, 5% CO₂. Just prior to use, slices were transferred to a 35 mm culture dish containing ECS and neurons isolated by manually tapping the CA1 region.

1.42.2. **Cell culture**

Primary cultures of mouse hippocampal neurons were prepared as described in (MacDonald *et al.*, 1989). In brief, fetuses were rapidly removed from time-pregnant (E17-18) Swiss mice and hippocampal tissue dissected and placed in cold Hanks’ solution before mechanical dissociation by trituration. Cells were plated at a density of <1 x 10⁶ cells/ml on 35 mm
collagen-coated culture dishes and maintained in serum. Neurons were used for electrophysiological recordings between 14 and 21 days *in vitro*.

### 1.42.3. Whole-cell recordings

Patch pipettes were constructed from thin-walled borosilicate glass (1.5 mm diameter, WPI, Sarasota, FL) using a two-stage vertical puller (PP-83, Narashige, Greenvale, NY). For recordings from acutely isolated neurons, patch electrodes had a resistance of 3.5 to 5.5 MΩ when filled with an internal solution containing in mM: 140 CsF, 10 HEPES, 11 EGTA, 1 CaCl₂, 2 MgCl₂, 2 TEACl and 2 K₂ATP, with pH adjusted to 7.3 using CsOH and osmolarity between 290 and 300 mosmol. For recordings from cultured neurons, CsF was replaced by 140 mM CsGluconate. In some experiments, EGTA and CaCl₂ were omitted from the patch pipette and replaced with 20 mM BAPTA. All experiments were performed at room temperature (20-22 °C). Whole-cell voltage-clamp recordings were carried out using a Multiclamp 700a amplifier (Molecular, Devices, Sunnyvale, CA). After formation of the whole-cell configuration, neurons were voltage-clamped at -60 mV and lifted into the stream of solution supplied by a computer-controlled multi-barreled perfusion system (SF-77B, Warner Institute, Hamden, CT, USA). Access resistance was monitored by making a voltage step of -10 mV prior to each application of TA9. Recordings where series resistance varied by more than 10% were not included for analysis. Data were digitized, filtered (2 kHz) and acquired on-line using pCLAMP 9 (Molecular Devices). Data are expressed as mean ± SEM and differences between groups analyzed using the Student’s *t*-test.
1.42.4. Salt Bridge

For ion substitution experiments, recordings were made using a 150 mM KCl in agar salt bridge contained within a glass pipette. This was done so as to minimize any artifact caused by the liquid junction potential between the extracellular (bath) solution and the intracellular (pipette) solution. Such artifacts are most likely to occur when there are large changes in the ionic composition of the bath solution throughout the experiment, as would occur during ion substitution experiments. Use of the salt bridge allows the ground reference electrode to “see” the same solution throughout the experiment, thereby providing a constant liquid junction potential. Furthermore, as the monovalent cation concentration was similar for both the internal and external solutions, “electrode symmetry” was achieved.
1.43. Results

1.43.1. Tyrphostin A9 activates an unidentified current that is sensitive to block by Gd$^{3+}$

Extracellular application of TA9 (10 µM, 3 sec) activated a robust yet unidentified inward current ($I_{TA9}$) ($-1183.7 \pm 227.6$ pA, $n = 6$) (Fig. 1A, 10 µM trace) in acutely isolated CA1 hippocampal neurons voltage-clamped ($E_m = -60$ mV) in the whole-cell configuration. The current was activated rapidly following a 3-sec TA9 application (Fig. 1A, 10 µM trace). As TA9 is a membrane-permeable PDGFR antagonist, one possible mechanism of $I_{TA9}$ activation is through an intracellular, receptor tyrosine kinase-mediated pathway. To explore whether $I_{TA9}$ activation was initiated at an extracellular or intracellular site, we dialyzed voltage-clamped neurons with TA9 by including it in the patch-pipette and monitored any changes in the holding current for 20 min. This time allowed for dialysis was sufficient to observe TA9-dependent inhibition of PDGFR-mediated effects in isolated hippocampal neurons (Kotecha et al., 2002). Intracellular application of TA9 (1 µM or 10 µM) failed to activate the current (data not shown), indicating that $I_{TA9}$ is likely activated at an extracellular site via a receptor tyrosine kinase-independent pathway. Furthermore, if $I_{TA9}$ were activated through an intermediary second-messenger system, the current should persist beyond the TA9 application period. However, the effects of extracellular TA9 (10 µM) were rapidly reversible, as the holding current returned to baseline within several seconds of the TA9 application (Fig. 1A). Taken together, these results suggest that the activation of $I_{TA9}$ reflects the direct gating of an, as yet, unidentified ion channel.

With this in mind, we first addressed the possibility that TA9 is a novel agonist of a well-characterized neuronal current. Acutely isolated hippocampal neurons express a wide
array of ion channels involved in regulating neuronal excitability, including AMPA- and NMDA-receptors. Like \( I_{\text{TA9}} \), both AMPA and NMDA currents are inward at \(-60\) mV in \( \text{Mg}^{2+} \)-free ECS and both exhibit rapid onset followed by desensitization or inactivation (Dingledine et al., 1999). Conveniently, the study of these channels is facilitated by the existence of highly specific antagonists to their activity. If \( I_{\text{TA9}} \) is in fact an AMPA- or NMDA-receptor-mediated current, inclusion of the appropriate antagonist should block the development of the TA9-activated current. We initially maintained cells in our standard ECS and added either CNQX (100 \( \mu \)M) or AP5 (40 \( \mu \)M), competitive antagonists of the AMPA- and NMDA-receptors respectively. Under these conditions, the peak current evoked by TA9 application (10 \( \mu \)M, 3 sec) did not differ significantly from control recordings (control, 95.4 ± 3.5\%, \( n = 6 \); CNQX, 96.7 ± 4.6\%, \( n = 4 \); AP5, 93.3 ± 2.6\%, \( n = 5 \)) (Fig. 1C).

Under the conditions of our recordings (i.e. CsF containing ICF), activation of inhibitory GABAA or glycine receptors, both of which are permeable to \( F^- \), will generate inward currents. Therefore, we tested the ability of antagonists of these receptors to inhibit the tyrphostin-activated current. However, antagonists of GABAA-receptors (bicuculline, 10 \( \mu \)M), glycine-receptors (strychnine, 5 \( \mu \)M) as well as voltage-gated L-type \( \text{Ca}^{2+} \) (nifedipine, 10 \( \mu \)M) channels failed to inhibit \( I_{\text{TA9}} \) (bicuculline, 95.9 ± 3.3\%, \( n = 4 \); strychnine, 96.5 ± 3.5\%, \( n = 4 \); nifedipine, 97.2 ± 4.8\%, \( n = 4 \)) (Fig. 1C), indicating that activation of these channels does not underlie the PDGFR-independent effects of TA9.

The trivalent cation gadolinium (Gd\(^{3+}\)) is a commonly used inhibitor of a wide range of non-selective cation channels including mechanosensitive (Hamill et al., 1996), \( \text{Mg}^{2+} \)-inhibited (Gwanyanya et al., 2004), \( \text{Ca}^{2+} \)-sensing (Xiong et al., 1997) and anoxia-activated (Aarts et al., 2003) currents. We therefore tested the effects of Gd\(^{3+}\) on \( I_{\text{TA9}} \). The addition of
10 µM Gd³⁺ to the ECS resulted in a 52.5 ± 8.6% ($n = 5$, $p<0.01$) reduction of the peak current activated by TA9 (Fig. 1 C and D). This suggests that $I_{TA9}$ is mediated, at least in part, by a non-selective cation channel.

Although our TA9 concentration of 10 µM was within the limits used when inhibiting PDGFR activity (Beazely et al., 2006; Oak et al., 2001) and provided us with a well-defined peak to study the effects of potential antagonists, we noted that considerable and continuous “run-down” of the peak current occurred following the initial drug application. While we accounted for the declining baseline by normalizing our results to the average of the first five drug applications, we worried that this would not be feasible for all experiments. We therefore constructed a concentration response curve to select an appropriate TA9 concentration for our ensuing experiments. $I_{TA9}$ had a threshold concentration of 100 nM and the amplitude of the evoked current increased in a concentration-dependent manner from 100 nM to 50 µM (Figs. 1A and B). Effects of higher concentrations of TA9 could not be ascertained as, in our solutions, the limits of solubility were reached at concentrations above 50 µM. Of importance, we noted that, despite the absence of a well-defined peak, the steady-state current evoked by 1 µM TA9 exhibited proportionally less “run-down” and remained stable following 1-3 drug applications. We therefore employed 1 µM TA9 for all future experiments.
Figure 1 – TA9 activates a Gd$^{3+}$-sensitive inward current in acutely isolated CA1 pyramidal hippocampal neurons. A, representative whole-cell recording demonstrating graded responses to TA9 applications (100 nM to 50 µM). Bar indicates period of TA9 application. B, summary dose-response data for TA9-induced inward currents. Each data point represents mean peak current amplitude ± SEM. C, bar graph of peak TA9 response in the presence of various pharmacological inhibitors of well-characterized neuronal currents. Currents were normalized to the average of the first 5 TA9 responses. Asterisk indicates significance (p < 0.05). D, sample, time-matched traces showing (i) “run-down” in control TA9 current and (ii) block of TA9-current by Gd$^{3+}$. Bar indicates period of TA9 application.
A. 

B. 

C. 

D. 

- **Control**
- **APV**
- **CNQX**
- **Bicuculline**
- **Strychnine**
- **Nipecotoline**
- **Gd**

**Normalized peak current**

- **i**
- **ii**

**Gd**
1.43.2. TA9 activates a Ca$^{2+}$- and voltage-dependent mixed conductance

If TA9 activates a non-selective cation channel, the evoked current should have a reversal potential ($E_{\text{rev}}$) near 0 mV, a hallmark feature of these channels. Accordingly, to begin to assess the ionic basis of $I_{\text{TA9}}$, neurons were voltage-clamped at -60 mV and a series of voltage steps (-80 mV to +20 mV) applied to the membrane (Fig. 2Ai). The resulting current-voltage (IV) relationship was linear and reversed at -42.6 mV ($\pm 3.8$ mV, $n=7$) (Fig. 2Bi and Ci). Despite the observed block by Gd$^{3+}$, the measured $E_{\text{rev}}$ appeared inconsistent with the TA9-mediated activation of a non-selective current. However, as the $E_{\text{rev}, \text{TA9}}$ was not equal to the $E_{\text{rev}}$ of any single ion in our solutions, we could not rule out the possibility that multiple conductances were activated following extracellular TA9 application to hippocampal neurons.

Calcium (Ca$^{2+}$) influx into neurons provides an important signaling function as rises in intracellular Ca$^{2+}$ ($[\text{Ca}^{2+}]_i$) regulate a wide array of cellular targets, including ion channels (Hotson et al., 1980; Peterson et al., 1999; Zhang et al., 1998). If TA9 is a Ca$^{2+}$-permeable, non-selective cation channel agonist, activation of a secondary, Ca$^{2+}$-dependent current may account for the measured non-zero $E_{\text{rev}}$. To determine if $I_{\text{TA9}}$ is dependent on the $[\text{Ca}^{2+}]_i$, we increased the buffering capacity of our ICS by replacing our standard intracellular Ca$^{2+}$ buffer (11 mM EGTA) with BAPTA (20 mM), a rapid and highly selective Ca$^{2+}$ chelator. Inclusion of 20 mM BAPTA in the patch pipette during voltage steps from -80 to +20 mV resulted in a positive shift in the $I_{\text{TA9}}$ $E_{\text{rev}}$ from -42.6 ± 3.8 to -21.3 ± 3.1 mV ($n=7$), indicating that there is a secondary Ca$^{2+}$-dependent component to the TA9-evoked current (Fig 2Ai, Bi and Ci). To further isolate the primary $I_{\text{TA9}}$ from contaminating secondary
conductances, we employed a depolarizing prepulse protocol so as to inactivate voltage-dependent currents with slow inactivation kinetics. During the prepulse period, the membrane was depolarized to the test potential and maintained at this voltage for 1 minute prior to recording $I_{TA9}$ (Fig. 2Aii). With 20 mM BAPTA in the patch pipette, a 1 min prepulse resulted in a right-shift of the IV relationship and depolarized $E_{rev}$ (prepulse + BAPTA, $-2.3 \pm 1.3$ mV, $n = 8$; BAPTA alone, $-21.3 \pm 3.1$ mV, $n = 7$) (Fig. 2Biii and Ci).
Figure 2 – TA9 activates a $\text{Ca}^{2+}$- and voltage-dependent mixed current in hippocampal neurons. A, (i) schematic voltage protocol employed in Bi,ii & Ci,ii and in (ii) Biii & Ci.ii. B, representative voltage step family with (i) 11 mM intracellular EGTA (ii) 20 mM intracellular BAPTA and (iii) 20 mM intracellular BAPTA + depolarizing prepulses. C, averaged I-V relationships from neurons exposed to with (i) 11 mM intracellular EGTA (ii) 20 mM intracellular BAPTA and (iii) 20 mM intracellular BAPTA + depolarizing prepulses.
To test the ionic basis of $I_{TA9}$ in more detail, a series of ion substitution experiments was carried out. Substitution of extracellular monovalent cations for the bulky ion N-methyl-D-glucinate (NMDG$^+$) did not alter the amplitude of $I_{TA9}$ recorded at a holding potential of -60 mV (NMDG$^+$: $-7\pm10\%$ control, n=4, $p>0.15$) nor the measured $E_{rev}$ in response to a series of voltage-steps from -80 to +20 mV (NMDG$^+$, $-38.4\pm2.7$ mV, $n=3$; control, $-35.9\pm2.7$ mV, $n=7$) (Fig. 3Ai,ii,iv). As $I_{TA9}$ was not dependent on the presence of extracellular Na$^+$ or K$^+$, we assayed the possibility that it is an anionic current. When extracellular Cl$^-$ was replaced with the large anion gluconate, a leftward shift in the reversal potential was observed (gluconate, $-55.7\pm5.1$ mV, $n=7$; control, $-35.9\pm2.7$ mV, $n=7$) (Fig. 3Ai,iii,iv). Under our recording conditions, the Cl$^-$ reversal potential was equal to -69 mV. This calculation was based on the assumption that the tyrphostin-activated channel was impermeable to the F- in our patch pipette. The shift in the reversal potential for $I_{TA9}$ towards the Cl$^-$ reversal potential is counterintuitive and suggests that $I_{TA9}$ is not an anionic current. However, if F- is assumed to permeate the tyrphostin-activated channel, the Cl$^-$/F-reversal potential is approximately 0 mV. In this case, a leftward shift in the measured reversal potential (away from the Cl$^-$/F-reversal potential) would be consistent with $I_{TA9}$ being mediated, at least in part, by anions. However, our isolated hippocampal neurons did not tolerate this substitution well, as demonstrated by large holding currents and unstable baselines, particularly at depolarized potentials. We reasoned that maintaining the extracellular Cl$^-$ concentration and instead substituting a bulky anion in the ICS would be less traumatic to the cells, as this is done routinely in our recordings from cultured hippocampal neurons. Furthermore, as the quality of high resistance seals in isolated neurons
deteriorates significantly in the absence of internal F-, we confirmed our findings in cultured hippocampal neurons. No significant difference in the reversal potential was observed in cultured neurons, irrespective of whether gluconate or F- was used as the intracellular anion (*data not shown*).

The failure of either small monovalent cations or anions to account entirely for $I_{TA9}$ suggested that TA9 activated an ion channel with significant Ca$^{2+}$-permeability, an idea supported by the positive shift in $E_{rev}$ with strong intracellular Ca$^{2+}$-buffering (Fig. 2). We further explored the Ca$^{2+}$-permeability of this unidentified channel by altering the concentration of Ca$^{2+}$ in our ECS [Ca$^{2+}$]e and applying a family of voltage steps to the membrane (-80 to +20 mV). No change in the measured $E_{rev}$ was observed upon increasing the [Ca$^{2+}$]e from 1.3 to 5.0 mM (1.3 mM Ca$^{2+}$, -44.2 ± 2.4 mV, $n = 3$; 5 mM Ca$^{2+}$, -46.9 ± 0.6 mV, $n = 3$) (Fig 3. Bi-iii). We worried that a 3.8 fold increase did not sufficiently increase the driving force on Ca$^{2+}$ into the cells. However, voltage-step families recorded in the presence of 10 mM extracellular Ca$^{2+}$ and 20 mM intracellular BAPTA did not yield an $E_{rev}$ that differed significantly from control (control, -16.8 ± 8.6 mV, $n = 5$; 10 mM Ca$^{2+}$, -19.4 ± 8.3 mV, $n = 5$) (Fig. 3 Ci-iii). The simplest explanation for these results is that TA9 activates a channel with effectively equal permeable to Na+, K+ and Ca$^{2+}$. 
Figure 3 – The tyrphostin-activated current is carried by both cations and anions.  

A, sample responses to a family of voltage steps recorded from neurons exposed to (i) control (ii) NMDG+ or (iii) gluconate- substituted ECS.  (iv) average I-V relationships for the TA9-current: solid line, control; dashed line, NMDG+; dotted line, gluconate- ECS.  

B, Ca²⁺-permeability of ITA9.  Sample response to a family of voltage steps from a neuron exposed to (i) control ECS, (ii) 5 mM Ca²⁺ ECS.  (iii) summary I-V data indicating no shift in the reversal potential for control (solid) vs. high Ca²⁺ (dashed) ECS.  

C, sample traces demonstrating response to family of voltage steps in (i) control and (ii) 10 mM Ca²⁺ ECS + 20 mM BAPTA ICS.  (iii) summary I-V data for control (solid) and high Ca²⁺ + increased intracellular buffering (dashed) groups.
1.43.4. **TA9 activates pannexin-like hemichannels in hippocampal neurons**

The linear IV relationship, reversal potential near 0 mV, permeability to Na+, K+ and Ca\(^{2+}\) strongly suggested that TA9 activated a non-selective cation current in hippocampal neurons. TRPM2, a Ca\(^{2+}\)-permeable member of the transient receptor potential melastatin family of ion channels, exhibits a linear IV relationship that reverses near 0 mV. Though no specific TRPM2 antagonists have been identified to date (Olah et al., 2009), the non-steroidal anti-inflammatory agent, flufenamic acid (FFA) was shown to inhibit TRPM2 currents (Hill et al., 2004). FFA also inhibits several other channel types including Ca\(^{2+}\)-activated Cl\(^-\) ion channels (White et al., 1990) and connexin gap junction channels (Eskandari et al., 2002). However, when \(I_{TA9}\) was recorded at a holding potential of -60 mV, application of 200 µM FFA failed to inhibit the current, indicating that the TA9 current is not mediated by TRPM2. Similarly, La\(^{3+}\) (100 µM), which either enhances or has no effect on TRPM2 currents but blocks many other non-selective cation channels (Mlinar et al., 1993; Sinkins et al., 1998), had no significant effect on \(I_{TA9}\).

Although \(I_{TA9}\) shares various biophysical and pharmacological properties with traditional non-selective cation currents, unlike \(I_{TA9}\), these currents are typically reduced at negative membrane potentials when NMDG\(^+\) is the major charge carrier. An alternate possibility is that TA9 activates a channel with high permeability to NMDG\(^+\). This idea lead us to consider the possibility that TA9 activates pannexin hemichannels, which are permeable to molecules up to 1.5 kD and may better account for our ability to record \(I_{TA9}\) in the presence of NMDG\(^+\) (and absence of extracellular Na\(^+\) and K\(^+\)). Pannexin hemichannels are large-pore, unapposed gap junction channels (Barbe et al., 2006). To determine if hemichannel activation mediated \(I_{TA9}\), we tested the ability of the gap junction and
hemichannel antagonist, carbenoxolone (CBX), to block the current. $I_{TA9}$ was recorded in standard ECS until a stable steady-state current was obtained. Upon extracellular application of CBX (100 µM), a slowly developing inhibition of $I_{TA9}$ was observed, which reached a maximum after 6 minutes of recording (-38.6 ± 0.1% versus control), $n = 5 \text{ or } 6$, $p < 0.05$).
Figure 4 – The TA9-activated current is sensitive to the hemichannel inhibitor carbenoxolone. A, samples traces demonstrating the evoked TA9-current at the start (dark) and end (light) of a 6 minute recording period in the absence (i) and presence (ii) of CBX. B, summary date of time-dependent development of CBX-mediated block of ITA9. C, bar graph demonstrating maximum block by 100 μM CBX of the tyrphostin-activated current.
1.44. Discussion

1.44.1. Tyrphostin A9 activates a pannexin-like current in hippocampal neurons

Pannexins are a small family of proteins with predicted structural similarity to the vertebrate gap junction proteins, connexins (Baranova et al., 2004). Although they can also act as intercellular communication conduits, recent evidence indicates that the predominant role of pannexins may be as non-junctional hemichannels (Thompson et al., 2008b).

Hemichannels are, open unapposed half gap junctions channels and have been demonstrated to function both in neurons and heterologous expression systems (Bruzzone et al., 2003; Thompson et al., 2006). Here, we demonstrate that the PDGFR inhibitor TA9 activates a Ca\(^{2+}\)-permeable, non-selective current in hippocampal neurons. Both the biophysical and pharmacological properties of the TA9-activated current resembled those of pannexin-containing hemichannels as both currents were (1) permeable to Ca\(^{2+}\), anions and the bulky cation, NMDG\(^{+}\), (2) sensitive to block by CBX and (3) insensitive to \([Ca^{2+}]_o\). To our knowledge, this is the first report of a tyrphostin compound acting as an agonist independently of its RTK inhibitory effects. Furthermore, this is the first demonstration that a pharmacological agent can activate a pannexin-like hemichannel.

1.44.2. Connexin- versus pannexin-containing hemichannels

Connexins and pannexins demonstrate overlapping sensitivity to pharmacological agents including CBX. Although Px1 was demonstrated to be approximately 5 to 10 times more sensitive to CBX than Cx46, suggesting that block by CBX can discriminate between
connexins and pannexins, it remains unknown whether this holds true for other gap junction subtypes (Bruzzone et al., 2005). A more useful tool may be the differing sensitivities to Ca$^{2+}$ as, unlike pannexins, connexins are blocked by physiological concentrations of extracellular Ca$^{2+}$ (Srinivas et al., 2005). Our standard extracellular solution contained either 1.3 mM Ca$^{2+}$ (recordings from acutely isolated neurons) or 2.0 mM Ca$^{2+}$ (recordings from cultured hippocampal neurons). In both cases, we were able to reliably activate ITA9, suggesting that it is not mediated by connexin-containing hemichannels. In this vein, further support for this conclusion stems from the finding that ITA9 was insensitive to increases in extracellular Ca$^{2+}$ up to 5 mM. Finally, TA9 is unlikely to activate a member of the TRP family of ion channels since these channels (a) exhibit significantly reduced currents or are blocked completely when extracellular cations are substituted with NMDG+ and (b) do not exhibit anion permeability.

1.44.3. Homomeric and heteromeric pannexin hemichannels

Our study does not distinguish between TA9-induced activation of Px1 homomeric or Px1/2 heteromeric hemichannels as transcripts for both Px1 and Px2 are expressed in the pyramidal cell layer of the hippocampus (Bruzzone et al., 2003; Vogt et al., 2005). In *Xenopus* oocytes, overexpression of Px1 or Px1/2 but not Px2 alone resulted in the formation of functional hemichannels. The homomeric and heteromeric channels differed from each other on the basis of gating kinetics and current amplitude (Bruzzone et al., 2003). However, Px1 and Px1/2 were equally sensitive to block by CBX and insensitive to increased extracellular Ca$^{2+}$, preventing us from distinguishing between the activation of homomeric and heteromeric hemichannels on the basis of pharmacology alone (Bruzzone et al., 2005).
1.44.4. **Pannexin hemichannel activity in the hippocampus**

Evidence of functional pannexin hemichannel activity in hippocampal neurons has been reported in response to oxygen-glucose deprivation (OGD) (Thompson *et al.*, 2006). The OGD-activated current had a single channel conductance similar to that of homomeric Px1 hemichannels when expressed in oocytes and was inhibited by CBX as well as La$^{3+}$, a non-specific blocker of connexin hemichannels (Bao *et al.*, 2004; Contreras *et al.*, 2002; Thompson *et al.*, 2006). More recently, NMDA receptor stimulation was found to activate a secondary current that was inhibited by either CBX or by knockdown of Px1 with RNAi (Thompson *et al.*, 2008a). Although $I_{TA9}$ was insensitive to block by La$^{3+}$, extracellular application of either CBX or Gd$^{3+}$ significantly reduced its peak amplitude. The difference in sensitivity to trivalent cation block may be attributed to differences in the mechanism of current activation in these two studies and to ensuing differences in the subtype(s) of hemichannel activated.

When ectopically expressed in HEK cells, neither La$^{3+}$ nor Gd$^{3+}$ reduced Px1-mediated currents at +60 mV or altered the corresponding I-V relationships, leading to the suggestion that the OGD-activated current is in fact mediated by Px1/Px2 heteromeric hemichannel activation (Pelegrin *et al.*, 2006). Px2 is highly expressed in neurons (Bruzzone *et al.*, 2003) and likely forms functional Px1/Px2 hemichannels in these cells, as suggested by evidence from recombinant systems (Bruzzone *et al.*, 2005). However, neither the single channel conductance of heteromeric hemichannels nor their sensitivity to block by trivalent cations has been ascertained in either recombinant or native systems, making distinctions between homomeric and heteromeric hemichannel activation difficult (Sohl *et al.*, 2005).
Given our current understanding of pannexins, it remains possible that either or both Px1 and Px1/Px2 hemichannels mediate $I_{TA9}$.

1.44.5. **Ca$^{2+}$-permeability of the TA9-activated current: A caveat**

Although $I_{TA9}$ was insensitive to increases in extracellular Ca$^{2+}$ up to 5 mM, we demonstrated that increasing the Ca$^{2+}$-buffering capacity of our intracellular solution shifted the $E_{rev,TA9}$. This result indicates that TA9 activates a Ca$^{2+}$-permeable pathway in hippocampal neurons. It is reasonable to conjecture that Ca$^{2+}$-influx through TA9-activated channels will activate Ca$^{2+}$-dependent signaling pathways. As TA9 is commonly applied extracellularly to assess the contribution of PDGF-mediated signaling to various cellular events, results from such experiments should be reconsidered in light of the concomitant TA9-mediated increase in [Ca$^{2+}$]$i$. 
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