CALCINEURIN-MEDIATED SIGNALING IN ISCHEMIC PRECONDITIONING AND NEURONAL CELL DEATH

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Stroke is a leading cause of morbidity and mortality in the United States and worldwide. However, few effective therapeutic interventions exist to treat this devastating disease. A detailed understanding of endogenous cell-adaptive mechanisms in ischemia, as well as signaling pathways leading to ischemic neurodegeneration, will critically aid in developing better treatments for stroke. In this dissertation, I investigated calcineurin-mediated signaling pathways in ischemic preconditioning and neuronal cell death. Calcineurin is a calcium/calmodulin-dependent phosphatase that regulates important neuronal functions. An ischemic preconditioning stimulus triggers calcineurin-dependent changes in the localization, phosphorylation status, and voltage-gated activation of Kv2.1 channels, which are involved in promoting neuronal tolerance in the context of otherwise lethal excitotoxic injury. Recent studies suggest that the signaling pathways mediating Kv2.1 channel regulation are complex, and may not be calcineurin activity-dependent in all cases. Additionally, how these processes contribute to neuroprotection is not well defined. In this thesis, I tested the hypothesis that Kv2.1 channel declustering may be sufficient to promote neuronal tolerance. I discovered that preconditioning leads to calcineurin-dependent increases in cyclin E1 protein levels in cortical neurons, which induces Kv2.1 dephosphorylation and dispersal of channel clusters without a concomitant shift in voltage-gated activation. Importantly, cyclin E1 over-expression reduces excitotoxic cell death in neurons. Although calcineurin is required for normal neuronal function, dysregulated calcineurin

activation may be neurotoxic. In the second part of my studies, I explored the isoform-specific effects of over-expressing Regulator of calcineurin 1 (RCAN1) in neurons. I found that RCAN1 mediates isoform-dependent, distinct neuroprotective and neurotoxic cell signaling pathways through calcineurin-dependent and independent mechanisms. Thus, the studies in this dissertation provide insight into calcineurin-mediated neuronal cell survival and neurotoxic signaling pathways that may be important in the pathology of stroke and other neurodegenerative disorders.

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ABBREVIATIONS

Aβ: amyloid-β

AMPA-R: post-synaptic AMPA receptors

ASK-1: apoptosis signal-regulating kinase 1

Ca^{2+}: calcium ion

CaMKII: Ca^{2+}-activated Ca^{2+}/calmodulin-dependent protein kinase II

cAMP: cyclic adenosine monophosphate

Cdk5: cyclin-dependent kinase 5

CGN: cerebellar granule neuron

Cl^-: chloride ion

CREB: cAMP response element-binding protein

CsA: cyclosporine A

Cyt-PTPε: cytoplasmic protein tyrosine phosphatase ε

DTDP: 2,2'-dithiodipyridine

EA-1: episodic ataxia type 1

GSK-3β: glycogen synthase kinase-3β

I_A: rapidly inactivating, A-type Kv channel-mediated K^+ currents

K^+: potassium ion

KCl: potassium chloride

KCN: potassium cyanide

Kv channels: voltage-gated potassium channels

Kv2.2-CT: Kv2.2 C-terminus

MLK: mixed-lineage kinase

NFAT: nuclear factor of activated T cells

NGF: nerve growth factor

NMDA: N-methyl-D-aspartate

NS5A: Hepatitis C virus nonstructural protein 5A

OGD: oxygen-glucose deprivation

PKA: protein kinase A

RCAN1: Regulator of calcineurin 1

SDF-1α: stromal cell-derived factor-1α

SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor

SNOC: S-nitrocysteine

SUDEP: sudden unexplained death in epilepsy

TEA: tetraethylammonium

TPEN: tetrakis-(2-pyridylmethyl)ethylenediamine

Zn^{2+}: zinc ion

4-AP: 4-aminopyridine

6-OHDA: 6-hydroxydopamine

PREFACE

There are several important people that I would like to thank for making this dissertation possible. First and foremost, I want to thank my thesis advisor Elias Aizenman. Elias demonstrated that rare and perfect balance as a mentor, giving me the freedom to explore my ideas and learn from my mistakes, while guiding me when I really needed direction. Elias' steadfast support and encouragement have been invaluable throughout my graduate training. I am continually inspired by his unique scientific insights, his enthusiastic and meticulous approach to both the day-to-day and the 'big picture' aspects of research, his unfailing work ethic, and his genuine and energetic commitment towards mentoring young scientists - qualities I hope to emulate as I move forward in my career. I am very proud and humbled to be graduating from the Aizenman lab.

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My deepest gratitude is for my family – my parents, my brother, Krishna, my sister, Nayna, and my husband, Vivan – for their endless love. My father was really my earliest

inspiration for pursuing an academic career, before I even knew what I wanted to do. He instilled in me a love for literature that has changed my life in countless ways, in academic pursuits and otherwise. My mother is and always will be a pillar of support and strength in my toughest times. My brother and sister are my strongest advocates and build my confidence every day.

Through the ups and downs of graduate school, I have been fortunate beyond words to have my husband, Vivan, by my side. His unwavering support, wonderful sense of humor, infallible insight on problems small and large, and his beautiful, optimistic outlook on life, inspire me every day. He is, quite simply, the best person in the world.

Finally, I would like to dedicate this thesis to my Ajji (my grandmother), a brilliant and tough woman who I think of every day. Not having had the opportunity for formal education herself, she understood the pursuit of knowledge to be noble, and strived tirelessly for her family to achieve great things.

1.0 GENERAL INTRODUCTION

1.1 K_V CHANNELS AT THE CROSSROADS OF NEURONAL FUNCTION, ISCHEMIC TOLERANCE, AND NEURODEGENERATION

Voltage-gated potassium (Kv) channels are widely expressed in the central and peripheral nervous system, and are crucial mediators of neuronal excitability. Importantly, these channels also actively participate in cellular and molecular signaling pathways that regulate the life and death of neurons. Injury-mediated increased K^+ efflux through Kv2.1 channels promotes neuronal apoptosis, contributing to widespread neuronal loss in neurodegenerative disorders such as Alzheimer's disease and stroke. In contrast, some forms of neuronal activity can dramatically alter Kv2.1 channel phosphorylation levels and influence their localization. These changes are normally accompanied by modifications in channel voltage-dependence, which may be neuroprotective within the context of ischemic injury. Kv1 and Kv7 channel dysfunction leads to neuronal hyperexcitability that critically contributes to the pathophysiology of human clinical disorders such as episodic ataxia and epilepsy. This introduction summarizes the neurotoxic, neuroprotective, and neuroregulatory roles of Kv channels, and highlights the consequences of Kv channel dysfunction on neuronal physiology. The studies described in this chapter thus underscore the importance of normal Kv channel function in neurons, and emphasize the

therapeutic potential of targeting Kv channels in the treatment of a wide range of neurological diseases.

1.2 STRUCTURE AND FUNCTION OF K_V CHANNELS

Voltage-gated potassium (Kv) channels are the largest gene family of potassium (K^+) channels, and are key regulators of neuronal excitability (Yellen 2002, Guan et al. 2007a, Guan et al. 2013). In humans, they are encoded by forty different genes and categorized into twelve sub-families, Kv1 through Kv12 (Gutman et al. 2005, Johnston et al. 2010). Mammalian Kv channels are tetramers, composed of four α-subunits that surround an ion conduction pore. Each α-subunit contains six α-helical transmembrane domains (S1-S6), a membrane-reentering P loop between S5 and S6, and intracellular N- and C-termini. Four S5-P-S6 segments line the ion conduction pore, while the S1-S4 sequences are critical for channel voltage-sensing and gating.

Kv channels mediate outward K^+ currents that contribute to membrane repolarization and hyperpolarization, thus generally serving to limit neuronal excitability. Characterizing the precise molecular correlates of Kv-mediated K^+ currents in different cell types has been difficult, owing to the assortment of channels generated from α-subunit heteromerization within Kv families. This diverse channel subunit composition produces a wide spectrum of Kv channels with differing biophysical and pharmacologic profiles. Furthermore, Kv α-subunits can bind to regulatory Kv β-subunits, as well as with other Kv channel-interacting proteins, which can strongly modify channel properties (Imbrici et al. 2006, Schulte et al. 2006, McKeown et al. 2008). Moreover, post-translational modifications such as phosphorylation, dephosphorylation,

glycosylation, and SUMOylation all have been shown to alter Kv channel properties significantly (Murakoshi et al. 1997, Park et al. 2006, Benson et al. 2007, Watanabe et al. 2007). Despite these challenges, through electrophysiological studies utilizing pharmacologic agents and Kv channel subunit-specific genetic manipulation, the general functions of Kv channel sub-families in neurons have been relatively well characterized. As such, low-voltage-activated channels such as Kv1 channels regulate the threshold potential for firing, and limit the number of action potentials generated in response to depolarization (Brew et al. 2003). In contrast, high-voltage-activated, slowly inactivating Kv2 channels play an important role in influencing action potential duration during periods of high frequency firing (Du et al. 2000, Malin and Nerbonne 2002, Misonou et al. 2005, Mohapatra et al. 2009). In addition to strongly shaping neuronal excitability, Kv channels also critically contribute to cell death and cell survival signaling pathways. In this introduction, the diverse neurotoxic, neuroprotective, and neuroregulatory roles of Kv channels will be discussed. Additionally, the implications of Kv channel dysfunction, particularly in the context of human neurological diseases, will also be addressed.

1.3 NEUROTOXICITY OF K_V CHANNELS

1.3.1 K^+ efflux is a requisite component of apoptotic cell death

Apoptotic cell death contributes significantly to the neuronal loss observed in a number of neurological disorders, including Alzheimer's disease and stroke (Linnik et al. 1993, Thompson 1995, Choi 1996, Ferrer et al. 2003, Lobysheva et al. 2009). Therefore, understanding the mechanisms of apoptotic signaling pathways is of paramount importance in order to successfully

develop therapeutic strategies for preventing or reducing neuronal damage. Apoptosis was first described as "shrinkage necrosis," due to the morphological features of shrunken cell size and fragmentation of nuclei, which distinguished apoptotic cells from the swollen appearance of necrotic cells (Kerr 1971). The key biochemical features of apoptosis have since been characterized, and include DNA fragmentation, mitochondrial damage, and caspase activation. Several critical components of apoptotic cascades occur only in the presence of a reduction in cell volume (termed apoptotic volume decrease), and decreased intracellular ionic strength, both of which are observed regardless of apoptotic stimulus and cell type (Kerr 1971, Beauvais et al. 1995, Benson et al. 1996, Bortner and Cidlowski 1996, Bortner et al. 1997, McCarthy and Cotter 1997, Bortner and Cidlowski 1998, Maeno et al. 2000, Yu and Choi 2000, Hernández-Enríquez et al. 2011). Because the net electrochemical gradient of the cell favors the exit of K^+, K^+ channel-mediated K^+ efflux was an early contender for promoting the apoptotic volume decrease and thus facilitating apoptotic signaling cascades. This idea is supported by several key findings:

(1) Physiological concentrations of K^+ inhibit, while lowered K^+ levels activate, apoptotic enzymes: In 1997, Cidlowski and colleagues identified a critical relationship between potassium concentrations and apoptotic enzyme activity. They incubated thymocyte nuclei with calcium (Ca^{2+}) and magnesium to activate autodigestion, a process that recapitulates apoptotic DNA degradation *in vitro*. Potassium chloride (KCl) inhibited DNA fragmentation in a dose-dependent fashion, indicating blockade of pro-apoptotic nuclease activity. Importantly, normal physiological levels of intracellular K^+ effected near-complete inhibition of nuclease activity (Hughes et al. 1997). Using cytoplasmic extracts from rats treated with dexamethasone to induce apoptosis, they also showed that caspase-3 activation was reduced with increasing concentrations

of KCl. In other *in vitro* systems of apoptosis, physiologic K^+ concentrations have been shown to mitigate DNA fragmentation and chromatin condensation (Dallaporta et al. 1998), as well as apoptosome formation (Cain et al. 2001). In neurons exposed to serum deprivation, low intracellular K^+ concentrations enhance the DNA binding activity of pro-apoptotic transcription factors and the mRNA expression of their target genes, while depressing the DNA binding activity of anti-apoptotic factors and mRNA expression of their target genes (Yang et al. 2006). This evidence strongly indicates that reduced intracellular K^+ concentrations provide a permissive environment for apoptotic signaling cascades.

(2) Apoptotic stimuli cause K^+ loss: Reduced K^+ concentrations are observed in cortical neurons following serum deprivation (Yu et al. 1997), and in other cell types following an assortment of apoptotic insults (Barbiero et al. 1995, Bortner et al. 1997, Hughes et al. 1997, McCarthy and Cotter 1997, Dallaporta et al. 1998). Important early flow cytometry studies in thymocytes demonstrated that K^+ loss after exposure to an apoptotic stimulus is restricted to cells exhibiting apoptotic features such as cell volume reduction, DNA fragmentation, and loss of mitochondrial membrane potential (Hughes et al. 1997, Dallaporta et al. 1998).

(3) K^+ efflux promotes apoptosis: K^+ efflux promotes apoptotic signaling and cell death in a range of cell types (Ojcius et al. 1991, Deckers et al. 1993, Perregaux and Gabel 1994, Walev et al. 1995, Yu et al. 1997, Nadeau et al. 2000, Abdalah et al. 2006). Ionophores that induce K^+ efflux, including nigericin and valinomycin, and the Na^+/K^+ ATPase inhibitor ouabain, activate LPS-stimulated, caspase-1-mediated maturation of interleukin-1β in phagocytes (Perregaux and Gabel 1994, Walev et al. 1995). Cortical neurons exposed to valinomycin undergo cell death, displaying the typical morphological and biochemical features of apoptosis (Yu et al. 1997).

High extracellular K^+ concentrations, by decreasing the K^+ gradient and thus blocking K^+ efflux, oppose apoptotic signaling and promote cell survival. This observation has been well characterized particularly in cerebellar granule neurons (CGNs) (Gallo et al. 1987, D'Mello et al. 1993, Yan et al. 1994, Galli et al. 1995, de Luca et al. 1996, Schulz et al. 1996, Jiao et al. 2007, Hernández-Enríquez et al. 2011). Neurons grown in 5 mM KCl exhibit indications of apoptotic cell death, as compared to neurons grown in 25 mM KCl, which are protected from DNA fragmentation and are resistant to TGF-β-induced apoptosis (Galli et al. 1995, de Luca et al. 1996, Jiao et al. 2007). Accordingly, switching mature CGNs from 25 mM KCl to 5 mM KCl induces vacuole formation, condensing of nuclei, cellular and neurite shrinkage, and apoptotic cell death (D'Mello et al. 1993). Cholesterol enhances apoptosis in CGNs cultured in low K^+ medium through a cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)/cAMP response element-binding protein (CREB) signaling pathway, but does not influence cell survival in CGNs incubated in high K^+ medium (Zhou et al. 2012). Similar results have been demonstrated in: *(i)* ciliary and dorsal root ganglion neurons, which display increased survival and differentiation in high extracellular K^+ media (Chalazonitis and Fischbach 1980, Collins et al. 1991); *(ii)* cortical neurons, which are protected by high extracellular K^+ from apoptosis induced by oxidants, staurosporine, glutamate, ceramide, neurotoxic amyloid-β (Aβ) peptides, and serum deprivation (Koh et al. 1995, Yu et al. 1997, Yu et al. 1998, Yu et al. 1999a, Aizenman et al. 2000); *(iii)* septal cholinergic cells, which in high K^+ media are resistant to Aβ-induced cell death (Colom et al. 1998); *(iv)* thymocytes, where high K^+ media limits pro-apoptotic caspase activation and DNA fragmentation (Hughes et al. 1997); and *(v)* human monocytes and mouse macrophages, where high K^+ growth media prevents interleukin-1β processing by caspase-1 (Perregaux and Gabel 1994, Walev et al. 1995). In agreement with these

findings, K^+ channel blockers attenuate apoptotic signaling cascades and cell death in numerous neuronal (Furukawa et al. 1996, Yu et al. 1997, Yu et al. 1998, Yu et al. 1999a, Yu et al. 1999b, Wang et al. 2000, Liu et al. 2003, Mei et al. 2004, Chen et al. 2005b, Hu et al. 2006, Yu et al. 2006, Shen et al. 2009, Chen et al. 2011) and non-neuronal systems (Beauvais et al. 1995, Wang et al. 1999b, Lu et al. 2003, Singleton et al. 2009).

Some studies have suggested that elevated extracellular K^+ mitigates apoptotic cell death by increasing Ca^{2+} entry through voltage-gated Ca^{2+} channels, rather than by eliminating pro-apoptotic K^+ efflux (Gallo et al. 1987, Koike et al. 1989, Franklin and Johnson Jr 1992, Johnson Jr et al. 1992, Enokido and Hatanaka 1993, Barbiero et al. 1995, Franklin et al. 1995, Galli et al. 1995, Koh et al. 1995, Lampe et al. 1995). In rat embryonic sympathetic neurons, withdrawal of Ca^{2+} from the media or treatment with Ca^{2+} channel blockers precludes high extracellular K^+-induced rescue from nerve growth factor (NGF) deprivation in some cases (Koike et al. 1989, Franklin et al. 1995, Lampe et al. 1995), while thapsigargin-induced Ca^{2+} influx restricts NGF deprivation-induced apoptosis (Lampe et al. 1995). Similarly, Ca^{2+} channel antagonists impede high K^+-mediated cell survival in CGNs (Gallo et al. 1987, Galli et al. 1995), and prevent rescue by increased extracellular K^+ of high oxygen-stimulated apoptotic toxicity in hippocampal neurons, and of staurosporine-mediated cell death in cortical neurons (Enokido and Hatanaka 1993, Koh et al. 1995). However, as noted by Yu and colleagues in a landmark paper (Yu et al. 1997), these studies do not rule out the possibility that reducing K^+ efflux inhibits apoptosis and promotes neuronal survival. In fact, increases in intracellular Ca^{2+} can promote neuronal apoptosis (Gwag et al. 1999, Song et al. 2013), and heightened Ca^{2+} levels are not always required for high extracellular K^+-facilitated survival of NGF-deprived sympathetic neurons

(Murrell and Tolkovsky 1993). Importantly, in cortical neurons, Ca^{2+} channel blockers do not eliminate neuroprotection by high extracellular K^+ or tetraethylammonium (TEA, a blocker of delayed rectifying Kv channels) in response to serum deprivation, N-methyl-D-aspartate (NMDA), Aβ peptide, or ceramide (Yu et al. 1997, Yu et al. 1998, Yu et al. 1999a, Yu et al. 1999b). Additionally, TEA analogs that ablate staurosporine-induced K^+ efflux, cell volume loss, caspase cleavage and activation, and neuronal apoptosis, also inhibit high threshold voltage-activated Ca^{2+} channels, supporting the idea that neuroprotection via K^+ channel inhibition does not occur by activation of Ca^{2+} channels (Wang et al. 2000). The specificity for K^+ efflux, rather than inhibition of Ca^{2+} influx, in the promotion of apoptotic signaling cascades has also been demonstrated in monocytes (Walev et al. 1995), leukocytes (Wang et al. 1999b), Chinese hamster ovary cells (Abdalah et al. 2006), and corneal epithelial cells (Lu et al. 2003, Singleton et al. 2009).

Chloride ion (Cl^-) efflux may accompany pro-apoptotic K^+ exit in order to maintain electroneutrality in the cell. In fact, Cl^- channel activation and Cl^- efflux are observed following an apoptotic stimulus in several cell types (Nilius et al. 1995, Szabò et al. 1998, Dupere-Minier et al. 2004, Shimizu et al. 2004, Okada et al. 2006). Furthermore, Cl^- channel blockers attenuate some features of apoptotic signaling and cell death in neurons and other cell types, although these blockers are not invariably as effective as K^+ channel inhibitors (Szabò et al. 1998, Rasola et al. 1999, Wei et al. 2004, Inoue et al. 2007). Cl^- exit, while insufficient to facilitate the completion of apoptotic programs, may promote pro-apoptotic K^+ efflux and thus contribute to cell death. Although beyond the scope of this discussion, Cl^- efflux in apoptosis merits further investigation for possible therapeutic intervention.

Finally, while K^+ efflux is a requisite event for many forms of apoptosis, it is not, in and of itself, completely sufficient to stimulate apoptotic cell death in all injurious contexts. In Chinese hamster ovary cells, which do not express endogenous Kv channels and are resistant to apoptosis induced by hypoxia or serum deprivation, treatment with the K^+ ionophore valinomycin stimulates massive cell death characterized by mitochondrial damage and caspase activation (Abdalah et al. 2006). In contrast, lymphocytes cultured under hypotonic conditions undergo a 50% drop in K^+ concentrations via a volume regulatory response, but this reduction alone is not sufficient to induce apoptosis (Bortner et al. 1997). Similarly, serum deprivation along with decreased extracellular K^+ is required to stimulate apoptosis in CGNs, while in cortical neurons, caspase activity inhibition blocks oxidant-induced apoptotic cell death, despite the presence of prominent increased outward K^+ currents (Gallo et al. 1987, D'Mello et al. 1993, Yan et al. 1994, Galli et al. 1995, Schulz et al. 1996, Gerhardt et al. 2001, Castel et al. 2006, Hu et al. 2006, Jiao et al. 2007).

1.3.2 Kv currents enable neuronal apoptosis

Delayed rectifier Kv channels are thought to be the principal conduits for the exit of K^+ in neuronal apoptosis (Yu et al. 1997, Yu et al. 1998, Yu et al. 1999a, Yu et al. 1999b, Aizenman et al. 2000, Wang et al. 2000, Huang et al. 2001, McLaughlin et al. 2001, Pal et al. 2003, Wei et al. 2003, Bossy-Wetzel et al. 2004, Aras and Aizenman 2005, Chen et al. 2005b, Redman et al. 2006, Yu et al. 2006, Jiao et al. 2007, Redman et al. 2007, Knoch et al. 2008, Shen et al. 2009, Yao et al. 2009), although other K^+ channels, including A-type K^+ channels (Beauvais et al. 1995, Walev et al. 1995, Wang et al. 1999b, Lu et al. 2003, Hu et al. 2005, Hu et al. 2006, Singleton et al. 2009, Chen et al. 2011), Ca^{2+}-activated K^+ channels (Furukawa et al. 1996,

Jalonen et al. 1997, McCarthy and Cotter 1997, Chen et al. 2013), K_{ATP} channels (Liu et al. 2003), and TASK leak K^+ channels (Lauritzen et al. 2003), may also play an important role in this context. Yu and coworkers have shown that cortical neurons deprived of serum, or exposed to staurosporine, neurotoxic Aβ peptide, or ceramide, manifest a TEA-sensitive increase in delayed rectifying Kv currents, without exhibiting an increase in other major K^+ currents, including inwardly rectifying, A-type (with the exception of serum deprivation, which increases these currents slightly), M type, or BK currents (Yu et al. 1997, Yu et al. 1998, Yu et al. 1999b). TEA or TEA analogs render neurons resistant to the above-mentioned apoptotic insults, while 4-aminopyridine (4-AP), a Kv1 channel inhibitor that opposes apoptosis in some neuronal and non-neuronal systems (Beauvais et al. 1995, Walev et al. 1995, Wang et al. 1999b, Hu et al. 2006), does not attenuate the rise in K^+ currents or confer neuroprotection against apoptotic stimuli in these studies (Yu et al. 1997, Yu et al. 1998, Yu et al. 1999b, Wei et al. 2004, Chen et al. 2005b). A study in septal cholinergic cells has similarly demonstrated Aβ-induced K^+ current increase and apoptotic cell death, both of which are blocked by TEA. In a dopaminergic cell line that does not manifest Aβ-induced increased K^+ currents, TEA is not protective, while septal cholinergic cells that exhibit minimal basal K^+ currents are not susceptible to Aβ-mediated toxicity, consistent with the requirement for increased K^+ currents in the completion of apoptotic signaling (Colom et al. 1998). In neurons, amplified apoptotic Kv channel currents that can be tempered by TEA, high extracellular K^+, Kv siRNA-mediated knockdown, and/or a dominant negative form of the Kv channel, have also been shown in response to peroxynitrite (Bossy-Wetzel et al. 2004), the apoptosis inducer thiol oxidant 2,2'-dithiodipyridine (DTDP) (McLaughlin et al. 2001, Pal et al. 2003, Aras and Aizenman 2005, Pal et al. 2006, Redman et al. 2007, Redman et al. 2009, Dallas et al. 2011), the nitric oxide donor S-nitrocysteine (SNOC)

(Bossy-Wetzel et al. 2004), low K^+/serum-free media (Hu et al. 2005, Jiao et al. 2007, Yao et al. 2009), 6-hydroxydopamine (Redman et al. 2006), glutamate (Zhao et al. 2006), and increased intracellular cholesterol (Zhou et al. 2012). These studies will be discussed in further detail in the next section.

K^+ efflux and changes in K^+ current behavior have also been observed following ischemic injury *in vitro* and *in vivo* (Hansen and Zeuthen 1981, Leblond and Krnjevic 1989, Jiang and Haddad 1991, Jiang and Haddad 1993, Jiang and Haddad 1994a, Jiang and Haddad 1994b, Jiang et al. 1994, Gido et al. 1997, Chi and Xu 2001, Yushmanov et al. 2013). For instance, delayed rectifying K^+ currents are increased in CA1 pyramidal neurons after transient forebrain ischemia (Chi and Xu 2000, Xuan Chi and Xu 2000). Moreover, two Kv channel antagonists, TEA and clofilium, are neuroprotective against cerebral ischemia in mice (Wei et al. 2003). In another study, TEA administered to rats post-forebrain ischemia significantly rescues neuronal density, shrunken cells, and nuclei condensation, while treatment with 4-AP does not prevent the apoptotic phenotype (Huang et al. 2001).

1.3.2.1 Kv2.1-mediated neuronal apoptosis

Kv2.1, the predominant mediator of delayed rectifying K^+ currents in neurons (Trimmer 1991, Murakoshi and Trimmer 1999, Malin and Nerbonne 2002), has been identified as the channel responsible for the pro-apoptotic K^+ current increase in cortical, hippocampal, and cerebellar granule neurons. Importantly, the increase in K^+ current amplitude occurs without changes in the voltage-gated activation or inactivation kinetics of the Kv2.1 channels (Yu et al. 1997, Pal et al.

2003, Aras and Aizenman 2005, Pal et al. 2006, Redman et al. 2006, Redman et al. 2007, Knoch et al. 2008, Yao et al. 2009, Dallas et al. 2011, Zhou et al. 2012).

A Kv2.1-mediated neuronal apoptotic pathway stimulated by oxidant treatment has been well characterized (Figure 1a and Figure 2, right). Oxidants, such as DTDP, induce an intracellular release of zinc (Zn^{2+}) from metal-binding proteins, which is required to activate two kinase signaling pathways that converge upon increased phosphorylation of Kv2.1 channels, enhanced plasma membrane delivery of Kv2.1 channels, and amplified Kv2.1 K^+ currents, producing an intracellular environment that enables DNA fragmentation, caspase activation, and apoptosis (McLaughlin et al. 2001, Pal et al. 2003, Bossy-Wetzel et al. 2004, Aras and Aizenman 2005, Pal et al. 2006, Redman et al. 2007, Redman et al. 2009, Sensi et al. 2011). In cortical neurons and Chinese hamster ovary cells, the increased Kv2.1-mediated K^+ currents are observed approximately three hours following a brief exposure to the apoptogenic stimulus.

Apoptotic enhancement of K^+ currents via Kv2.1 channels occurs upstream of caspase activation and requires coordinate channel phosphorylation at two amino acid residues, C-terminal Ser800 and N-terminal Tyr124, by p38 kinase and Src kinase, respectively (McLaughlin et al. 2001, Redman et al. 2009). The oxidant-stimulated Zn^{2+} release is a necessary early event for p38 kinase activation, via either apoptosis signal-regulating kinase 1 (ASK-1) (Aras and Aizenman 2005) or mixed-lineage kinase (MLK) (Amako et al. 2013), and for consequent, p38 kinase-mediated Ser800 phosphorylation (Aras and Aizenman 2005, Redman et al. 2007, Redman et al. 2009). Inhibiting p38 kinase activity blocks oxidant-induced Ser800 phosphorylation, increased Kv2.1 currents, caspase activation, and toxicity (McLaughlin et al.

2001). Zn^{2+} also permits the second, Src kinase-mediated phosphorylation step by inhibiting the activity of cytoplasmic protein tyrosine phosphatase ε (Cyt-PTPε), which is normally responsible for dephosphorylating Kv2.1 channels at the Src kinase-phosphorylated site Tyr124 (Sobko et al. 1998, Tiran et al. 2003, Redman et al. 2009). In fact, over-expression of Cyt-PTPε blocks the increase in K^+ currents and is neuroprotective, while Src kinase activity inhibition blocks the apoptotic K^+ current surge (Redman et al. 2009). The coordinate, oxidant-induced phosphorylation of Kv2.1 channels at the Ser800 and Tyr124 residues permits Kv2.1 channels to interact with soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins via a proximal C-terminal region of the channel (Leung et al. 2003, Tiran et al. 2003, Pal et al. 2006). This SNARE-Kv2.1 channel interaction, which requires Ca^{2+}-activated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activation, facilitates Kv2.1 channel delivery to the cell surface, enabling pro-apoptotic K^+ currents through Kv2.1 channels (McCord and Aizenman 2013). Accordingly, oxidant-stimulated Kv2.1 trafficking to the plasma membrane is blocked by co-expression of botulinum toxin fragments, expression of a Kv2.1 Ser800Ala mutant, or treatment with p38 kinase inhibitor (Pal et al. 2006, Redman et al. 2007). In summary, interfering with any one of multiple steps of this apoptotic pathway, including reactive oxygen species production, intracellular Zn^{2+} release, CAMKII activation, Src- and p38-mediated Kv2.1 phosphorylation, or SNARE-dependent membrane insertion of new Kv2.1 channels, precludes the pro-apoptotic K^+ current rise and rescues neurons from oxidant-mediated toxicity. This injurious pathway has also been validated in neurons exposed to activated microglia, which generate peroxynitrite, a well-established Zn^{2+}-liberating agent (Zhang et al. 2004, Knoch et al. 2008).

Neuronal cell death facilitated by a range of other apoptotic stimuli share several features of DTDP-mediated neurotoxicity, particularly the Kv2.1-mediated current increase, providing a compelling argument for the convergence of apoptotic signaling pathways on a requisite, Kv2.1-facilitated rise in K^+ currents in neurons. In CGNs, increased K^+ currents and apoptosis follow incubation in low K^+, serum-free media, while silencing Kv2.1 gene expression via siRNA knockdown reduces K^+ current amplitudes and increases cell viability (Jiao et al. 2007). Increased intracellular cholesterol potentiates the low K^+/serum deprivation-stimulated Kv2.1 current rise, DNA fragmentation, and consequent apoptosis in CGNs, all of which are blocked by TEA or MβCD, a cholesterol-binding agent (Zhou et al. 2012). The elevated K^+ currents are attenuated by inhibition of endoplasmic reticulum/Golgi transport (Zhou et al. 2012), indicating a role for *de novo* Kv channel plasma membrane insertion in propagating pro-apoptotic K^+ efflux, similar to that seen in DTDP-treated neurons (Pal et al. 2006). Treatment of cerebrocortical neurons with the nitric oxide donor SNOC facilitates apoptosis characterized by K^+ efflux, cell shrinkage, and activation of TEA-sensitive K^+ channels. In agreement with the cell death pathway observed in DTDP-treated cortical neurons, this process involves nitric oxide-mediated Zn^{2+} release, leading to further oxidative injury, mitochondrial function impairment, and p38 kinase activation-mediated enhanced Kv currents, all of which are required for neurotoxicity (Bossy-Wetzel et al. 2004). p38 kinase activation and Kv2.1 K^+ current-mediated apoptosis are also observed in hippocampal neurons following sustained treatment with the chemokine stromal cell-derived factor-1α (SDF-1α) or exposure to HIV-1 glycoprotein gp120 (Shepherd et al. 2012), in 6-hydroxydopamine (6-OHDA)-treated dopaminergic neurons, and in dopamine transporter-expressing non-dopaminergic neurons after incubation with 6-OHDA (Redman et al. 2006). In another report, serum deprivation in cortical neurons was shown to provoke Kv2.1 K^+

current surge-mediated apoptosis that is dependent on SNARE-facilitated channel membrane insertion: the apoptotic stimulus enhances interaction of Kv2.1 and SNARE protein SNAP-25, while blocking this interaction with botulinum toxin completely blocks the serum deprivation-associated enhancement of K^+ currents (Yao et al. 2009).

Additionally, most features of this Kv2.1-facilitated apoptotic pathway have been recapitulated in heterologous expression systems, strongly implicating Kv2.1 channels in an apoptogen-stimulated, requisite K^+ current surge that is sufficient for caspase activation and completion of apoptosis (McLaughlin et al. 2001, Pal et al. 2003, Aras and Aizenman 2005, Pal et al. 2006, Redman et al. 2007, Redman et al. 2009, Yuan et al. 2011, Al-Owais et al. 2012, Shepherd et al. 2012). Transfection of Kv2.1 in Chinese hamster ovary or HEK293 cells, for example, renders them newly susceptible to apoptosis induced by DTDP or oxygen-glucose deprivation, respectively (Pal et al. 2003, Yuan et al. 2011). Further, these studies have confirmed the involvement of pro-apoptotic p38- and Src-mediated Kv2.1 phosphorylation, as well as *de novo* Kv2.1 channel membrane insertion (Pal et al. 2006, Shepherd et al. 2012).

Other signaling components that may participate in Kv2.1-mediated neuronal apoptosis have been identified, but have not yet been thoroughly investigated. For example, the cAMP/PKA/CREB pathway has been implicated in K^+ channel-mediated apoptosis. In CGNs, cAMP-promoting agents reduce Kv channel-facilitated apoptosis induced by low extracellular K^+ or ethanol treatment (D'Mello et al. 1993, Galli et al. 1995, Mei et al. 2004, Castel et al. 2006, Jiao et al. 2007). In contrast, cAMP/PKA/CREB activation promotes the Kv2.1-mediated rise in K^+ currents and subsequent cell death in cholesterol-enhanced, low K^+-mediated apoptosis (Zhou

et al. 2012). Kv2.1-facilitated K^+ efflux and consequent neuronal apoptosis following exposure to SDF-1α or HIV-1 glycoprotein gp120 depend on calcineurin signaling, and are accompanied by a shift in Kv2.1 voltage-gated kinetics that is not normally observed in oxidant-mediated neurotoxicity (Shepherd et al. 2012).

An alternate mechanism of Kv2.1-mediated neuronal apoptosis has been proposed. In this model, oxidant-mediated oligomerization of Kv2.1 channels leads to a rapid decrease, rather than an increase, of Kv2.1 K^+ currents that is absent in cells expressing an oxidation-resistant Kv2.1 cysteine mutant. Neurons expressing the mutant are protected from neurotoxic Aβ peptide-stimulated apoptosis, and, interestingly, increased oxidation of Kv2.1 channels is observed in Alzheimer's disease mouse model brain (Cotella et al. 2012). Oxidant-induced toxicity is postulated to proceed via defective Kv2.1 internalization and consequent Kv2.1 oligomer formation, leading to activation of the Src/JNK signaling pathway, although the data does not unequivocally place Kv2.1 oligomerization upstream of Src/JNK activation (Wu et al. 2013). Further, while decreased K^+ currents are observed acutely following DTDP treatment in this study, the previously described, pro-apoptotic, Kv2.1 K^+ current increase is detected approximately three hours after oxidant treatment (Aizenman et al. 2000, McLaughlin et al. 2001, Pal et al. 2003, Aras and Aizenman 2005, Redman et al. 2007, Redman et al. 2009). The results from these studies, therefore, are not irreconcilable; in fact, there may be oxidation of Kv2.1 channels and reduction of currents immediately following oxidative insult (Cotella et al. 2012, Wu et al. 2013), followed by SNARE-dependent trafficking of Kv2.1 channels to the plasma membrane, resulting in K^+ current enhancement, caspase activation, and apoptotic cell death (Aizenman et al. 2000, McLaughlin et al. 2001, Pal et al. 2003, Bossy-Wetzel et al. 2004,

Pal et al. 2006, Redman et al. 2006, Zhao et al. 2006, Redman et al. 2007, Knoch et al. 2008, Redman et al. 2009, Yao et al. 2009, Zhou et al. 2012).

Evidence collected thus far from numerous studies certainly points to the existence of disparate cell death signaling events in neurons, potentially depending on the nature of apoptotic stimulus and neuronal cell type. However, the fact that several early (e.g. Zn^{2+} release) and late pro-apoptotic processes are elicited by such a diverse range of toxic stimuli, converging on Kv2.1-mediated K^+ current enhancement, strongly suggests that this step represents a key mechanism in neuronal apoptosis that could be therapeutically targeted. In this regard, the hepatitis C virus nonstructural protein 5A (NS5A) was recently discovered to attenuate pro-apoptotic Kv2.1 K^+ current enhancement in hepatocytes and cortical neurons (Mankouri et al. 2009, Norris et al. 2012, Amako et al. 2013). This K^+ current blockade has been suggested to occur through NS5A-mediated inhibition of MLK3, a MAP kinase kinase kinase which promotes the activation of p38 kinase (Amako et al. 2013). As described above, p38 kinase is required for Kv2.1 Ser800 phosphorylation, enabling the pro-apoptotic K^+ current increase. However, in another study, NS5A was shown to block Src kinase-facilitated phosphorylation of the Tyr124 residue, without affecting channel phosphorylation of Ser800 by p38 kinase. In fact, pseudo-phosphorylation of Kv2.1 channels at Ser800, through expression of a Kv2.1 Ser800E mutant, does not eliminate NS5A-induced inhibition of K^+ currents, whereas Kv2.1 channels expressing a phospho-mimetic substitution at Tyr124Phe are no longer susceptible to K^+ current attenuation by NS5A, strongly indicating that NS5A exerts its inhibition of Kv2.1 currents and neuroprotective effects through preventing Src kinase-mediated Tyr124 phosphorylation rather than by blocking p38 kinase-induced Ser800 phosphorylation (Norris et al. 2012). This

mechanism warrants further exploration, as NS5A could serve as a model for new neuroprotective agents specifically targeting pro-apoptotic Kv2.1-mediated K^+ currents.

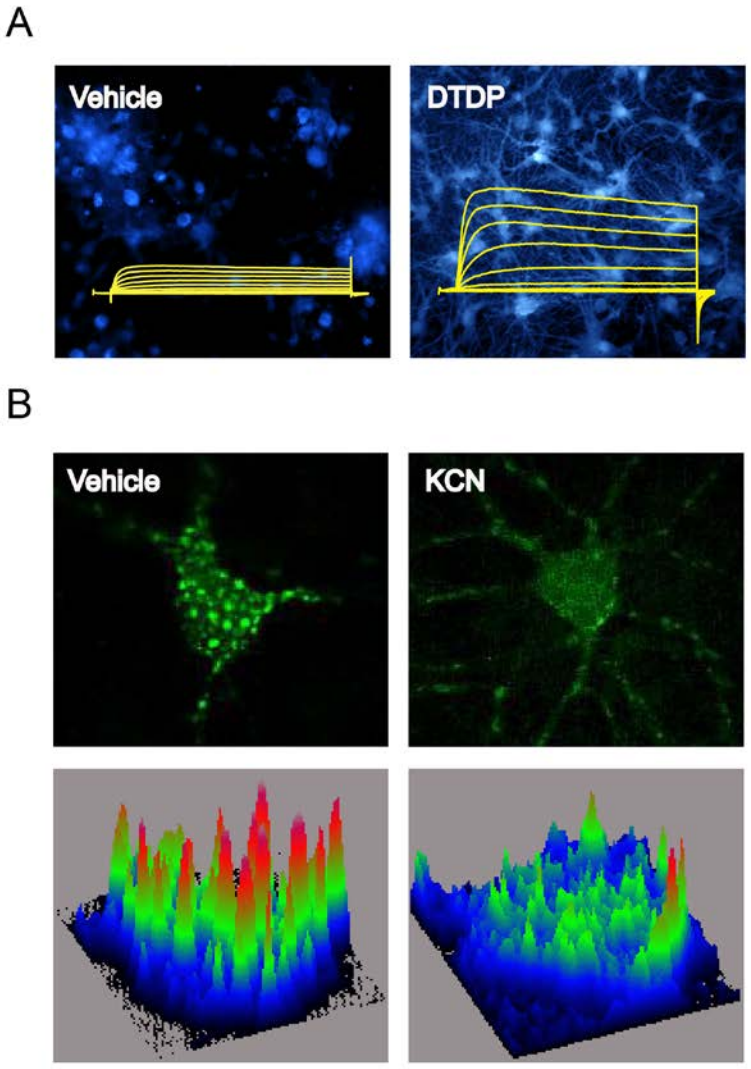

Figure 1 – Distinct mechanisms of Kv2.1 channel regulation following apoptotic stimuli or ischemic preconditioning

Figure 1. A, Oxidant exposure in neurons liberates Zn^{2+} from intracellular metal binding proteins (as detected by an increase in fluorescence using a Zn^{2+}-sensitive indicator such as FluoZin-3), which produces a proapoptotic enhancement of Kv2.1 K^+ currents. Reprinted with permission and adapted from Sensi et al. 2011. **B,** In contrast, neuronal activity or sublethal ischemia stimulates Kv2.1 channel dephosphorylation-dependent declustering, which, along with hyperpolarizing voltage-gated activation, induces neuronal tolerance to ischemic or epileptic challenge. Shown are confocal micrographs of rat cortical neurons transfected with plasmid vectors encoding GFP-labeled Kv2.1 channels. Below are fluorescence surface intensity maps used to quantify the number of clusters present in neurons.

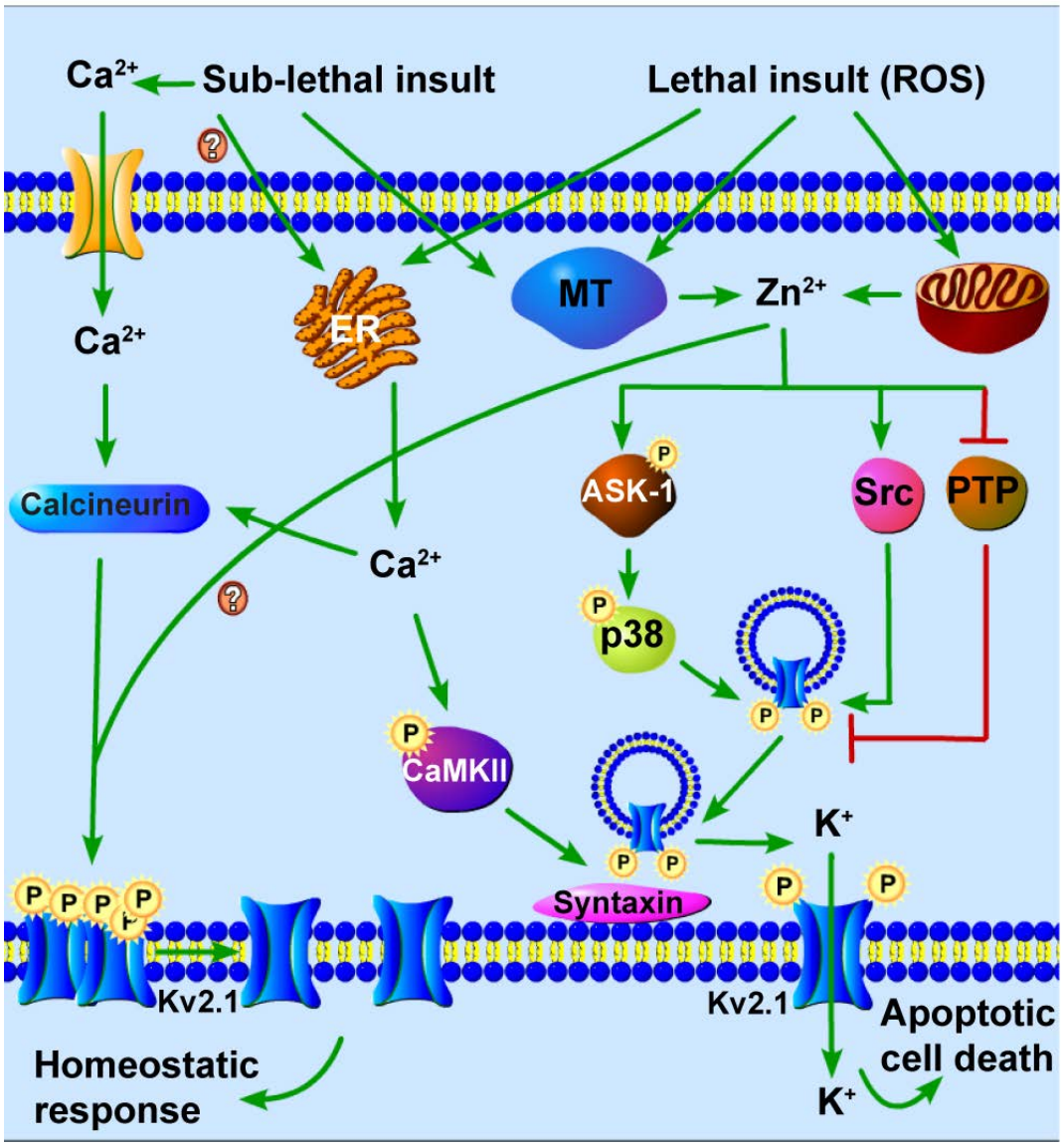

Figure 2 – Kv2.1-mediated pathways of neuronal apoptosis and neuronal tolerance

Figure 2. Right, An oxidant stimulus induces the release of Zn^{2+} from mitochondrial stores and metal-binding proteins, such as metallothionein (MT). Zn^{2+} activates ASK-1, leading to the phosphorylation and activation of p38 kinase. Zn^{2+} also inhibits PTPε and activates Src kinase. The combined action of both kinase systems results in increased phosphorylation of Kv2.1 channel residues Ser800 (by p38 kinase activation) and Tyr124 (by Src kinase activation and PTPε inhibition). Oxidant injury additionally stimulates release of Ca^{2+} from endoplasmic reticulum (ER) stores, which activates CaMKII. Coordinate phosphorylation of Kv2.1 channels at Ser800 and Tyr124 and the interaction of CaMKII with syntaxin facilitate Kv2.1 channel-syntaxin binding and subsequent channel delivery to the plasma membrane. Increased K^+ currents through these newly inserted Kv2.1 channels permit the completion of the apoptotic signaling pathway by mediating cytoplasmic K^+ loss. **Left,** Neuronal activity or sublethal ischemia induces Ca^{2+} influx through glutamate receptors or intracellular Ca^{2+} release from the ER and release of free Zn^{2+} from metal-binding proteins. Ca^{2+} increases calcineurin activity, leading to dephosphorylation and declustering of Kv2.1 channels. These changes are accompanied by a hyperpolarizing shift in the channel's voltage-gated activation profile. Zn^{2+} is required for channel declustering and the voltage-gated activation shift, but not for Kv2.1 channel dephosphorylation. These changes in Kv2.1 channels reduce neuronal excitability in the context of an ischemic or epileptic insult, and render neurons resistant to excitotoxic or other forms of injury.

1.3.2.2 Other Kv channels involved in neuronal damage and cell death

In addition to Kv2.1 channels, Kv1.5 channels, which also mediate delayed rectifying K^+ currents, have been implicated in playing a role in neuronal cell death, particularly in the context of ischemia. Cell viability following ischemia is increased in rat cortical neurons lacking Kv1.5 and the auxiliary β-subunit Kvβ2 (Stapels et al. 2010). Ischemic preconditioning *in vivo*, which limits infarct size following lethal ischemia, produces a decrease in Kv1.5 and Kvβ2 mRNA and protein expression in rat cortex, while preconditioning in rat cortical neurons reduces delayed rectifying K^+ currents, suggesting that inhibition of Kv1.5 channel-mediated K^+ currents is neuroprotective, and may be a viable therapeutic strategy for reducing neuronal damage and cell death in ischemic stroke (Stenzel-Poore et al. 2003).

Apoptotic stimuli that enhance delayed rectifier Kv currents have also been shown to increase rapidly inactivating, A-type Kv channel-mediated K^+ currents (I_A), implicating these currents in promoting apoptosis, although the molecular mechanisms underlying these processes have not yet been thoroughly characterized (Beauvais et al. 1995, Walev et al. 1995, Wang et al. 1999b, Jiao et al. 2004, Hu et al. 2005, Ogita et al. 2005, Pannaccione et al. 2005, Hu et al. 2006, Pannaccione et al. 2007, Hu et al. 2010, Pieri et al. 2010). Activated macrophages and conditioned media from these inflammatory cells induce an increase in I_A and in apoptotic cell death in hippocampal neurons (Hu et al. 2010). Similarly, the HIV-1 glycoprotein gp120 causes a rise in I_A and protein kinase C-mediated apoptotic cell death (Chen et al. 2011). In both studies, the I_A increase and toxicity are attenuated by 4-AP. 4-AP also reduces low K^+/serum deprivation-mediated I_A current increase and augments viability in CGNs (Jiao et al. 2004, Hu et al. 2005, Hu

et al. 2006), and in UV-treated epithelial cells (Lu et al. 2003, Singleton et al. 2009). However, 4-AP inhibits a relatively broad spectrum of Kv channels that mediate currents which include but are not limited to rapidly inactivating, A-type K^+ currents (Gutman et al. 2005), underscoring the need for further exploration of the role of A-type Kv currents in apoptotic cell death pathways.

A-type Kv currents may be particularly relevant in Alzheimer's disease (AD) as neurotoxic Aβ peptides have been shown to provoke an increase in I_A (Pannaccione et al. 2005, Pannaccione et al. 2007, Pieri et al. 2010). A specific inhibitor of Kv3.4 channels, which mediate I_A, reduces Aβ peptide-stimulated I_A enhancement and apoptotic nuclear morphology in hippocampal neurons (Pannaccione et al. 2007). Kv3.4 co-localizes with Aβ plaques, and its mRNA and protein expression is increased in AD mouse model brain, neurotoxic Aβ-treated PC-12 cells and rat hippocampal neurons, and in post-mortem frontal cortex tissue from patients with early and late AD (Angulo et al. 2004, Pannaccione et al. 2005, Pannaccione et al. 2007). mRNA and protein expression of Kv4.2, another channel responsible for A-type K^+ currents, is also enhanced in the cortex of rats whose spatial memory is compromised due to an intracerebroventricular injection of Aβ peptide (Pan et al. 2004). Of note, increased Kv1.4 and Kv2.1 channel expression is also observed in the hippocampus of these Aβ-injected animals, and in CGNs, the neuroprotective peptide substance P blocks Aβ-induced increases in both delayed rectifier and rapidly inactivating K^+ currents, suggesting that both types of K^+ currents may be involved in Aβ-mediated neurotoxicity (Yu et al. 1998, Yu et al. 2006, Pieri et al. 2010). In contrast to these observations, several groups have suggested a normal physiological role for Aβ in modulating Kv currents in a neuronal cell type-specific manner. One study has shown that aggregated, neurotoxic Aβ peptide has no effect on K^+ currents in cortical neurons or CGNs.

Non-toxic, unaggregated Aβ peptide, however, increases Kv4.2 protein expression, and A-type and Ca^{2+}-activated delayed rectifier K^+ currents in CGNs, while inhibition of endogenous Aβ production decreases Kv4.2 expression and inhibits K^+ currents (Ramsden et al. 2001, Plant et al. 2006).

Kv1.1 channels have also been implicated in I_A-mediated neuronal apoptosis (Hu et al. 2008, Koeberle et al. 2009, Koeberle and Schlichter 2010). siRNA knockdown of Kv1.1 blocks I_A in CGNs, and prevents rises in I_A and rescues cell viability in low K^+/serum-deprived CGNs (Hu et al. 2008). This apoptotic pathway is promoted by protein kinase C signaling, which is sufficient to activate I_A and apoptosis, effects that are mitigated by decreasing Kv1.1 expression. Further, Kv1-specific blockers reduce retinal ganglion cell degeneration after axotomy, while siRNA knockdown of Kv1.1 or Kv1.3 channels augments cell survival (Koeberle et al. 2009, Koeberle and Schlichter 2010).

1.4 NEUROPROTECTIVE AND NEUROREGULATORY ROLES FOR K_V CHANNELS

1.4.1 Kv channels in ischemic neuroprotection

As described above, Kv2.1 channels critically contribute to oxidant injury-induced neuronal apoptosis. As the major mediators of delayed rectifying, outward K^+ currents in neurons, Kv2.1 channels also play a key role in maintaining intrinsic neuronal excitability, primarily by promoting slow after-hyperpolarization and by regulating action potential repolarization during

high frequency stimulation (Trimmer 1991, Baranauskas et al. 1999, Murakoshi and Trimmer 1999, Bekkers 2000, Du et al. 2000, Kang et al. 2000, Korngreen and Sakmann 2000, Malin and Nerbonne 2002, Pal et al. 2003, Baranauskas 2007, Guan et al. 2007b, Guan et al. 2013). Excitatory stimuli, such as glutamate treatment, exposure to convulsants, or ischemia, trigger dramatic changes in Kv2.1 voltage-gated activation, in addition to affecting cellular localization of the channel (Figure 1b). Emerging evidence indicates that these modifications aid in reducing neuronal excitotoxicity in the context of an injurious stimulus (Figure 2, left).

Trimmer and coworkers first showed that Kv2.1 channels are maintained in highly phosphorylated, somatodendritic clusters in neurons (Trimmer 1991, Trimmer 1993, Shi et al. 1994, Murakoshi et al. 1997). An excitatory stimulus induces bulk Kv2.1 dephosphorylation *in vivo*, in rats subjected to kainate-induced seizures or CO_2 exposure, for example, and *in vitro*, in cultured hippocampal or cortical neurons treated with glutamate, NMDA, or chemical ischemia. This dephosphorylation is thought to be critical in promoting two concomitant changes in the channels: dispersal of Kv2.1 channel clusters, and a hyperpolarizing shift in voltage-gated activation of the channel (Perozo and Bezanilla 1990, Murakoshi et al. 1997, Misonou et al. 2004, Misonou et al. 2005, Misonou et al. 2006, Mohapatra and Trimmer 2006, Park et al. 2006, Misonou et al. 2008, Aras et al. 2009b, Mohapatra et al. 2009, Shepherd et al. 2012). Several lines of evidence support this concept. Phosphorylation of Kv channels promotes depolarizing shifts in voltage dependence, possibly due to an increase in the density of negative surface charges near the voltage sensor, explaining why dephosphorylation may induce a hyperpolarizing shift in the activation voltage (Perozo and Bezanilla 1990). Phospho-mimetic substitutions of seven, normally phosphorylated serine residues on the cytosolic Kv2.1 C-

terminus reduce the hyperpolarizing effects of excitatory stimuli, while serine-to-alanine mutations, which render the residues non-phosphorylatable, result in hyperpolarized voltage-gated activation. Similarly, blocking phosphorylation or inducing dephosphorylation of Kv2.1 channels results in declustering as well as hyperpolarizing voltage-gated activation (Park et al. 2006, Cerda and Trimmer 2011).

What signaling mechanisms govern these neuronal activity-induced changes in Kv2.1 channels? Several studies have demonstrated an early requirement for intracellular Zn^{2+} release and the Ca^{2+}/calmodulin-dependent phosphatase calcineurin. Chelating Zn^{2+} blocks the hyperpolarizing shift and cluster dispersal, but not the channel dephosphorylation in cortical neurons (Aras et al. 2009b). Ca^{2+} influx via a Ca^{2+} ionophore is sufficient to induce Kv2.1 dephosphorylation, declustering, and the hyperpolarizing activation shift, while inhibiting either Ca^{2+} influx or calcineurin activity blocks these changes in Kv2.1 in response to an excitatory stimulus in hippocampal and cortical neurons (Misonou et al. 2004, Misonou et al. 2005, Misonou et al. 2006, Mohapatra and Trimmer 2006, Park et al. 2006, Aras et al. 2009b). One C-terminal serine residue in particular, Ser603, is highly sensitive to excitatory stimuli-induced, calcineurin-mediated dephosphorylation (Misonou et al. 2006). Recently, cyclin-dependent kinase 5 (Cdk5) was shown to phosphorylate this residue. Pharmacologic inhibition of Cdk5 kinase activity blocks Kv2.1 Ser603 phosphorylation and stimulates dispersal of channel clusters (Cerda and Trimmer 2011). Further, neuronal activity blockade promotes precipitous increases in Ser603 phosphorylation, whereas activity-inducing stimuli trigger its dephosphorylation. As the phosphorylation status of Ser603 critically regulates voltage-dependent gating of the channel (Park et al. 2006), this residue may serve as a bidirectional sensor of neuronal activity, mediating

changes in Kv2.1 channel gating kinetics, and thus regulating neuronal excitability in response to excitatory or inhibitory stimuli.

As described above, calcineurin signaling is thought to regulate Kv2.1 channel localization and function in ischemic preconditioning. Calcineurin also plays an important role in normal neuronal function. However, calcineurin over-activation may promote neuronal cell death in stroke and in other neurodegenerative disorders (Wang et al. 1999, Terada et al. 2003, Shioda et al. 2006). Therefore, it is important to understand both physiological and pathological regulation of calcineurin activity. In Chapter 3, I investigated mechanisms of neuroprotection and neuronal cell death mediated by the two brain isoforms of RCAN1, an endogenous regulator of calcineurin (Fuentes et al. 1995).

Although RCAN1 was originally identified as an endogenous inhibitor of calcineurin, RCAN1 may in fact regulate calcineurin through inhibitory or facilitative mechanisms depending on cell type, cytoplasmic environment, protein levels and phosphorylation status of RCAN1, and, particularly relevant to this thesis, the RCAN1 isoform under study. *In vitro* studies have clearly demonstrated that RCAN1 binds to and inhibits calcineurin, and over-expression of RCAN1 in mammalian cell lines impedes calcineurin/nuclear factor of activated T cells (NFAT) signaling (Görlach et al. 2000, Kingsbury and Cunningham 2000, Rothermel et al. 2000, Martínez-Martínez et al. 2009). However, differing findings indicate that RCAN1 may facilitate calcineurin phosphatase activity in certain circumstances. Loss of RCAN1 expression in yeast and mouse fibroblasts, for example, diminishes calcineurin signaling (Kingsbury and Cunningham 2000, Sanna et al. 2006). Shin et al provided evidence that at low levels, RCAN1

may function as a calcineurin inhibitor, but at high levels of expression it acts as a facilitator of calcineurin signaling (Shin et al. 2011). Additionally, phosphorylation of RCAN1 may be required for induction of calcineurin activity. However, whether this facilitation occurs through direct activation of calcineurin, or through release of calcineurin inhibition by binding to other proteins or degradation of phosphorylated RCAN1, is unresolved (Vega et al. 2002, Hilioti et al. 2004, Abbasi et al. 2006, Kishi et al. 2007, Liu et al. 2009).

Alternate promoter usage and splicing of RCAN1 mRNA produce two distinct brain isoforms, RCAN1.1 and RCAN1.4. The proteins differ only in the N-terminus: RCAN1.1 contains the protein sequence encoded by exons 1, 5, 6, 7, while RCAN1.4 is encoded by exons 4, 5, 6, and 7 (Fuentes et al. 1997). Protein expression levels of RCAN1.1 and RCAN1.4 have been shown to be regulated differently based on distinct response elements in the sequences upstream of exons 1 and 4 respectively. In neurons and non-neuronal cells, RCAN1.1 protein expression is increased in response to glucocorticoid exposure (Hirakawa et al. 2009, Sun et al. 2011), while RCAN1.4 levels are up-regulated by Ca^{2+}/calcineurin signaling (Yang et al. 2000, Mitchell et al. 2007).

RCAN1.1 and RCAN1.4 share a high degree of similarity in their protein sequences. In fact, most of the calcineurin-binding domains are contained in the sequence encoded by exons 5, 6, and 7 (Vega et al. 2002, Mehta et al. 2009), and are therefore identical between RCAN1.1 and RCAN1.4. Despite these similarities, the two isoforms have been reported to activate distinct cell-signaling mechanisms, including those relevant to cell survival and cell death. For example, whereas RCAN1.4 may be cytotoxic under certain conditions, this isoform is up-regulated in

astrocytes and may decrease infarct volume following ischemic injury in mice through a calcineurin inhibition-dependent mechanism (Sobrado et al. 2012). RCAN1.4 also may reduce Zn^{2+} neurotoxicity (Lee et al. 2007). Chronically elevated RCAN1.1 levels are implicated in promoting Alzheimer's disease and Down syndrome-related neurodegeneration (Ermak et al. 2001, Sun et al. 2011), but a contrasting study reported that increased RCAN1.1 protein levels may reduce neuronal ischemic damage (Brait et al. 2012).

Additionally, RCAN1 may promote cell-signaling pathways independently of calcineurin regulation (Strippoli et al. 2000, Chang and Min 2005, Keating et al. 2008, Martin et al. 2012). Although these mechanisms have not been elucidated in detail, a few studies suggest that they may be relevant in RCAN1 regulation of neuronal function and cell viability (Keating et al. 2008, Brait et al. 2012, Martin et al. 2012). Finally, our understanding of RCAN1-regulated cell signaling pathways is further complicated by unclear or incorrect isoform identification in several previously published reports. Thus, many unanswered questions remain as to the isoform-specific functions of RCAN1 in regulating neuronal viability. We have begun to address these questions in Chapter 3 by investigating the effects of RCAN1.1 and RCAN1.4 over-expression on cell viability and toxicity in primary cortical neurons.

In addition to calcineurin signaling, ischemia-induced changes in Kv2.1 channel properties may be dependent on specific neuronal-glial interactions. In the rat cerebral cortex, Kv2.1 channel clusters are located in the extra-synaptic zone, adjacent to astrocytic processes that contain a high concentration of glutamate transporters (Du et al. 1998, Misonou et al. 2008). During ischemia, excessive glutamate accumulation in the extracellular space due to

compromised glutamate uptake in damaged astrocytes may be responsible for promoting Kv2.1 channel dephosphorylation, cluster dispersal, and hyperpolarizing shifts in voltage-gated activation following NMDA receptor activation (Misonou et al. 2008, Mulholland et al. 2008, Mohapatra et al. 2009, Mulholland et al. 2009). Indeed, NMDA exposure or selective inhibition of astrocytic glutamate uptake in cortical or hippocampal slices is sufficient to promote neuronal Kv2.1 dephosphorylation. Accordingly, NMDA receptor antagonists block the dephosphorylation and hyperpolarizing gating shift activated by exogenous glutamate treatment or inhibition of astrocytic glutamate uptake (Misonou et al. 2008, Mulholland et al. 2008, Mohapatra et al. 2009, Mulholland et al. 2009).

The hyperpolarizing shift in Kv2.1 channel voltage-gated activation is thought to reduce excitability and, consequently, excitotoxicity in neurons facing an ischemic or epileptic challenge. Sub-lethal chemical ischemia, which renders rat cortical neurons resistant to subsequent NMDA receptor-mediated excitotoxicity (McLaughlin et al. 2003, Aras et al. 2009a), induces Kv2.1 channel dephosphorylation and declustering, and produces a hyperpolarized shift in voltage-gated activation, implicating these channel modifications in promoting neuroprotection (Aras et al. 2009b). In hippocampal neurons, ischemia or glutamate treatment reduces spontaneous Ca^{2+} transients, and spontaneous and current-evoked firing. Combining Kv2.1 channel block with either of these treatments promotes an increase in Ca^{2+} overload and in firing frequency, suggesting the involvement of Kv2.1 channel-mediated K^+ currents in reducing neuronal hyperexcitability within the context of ischemia (Du et al. 2000, Misonou et al. 2005, Mohapatra et al. 2009).

As described above, the changes in Kv2.1 localization, phosphorylation status, and voltage gating have been observed in response to a range of excitatory stimuli *in vitro* and *in vivo*. Further, the dephosphorylation and hyperpolarization of voltage-gated activation have been linked to reduction of intrinsic excitability and neuronal tolerance to otherwise-lethal injury. However, little is known about the mechanism and significance of Kv2.1 channel clustering, and the specific contribution of Kv2.1 declustering towards mediating neuronal hyperactivity. Four C-terminal residues, Ser583, Ser586, Phe587, and Ser589, are critical for Kv2.1 channel clustering. A C-terminal portion of Kv2.1 channels possessing all four of these residues confers Kv2.1-like clustered localization on other Kv channels subtypes, such as Kv2.2 and Kv1.5 (Scannevin et al. 1996, Lim et al. 2000, Mohapatra and Trimmer 2006). Additionally, a cytoplasmic N-terminal/C-terminal interaction is required for proper channel surface expression and phosphorylation-driven modulation of activation kinetics (Mohapatra et al. 2008). As mentioned above, it has been postulated that channels in clusters located at extra-synaptic locations and adjacent to astrocytic processes may be important in sensing ischemia-induced glial dysfunction through glutamate signaling, while the channel declustering following calcineurin activity-dependent dephosphorylation would remove the Kv2.1 channels from the site of Ca^{2+} release, initiating recovery and precluding a potentially detrimental, prolonged response. This cluster dispersal may occur through excess glutamatergic stimulation of extrasynaptic rather than synaptic NMDA receptors, prompting relocation of Kv2.1 channels to synaptic zones (Misonou et al. 2008, Mulholland et al. 2008, Mulholland et al. 2009). However, the cellular and molecular mechanisms involved in these processes require further exploration.

Tamkun and colleagues have proposed a somewhat different role for Kv2.1 channel clusters. They have reported that clustered Kv2.1 channels are non-conducting, but retain gating currents that display a hyperpolarized activation profile when compared to that of Kv2.1 ionic currents (O'Connell et al. 2010). Because the channels would detect membrane depolarization at a lower threshold, these studies suggest that Kv2.1 channels clusters may serve as voltage sensors of neuronal activity that convey changes in membrane potential to cytosolic signaling pathways. Supporting this hypothesis is the demonstration that Kv2.1 channel clusters are insertion platforms for trafficking of Kv2.1 and other channels to the plasma membrane, indicating that clustered Kv2.1 channels could be sites of depolarization-driven vesicle trafficking and neurotransmitter release (O'Connell and Tamkun 2005, O'Connell et al. 2006, Deutsch et al. 2012). In fact, Lotan and co-workers have shown that in neuroendocrine cells, Kv2.1 channels play an important role in depolarization-induced exocytosis that is independent of their ion conducting properties (Singer-Lahat et al. 2008, Feinshreiber et al. 2009). However, these investigations have been conducted in recombinant cell expression systems and future studies examining these properties in neurons are necessary. Importantly, it was demonstrated recently that the majority of Kv2.1 channels in hippocampal neurons are non-conducting, lending further credence to the theory that Kv2.1 channel clusters may regulate key neuronal functions unrelated to their ion conducting properties (Fox et al. 2013).

Other Kv channels may be involved in reducing neuronal excitability and cell death in the context of ischemic injury. Following ischemia, Kv1-mediated delayed rectifying K^+ currents increase in large aspiny neurons, which are highly resistant to anoxic cell death (Deng et al. 2005). Ischemic injury shortens spike duration in these neurons, which could limit Ca^{2+} influx

and thus mitigate excitotoxicity. Importantly, blocking Kv1 channel function restores action potentials to normal duration in anoxia-treated cells, suggesting a role for Kv1-facilitated K^+ currents in regulating neuronal excitability in ischemia. Further, increased Kv1.2 subunit expression is observed in rat brain following transient focal ischemia (Chung et al. 2001). An ischemic injury-promoted rise in A-type K^+ currents may also be responsible for decreasing excitability and thus limiting excitotoxic cell death in large aspiny neurons (Deng et al. 2011). Medium spiny neurons, which are more vulnerable to ischemic neuronal damage, do not manifest an increase in I_A following ischemic injury. Importantly, over-expression of I_A-mediating Kv1.4 or Kv4.2 channels in medium spiny neurons reduces oxygen-glucose deprivation-induced toxicity, while neurons lacking Kv1.4 or Kv4.2 channel expression are more sensitive to ischemic cell death (Deng et al. 2011). Increased I_A is also observed in CA1 hippocampal neurons after transient forebrain ischemia (Chi and Xu 2000).

1.4.2 Loss of Kv1 or Kv7 channel function mediates neuronal hyperexcitability disorders

Kv1 and Kv7 encode K^+ channels that are also important contributors to neuronal excitability, with functions including maintenance of resting membrane potential, action potential repolarization and after-hyperpolarization, and regulation of neurotransmitter release (Southan and Robertson 1998, Geiger and Jonas 2000, Jentsch 2000, Bekkers and Delaney 2001, Lambe and Aghajanian 2001, Brew et al. 2003, Dodson et al. 2003, Dodson and Forsythe 2004, Peters et al. 2004, Gu et al. 2005, Vervaeke et al. 2006, Guan et al. 2007a, Kole et al. 2007, Shu et al. 2007, Goldberg et al. 2008, Tzingounis and Nicoll 2008, Brown and Passmore 2009, Heeroma et al. 2009, Hsiao et al. 2009, Tzingounis et al. 2010, Foust et al. 2011, Higgs and Spain 2011).

Accordingly, loss of proper function of these channels is generally associated with hyperexcitability phenotypes such as episodic ataxia type 1 (EA-1) and epilepsy.

1.4.2.1 Kv1 channels and EA-1

EA-1 is a rare, autosomal dominant disorder characterized by generalized ataxia attacks and spontaneous muscle quivering (Gancher and Nutt 1986). In 1994, Browne and colleagues discovered four mutations in Kv1.1 in each of four families that had multiple members affected by EA-1 (Browne et al. 1994). Since then, more than a dozen Kv1.1 mutations have been identified in EA-1 patients with variable symptomatic presentations (Browne et al. 1994, Browne et al. 1995, Çomu et al. 1996, D'Adamo et al. 1998, Scheffer et al. 1998, Zerr et al. 1998b, Zerr et al. 1998a, Spauschus et al. 1999, Zuberi et al. 1999, Herson et al. 2003, Klein et al. 2004, Lee et al. 2004, Poujois et al. 2006, Rajakulendran et al. 2007, Shook et al. 2008, Tomlinson et al. 2010, Zhu et al. 2012). Most of these are point mutations in highly conserved channel residues that generate Kv1.1 loss-of-function phenotypes of varying degrees. For several EA-1 Kv1.1 mutations, the extent of disease in patients correlates to the magnitude of altered channel properties in *Xenopus* oocyte expression experiments, strongly implicating Kv1.1 channel dysfunction in the pathogenesis of EA-1 (Spauschus et al. 1999, Zuberi et al. 1999, Eunson et al. 2000, Rea et al. 2002).

When expressed in oocytes or mammalian cells, the majority of EA-1 Kv1.1 channel mutants exhibit undetectable or reduced K^+ currents, compared to expression of wild-type Kv1.1 channels (D'Adamo et al. 1998, Scheffer et al. 1998, Zerr et al. 1998b, Zerr et al. 1998a, Spauschus et al. 1999, Zuberi et al. 1999, Zhu et al. 2012). Dysfunctional post-translational

modifications and improper plasma membrane trafficking may mediate the reduced currents (Bretschneider et al. 1999, Eunson et al. 2000, Manganas et al. 2001, Rea et al. 2002, Zhu et al. 2012). Arg417stop Kv1.1 channels, for example, lack a C terminal targeting determinant, and undergo inefficient phosphorylation and N-glycosylation, forming large intracellular membranous aggregates in COS cells and mammalian neurons (Manganas et al. 2001).

Other modifications that are observed in several EA-1 Kv1.1 mutant channels expressed in oocytes, such as slowed activation kinetics and a depolarizing shift in voltage-gated activation, implicate gating defects as the source of Kv1.1 dysfunction (Adelman et al. 1995, D'Adamo et al. 1998, Zerr et al. 1998b, Zerr et al. 1998a, Spauschus et al. 1999, Eunson et al. 2000, Maylie et al. 2002, Imbrici et al. 2003, Imbrici et al. 2006, Peters et al. 2011). Given the importance of Kv1 channels in limiting neuronal excitability, these alterations in Kv1.1 channel kinetics would be expected to increase neuronal activity, providing a possible explanation for the hyperexcitable EA-1 phenotype. Indeed, expressing Kv1.1 Arg417stop or Thr226Arg mutant channels in hippocampal neurons elicits a lower current threshold for action potential firing, and increased neurotransmitter release compared to expression of wild-type Kv1.1 channels (Heeroma et al. 2009). Another EA-1 Kv1.1 mutation, Val408Ala, confers a range of channel gating defects in heterologous expression systems (Browne et al. 1994, Adelman et al. 1995, D'Adamo et al. 1998, Zerr et al. 1998b, Bretschneider et al. 1999, Maylie et al. 2002, Imbrici et al. 2006, Peters et al. 2011). Val408Ala heterozygous mice show increased frequency and amplitude of cerebellar Purkinje cell inhibitory post-synaptic currents, spontaneous neuromuscular activity, and importantly, stress-induced motor deficits, similar to EA-1 patients (Herson et al. 2003, Brunetti et al. 2012). Two other Kv1.1 mutant mouse models that demonstrate variable EA-1 phenotypes

have also been reported (Petersson et al. 2003, Ishida et al. 2012). However, as most EA-1 Kv1.1 mutational analysis has been conducted in oocyte expression systems, a thorough investigation into the biophysical properties of neurons expressing EA-1 Kv1.1 channel mutations is warranted, given the key role Kv1.1 dysfunction likely plays in this disorder.

1.4.2.2 Kv1 channels and epilepsy

A subset of patients with familial EA-1 is affected with epileptic seizures, suggesting that Kv1 channel dysfunction may play a role in the pathophysiology of epilepsy (Spauschus et al. 1999, Zuberi et al. 1999, Eunson et al. 2000). Several reports have also identified patients who are heterozygous for Kv1.1 mutations, and suffer epileptic seizures concomitant with other neurologic abnormalities such as cognitive delay (Liguori et al. 2001, Demos et al. 2009). Injection of dendrotoxin, a Kv1 channel antagonist, into rat hippocampus induces neuronal hyperexcitability, seizures, and cell death (Bagetta et al. 1992, Lalic et al. 2011). Importantly, Kv1.1-null mice exhibit an epileptic phenotype, undergoing spontaneous behavioral seizures once or twice every hour, which are consistently accompanied by ictal electroencephalographic (EEG) patterns. The threshold for seizure initiation is determined by Kv1.1 gene dosage. Homozygous Kv1.1-null mice are more rapidly susceptible to convulsant-induced seizures than heterozygous Kv1.1-null mice, which are in turn more sensitive than their wild-type littermates (Smart et al. 1998, Rho et al. 2011). On the cellular level, loss of Kv1.1 channel function in Kv1.1-null mice produces a neuronal hyperexcitability phenotype that is commonly observed in epilepsy models, in the hippocampus, a brain region highly susceptible to epileptogenic activity (Smart et al. 1998, Zhou et al. 1998, Zhang et al. 1999, Zhou et al. 1999, Brew et al. 2003, Kopp-Scheinpflug et al. 2003, Lopantsev et al. 2003, Baraban et al. 2009, Heeroma et al. 2009, Wykes

et al. 2012, Simeone et al. 2013). Neuronal hyperexcitability in Kv1.1-null mice has also been observed in myelinated nerves (Zhou et al. 1998, Zhou et al. 1999), cerebellar basket neurons (Zhang et al. 1999, Chen et al. 2005a), and medial nucleus of the trapezoid body neurons in the brainstem (Brew et al. 2003, Kopp-Scheinpflug et al. 2003). Decreasing network excitability by impairing P/Q-type Ca^{2+} channel function, or providing inhibitory synaptic input by grafting medial ganglionic GABAergic neuron precursors into the cortex of Kv1.1-null mice, lowers the duration and frequency of spontaneous seizures (Glasscock et al. 2007, Baraban et al. 2009). In agreement with these findings, lentiviral-mediated delivery of Kv1.1 channels to motor cortex pyramidal neurons attenuates neuronal hyperexcitability and prevents EEG-measured epileptic activity in a rodent model of tetanus toxin-induced neocortical epilepsy (Wykes et al. 2012).

Kv1.2 channel dysfunction in neuronal hyperexcitability has also been reported. Early studies revealed that Kv1.1 α-subunits co-localize and likely form heteromers with Kv1.2 channel subunits in most parts of the brain where both channels are expressed (Wang et al. 1993, Wang et al. 1994, Rhodes et al. 1997, Southan and Robertson 1998, Southan and Robertson 2000, Monaghan et al. 2001). When co-expressed in fibroblast cells, EA-1 Arg417stop Kv1.1 mutant channel and wild-type Kv1.2 channel trafficking is impaired, implying heteromerization and suggesting that loss of Kv1.2 channel function, as a result of Kv1.1 mutations, may play a role in familial EA-1 (Manganas et al. 2001). Further, most pharmacologic agents that block Kv1.1 channels and induce neuronal hyperexcitability, inhibit Kv1.2 channels as well (Bagetta et al. 1992, Bekkers and Delaney 2001, Shu et al. 2007). Several studies indicate that loss of Kv1.2 channel function alone is sufficient to promote neuronal hyperexcitability, and may mediate epileptic pathology. For example, Kv1.2-specific inhibitors instigate hyperexcitability in

cerebellar and brainstem neurons (Southan and Robertson 1998, Dodson et al. 2003). Additionally, decreased Kv1.2 protein expression, which can be rescued by anti-convulsant agents, is detected in the hippocampus of seizure-prone or convulsant-treated mice (Tsaur et al. 1992, Petersson et al. 2003). Although no Kv1.2 mutations have been detected in patients with epilepsy, Kv1.2-null mice display increased susceptibility to seizures and decreased life span (Brew et al. 2007). In contrast to studies demonstrating impairment of Kv1.2 channel function due to Kv1.1 dysfunction in EA-1 (Manganas et al. 2001), some investigators have suggested that Kv1.2 subunits may play a compensatory role in neurons when Kv1.1 function is compromised (Brew et al. 2003).

As described above, neuronal hyperexcitability due to Kv1 channel loss-of-function is associated with pathogenesis of some forms of epilepsy. However, epilepsy is a complex disorder that encompasses network excitability abnormalities arising from dysfunction of a wide range of molecular components in various cell types and in different brain regions. The effects of reduced Kv1 K^+ currents on epileptic pathology, therefore, may be varied depending on the location of the epileptogenic focus, and the affected neuronal cell type. Kv1.1 channel loss-of-function is associated with promotion of epileptic activity in the hippocampus, whereas in an animal model of absence epilepsy associated with defects in thalamocortical circuitry, eliminating Kv1.1 channel function rescues the seizure phenotype (Glasscock et al. 2007). Moreover, decreased intrinsic excitability in cortical, fast-spiking inhibitory neurons, via up-regulation of Kv1.1 channel activity, may promote seizure susceptibility (Lau et al. 2000, Li et al. 2011).

Spinal cord injury and multiple sclerosis are additional examples of clinical disorders in which increased neuronal signaling via blockade of Kv1 channel activity may be beneficial. In these diseases, outward K^+ currents through exposed Kv1 channels along damaged, demyelinated axons may impair action potential propagation. In fact, fampridine, a slow-release formulation of the Kv channel blocker 4-AP, was recently approved by the Food and Drug Administration (FDA) to improve walking in patients with multiple sclerosis (Preiningerova et al. 2013).

1.4.2.3 A success story: Kv7 channel activators in the therapeutic management of epilepsy

Heteromeric Kv7.2/Kv7.3 channels mediate the slowly activating, non-inactivating M currents in central and peripheral neurons (Wang et al. 1998, Shah et al. 2002, Brown and Passmore 2009). These channels critically contribute to the after-hyperpolarizing potential, aid in maintaining resting membrane potential and firing thresholds, and importantly, reduce intrinsic burst firing and repetitive action potential firing in response to excitatory stimuli (Wang et al. 1998, Cooper et al. 2000, Jentsch 2000, Cooper et al. 2001, Gu et al. 2005, Shah et al. 2008, Tzingounis and Nicoll 2008, Brown and Passmore 2009, Tzingounis et al. 2010, Sun and Kapur 2012, Miranda et al. 2013). Increasing Kv7 channel function decreases excitability, while suppressing Kv7 channel K^+ currents enhances excitability in hippocampal pyramidal, and superior cervical and dorsal root ganglionic neurons, and promotes epileptiform activity in hippocampal neurons (Otto et al. 2004, Peters et al. 2004, Otto et al. 2006, Peña and Alavez-Pérez 2006, Otto et al. 2009, Andreasen and Nedergaard 2012, Sun and Kapur 2012, Maslarova et al. 2013, Miranda et al. 2013). Mice expressing dominant negative mutant Kv7.2 channels display spontaneous seizures,

behavioral hyperactivity, and increased neuronal excitability as well as cell death in the hippocampus (Peters et al. 2004).

Mutations in Kv7.2 and Kv7.3 channels are associated with sporadic neonatal seizures and benign familial neonatal convulsions (BFNC), an autosomal dominant disease of frequent generalized epileptic seizures beginning in the first week of life and generally disappearing within a few months (Biervert et al. 1998, Charlier et al. 1998, Singh et al. 1998, Hirose et al. 2000, Singh et al. 2003, Sadewa et al. 2008, Ishii et al. 2009, Miceli et al. 2011). However, several neonatal seizure-associated Kv7.2 mutations are linked to more severe abnormalities in patients, such as increased risk of seizures and therapy-refractory epilepsy later in life, epileptic encephalopathy, myokymia, and slowed psychomotor development (Dedek et al. 2001, Dedek et al. 2003, Borgatti et al. 2004, Schmitt et al. 2005, Zhou et al. 2006, Steinlein et al. 2007, Wuttke et al. 2007, Weckhuysen et al. 2012). These studies further confirm the involvement of Kv7 channel dysfunction in some forms of epilepsy, and implicate central and peripheral neuronal Kv7 channel dysfunction in diverse clinical phenotypes generally correlating with neuronal hyperexcitability.

Most Kv7.2 and Kv7.3 mutations associated with BFNC and more severe disorders occur in the cytosolic C-terminus, voltage-sensing domain, or pore-forming region. Expression of mutant channels in oocytes or hippocampal neurons reveals a range of channel defects. Several mutations, particularly those in the voltage-sensing domain of the channel, confer slower activation kinetics and depolarizing shifts in voltage-gated activation (Dedek et al. 2001, Castaldo et al. 2002, Singh et al. 2003, Wuttke et al. 2007, Uehara et al. 2008, Volkers et al.

2009), while C-terminal frameshift, insertion, or truncation mutant Kv7 channels exhibit reduced current amplitudes due to intracellular trafficking defects, inefficient membrane targeting, or increased degradation (Lerche et al. 1999, Schwake et al. 2000, Singh et al. 2003, Chung et al. 2006, Volkers et al. 2009, Su et al. 2011). Two transgenic BFNC mouse models, expressing Kv7.2 Ala306Thr or Kv7.3 Gly311Val channels, present with generalized seizures likely of hippocampal origin, but display minimal synaptic reorganization or permanent neuronal damage in the hippocampus, recapitulating the major features of human BFNC. Additionally, Kv7 current density in homozygous mutant hippocampal slices is decreased, while deactivation kinetics are accelerated (Singh et al. 2008, Otto et al. 2009). Heterozygous adult mice show reduced threshold to electroconvulsant-induced seizures and similar, albeit less severe, Kv7 current alterations to homozygous mice.

Retigabine, also known as ezogabine, is a Kv7 channel activator that was approved by the FDA in 2011 for adjuvant treatment of partial-onset seizures in adults (Blackburn-Munro et al. 2005, Miceli et al. 2011, Weisenberg and Wong 2011, Orhan et al. 2012, Amabile and Vasudevan 2013), following demonstration of seizure reduction in animal models of epilepsy (Rostock et al. 1996, Tober et al. 1996) and in human clinical trials (Porter et al. 2007, Brodie et al. 2010, French et al. 2011). Retigabine enhances Kv7 channel activation by inducing a hyperpolarizing effect on voltage-gated channel activation. This mechanism of action limits neuronal excitability, as evidenced by the reduction of depolarization-induced action potential firing in neurons treated with retigabine (Tatulian et al. 2001, Wuttke et al. 2005). Since the discovery of retigabine's anticonvulsant properties, numerous novel Kv7 activators are being explored for their therapeutic potential in treating epilepsy (Roeloffs et al. 2008, Wulff et al.

2009, Miceli et al. 2011, Qi et al. 2011, Dalby-Brown et al. 2013, Kasteleijn-Nolst Trenité et al. 2013). Notably, in addition to epilepsy, Kv7 channel activators may also be effective in treating other diseases in which neuronal hyperexcitability represents a primary pathological component, including inflammatory or neuropathic pain (Hirano et al. 2007, Munro and Dalby-Brown 2007, Bi et al. 2011), tinnitus (Li et al. 2013), as well as neuropsychiatric disorders (Redrobe and Nielsen 2009, Sotty et al. 2009).

1.4.3 A role for Kv channels in neuro-cardiac regulation

Recently, Kv channels have been associated with sudden unexplained death in epilepsy (SUDEP), an event which occurs in two to eighteen percent of chronic, idiopathic epileptic patients, and is thought to arise from neurologically-driven cardiac dysfunction (Stöllberger and Finsterer 2004, Nashef et al. 2007, Tomson et al. 2008, Goldman et al. 2009). Kv1.1-null mice display a range of cardiac abnormalities, some of which are ameliorated by inhibiting parasympathetic innervation from the vagus nerve (where Kv1.1 is normally expressed) to the heart (Glasscock et al. 2010). Additionally, about half of Kv1.1-null mice die suddenly between the third and fourth week of life, with several of these mice exhibiting severe generalized seizures prior to death (Smart et al. 1998, Rho et al. 2011), suggesting that they may be experiencing SUDEP. In another study, mice carrying a human long QT syndrome mutation in Kv7.1 channels exhibit cardiac arrhythmias and epileptiform activity, with a mouse in this report experiencing seizures that developed into status epilepticus accompanied by severe cardiac abnormalities, culminating in cardiac arrest (Goldman et al. 2009). These studies implicate Kv channels in the pathophysiology of a disastrous complication of epilepsy, highlighting the importance of Kv channels in neurological regulation of cardiac function.

1.5 SUMMARY

The Kv channel family is a diverse group of channels mediating outward K^+ currents that play important roles in normal and pathological processes in neurons. Increased efflux of currents through Kv2.1 channels promotes apoptotic signaling (Figure 1a and Figure 2, right), while neuronal activity-regulated alterations in channel localization, phosphorylation, and voltage-gated activation reduce neuronal excitability, suggesting a role for these modifications in neuroprotection against ischemic or epileptic injury (Figure 1b and Figure 2, left). Loss of Kv1 or Kv7 promotes neuronal hyperexcitability, which manifests pathological consequences in disorders such as epilepsy or EA-1. Further, Kv channelopathy is likely to contribute to the pathophysiology of several other neurological diseases, including spinal cord injury, multiple sclerosis, inflammatory and neuropathic pain, and neuropsychiatric disorders (Table 1). Significant challenges, however, exist for developing Kv channel-directed therapeutic agents. Kv channels are widely expressed in most organs, including the brain, heart, liver, lungs, pancreas, and kidney (Gutman et al. 2005, Wulff et al. 2009). As such, drugs targeting these channels in neuronal diseases may cause potentially harmful effects. Additionally, the precise molecular composition of Kv channels mediating specific K^+ currents in different neuronal cell types is often difficult to pinpoint, given the diversity of α-subunit heteromerization patterns and the presence of modulatory binding partners. However, as evidenced by the successful clinical use of retigabine to activate Kv7 channels in treating epilepsy, targeting Kv channels is likely to be a viable therapeutic strategy for a wide range of neurological diseases in the near future. To this end, the studies in Chapter 2 of this thesis explore mechanisms of Kv2.1 channel regulation that may promote neuroprotection in stroke.

Table 1 – Kv channels implicated in neuronal pathology and neurological disease

Subtype	K^+ current type	Associated pathology
Kv1	Delayed rectifying (Kv1.1-1.3, Kv1.5-1.8)	Episodic ataxia, epilepsy (Kv1.1, Kv1.2, Kv1.4)
	A-type (Kv1.4)	Neuronal apoptosis (Kv1.1, Kv1.3)
		Ischemic cell death (Kv1.5)
Kv2	Delayed rectifying	Neuronal apoptosis (Kv2.1)
Kv3	Delayed rectifying (Kv3.1, Kv3.2)	Epilepsy (Kv3.2)
	A-type (Kv3.3, Kv3.4)	Alzheimer's disease (Kv3.4)
Kv4	A-type	Alzheimer's disease (Kv4.2)
		Epilepsy (Kv4.2, Kv4.3)
Kv7	Delayed rectifying, M-type	Epilepsy, tinnitus, pain, neuropsychiatric disorders (Kv7.1-7.5)

1.6 THESIS GOALS

The goal of this dissertation was to explore calcineurin-mediated signaling pathways in ischemic preconditioning and neuronal cell death. Previous studies from our laboratory and other groups have shown the importance of calcineurin-triggered modifications in Kv2.1 channel phosphorylation and localization, and the effects of these changes on voltage-gated activation of the channel. However, the signaling pathways contributing to these channel alterations, as well as the role of these mechanisms in promoting neuronal tolerance in ischemia, are not well understood. In the first part of my study, I hypothesized that Kv2.1 channel declustering may be sufficient to confer neuronal resistance to excitotoxic injury. I found that two distinct mechanisms of channel declustering, cyclin E1 inhibition of Cdk5-mediated Kv2.1 phosphorylation, or over-expression of the Kv2.2 C-terminus, were independently sufficient to reduce excitotoxic neuronal cell death. Furthermore, preconditioned neurons exhibit calcineurin activity-mediated up-regulation of cyclin E1 protein levels, suggesting that cyclin E1-triggered channel declustering may be an endogenous mechanism that promotes cell survival in response to an excitotoxic insult.

Although calcineurin is required for normal cellular processes, calcineurin dysregulation and over-activation may be neurotoxic. In the second part of my thesis, I focused on this injurious aspect of calcineurin signaling. RCAN1, an endogenous regulator of calcineurin, has been implicated in conflicting cell survival promoting and cell death-inducing signaling pathways. Further, the isoform-specific functions of this protein remain unclear. The findings in Chapter 3 establish distinct neurotoxic and neuroprotective signaling pathways mediated by the two principal brain isoforms of this protein, RCAN1.1 and RCAN1.4, through both calcineurin

regulation-dependent and potentially independent mechanisms. Together, the results presented in this dissertation further our understanding of Kv channel regulation in ischemia, and point to complex and critical roles for calcineurin signaling in ischemic preconditioning and in neuronal cell death.

2.0 CYCLIN E1 REGULATES K_V2.1 CHANNEL PHOSPHORYLATION AND LOCALIZATION IN NEURONAL ISCHEMIA

2.1 ABSTRACT

Kv2.1 is a major delayed rectifying K^+ channel, normally localized to highly phosphorylated somato-dendritic clusters in neurons. Excitatory stimuli induce calcineurin-dependent dephosphorylation and dispersal of Kv2.1 clusters, with a concomitant hyperpolarizing shift in the channel's activation kinetics. We have previously shown that sub-lethal ischemia, which renders neurons transiently resistant to excitotoxic cell death, can also induce Zn^{2+}-dependent changes in Kv2.1 localization and activation kinetics, suggesting that activity-dependent modifications of Kv2.1 may contribute to cellular adaptive responses to injury. Recently, cyclin-dependent kinase 5 (Cdk5) was shown to phosphorylate Kv2.1, with pharmacological Cdk5 inhibition being sufficient to decluster channels. In another study, cyclin E1 was found to restrict neuronal Cdk5 kinase activity. We show here that cyclin E1 regulates Kv2.1 cellular localization via inhibition of Cdk5 activity. Expression of cyclin E1 in human embryonic kidney cells prevents Cdk5-mediated phosphorylation of Kv2.1, and cyclin E1 over-expression in rat cortical neurons triggers dispersal of Kv2.1 channel clusters. Sub-lethal ischemia in neurons induces calcineurin-dependent up-regulation of cyclin E1 protein expression and cyclin E1-dependent Kv2.1 channel declustering. Importantly, over-expression of cyclin E1 in neurons is sufficient to

reduce excitotoxic cell death. These results support a novel role for neuronal cyclin E1 in regulating the phosphorylation status and localization of Kv2.1 channels, a likely component of signaling cascades leading to ischemic preconditioning.

2.2 INTRODUCTION

Voltage-gated Kv2.1 channels mediate a significant component of delayed rectifying K^+ currents in neurons. As such, they critically regulate neuronal excitability, particularly during periods of high-frequency synaptic transmission (Murakoshi and Trimmer 1999, Du et al. 2000, Malin and Nerbonne 2002, Guan et al. 2013). Approximately half of the Kv2.1 channels present on cortical and hippocampal neuronal cell membranes are maintained in highly phosphorylated, somato-dendritic clusters (Fox et al. 2013). Excitatory or injurious stimuli trigger calcineurin-dependent channel dephosphorylation at multiple C-terminal serine residues, which is accompanied by cluster dispersal and a hyperpolarizing shift in channel voltage-gated activation (Misonou et al. 2004, Mulholland et al. 2008, Aras et al. 2009b, Baver and O'Connell 2012, Shepherd et al. 2012, Shah and Aizenman 2013). Calcineurin-mediated dephosphorylation at several C-terminal residues is thought to mediate the hyperpolarizing activation shift (Park et al. 2006), which may mitigate neuronal damage and cell death within the context of excitotoxic injury by reducing neuronal excitability (Aras et al. 2009a, Aras et al. 2009b, Mohapatra et al. 2009, Shepherd et al. 2012).

The phosphorylation status of one Kv2.1 C-terminal serine residue in particular, Ser603, is highly sensitive to changes in neuronal activity. Glutamate stimulation triggers a precipitous

decrease in Ser603 phosphorylation, while acute activity blockade results in Ser603 hyper-phosphorylation (Misonou et al. 2006). Ser603 is phosphorylated by cyclin dependent kinase 5 (Cdk5), a kinase highly expressed in post-mitotic neurons that regulates many critical physiological functions (Cheung et al. 2006, Cerda and Trimmer 2011). Pharmacologic inhibition of Cdk5 kinase activity causes Ser603 dephosphorylation and channel declustering, and prevents recovery of Ser603 phosphorylation and channel re-clustering following washout of glutamate treatment (Cerda and Trimmer 2011).

Here, we identify cyclin E1 as a novel regulator of Kv2.1 localization via inhibition of Cdk5 kinase-mediated phosphorylation of the channel. Our studies suggest that cyclin E1 facilitates Kv2.1 channel dephosphorylation and declustering in neurons subjected to sub-lethal ischemia, and may be critical for cell survival mechanisms following excitotoxic injury.

2.3 MATERIALS AND METHODS

Cell culture, transfection, and ischemic preconditioning: Cortical neuronal cultures were prepared from embryonic day 16-17 rat embryos of either sex (Aras et al. 2009b) and transfected at 21-25 DIV using Lipofectamine 2000 (Invitrogen; Aras et al. 2009a). Cultures were preconditioned with 3 mM potassium cyanide (KCN) in a glucose-free balanced salt solution (in mM: 150 NaCl; 2.8 KCl; 1 $CaCl_2$; and 10 HEPES; pH 7.2) for 90 minutes at 37°C (McLaughlin et al. 2003). For viability assays in transfected neurons, 24h after transfection or preconditioning, neurons were treated with 10 µM glycine (vehicle) ± 75 µM NMDA for 30 minutes, and assayed for viability after 24h as reported previously (Aras et al. 2009a).

Confocal imaging: 24h following transfection with GFP-tagged Kv2.1, cyclin E1, Cdk5-DN, Kv2.2 C-terminus (Kv2.2-CT), or corresponding vector (pCDNA3 for cyclin E1 and Cdk5 DN, pBK for Kv2.2-CT), live imaging of transfected neurons was performed on a Nikon A1+ confocal microscope at 60x. Five–ten optical sections (0.5 μm) were acquired to generate a maximum intensity projection image that was analyzed using NIH image processing software (ImageJ). Following background subtraction, a plot displaying a three-dimensional graph of pixel intensity over the neuronal soma was used to show Kv2.1 localization (Figure 3B). Channel clusters appearing as orange–red peaks in pixel intensity were counted. In several control cells, there were large-density peaks that likely represented multiple channel clusters; these peaks were counted as one cluster to preclude any possibility of over-counting for these cells. Therefore, we may have underestimated the cluster number under control conditions. Nikon Instruments Software (NIS)-Elements Advanced Research was employed to measure cluster surface area, and to confirm channel cluster counts.

Immunoblotting: Protein samples harvested from transfected human embryonic kidney (HEK) 293T cells or neuronal cultures were incubated with mouse monoclonal anti-Kv2.1 antibody (1:1000; NeuroMab), rabbit polyclonal anti-phosphorylated Ser603 Kv2.1 antibody (1:500; gift from Dr. James Trimmer, University of California Davis), rabbit polyclonal anti-cyclin E1 antibody (1:500; Santa Cruz Biotechnology), or mouse monoclonal anti-GAPDH antibody (1:1000; Novus Biologicals) as a loading control. Blots were quantified by infrared fluorimetry (Li-Cor).

Immunofluorescence: 48h following transfection, neurons were washed three times in PBS and fixed with 4% paraformaldehyde for 18 min. Neurons were washed three times with PBS, and permeabilized for 5 min in PBS containing 0.3% Triton X-100. Following three washes in PBS, neurons were incubated in PBS containing 1% bovine serum albumin (BSA) for 5 min. Neurons were incubated overnight at 4 °C with anti-Kv2.1 rabbit polyclonal antibody (1:500; Alomone Labs), and then with AlexaFluor555 donkey anti-rabbit IgG (1:1000; Life Technologies) at room temperature for 1 h. Coverslips containing neurons were then mounted onto glass slides and allowed to air-dry before imaging.

Electrophysiology: Whole-cell recordings from cortical neurons were obtained with 2–3 MΩ electrodes (Aras et al. 2009b). The extracellular solution contained (in mM): 115 NaCl, 2.5 KCl, 2.0 $MgCl_2$, 1.0 $CaCl_2$, 10 HEPES, 10 D-glucose, 0.25 µM tetrodotoxin; pH 7.2. The electrode contained (in mM): 100 K-gluconate, 10 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 2.2 Mg_2·ATP, 0.33 GTP, 11 EGTA, 10 HEPES; pH 7.2. Measurements were obtained under voltage clamp with an Axopatch 200B amplifier and pClamp software (Molecular Devices). 80% compensation for series resistance was provided. Currents were filtered at 2 kHz and digitized at 10 kHz. K^+ currents were evoked with a series of 200 ms voltage steps from a holding potential of -80 mV to +80 mV in 10 mV increments. Before depolarization, a single 30 ms prepulse to +10 mV was used to inactivate A-type K^+ currents. Peak conductance (G) was calculated from peak steady-state current amplitudes (I) using the equation $G = I / (V-E_K)$ (E_K = Nerst K^+ equilibrium potential), and plotted against the potential (V) and fitted to a single Boltzmann function $G = Gmax / (1 + \exp[-(V - V_{1/2}) / k])$: G_{max} = maximum conductance, $V_{1/2}$ = potential at half-maximal conductance, k = slope of activation curve.

2.4 RESULTS

2.4.1 Cyclin E1 inhibits Cdk5-mediated phosphorylation and clustering of Kv2.1 channels

Cyclin E1 was recently shown to influence dendritic spine density and synaptic function by regulating the kinase activity of Cdk5 (Odajima et al. 2011). We first determined whether cyclin E1 expression could inhibit Cdk5-facilitated Kv2.1 Ser603 phosphorylation. HEK293T cells were transfected with Kv2.1 and Cdk5, together with Cdk5 kinase co-activator p35, which yielded an increase in Ser603 phosphorylation that was significantly reduced by cyclin E1 co-expression (Figure 3A). Similarly, as shown previously (Cerda and Trimmer 2011), the Cdk5 inhibitor roscovitine (30 μM, 3h) blocked Cdk5-mediated Kv2.1 Ser603 phosphorylation.

Previous studies have demonstrated that injury-induced Kv2.1 dephosphorylation is accompanied by dispersal of channel clusters (Misonou et al. 2004, Misonou et al. 2006, Mulholland et al. 2008, Aras et al. 2009b, Shepherd et al. 2012, Shepherd et al. 2013). We hypothesized that cyclin E1 over-expression, by blocking Cdk5-mediated Kv2.1 Ser603 phosphorylation, would be sufficient to induce channel declustering in cortical neurons. We transfected rat cortical neurons with a GFP-tagged Kv2.1 construct, which exhibits somato-dendritic clustering similar to endogenous Kv2.1 channels (O'Connell et al. 2006; Figure 3B), along with cyclin E1 or empty vector. Neurons over-expressing cyclin E1 displayed a significantly reduced number of channel clusters (Figure 3B and C), which we verified using Nikon image analysis software. We also observed reduced cluster surface area in cyclin E1-expressing cells (vector: $4.0 \pm 1.2\ \mu m^2$, $n = 23$; cyclin E1: $1.6 \pm 0.5\ \mu m^2$, $n = 30$; $p < 0.05$, two-tailed unpaired t-test). To confirm that Cdk5 kinase activity inhibition promotes channel

declustering, we determined that over-expression of Cdk5-DN, a kinase-inactive, dominant negative Cdk5 mutant (Nikolic et al. 1996), declustered Kv2.1 channels, and verified that roscovitine treatment triggered dispersal of channel clusters (Figure 3C; Cerda and Trimmer 2011).

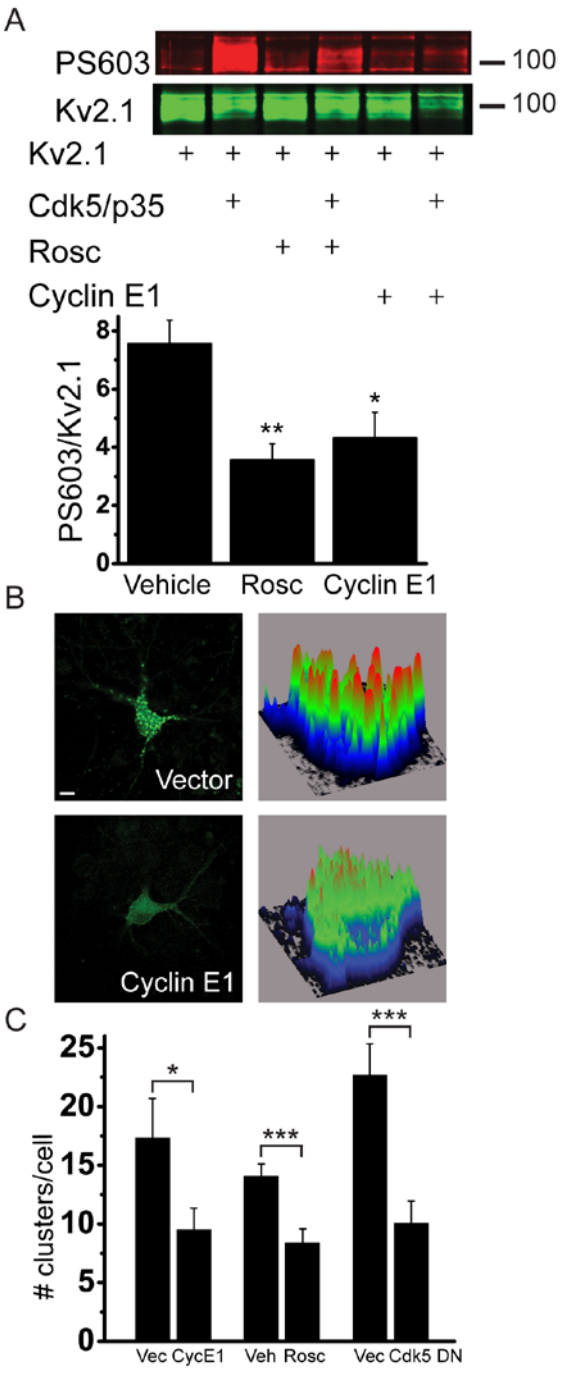

Figure 3 – Cyclin E1 over-expression blocks Cdk5/p35-mediated Kv2.1 phosphorylation, and induces channel declustering

Figure 3. A, HEK293T cells were co-transfected with Kv2.1, Cdk5/p35 or vector, and cyclin E1 or vector. 21h after transfection, cells were treated with vehicle (DMSO) or roscovitine (rosc) for 3h, and proteins were harvested immediately following exposure. **Top**, Representative immunoblot; duplicate membranes were probed with phosphorylated Ser603 Kv2.1 antibody (PS603) or Kv2.1 antibody. Numbers to the right indicate mobility of molecular mass standard (kDa). **Bottom,** Summary of 6 independent experiments; values represent Cdk5/p35-mediated increase in PS603 normalized to total Kv2.1**,** expressed as ratio of PS603/total Kv2.1 (+Cdk5/p35) to corresponding PS603/total Kv2.1 (no Cdk5/p35), e.g. column 1 in bar graph is PS603/total Kv2.1 (lane 2): PS603/total Kv2.1 (lane 1) (mean ± SEM, * $p < 0.05$, ** $p < 0.01$, ANOVA/Dunnett versus vehicle). **B,** Neuronal cultures were transfected with GFP-Kv2.1 and cyclin E1, dominant negative Cdk5 (Cdk5-DN), or vector, or transfected with GFP-Kv2.1 and treated with DMSO or roscovitine 23h after transfection. Neurons were imaged 24h after transfection. Shown are representative neurons and their associated background-subtracted surface maps, which show relative Kv2.1 staining intensity values plotted along the cell body area. Scale bar, 10 μM. **C,** Clusters that appeared as orange–red peaks in pixel intensity were counted for 24-42 cells per group from 4-7 independent experiments. Data points represent average number of clusters per cell (mean ± SEM; * $p < 0.05$, *** $p < 0.001$, two-tailed unpaired t-test versus vector or vehicle). Vec = vector, CycE1 = Cyclin E1, Veh = Vehicle, Rosc = Roscovitine.

Stimuli that activate Kv2.1 dephosphorylation and declustering induce a concomitant hyperpolarizing shift in the channel's voltage-gated activation. Co-treatment with calcineurin inhibitors in neurons (Misonou et al. 2004, Mohapatra and Trimmer 2006, Aras et al. 2009b, Mohapatra et al. 2009, Shepherd et al. 2012, Shepherd et al. 2013), and mutational analysis of calcineurin dephosphorylation-dependent residues in HEK cells (Park et al. 2006) strongly suggest that channel dephosphorylation is closely associated with the activation shift. Therefore, we explored whether cyclin E1 over-expression or roscovitine exposure would promote a hyperpolarizing channel activation shift. We measured whole-cell K^+ currents in neurons over-expressing cyclin E1 or exposed to roscovitine (30 μM, 1h, followed by removal from roscovitine-containing media as it has been shown to directly block Kv2.1 channels; Buraei et al. 2007). We found, however, that neither condition shifted Kv2.1 channel voltage-gated activation ($V_{1/2}$: 13.7 ± 1.1 mV, vector; 10.5 ± 2.3 mV, cyclin E1; 14.2 ± 1.5 mV, vehicle; 18.5 ± 1.5 mV, roscovitine; n = 9, 10, 5, 7 cells, respectively). We also noted no changes in current density; current densities at +10 mV in pA/pF: 284.5 ± 22.9 (vector); 228.4 ± 37.0 (cyclin E1); 181.3 ± 19.0 (vehicle); 145.2 ± 27.3 (roscovitine); n = 6, 7, 3, 6 cells, respectively. These results suggest that, as previously noted (Aras et al. 2009b, Baver and O'Connell 2012), Kv2.1 dephosphorylation and declustering may not be unequivocally linked to changes in channel activation kinetics in all cases.

2.4.2 Neuronal ischemic preconditioning *in vitro* induces calcineurin-dependent up-regulation of cyclin E1 expression

We have previously shown that chemical ischemic preconditioning, which mitigates subsequent excitotoxic neuronal injury, induces Kv2.1 channel dephosphorylation and declustering in

cortical neurons (Aras et al. 2009a, Aras et al. 2009b). Rapid Ser603 dephosphorylation is observed following ischemic or epileptic injury in vivo, and after glutamate treatment in cultured hippocampal neurons (Misonou et al. 2006, Cerda and Trimmer 2011). We found that ischemic preconditioning in cortical neurons also reduced Ser603 phosphorylation in native Kv2.1 channels by 82% (Figure 4A; phosphorylated Ser603 Kv2.1 normalized to total Kv2.1 expression is 0.18 ± 0.03 relative units (r.u.) in vehicle-exposed neurons, compared to 0.03 ± 0.02 r.u. in KCN-treated neurons; $n = 3$; $p < 0.05$, two-tailed paired t-test). We thus hypothesized that preconditioning-stimulated signaling pathways may lead to up-regulation of cyclin E1 protein expression, which would contribute to Ser603 dephosphorylation and channel declustering by reducing Cdk5 kinase activity. Therefore, we measured cyclin E1 protein expression immediately following, and 24 hours after preconditioning, at which point Kv2.1 channels revert to the phosphorylated and clustered state (Aras et al. 2009b). Ischemic preconditioning triggered an increase in cyclin E1 protein expression immediately following treatment (Figure 4B). Importantly, we also observed a return to control cyclin E1 levels 24 hours after preconditioning. Thus, ischemia induced-changes in cyclin E1 coincide temporally with the modifications in phosphorylation and localization of Kv2.1 channels in neurons.

Kv2.1 channel dephosphorylation and declustering following ischemic or epileptic stimuli are dependent on the Ca^{2+}-activated phosphatase calcineurin, which has been suggested to directly dephosphorylate Kv2.1 channels (Misonou et al. 2004, Park et al. 2006, Shepherd et al. 2012; although, see Mulholland et al. 2008, Aras et al. 2009b). We explored the possibility that calcineurin may also be required for the preconditioning-mediated rise in cyclin E1 expression, as restricting calcineurin activity blocks growth factor-stimulated up-regulation of

cyclin E in fibroblasts (Tomono et al. 1998). Accordingly, we found that co-treatment with the calcineurin inhibitor FK520 (5 μM) blocked the increase in cyclin E1 protein expression in preconditioned neurons (Figure 4B). We confirmed that calcineurin activation occurs upstream of cyclin E1 up-regulation by measuring cyclin E1 expression in neurons exposed to the Ca^{2+} ionophore A23187 (1 μM, 10 minutes) with or without FK520: cyclin E1 expression normalized to loading control increases 1.5 ± 0.18-fold with A23187 treatment, compared to 0.78 ± 0.15-fold in A23187-exposed cells co-treated with FK520 ($n = 3$, $p < 0.01$, two-tailed paired t-test).

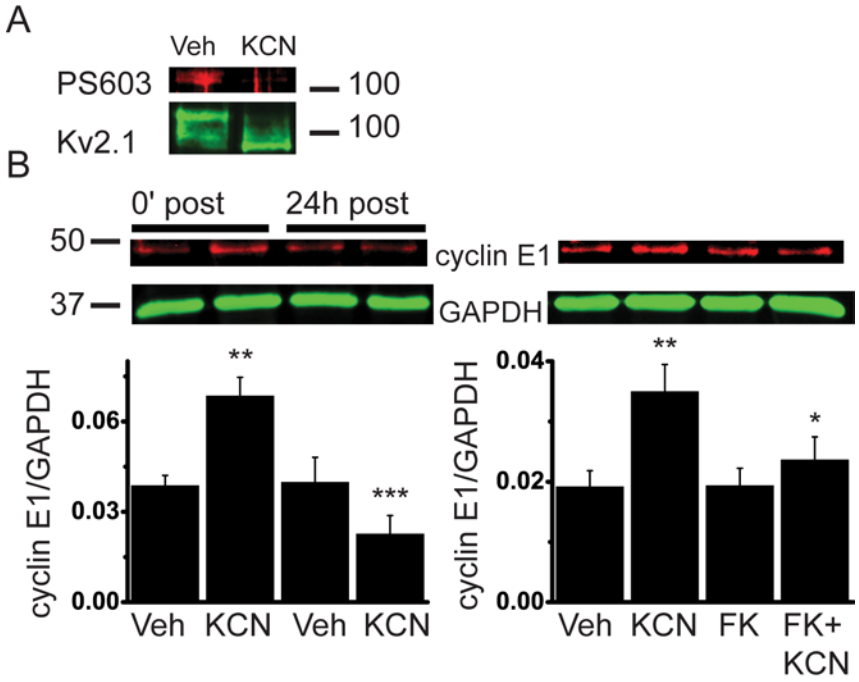

Figure 4 – Chemical ischemia induces calcineurin activity-dependent transient increase in neuronal cyclin E1 expression

Figure 4. Neurons were exposed to 3 mM KCN with vehicle (Veh) or FK520 (FK; 5 µM) for 90 minutes. Cell lysates were harvested immediately (0') or 24h following exposure. **A,** Membranes were probed with anti-PS603 Kv2.1 or anti-Kv2.1 antibody. Representative blot from one of three independent experiments is shown; phosphorylated Ser603 Kv2.1 normalized to total Kv2.1 expression is 0.18 ± 0.03 r.u. (vehicle), compared to 0.03 ± 0.02 r.u. (KCN); n = 3; $p < 0.05$, two-tailed paired t-test. **B,** Membranes were probed with anti-cyclin E1 or anti-GAPDH antibody. **Top**, representative immunoblots are shown. **Bottom**, summary of 4 (**left**) or 5 (**right**) independent experiments; cyclin E1 values are normalized to loading control GAPDH; (mean ± SEM, * $p < 0.05$ versus KCN, ** $p < 0.01$ versus vehicle at 0' post treatment, *** $p < 0.001$ versus KCN at 0' post treatment; ANOVA/Bonferroni).

2.4.3 p35 over-expression blocks ischemic preconditioning-mediated Kv2.1 channel declustering

Cyclin E1 restricts Cdk5 kinase activity by out-competing binding of Cdk5 to p35, and forming a catalytically inactive complex with Cdk5 (Odajima et al. 2011). If ischemic preconditioning-triggered Kv2.1 declustering is dependent on cyclin E1-mediated inhibition of Cdk5 kinase activity, then over-expressing p35 should restore channel clustering in preconditioned neurons. Indeed, we found that p35-over-expressing preconditioned neurons retain Kv2.1 channel clusters (Figure 5), strongly suggesting that increased cyclin E1 expression and consequent inhibition of Cdk5 kinase activity contribute significantly to preconditioning-induced cluster dispersal.

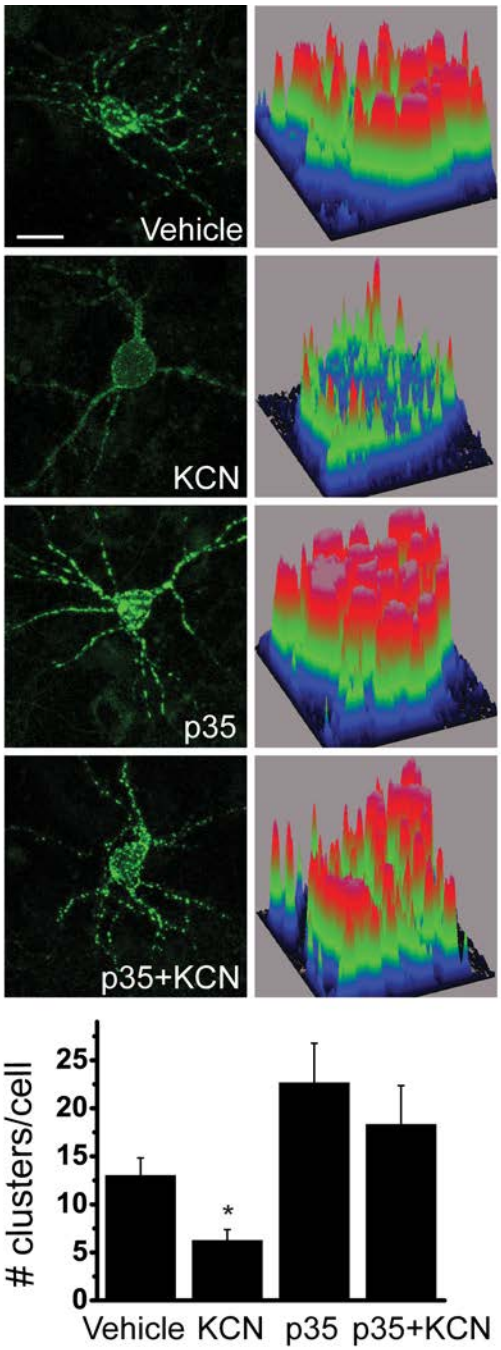

Figure 5 – Over-expression of Cdk5 co-activator p35 blocks KCN-induced Kv2.1 channel declustering in cortical neurons

Figure 5. **Top,** Neurons were transfected with GFP-Kv2.1 and p35 or vector, preconditioned 24h after transfection, and imaged immediately following preconditioning. Scale bar, 10 μM. **Bottom**, Number of clusters per cell was counted and averaged for 15-39 cells per group from six independent experiments (mean ± SEM; * $p < 0.05$, ANOVA/Bonferroni versus vehicle).

2.4.4 Cyclin E1 over-expression reduces excitotoxic cell death

We next investigated whether cyclin E1 over-expression, which produces similar changes in Kv2.1 channel phosphorylation status and clustering as ischemic preconditioning (Figure 3 and 4; Aras et al. 2009b), could alone promote neuronal tolerance to excitotoxic cell death. As shown in Figure 6, over-expressing cyclin E1 in cortical neurons mitigates excitotoxicity in NMDA-treated neurons at levels highly comparable to the neuronal tolerance elicited by ischemic preconditioning. Moreover, we confirmed that limiting Cdk5 kinase activity with the Cdk5-DN construct similarly reduces excitotoxic injury.

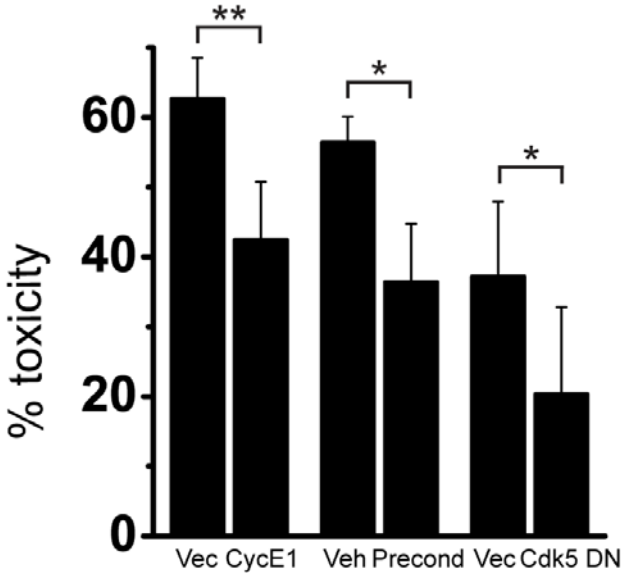

Figure 6 – Cyclin E1 over-expression decreases NMDA receptor-mediated excitotoxicity

Figure 6. Neurons were transfected with cyclin E1 or vector **(1st two columns),** or Cdk5-DN or vector **(last two columns),** and a luciferase reporter gene (PUHC 13-3), or transfected with vector and luciferase reporter gene and treated with KCN 24h following transfection **(2nd two columns)**. 24h after transfection or preconditioning treatment, neurons were exposed to 10 μM glycine ± 75 μM NMDA (30 min), 24h prior to viability assay. Mean (± SEM) values expressed as % toxicity of NMDA-treated relative to vehicle-treated neurons is shown. (* $p < 0.05$, ** $p = 0.01$, versus corresponding vector or vehicle, two-tailed paired t test, n = 5, 6, 4 for vector/cyclin E1**,** vehicle/preconditioning**,** vector/Cdk5-DN, respectively). Note slight differences in NMDA toxicity between vectors for cyclin E1 and for Cdk5-DN are due to differences in inherent toxicity of transfected vectors. Precond = preconditioning

2.4.5 The Kv2.2 C-terminus declusters Kv2.1 channels and reduces excitotoxicity

Cyclin E1 over-expression induced dispersal of Kv2.1 channel clusters (Figure 3), but did not modify Kv2.1 voltage-gated activation. We also found that it reduced excitotoxic cell death in neurons (Figure 6). We suggest that this neuroprotection is achieved at least partially through declustering of Kv2.1 channels. However, we cannot rule out the possibility that cyclin E1 reduces toxicity independently of Kv2.1 cluster dispersal. For example, neuroprotection may occur through inhibition of Cdk5-mediated phosphorylation of other targets, such as NMDA receptors (Rashidian et al. 2009). Therefore, to further validate our hypothesis, we investigated whether declustering of Kv2.1 channels through a different mechanism could also confer excitotoxic neuronal tolerance. O'Connell and colleagues have demonstrated that the C-terminus of Kv2.2 channels (Kv2.2-CT) displaces Kv2.1 from clusters, but does not alter surface expression or electrophysiological properties of the channel (Baver and O'Connell 2012). Importantly, Kv2.2-CT expression does not eliminate the glutamate-induced hyper-polarizing shift in the channel's voltage-gated activation. We confirmed that Kv2.2-CT induced cluster dispersal of both exogenously expressed and native Kv2.1 channels in neurons (Figure 7, A and B). We then tested whether Kv2.2-CT expression would reduce NMDA receptor-mediated neurotoxicity. We found that cell death is, in fact, reduced in Kv2.2-CT-expressing neurons exposed to NMDA (Figure 7C).

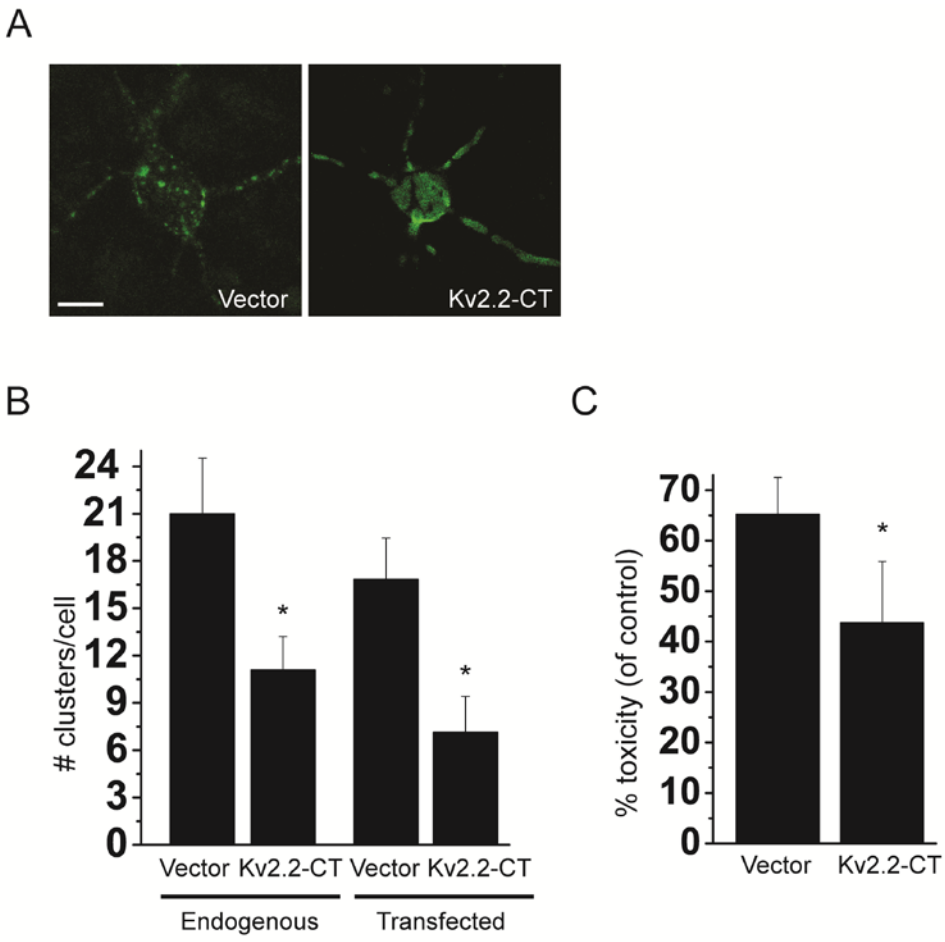

Figure 7 – The C-terminus of Kv2.2 declusters Kv2.1 channels and is neuroprotective

Figure 7. A, Neurons were transfected with GFP-Kv2.1, and Kv2.2-CT or vector, and imaged 48h after transfection. Scale bar, 10 μM. **B**, Number of clusters per cell was counted and averaged for 6-12 cells per group from 1-2 independent experiments. The 1st two columns represent values from neurons transfected with Kv2.2-CT or vector, and tomato (Addgene plasmid 22799), immunostained for Kv2.1, and imaged; 2nd two columns are values from neurons transfected with GFP-Kv2.1 and Kv2.2-CT or vector and imaged live 48h after transfection; (mean ± SEM; * $p < 0.05$, unpaired t-test vs vector). **C,** Neurons were transfected with Kv2.2-CT or vector, and a luciferase reporter gene. 24h after transfection, neurons were exposed to 10 μM glycine ± 50 μM NMDA (1h), 24h prior to viability assay. Mean (± SEM) values expressed as % toxicity of NMDA-treated relative to vehicle-treated neurons is shown (* $p < 0.05$ versus vector, two-tailed paired t test, $n = 4$).

2.5 DISCUSSION

Modulation of Kv2.1 channel phosphorylation, localization, and function, elicited by a range of injurious stimuli, may be a critical component of endogenous neuroprotective signaling pathways that reduce neuronal damage and cell death (Aras et al. 2009b, Mohapatra et al. 2009, Shepherd et al. 2013). We have identified cyclin E1 as a key regulator of Kv2.1 channel phosphorylation and localization in neuronal ischemia. Cyclin E1 blocks Cdk5-mediated Kv2.1 Ser603 phosphorylation, and promotes the dispersal of Kv2.1 channel clusters in cortical neurons.

Interestingly, neither cyclin E1 over-expression nor exposure to roscovitine produced hyperpolarizing changes in Kv2.1 channel voltage-gated activation. In contrast, a previous study showed that Kv2.1 voltage-gated activation was closely linked to the phosphorylation status of Ser603 (Park et al. 2006). However, Park et al. (2006) utilized recombinant expression of a single population of channel mutants, which may not be completely representative of native Kv2.1 channels with varying degrees of phosphorylation in each of the four subunits that assemble to form functional ion channels in neurons. In fact, in neurons, injury-induced Kv2.1 channel activation shifts span a wide range of half-maximal activation voltages, from near 9 mV to 30 mV, depending on the stimulus and neuronal cell type under study (Misonou et al. 2004, Misonou et al. 2008, Mulholland et al. 2008, Aras et al. 2009b, Shepherd et al. 2013). Additionally, neuronal signaling pathways that are activated by excitatory or ischemic stimuli, but not associated with reducing Cdk5 kinase activity alone, may be required to alter the

channel's function, including Zn^{2+}-activated processes (Aras et al. 2009b), calcineurin activity-independent channel dephosphorylation (Mulholland et al. 2008), or post-translational modifications at channel regions other than the C-terminus (Baver and O'Connell 2012).

We demonstrate here that ischemic preconditioning induces calcineurin activity-dependent up-regulation of cyclin E1 protein expression in cortical neurons. This increase in cyclin E1 expression may occur through calcineurin-mediated dephosphorylation of cyclin E1 at ubiquitin ligase-binding sites, preventing its degradation (Hwang and Clurman 2005), but this potential signaling pathway remains to be characterized.

Our results point to a calcineurin activity-driven mechanism that ensures Kv2.1 dephosphorylation in preconditioned neurons, both by dephosphorylating the channel (Misonou et al. 2004, Park et al. 2006, Aras et al. 2009b, Shepherd et al. 2012, Shepherd et al. 2013), and by promoting cyclin E1-mediated inhibition of Cdk5 kinase activity, as we have demonstrated in this study. We postulate that neuroprotection through increased cyclin E1 levels, as reported here, occurs at least in part through Kv2.1 dephosphorylation and declustering in preconditioned neurons, phenomena that have been closely tied to cell survival in a wide range of injury models (Aras et al. 2009a, Aras et al. 2009b, Mohapatra et al. 2009, Shepherd et al. 2012, Shepherd et al. 2013). Naturally, other mechanisms in addition to Kv2.1 channel modifications may aid in this process, such as modulation of NMDA receptors by Cdk5 (Wang et al. 2003a, Rashidian et al. 2009). However, we showed that Kv2.1 declustering by Kv2.2-CT over-expression also reduces NMDA neurotoxicity. Thus, two different stimuli that trigger dispersal of Kv2.1 channel clusters

by different mechanisms both activate excitotoxic neuroprotection. These findings strongly support a role for channel declustering in ischemic preconditioning.

The specific contribution of Kv2.1 declustering to limiting neuronal hyperactivity in ischemia is unknown. Channel cluster localization adjacent to astrocytic processes (Du et al. 1998) may enable them to sense ischemic glial dysfunction due to compromised glutamatergic uptake, leading to excessive neuronal glutamate signaling and Ca^{2+} influx, which would facilitate calcineurin-dependent dephosphorylation and the hyperpolarizing shift in channel activation (Misonou et al. 2008, Mulholland et al. 2008, Mohapatra et al. 2009). Subsequent channel declustering would remove the channels from the site of Ca^{2+} release, initiating recovery. Alternatively, it was recently reported that the majority of clustered Kv2.1 channels are non-conducting (O'Connell et al. 2010, Fox et al. 2013), and may play roles in depolarization-driven vesicle trafficking (Feinshreiber et al., 2009). In fact, Kv2.1 channel clusters may serve as insertion platforms for targeting of new channels to the cell surface (Deutsch et al. 2012). In cortical neurons, an oxidative injury-triggered K^+ current surge, mediated by newly inserted Kv2.1 channels at the plasma membrane, leads to apoptotic cell death (Pal et al. 2003, Pal et al. 2006, McCord and Aizenman 2013). This cell death mechanism may also be critical in promoting NMDA receptor-mediated excitotoxicity (Yao et al. 2009). Cyclin E1-mediated dispersal of channel clusters may thus prevent pro-apoptotic insertion of new Kv2.1 channels at the cell surface, reducing neuronal damage and cell death in excitotoxic injury.

3.0 ISOFORM-SPECIFIC DIVERGENT ROLES FOR RCAN1.1 AND RCAN1.4 IN REGULATING NEURONAL VIABILITY

3.1 ABSTRACT

Alternate promoter usage and splicing of the RCAN1 gene yields two major brain isoforms, RCAN1.1 and RCAN1.4. Previous studies have reported conflicting RCAN1 isoform-dependent roles in modulating cell viability. RCAN1.4 protects against astrocytic ischemic injury, as well as Zn^{2+} neurotoxicity, but may be cytotoxic within certain contexts. Increased RCAN1.1 levels are implicated in promoting Alzheimer's disease and Down syndrome-related neurodegeneration, whereas the same protein may be protective against oxidative stress, ischemia, and Huntington's disease-associated neurotoxicity. The roles of RCAN1.1 and RCAN1.4 in regulating cell survival, particularly in neurons, are therefore not well defined. Here, we identify RCAN1 divergent, isoform-specific neuroprotective and neurotoxic processes in a well-characterized neuronal preparation. We demonstrate that cortical neurons up-regulate RCAN1.4 protein, relative to RCAN1.1, in response to a neuroprotective sub-lethal ischemic stimulus. Moreover, RCAN1.4 over-expression in neurons is protective against NMDA excitotoxicity and microglia-induced neurotoxicity, two forms of injury that are closely associated with ischemic cell death. In contrast, neuronal RCAN1.1 over-expression induces a cell death signaling pathway that is partially mediated by calcineurin as well as Zn^{2+}, two

components that have been previously linked to ischemic neuronal damage. Our studies suggest that RCAN1.1 and RCAN1.4 critically regulate neuronal cell viability within the context of ischemia.

3.2 INTRODUCTION

Calcineurin is a Ca^{2+}/calmodulin-dependent phosphatase that regulates important physiological processes in neurons, including synaptic plasticity and cytoskeletal stability (Winder and Sweatt 2001). However, calcineurin can be over-activated in neuronal injury, and dysregulation of this phosphatase is implicated in neuronal cell death (Asai et al. 1999, Wang et al. 1999a, Shioda et al. 2006). Importantly, blocking calcineurin hyper-activation may be a potential early strategy in slowing or preventing neuronal ischemic damage (Sharkey and Butcher 1994, Uchino et al. 2002, Shioda et al. 2006).

RCAN1 is an endogenous regulator of calcineurin that is widely expressed in the cerebral cortex, hippocampus, and striatum (Mitchell et al. 2007, Porta et al. 2007). Alternate promoter usage and splicing of RCAN1 mRNA produces two major brain isoforms, RCAN1.1 and RCAN1.4, which differ in the first exon, and share the last three exons (Fuentes et al. 1997). Reports regarding RCAN1 isoform-specific effects on cell viability have been contradictory. Although RCAN1.4 may be cytotoxic under certain conditions (Lee et al. 2008), a primarily protective role has been suggested for this isoform (Lee et al. 2007, Cho et al. 2008, Sobrado et al. 2012). Following *in vitro* and *in vivo* ischemia, RCAN1.4 protein and mRNA expression is up-regulated in astrocytes, which may limit inflammatory injury (Sobrado et al. 2012). In

neurons, RCAN1.4 protects against Zn^{2+}-induced toxicity (Lee et al. 2007), an important observation as this metal plays a critical role in neuronal ischemic injury (Koh et al. 1996, Aras et al. 2009a, Medvedeva et al. 2009, Shuttleworth and Weiss 2011). In contrast, RCAN1.1 expression is increased in Alzheimer's disease and Down syndrome brain tissue, and may contribute to neurodegeneration associated with these diseases (Ermak et al. 2001, Sun et al. 2011). RCAN1.1 also has been reported to promote caspase activation, mitochondrial autophagy, and apoptotic cell death under certain conditions (Sun et al. 2011, Ermak et al. 2012, Wu and Song 2013), whereas it may be protective in post-ischemic neuronal injury (Brait et al. 2012) and Huntington's disease-associated neurotoxicity (Ermak et al. 2009).

Whether RCAN1 regulates neuronal cell survival by modulating calcineurin activation is not well understood (Ermak et al. 2009, Brait et al. 2012). Depending on the isoform, expression levels, and phosphorylation status of RCAN1, and on cell type and cytoplasmic environment, RCAN1 regulation of calcineurin activity may be inhibitory (Fuentes et al. 2000, Kingsbury and Cunningham 2000, Rothermel et al. 2000, Vega et al. 2002, Rothermel et al. 2003, Kishi et al. 2007, Liu et al. 2009, Martínez-Martínez et al. 2009, Mehta et al. 2009, Mulero et al. 2009, Shin et al. 2011), or facilitative (Hilioti et al. 2004, Fox and Heitman 2005, Abbasi et al. 2006, Sanna et al. 2006, Liu et al. 2009, Mehta et al. 2009). Additionally, potential calcineurin regulation-independent roles for RCAN1 have been described (Strippoli et al. 2000, Chang and Min 2005, Keating et al. 2008).

In this study, we show that neurons alter the relative levels of the two RCAN1 isoforms in response to a neuroprotective, sub-lethal ischemic stimulus. In light of this discovery, we

explored the hypothesis that RCAN1 mediates isoform-specific, distinct cell death and survival signaling mechanisms in primary cortical neurons, by modulating calcineurin activation or Zn^{2+}-triggered injurious processes, two cellular signaling components that promote neuronal injury following ischemia.

3.3 MATERIALS AND METHODS

Cell culture and transfection: Cortical neuronal cultures were prepared from embryonic day 16-17 rat embryos of either sex, and transfected with RCAN1.1, RCAN1.4, or corresponding control vector, and a luciferase reporter gene, at 21-25 DIV using Lipofectamine 2000 (Invitrogen; Aras et al. 2009a).

Ischemic preconditioning and toxicity assays: Cultures were preconditioned with 90 minutes of oxygen-glucose deprivation (OGD) by placing them in an ischemia chamber (Billups-Rothenberg, Inc.; 95% N_2, 5% CO_2) in a glucose-free balanced salt solution (composition, in mM: 116 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1 NaH_2PO_4, 1.8 $CaCl_2$, 26 $NaHCO_3$, 10 HEPES). For microglia toxicity assays, transfected neurons were exposed to activated microglia (0.25×10^6 cells/well with 10 U/ml Interferon-γ and 1 μg/mL lipopolysaccharide) for 24h as described previously (Knoch et al. 2008). For NMDA excitotoxicity, 24h after transfection or 32h after preconditioning, neurons were treated with glycine (vehicle) ± NMDA. Transfected cells were assayed for viability 24h following NMDA/glycine treatment or activated microglia exposure using luciferase reporter activity as a marker for cell viability as reported previously (Aras et al.

2009a). For non-transfected neurons, lactate dehydrogenase (LDH) release was measured as an index of cell death.

Immunoblotting: Protein samples were obtained from neuronal cultures harvested immediately or 24h following preconditioning. Cells were washed three times with PBS and incubated with lysis buffer (Cell Extraction Buffer, Invitrogen) supplemented with protease inhibitor mixture (Roche Diagnostics) and 1 mM phenylmethylsulphonyl fluoride, for 5 min on ice. Cell lysates were separated on a 12% SDS-PAGE gel and transferred onto nitrocellulose membranes. Membranes were blocked with 1% BSA in PBS with 0.05% Tween 20, and incubated with rabbit polyclonal anti-RCAN1 antibody (Sigma Aldrich; 1:1000) or mouse monoclonal anti-Tuj1 antibody (Covance; 1:1000) as a loading control. RCAN1 signals were quantified by infrared fluorimetry (Li-Cor).

Statistical procedures: All data was quantified from 3-7 independent experiments, expressed as mean ± SEM, and analyzed with pair-wise comparisons.

3.4 RESULTS

3.4.1 Neuronal ischemic preconditioning *in vitro* induces a change in the ratio of RCAN1.4 to RCAN1.1 protein

We have previously demonstrated that chemically-induced ischemic preconditioning (as described in Chapter 2), which promotes neuronal resistance against excitotoxic injury, induces calcineurin-mediated cellular processes that may limit neuronal damage (Aras et al. 2009a, Aras et al. 2009b, Shah et al. 2014). We hypothesized that preconditioned neurons may alter endogenous RCAN1.1 and RCAN1.4 protein levels in association with cell survival-promoting processes. To test this hypothesis, we first characterized the conditions necessary for oxygen-glucose deprivation (OGD)-mediated preconditioning in our system. We chose OGD as a more physiologically relevant ischemic stimulus compared to the chemical ischemic preconditioning used previously in our work (Chapter 2; McLaughlin et al. 2003, Aras et al. 2009). We exposed cortical neurons to increasing durations of OGD, and found that a 90 minute exposure to OGD was the most extensive sub-lethal stimulus tolerated by the cells. We then tested whether this stimulus would be neuroprotective against NMDA receptor-mediated excitotoxicity, and found that 90 minutes OGD preconditioning significantly reduces NMDA neurotoxicity (Figure 8A).

We then measured RCAN1.1 and RCAN1.4 protein levels in neurons immediately after, and twenty-four hours following preconditioning in sister cultures to identify possible changes in levels of the RCAN1 isoforms. As shown in Figure 8B, we found an up-regulation of RCAN1.4 protein levels relative to RCAN1.1 twenty-four hours following 90 minutes of OGD, which

suggested to us that relative changes in RCAN1 isoform levels may be involved in cell signaling events that promote neuronal tolerance.

3.4.2 RCAN1.4 over-expression reduces neuronal ischemic injury

The endogenous change in the RCAN1.4/RCAN1.1 protein ratio in preconditioned neurons led us to test the effects of changes in RCAN1 isoform levels on cell survival in excitotoxicity. First, we investigated whether neuronal transfection of an RCAN1.4-expressing plasmid alone would be sufficient to decrease neurotoxicity in response to exogenous injury. As ischemic preconditioning reduces NMDA-mediated excitotoxic cell death (Figure 8A), we exposed RCAN1.4-over-expressing neurons to NMDA-induced injury and found that, indeed, RCAN1.4 over-expression significantly reduced excitotoxic neuronal cell death (Figure 8C).

We then tested whether RCAN1.4 could confer neuroprotection in other models of neuronal injury. We and others have previously established intracellular Zn^{2+} dysregulation as an important trigger of ischemic neuronal injury (Koh et al. 1996, Aras et al. 2009a, Medvedeva et al. 2009, Shuttleworth and Weiss 2011). Furthermore, a previous study demonstrated that RCAN1.4 over-expression mitigates exogenous Zn^{2+}-induced neuronal cell death (Lee et al. 2007). We therefore explored whether RCAN1.4 could render neurons resistant to a Zn^{2+}-mediated neuronal injury cascade instigated by activated microglia (Knoch et al. 2008). In fact, activated microglia have been suggested to be an important contributor to neurotoxic signaling pathways in the ischemic brain (Wang et al. 2007). We found a robust decrease in neuronal cell death in RCAN1.4-over-expressing neurons exposed to activated microglia, when compared to vector-expressing cells (Figure 8C). Thus, in addition to reducing NMDA excitotoxicity,

RCAN1.4 is also significantly neuroprotective against an injurious stimulus that has been closely associated with Zn^{2+}-mediated toxicity (Knoch et al. 2008).

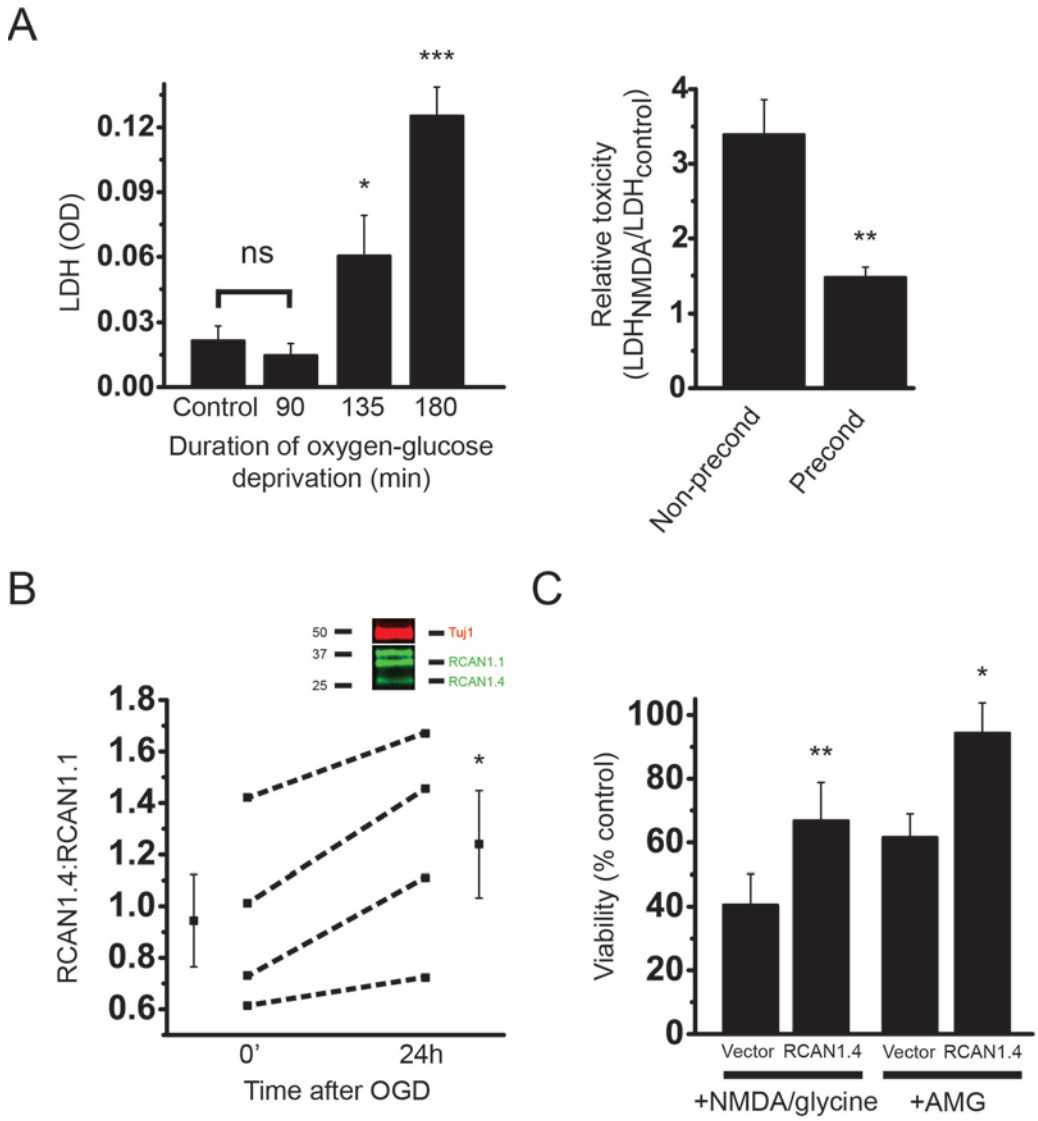

Figure 8 – RCAN1.4 is up-regulated relative to RCAN1.1 in preconditioned neurons, and reduces NMDA excitotoxicity and activated microglial toxicity

Figure 8. A, **Top:** 24h post OGD, cell toxicity is assayed in cortical neurons via quantifying lactate dehydrogenase release (LDH). *** $p < 0.001$ compared to control and 90 minutes, * $p < 0.05$ compared to 180 minutes, ANOVA, n = 5, 3, 3, 5 for control, 90, 135, 180 min, respectively. **Bottom:** Cells were incubated in OGD (preconditioning) or control for 90 minutes, treated 32h later with 10 μM glycine (vehicle) ± 30 μM NMDA for 30 minutes, and assayed for toxicity 24h later. Shown is mean (± SEM) toxicity in non-preconditioned or preconditioned neurons exposed to NMDA, relative to vehicle-treated cells; * $p < 0.05$, two-tailed paired t-test, n = 4. **B**, **Inset**: lysates from preconditioned neurons were probed with anti-RCAN1 (green) or Tuj1 (loading control, red) antibody. At each time point, RCAN1.1 or RCAN1.4 of OGD was normalized to RCAN1.1 or RCAN1.4, respectively, of control. Results of 4 such experiments, each indicated by a line, as well as mean (± SEM), are shown; * $p < 0.05$; two-tailed paired t-test. **D**, Neurons transfected with RCAN1.4 or empty vector, and luciferase reporter gene are exposed to 10 μM glycine ± 50 μM NMDA (1st 2 columns) for 1h, or activated microglia (2nd two columns), 24h following transfection and 24h prior to viability assay. Shown is mean (± SEM) viability, expressed as % of vehicle-treated cells, in neurons treated with NMDA or exposed to activated microglia (AMG); ** $p < 0.01$, n = 6; * $p < 0.05$, n = 7, respectively; two-tailed paired t-test.

3.4.3 Over-expressing RCAN1.1 is neurotoxic via a calcineurin- and Zn^{2+}-dependent cell death mechanism

We next tested the effects of over-expressing RCAN1.1 in primary neurons. We found that over-expressing RCAN1.1 alone caused significant neuronal cell death (~50% toxicity), whereas RCAN1.4, as expected, did not alter neuron viability (Figure 9A). We then designed studies to examine the mechanism of RCAN1.1-mediated cell death.

Dysregulated calcineurin activity has been implicated in neuronal cell death (Asai et al. 1999, Wang et al. 1999a). Studies have demonstrated both inhibition and activation of calcineurin activity by RCAN1 (Fuentes et al. 2000, Kingsbury and Cunningham 2000, Rothermel et al. 2000, Sanna et al. 2006, Liu et al. 2009, Shin et al. 2011). Although several studies demonstrate that either RCAN1 isoform can act as a potent inhibitor of calcineurin phosphatase activity, a study comparing the role of these two isoforms in angiogenesis has suggested that in this context, RCAN1.4 inhibits calcineurin, whereas RCAN1.1 potentiates calcineurin-dependent responses (Qin et al. 2006). Our studies likewise suggest opposing roles for RCAN1.4 and RCAN1.1 (Figure 8, Fig 9A). Therefore, we investigated whether RCAN1.1 induces neurotoxicity via a calcineurin-dependent process. We treated neurons over-expressing RCAN1.1 with deltamethrin, a calcineurin inhibitor (Enan et al. 1992, Misonou et al. 2004, Hayashi et al. 2009, although see Swingle et al. 2007). As shown in Figure 9B, deltamethrin treatment of RCAN1.1 over-expressing neurons significantly improved cell survival, suggesting that RCAN1.1 over-expression may act to stimulate calcineurin activity, and as such, promote cell death. Although deltamethrin is a less commonly used calcineurin inhibitor, we chose to use this drug in this experiment after finding that two more commonly used inhibitors, FK506 and

cyclosporine A (CsA), were neurotoxic following an overnight incubation (data not shown). In contrast, deltamethrin exposure was not neurotoxic, and has been previously used in primary neuronal cultures to block calcineurin activation (Misonou et al. 2004, Hayashi et al. 2009).

Dysregulation of Zn^{2+} homeostasis has also been shown to contribute to neuronal cell death, particularly in ischemia (Koh et al. 1996, Aras et al. 2009a, Medvedeva et al. 2009, Shuttleworth and Weiss 2011). RCAN1.4 over-expression protects against exogenous Zn^{2+}-induced cell death (Lee et al. 2007), and Zn^{2+}-mediated microglial injury (Knoch et al. 2008; Figure 8D), in primary neurons. The contrasting effects of the two isoforms (Figure 8; Figure 9A) led us to test whether RCAN1.1 neurotoxicity acts via a Zn^{2+}-dependent mechanism. Accordingly, RCAN1.1-over-expressing neurons exposed to TPEN, a high-affinity, cell-permeable Zn^{2+} chelator that is neuroprotective against ischemic injury (Aras et al. 2009a), exhibit significantly increased cell survival compared to vehicle-treated, RCAN1.1-expressing neurons (Figure 9B). Thus, RCAN1.1-mediated neuronal cell death occurs via a calcineurin- and Zn^{2+}-activated injurious signaling pathway. Given the previously described roles of calcineurin dysregulation and increased intracellular Zn^{2+} in promoting ischemic injury, RCAN1.1 may be a component of neuronal ischemic cell death signaling cascades.

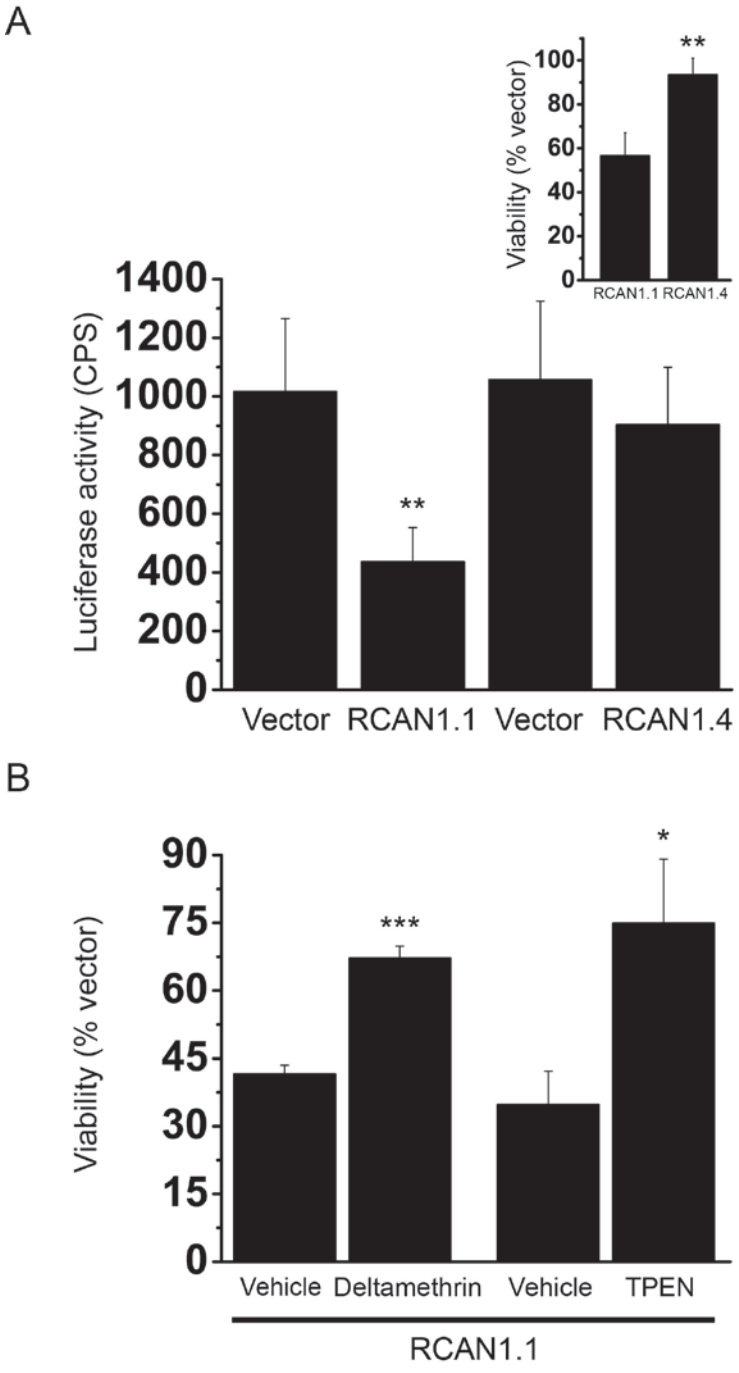

Figure 9 – RCAN1.1 toxicity is partially dependent on calcineurin and zinc signaling

Figure 9. A, Cortical neurons were transfected with RCAN1.1, RCAN1.4, or corresponding empty vector and assayed for viability 24-48h later. Shown are mean ± SD luciferase values (counts per second, CPS) of a representative experiment, performed in quadruplicate; ** $p < 0.01$, two-tailed unpaired t-test. **Inset**, Summary of experiments; mean ± SEM is expressed as % viability of corresponding vector; ** $p < 0.01$, two-tailed unpaired t-test, n = 19 for RCAN1.4, n = 13 for RCAN1.1. **B,** Neurons over-expressing RCAN1.1 or empty vector were treated with vehicle, 5 μM deltamethrin, or 3 μM TPEN 5h post transfection and assayed for viability 19h later. Mean ± SEM is expressed as % viability of corresponding vehicle-treated cells; * $p < 0.05$, *** $p < 0.01$, two-tailed paired t-test, n = 4.

3.5 DISCUSSION

We found that neurons increase the abundance of the endogenous calcineurin-regulatory protein RCAN1.4, relative to RCAN1.1, in response to a sub-lethal ischemic stimulus that induces excitotoxic neuronal tolerance. Importantly, in agreement with this change in RCAN1 isoform levels, RCAN1.4 over-expression was sufficient to significantly limit NMDA-induced, as well as, activated microglia-mediated neurotoxicity.

Although blocking calcineurin activity has been shown to limit ischemic brain damage (Uchino et al. 2002, Shioda et al. 2006), we postulate that RCAN1.4 is not acting solely via its ability to inhibit calcineurin. We have ascertained that pharmacologic inhibition of calcineurin is not sufficient to reduce excitotoxic cell death in our preparation (data not shown). Therefore, whereas calcineurin dysregulation may contribute significantly to ischemic neuronal cell death, our findings, in agreement with a previous report (Butcher et al. 1997), indicate that additional injurious signaling mechanisms may promote the excitotoxic injury component of ischemia. Indeed, RCAN1 may affect several cellular processes independently of its calcineurin-regulatory function, such as post-transcriptional events (Strippoli et al. 2000), vesicle exocytosis (Keating et al. 2008), and mitochondrial metabolism and morphology (Chang and Min 2005). Whether RCAN1.4-mediated changes in these processes are important for neuronal excitotoxic tolerance remains to be investigated.

Our results showing RCAN1.1 over-expression-induced neurotoxicity are consistent with those showing that primary neurons transiently over-expressing RCAN1.1 exhibit enhanced sensitivity to caspase-dependent apoptotic cell death (Sun et al. 2011). However, a recent study demonstrated that mice globally over-expressing RCAN1.1 exhibit increased resistance, compared to wild-type animals, in an *in vivo* model of stroke. Neurons from RCAN1.1 transgenic mice are also less susceptible to OGD-induced apoptotic cell death (Brait et al. 2012). Whether this discrepancy is due to differences in the experimental models, the duration of RCAN1.1 over-expression, or activation of compensatory mechanisms in the transgenic animals will require further study.

The sole difference between the RCAN1.1 and RCAN1.4 proteins lies in the N-termini, as a result of distinctive first exons (Fuentes et al. 1997), and in fact, both RCAN1 isoforms contain the same C-terminal calcineurin-binding and inhibition domains. There are few studies examining possible functional differences of the divergent N-termini. One study demonstrated that a site within the RCAN1.4 N-terminus undergoes calpain-mediated cleavage, which modulates its interaction with calcineurin and with Raf-1 kinase (Cho et al. 2005), although the potential significance of this mechanism is unknown. Alternatively, a specific component of the RCAN1.1 N-terminus may trigger cell death signaling pathways that over-ride RCAN1-mediated protection under conditions of RCAN1.1 over-expression, but that are out-competed by RCAN1.4 over-expression. It is also possible that there are N- and C-terminal interactions within the RCAN1.1 protein that are necessary for the neurodestructive actions of this isoform.

We have identified important functional distinctions between the neuronal RCAN1 isoforms. Our findings suggest that RCAN1 isoform-specific signaling pathways, via calcineurin regulation-independent and dependent mechanisms, as well as through Zn^{2+}-triggered processes, contribute significantly to neuronal cell death and survival within the context of ischemic injury.

4.0 GENERAL DISCUSSION

The goal of this dissertation was to explore calcineurin-mediated signaling in neuronal ischemic preconditioning and cell death. Calcineurin activity-induced Kv2.1 channel regulation in ischemia had been previously described, but the cell signaling mechanisms had not been fully understood. Our work has uncovered a novel role for a calcineurin-dependent increase in cyclin E1 protein levels in neuronal ischemic preconditioning. Cyclin E1 promotes Kv2.1 channel dephosphorylation and declustering by blocking Cdk5 kinase-dependent phosphorylation of Kv2.1 channels. Importantly, cyclin E1 over-expression is sufficient to promote excitotoxic neuronal tolerance, suggesting that channel declustering may be an adaptive mechanism in cells undergoing excitotoxic injury.

While calcineurin-mediated cellular processes may be important in neuroprotection, dysregulated calcineurin activity can be neurotoxic. Conflicting evidence exists on the role of calcineurin dysregulation, as well as the calcineurin regulatory protein RCAN1, in mediating neuronal viability. We found that the two brain RCAN1 isoforms mediate divergent effects on neuronal viability through calcineurin-dependent as well as through potentially calcineurin-independent cellular signaling cascades. In this discussion, I will describe the role of neuronal calcineurin activity in physiological and pathological contexts, and in ischemic preconditioning. Further, I will highlight the evidence supporting calcineurin inhibition as a therapeutic target for

stroke, and discuss the important caveats that exist in moving forward with this therapeutic strategy.

4.1 CALCINEURIN REGULATES PHYSIOLOGICAL FUNCTIONS IN NEURONS

Physiological calcineurin signaling, mediated by dephosphorylation of numerous molecular targets, is crucially important for a wide range of normal neuronal processes. These functions of calcineurin are discussed in the sections below, including a few illustrative examples of molecular targets that regulate each function.

4.1.1 Neuronal structure

Calcineurin dephosphorylates and consequently regulates several proteins that constitute the neuronal cytoskeleton. Calcineurin-mediated dephosphorylation of MAP2 and tau increases the affinity of these proteins for microtubules, thus contributing to stabilization of microtubule length. Additionally, calcineurin dephosphorylates tubulin, increasing its ability to assemble into microtubules (Goto et al. 1985, Mandelkow et al. 1995, Groth et al. 2003).

4.1.2 Neuro-transmission and synaptic plasticity

Generally, calcineurin negatively regulates synaptic transmission. As such, calcineurin activity is thought to oppose long-term potentiation (LTP) and promote long-term depression (LTD) by dephosphorylating and thus modifying the activity and/or localization of a number of proteins

that mediate these mechanisms. However, calcineurin has a wide range of molecular targets at both excitatory and inhibitory synapses. Therefore, the effects of the phosphatase on synaptic plasticity, and consequently, on learning and memory, can be complex and depend on factors such as cell type, post-synaptic receptor, and the nature of the synaptic stimulus (Groth et al. 2003, Baumgärtel and Mansuy 2012).

Calcineurin indirectly regulates synaptic function by dephosphorylating Inhibitor-1, releasing its inhibition of protein phosphatase-1. Activated protein phosphatase-1, in turn, dephosphorylates numerous proteins that influence synaptic plasticity as well as other cellular processes (Baumgärtel and Mansuy 2012). Calcineurin also directly modifies synaptic function by dephosphorylating several pre-and post-synaptic proteins. Post-synaptic AMPA receptors (AMPA-R), for example, are important targets of calcineurin-mediated dephosphorylation. AMPA-R internalization is a key regulatory mechanism that promotes an NMDA receptor-dependent form of LTD. Phosphorylation of serine residue 845 (Ser845) of the AMPA-R GluR1 subunit increases channel open time and promotes receptor trafficking to the membrane, whereas dephosphorylation activates AMPA-R internalization and weakens synaptic transmission. Through restricting synaptic AMPA-R localization both by direct dephosphorylation of GluR1 Ser845, and by aiding in NMDA receptor-activated redistribution of protein kinase A, a GluR1-phosphorylating kinase, calcineurin signaling is an important mechanism for LTD induction and expression (Morishita et al. 2005, Baumgärtel and Mansuy 2012, Sanderson et al. 2012).

A-type GABA receptors are another group of post-synaptic receptors that are dephosphorylated by calcineurin in an activity-dependent LTD signaling pathway in CA1

interneurons (Wang et al. 2003b). Other targets of calcineurin dephosphorylation that regulate synaptic function include Kv4.2 channels, the metabotropic glutamate receptor mGluR5, the NR2A subunit of NMDA receptors, and the tyrosine phosphatase STEP (Baumgärtel and Mansuy 2012).

4.1.3 Neuronal system development

Studies across different species strongly suggest a critical role for calcineurin in maintaining normal development of the nervous system. Calcineurin-mediated NFAT dephosphorylation significantly influences axonal outgrowth, dendritic morphogenesis, and synapse maturation in developing neurons (Graef et al. 2003, Schwartz et al. 2009). Recent reports have also uncovered an interesting role for calcineurin/NFAT-mediated transcriptional repression of specific developmental genes in order to ensure proper neuronal outgrowth within the correct developmental time window (Nguyen et al. 2009, Ding et al. 2013). Calcineurin/NFAT-driven gene transcription additionally participates in an NMDA receptor/BDNF-signaling pathway that promotes neuronal cell survival in the developing rat cortex (Vashishta et al. 2009). Finally, in addition to NFAT, several other molecular targets are dephosphorylated by calcineurin in the developing nervous system. Calcineurin-dephosphorylated SMAD1/5 proteins, for example, are crucial in promoting neural induction in human and murine embryonic stem cells (Cho et al. 2014). MEF2, dephosphorylated and activated by calcineurin, regulates activity-dependent synaptic differentiation in rat hippocampal and cerebellar granule neurons (Flavell et al. 2006, Shalizi et al. 2006).

4.2 CALCINEURIN SIGNALING IN ISCHEMIC NEUROPROTECTION

In addition to regulating normal cellular processes, calcineurin also mediates adaptive, cell survival-promoting signaling pathways in response to injury. It has been demonstrated that excitatory stimuli or a sub-lethal ischemic insult induces calcineurin-mediated Kv2.1 channel dephosphorylation, dispersal of somatodendritic channel clusters, and a concomitant hyper-polarizing shift in the channel's voltage-gated activation. These changes are thought to promote cell survival within the context of ischemic or excitotoxic injury (Misonou et al. 2006, Aras et al. 2009b, Mohapatra et al. 2009, Shepherd et al. 2013). In this section, I will discuss calcineurin-dependent and independent signaling pathways that regulate Kv2.1 localization and function, as well as the contribution of these mechanisms towards establishing ischemic neuronal tolerance.

All three Kv2.1 channel modifications – dephosphorylation, declustering, and the hyper-polarizing shift – occur in response to a wide range of stimuli in neurons: in cultured rat hippocampal neurons exposed to glutamate (Misonou et al. 2004), NMDA (Mulholland et al. 2008), carbachol (Mohapatra and Trimmer 2006), chemical ischemia (Misonou et al. 2005), SDF-1α (Shepherd et al. 2012), or HIV glycoprotein-120 (Shepherd et al. 2013); in cultured rat cortical neurons treated with an ischemic preconditioning stimulus (Aras et al. 2009a, Aras et al. 2009b); and in ischemic rat cortical slices (Misonou et al. 2008). All three changes can also be induced in HEK cells in response to cholinergic stimulation or Ca^{2+} influx (Mohapatra and Trimmer 2006). *In vivo*, Kv2.1 dephosphorylation and dispersal of channel clusters in the rat brain are triggered following CO_2 inhalation or seizure-inducing kainate injections (Misonou et al. 2004, Misonou et al. 2005).

Park et al showed that the phosphorylation status of at least seven, calcineurin dephosphorylation-sensitive, C-terminal residues critically regulates the voltage-gated activation of recombinant Kv2.1 channels expressed in HEK cells (Park et al. 2006). However, the findings presented in our study indicate that Kv2.1 channel dephosphorylation and declustering can occur without a concomitant hyperpolarizing shift in voltage-gated activation under certain conditions. Over-expression of cyclin E1 or exposure to roscovitine, both of which trigger channel declustering by blocking Cdk5-mediated Kv2.1 Ser603 phosphorylation (Figure 3; Cerda and Trimmer 2011, Odajima et al. 2011), did not modify the channel's voltage-gated activation. In light of these findings, we suspect that the Kv2.1 hyper-polarizing activation shift may require multiple converging signaling pathways that are activated following excitatory or ischemic stimuli (Misonou et al. 2004, Aras et al. 2009b), but not by Cdk5 inhibition alone. Additionally, these pathways may not, in all cases, involve calcineurin-dependent dephosphorylation of the channel's C-terminal residues (Park et al. 2006). In fact, recent studies have identified several such potential signaling pathways:

Sub-lethal intracellular Zn^{2+} release: Previous investigations in our laboratory have demonstrated a requirement for sub-lethal intracellular Zn^{2+} release for preconditioning-induced Kv2.1 channel declustering and hyper-polarized voltage-gated activation, but not for Kv2.1 dephosphorylation (Aras et al. 2009b). In other words, in preconditioned neurons exposed to TPEN, dephosphorylation of the channel still occurs, whereas channel declustering and the hyperpolarizing shift in voltage-gated channel activation are blocked. Possibly, in cyclin E1-over-expressing neurons, Cdk5 inhibition may be sufficient to mediate Kv2.1 dephosphorylation and declustering, but without intracellular Zn^{2+} release, the channel's voltage-gated activation

remains unchanged. Interestingly, *in vitro* assays demonstrated that addition of Zn^{2+} or TPEN did not affect calcineurin phosphatase activity, while calcineurin activity inhibition did not modify levels of preconditioning-induced Zn^{2+} release in neurons (Aras et al. 2009b). We are currently conducting studies to better understand the role of Zn^{2+} in modulating Kv2.1 localization and function, and to define the possible convergence of Zn^{2+}- and Ca^{2+}/calcineurin-activated signaling pathways in neuronal ischemia.

Kv2.1 channel SUMOylation: SUMOylation is a post-translational modification that involves the covalent linkage of the small ubiquitin-like modifier (SUMO) peptide to a lysine residue that lies within a consensus target motif. This modification may affect the stability, activity or intracellular localization of the SUMOylated protein (Silveirinha et al. 2013). A recent study demonstrated that native Kv2.1 channels interact with SUMO proteins in neurons, and are reversibly modulated by SUMOylation and deSUMOylation (Plant et al. 2011). Exogenous expression of SUMO proteins depolarizes neuronal Kv2.1 voltage-gated activation and decreases peak currents. De-SUMOylation has the opposite effect, causing a hyperpolarizing shift in Kv2.1 activation and increasing peak currents. Importantly, expression of a de-SUMOylating enzyme decreases neuronal excitability, suggesting that SUMOylation and de-SUMOylation of Kv2.1 channels could play a role in mediating neuronal excitability (Plant et al. 2011). Although the role of SUMOylation in ischemia is not fully understood, increased SUMOylation is observed in several experimental models of ischemia, and SUMOylation is generally thought to promote neuronal tolerance (Silveirinha et al. 2013). However, specific SUMOylation and de-SUMOylation of Kv2.1 channels in ischemia remain largely unexplored. As such, de-SUMOylation represents a possible mechanism of hyper-polarizing the channel's voltage-gated

activation that is entirely independent of calcineurin-mediated channel dephosphorylation. This mechanism may be activated in neurons exposed to excitatory stimuli or ischemic preconditioning.

Calcineurin-independent Kv2.1 channel dephosphorylation: Glutamate exposure and consequent NMDA receptor-mediated dephosphorylation and declustering of Kv2.1 channels has been demonstrated to require calcineurin phosphatase activity in cultured hippocampal neurons (Misonou et al. 2004). In contrast, treating organotypic rat hippocampal slices with NMDA induces calcineurin-independent dephosphorylation of Kv2.1 channels, along with dispersal of channel clusters and a hyper-polarizing shift in the channel's voltage-gated activation (Mulholland et al. 2008). These channel modifications are thought to proceed via activation of extrasynaptic, rather than synaptic, NMDA receptors (Misonou et al. 2008, Mulholland et al. 2008. Whether the Kv2.1 hyperpolarizing shift is dependent on calcineurin activation was not examined in this study. Nonetheless, this study illustrates that possibly different populations of Kv2.1 channels may be modified via distinct dephosphorylation-dependent signaling pathways, and that the hyperpolarizing shift in the channel's voltage-gated activation may require calcineurin-independent dephosphorylation in certain circumstances. Neuronal cyclin E1 over-expression blocks the phosphorylation of Ser603, a calcineurin dephosphorylation-sensitive residue that critically regulates channel function (Fig 3; Misonou et al. 2006, Park et al. 2006, Cerda and Trimmer 2011). However, it is possible that other, unknown channel residues subject to calcineurin-independent dephosphorylation remain phosphorylated, and thus the channel's activation kinetics are unchanged.

Several additional channel phosphorylation and dephosphorylation mechanisms exist that instigate changes in Kv2.1-mediated K^+ currents without altering voltage-gated activation. As described earlier, phosphorylation at Ser800 and Tyr124 promotes an oxidant-induced, Kv2.1-mediated K^+ current surge and consequent apoptotic cell death in neurons (Pal et al. 2003, Redman et al. 2009). Another study has shown that serum deprivation triggers an NMDA receptor-mediated enhancement of Kv2.1 channel K^+ currents that promotes apoptotic cell death. Interestingly, increased surface expression of Kv2.1 channels in this pro-apoptotic environment requires calcineurin-independent, PP1/PP2A-dependent channel dephosphorylation (Yao et al. 2009). The relationship between pro-apoptotic functions of Kv2.1 and neuroprotective channel modulations has not yet been well characterized. In this regard, Shepherd et al have demonstrated that both mechanisms may occur in response to different exposure durations of the same type of injury (Shepherd et al. 2012).

Somewhat to our surprise, we found that despite having no effect on Kv2.1 channel voltage-gated activation, cyclin E1 over-expression reduced neuronal excitotoxicity to a similar extent as ischemic preconditioning (Figure 6). Furthermore, over-expression of the Kv2.2 C-terminus, which displaces Kv2.1 from clusters (Figure 7) without changing the channel's voltage-gated activation (Baver and O'Connell 2012), also reduces NMDA-mediated neurotoxicity. These findings indicate channel declustering may be sufficient to promote excitotoxic neuroprotection in some instances. In light of these findings, we suggest that Kv2.1 declustering and the activation shift may represent two related, but not unequivocally linked, mechanisms of channel regulation that reduce neuronal cell damage after ischemic injury. For example, Kv2.1 declustering may promote cell survival in excitotoxicity independently of the

channel's ion conducting properties, by precluding pro-apoptotic insertion of new Kv2.1 channels at the membrane (Deutsch et al. 2012). In fact, a K^+ current surge mediated by Kv2.1 channels may play a critical role in serum deprivation-induced, NMDA receptor-mediated, apoptotic cell death in neurons (Yao et al. 2009). The hyper-polarizing activation shift may occur in an entirely different population of non-clustered, conducting channels (O'Connell et al. 2010), thus reducing membrane excitability and limiting excitotoxic neuronal cell damage. A stimulus such as ischemic preconditioning would trigger both changes, presumably by increasing direct calcineurin-mediated channel dephosphorylation and the consequent hyper-polarizing activation shift (Aras et al. 2009b), and by promoting cyclin E1-dependent Kv2.1 channel declustering (Figure 3), thus significantly reducing neurotoxicity. In fact, although cyclin E1 over-expression reduced NMDA-mediated neurotoxicity to the same extent as ischemic preconditioning in our experiments (Figure 6), we found that cyclin E1 was not effective against higher concentrations of NMDA. In contrast, chemical ischemic preconditioning promotes cell survival against higher concentrations and duration of NMDA exposure (McLaughlin et al. 2003, Aras et al. 2009a), suggesting that at increased levels of excitotoxic injury, channel declustering may not be sufficient for neuroprotection, and additional mechanisms that reduce hyper-excitability, such as shifting Kv2.1 channel voltage-gated activation, may be required.

A number of studies support the concept that dephosphorylation, declustering, and the change in biophysical properties of Kv2.1 channels may represent distinct modes of regulation. Although injury-induced channel declustering is blocked by calcineurin inhibitors (Misonou et al. 2004, Misonou et al. 2005, Aras et al. 2009b, Shepherd et al. 2013), dispersal of Kv2.1 clusters can be triggered independently by a range of stimuli. Importantly, these declustering

mechanisms may not require channel dephosphorylation by calcineurin. Actin depolymerization, for example, declusters Kv2.1 channels in HEK cells without modifying the channel's biophysical properties (Tamkun et al. 2007, O'Connell et al. 2010). Cholesterol depletion causes the formation of larger channel clusters compared to the Kv2.1 clusters in un-treated cells, but the effect of this manipulation on channel function has not been tested (O'Connell and Tamkun 2005). Indeed, it would be interesting to uncover a stimulus that activates Kv2.1 dephosphorylation and modulates the channel's electrophysiological properties without disrupting the channel clusters.

As alluded to earlier, studies are also needed to identify different populations of Kv2.1 channels, and to determine exactly which subset is modified in response to different stimuli. In this regard, Sarmiere and colleagues have recently investigated a population of Kv2.1 channels at the axon initial segment (AIS) in cortical and hippocampal mammalian neurons (Sarmiere et al. 2008). Kv2.1 channels may traffic to the AIS through a mechanism that is distinct from delivery of channels to the cell body and proximal dendrites. Ser586Ala, a channel mutant that does not cluster in neurons (Lim et al. 2000), traffics differently than wild-type channels to dendrites, displaying modified vesicle velocity and abnormal, distal dendritic localization. Trafficking of the mutant to the AIS, by comparison, is unhindered and identical to delivery of the wild-type channel to the AIS (Jensen et al. 2014). Remarkably, channel clusters in the AIS remain intact following *in vivo* injury that triggers both dephosphorylation of channels in the AIS, as well as dephosphorylation and declustering of Kv2.1 channels in the soma and dendrites (King et al. 2014). This result again strongly indicates that channel dephosphorylation does not unequivocally lead to channel declustering. Given the critical role of Kv2.1 channels in

restraining neuronal excitability, particularly by modulating activity-dependent action potential frequency (Du et al. 2000, Mohapatra et al. 2009, Guan et al. 2013), localization-specific modifications of the channel are likely to be highly relevant in modulating excitotoxic cell signaling cascades.

4.3 CALCINEURIN DYSREGULATION AND NEUROTOXICITY

The results presented in this thesis demonstrate that ischemic preconditioning triggers an increase in the protein levels of RCAN1.4, relative to RCAN1.1, in cortical cultures (Figure 8). Further, neuronal over-expression of RCAN1.4 reduces NMDA excitotoxicity and activated microglia-induced neuronal cell death, two types of neuronal injury that are closely associated with ischemia. Although calcineurin signaling is required for important physiological processes in neurons, dysregulated calcineurin phosphatase activity may be detrimental to cell survival. As an endogenous regulator of calcineurin, RCAN1.4 may promote neuronal tolerance in injury models by inhibition of calcineurin over-activation. This section will focus on calcineurin dysregulation in neuronal cell death, and highlight the potential roles of RCAN1.1 and RCAN1.4 in regulating calcineurin activity.

4.3.1 Calcineurin dysregulation is a potential therapeutic target in stroke

Dysregulated calcineurin activation promotes neurotoxic signaling pathways, particularly processes involved in apoptotic cell death. Exogenous expression of a constitutively active calcineurin mutant enhances cellular caspase-3 activity and LDH release, and renders neurons

and non-neuronal cells more susceptible to apoptotic cell death triggered by normally sub-lethal injurious stimuli (Shibasaki and McKeon 1995, Asai et al. 1999). Further, forced high expression of constitutively active calcineurin is sufficient to activate apoptosis in rat cortical neurons (Asai et al. 1999).

The best-characterized mechanism of calcineurin-dependent apoptotic cell death involves injury-induced, calcineurin-mediated dephosphorylation of Bcl-2-associated death promoter (BAD). Dephosphorylated BAD dissociates from 14-3-3 proteins in the cytosol, and translocates to the mitochondria, where it dimerizes with Bcl-xl. This dimerization promotes apoptotic cell death by sequestering Bcl-xl and preventing its anti-apoptotic functions (Wang et al. 1999a). Various components of this apoptotic signaling cascade have been validated in several different neuronal injury models: *(i)* in rat hippocampal neurons exposed to glutamate (Wang et al. 1999a), ammonia (Yang et al. 2004), or homocysteine (Wang et al. 2012); *(ii)* in mouse motor neurons following spinal cord injury (Springer et al. 2000); *(iii)* in retinal ganglionic neurons of mice subjected to elevated intraocular pressure (Huang et al. 2005); and *(iv)* in the mouse brain following prion protein injection (Mukherjee et al. 2010). In addition to BAD dephosphorylation, calcineurin over-activation may promote the expression of pro-apoptotic genes in neurons by directly dephosphorylating or indirectly altering the phosphorylated levels of the transcription factors NFAT (Terada et al. 2003, Jayanthi et al. 2005, Lee et al. 2005), CREB (Sée and Loeffler 2001, Lee et al. 2005, Mukherjee et al. 2010), or FKHR (Shioda et al. 2007). Other potential targets of calcineurin-mediated dephosphorylation in neuronal cell death include death-associated protein kinase (Shamloo et al. 2005), neuronal nitric oxide synthase (Rameau et al. 2003), and Akt kinase (Uchino et al. 2002, Park et al. 2008; although see Shioda et al. 2007).

What is the specific contribution of calcineurin over-activation to excitotoxic cell death in neurons? An important study by Wu and colleagues demonstrated that in primary rat cortical and hippocampal neurons, glutamate induces calpain-dependent cleavage of calcineurin, producing a constitutively active calcineurin protein product with a concomitant increase in Ca^{2+}-independent calcineurin phosphatase activity. Neuronal caspase-3 activity and apoptotic cell death, activated by glutamate exposure or by transfection of the calcineurin mutant, are diminished by treatment with FK506, a pharmacological inhibitor of calcineurin (Wu et al. 2004). Other studies in cortical and hippocampal neurons, and in CGNs, have similarly demonstrated that inhibiting calcineurin phosphatase activity with CsA, FK506, deltamethrin, or a calcineurin auto-inhibitory peptide (Terada et al. 2003), blocks the pro-apoptotic BAD dephosphorylation signaling cascade described above (Wang et al. 1999a), and reduces NMDA receptor-mediated cell death (Ankarcrona et al. 1996, Wang et al. 1999a, Marshall et al. 2003, Rameau et al. 2003, Terada et al. 2003).

In vivo studies have also demonstrated the role of calcineurin dysregulation in promoting ischemic neuronal damage. Middle cerebral artery occlusion (MCAO) in mice and increased intraocular pressure in the rat retina, both causing neuronal ischemic injury, lead to the generation of constitutively active calcineurin (Huang et al. 2005, Shioda et al. 2006, Shioda et al. 2007, Park et al. 2008, Brait et al. 2012). FK506 administration blocks cell death-inducing signaling processes, such as NFAT nuclear translocation-dependent FasL expression, BAD dephosphorylation, and caspase-3 activation, as well as reducing ischemic neuronal cell death and brain infarct volume in these ischemic injury models (Sharkey and Butcher 1994, Uchino et al. 2002, Huang et al. 2005, Shioda et al. 2006, Shioda et al. 2007, Park et al. 2008). Indeed, both

in vivo and *in vitro* studies point to calcineurin activity inhibition as a potential therapeutic target for reducing excitotoxic cell death and ischemic neuronal damage.

We found that RCAN1.4 over-expression in neurons reduced excitotoxic cell death, and considering the aforementioned studies, the protection may ensue through inhibition of calcineurin over-activation. RCAN1.4 increases in neurons and astrocytes following ischemic injury, and has been previously implicated in down-regulating injurious processes that occur in ischemia (Cho et al. 2008, Sobrado et al. 2012). The role of RCAN1.1 in ischemic toxicity, however, is not well-explored. In contrast to RCAN1.4, RCAN1.1 over-expression in cortical neurons provoked neurotoxicity that was partially blocked by pharmacological inhibition of calcineurin activation (Figure 9). Our RCAN1.1 findings are inconsistent with a recent study in which RCAN1.1 exhibits ischemic neuroprotection. RCAN1.1-over-expressing mice demonstrate resilience to post-ischemic neuronal injury, and cultured neurons from these mice are less vulnerable to oxygen-glucose deprivation-activated cell death compared to wild-type mice (Brait et al. 2012). No increase in RCAN1.4 protein expression is observed following ischemia, in contrast to previously published studies (Cho et al. 2008, Sobrado et al. 2012). Interestingly, calcineurin activity is increased following ischemic injury in this study, but does not differ between wild-type and RCAN1-transgenic mice, suggesting that neuroprotection in RCAN1.1-over-expressing mice occurs through a mechanism that is independent of calcineurin activity regulation. However, the authors measured calcineurin activity solely by quantifying levels of the constitutively active calcineurin isoform, and therefore cannot be absolutely certain that calcineurin phosphatase activity is unchanged in RCAN1.1-over-expressing mice relative to wild-type animals. Potential compensatory mechanisms and duration of RCAN1.1 over-

expression could also explain the calcineurin-independent, neuroprotective effect of RCAN1.1, in contrast to calcineurin activation-dependent neurotoxicity induced by transient RCAN1.1 over-expression, as demonstrated in our study.

How might RCAN1.1 and RCAN1.4 mediate distinct mechanisms of neurotoxicity and cell survival? Possibly, RCAN1.1 over-activates calcineurin (Figure 9), whereas RCAN1.4 blocks calcineurin over-activation in excitotoxicity. Qin et al have similarly shown opposing actions of RCAN1.1 and RCAN1.4 on calcineurin activity in angiogenesis (Qin et al. 2006). RCAN1.4 may concurrently activate additional cell survival-supporting processes in response to injury. For example, RCAN1.4 interacts with Raf-1 kinase (Cho et al. 2005), a protein associated with promoting cell survival that opposes the calcineurin-mediated, pro-apoptotic BAD dephosphorylation signaling cascade described earlier (Wang et al. 1999a). Although the potential significance of this interaction is not known, RCAN1.1 does not contain an N-terminal binding site for Raf-1 kinase. Thus, this putative mechanism could further explain the divergent effects of the two RCAN1 isoforms on neuronal viability: without counteracting cell survival-promoting events, over-activation of calcineurin and dysregulation of Zn^{2+} homeostasis together may lead to RCAN1.1-mediated cell death, in contrast to the effects of the RCAN1.4 isoform.

4.3.2 Critical caveats to the therapeutic potential of calcineurin inhibition in stroke

Although the studies discussed above indicate a critical role for calcineurin over-activation in promoting ischemic cell death, the therapeutic potential for calcineurin inhibition to reduce excitotoxic neuronal damage in stroke has several limitations. The calcineurin inhibitors FK506 and CsA have been shown to influence cellular processes distinct from their regulation of

calcineurin activity, which may contribute significantly to their neuroprotective efficacy. CsA, for example, forms a complex with the mitochondrial matrix protein cyclophilin D. This complex, in addition to inhibiting calcineurin activity, blocks the mitochondrial permeability transition pore, a putative key contributor to Ca^{2+}-dependent ischemic neuronal injury (Klettner and Herdegen 2003). Several studies indicate that this mechanism contributes significantly to CsA-mediated neuroprotection in ischemia and other types of neuronal injury, particularly in cases where FK506 is ineffective (Friberg et al. 1998, Pavlov et al. 2010). Similarly, Uchino et al (2002) have demonstrated that while both inhibitors block calcineurin phosphatase activity with equal efficacy, CsA is much more potent than FK506 as a neuroprotective agent when administered before inducing transient forebrain ischemia in rats. The increased potency of CsA is likely due to its preservation of mitochondrial function (Uchino et al. 2002). Finally, a derivative of CsA, which inhibits the mitochondrial permeability transition but does not inhibit calcineurin, is neuroprotective in transient focal cerebral ischemia (Korde et al. 2007).

The mechanism of FK506 neuroprotection in excitotoxicity may also involve calcineurin inhibition-independent processes, although these mechanisms are less well characterized. FK506 is thought to mediate nerve regeneration following injury, for example, via a mechanism that is completely distinct from inhibition of calcineurin activity (Toll et al. 2011). Studies in cultured neurons suggest that FK506 reduces NMDA receptor-mediated toxicity by blocking calcineurin-dependent neuronal nitric oxide synthase activation and nitric oxide production (Dawson et al. 1993, Rameau et al. 2003). *In vivo*, FK506 affords robust neuroprotection in focal cerebral ischemia, but does not affect nitric oxide production, arguing against this mechanism of excitotoxic protection (Toung et al. 1999). Further, Butcher and colleagues have demonstrated

that while FK506 protects against ischemic damage in the rat forebrain, it is not effective against excitotoxic lesions (Butcher et al. 1997).

Another interesting mechanism by which FK506 may mediate neuroprotection is through inhibition of outward delayed rectifying K^+ currents, which has been demonstrated in rat CA1 hippocampal neurons, as well as in several other experimental systems (DuBell et al. 1997, Ahn et al. 2007, Yu et al. 2007). In fact, increased K^+ currents mediated by Kv channels, as discussed earlier, contribute to several cell death pathways in neurons, potentially including excitotoxicity in stroke (Pal et al. 2003, Wei et al. 2003, Yao et al. 2009). FK506-mediated Kv channel inhibition may proceed through direct inhibition of the channel rather than through the drug's actions on calcineurin phosphatase activity (Ahn et al. 2007, Yu et al. 2007), although this mechanism remains to be thoroughly characterized.

Thus, calcineurin inhibition may not, in all cases, be sufficient for blocking excitotoxic neuronal damage in ischemia. In fact, we did not observe neuroprotection against excitotoxicity in our neuronal system with any of three different calcineurin inhibitors (CsA, FK506, or deltamethrin). Thus, the RCAN1.4-mediated neuroprotection we have demonstrated may proceed by a calcineurin activity-independent mechanism. Given that overexpression of RCAN1.4 is protective against exogenous Zn^{2+}-induced neurotoxicity (Lee et al. 2007), and that intracellular Zn^{2+} release is implicated both in inflammation-triggered neuronal injury (Knoch et al. 2008) and excitotoxic neuronal cell death (Aras et al. 2009a, Medvedeva et al. 2009), the mechanism of RCAN1.4 protection in our studies may involve blocking toxic Zn^{2+} release or downstream effectors of the metal.

Finally, when used clinically for immunosuppressive therapy, the calcineurin inhibitors FK506 and CsA can have pronounced toxic side effects in patients (Paul 2001). Thus, there is a crucial need to identify calcineurin inhibitors that possess minimal non-specific mechanisms of action, to improve current immunosuppressive therapy, and for potential future treatment of neurodegenerative diseases. In this regard, a recent study identified an RCAN1 peptide that specifically blocks calcineurin-NFAT signaling without affecting general calcineurin phosphatase activity (Mulero et al. 2009). The authors used the RCAN1 peptide-calcineurin interaction to identify dipyridamole, which is currently used to prevent thrombus formation in heart valve patients, as an agent that similarly blocked calcineurin-NFAT pathways with no effect on general phosphatase activity. Our studies show that RCAN1.4 over-expression is relatively non-toxic to neurons, and is neuroprotective against excitotoxicity (Figure 8). Thus, characterizing the mechanisms of RCAN1.4-mediated protection in ischemic cell death, as we and others have begun to do, may enable development of therapeutic agents that block calcineurin dysregulation-mediated injurious signaling pathways with few non-specific effects, in stroke and other non-neurological diseases.

4.3.3 Calcineurin dysregulation contributes to Alzheimer's disease-associated neurotoxicity

In addition to its role in enabling neuronal injury in stroke, disruption of normal calcineurin signaling may be a key step that triggers neuronal damage in Alzheimer's disease (AD). While this discussion will focus on calcineurin-mediated signaling pathways specifically triggering AD-related neuronal cell death, calcineurin over-activation is also strongly implicated in promoting AD-associated synaptic dysfunction.

Increased calpain-mediated truncation of calcineurin, resulting in calcineurin proteins that exhibit heightened phosphatase activity, is observed in human AD brains (Liu et al. 2005), and in the hippocampi of subjects with mild cognitive impairment (Mohmmad Abdul et al. 2011). Importantly, the levels of truncated calcineurin correlate with severity of neurofibrillary tangle pathology (Liu et al. 2005). Similarly, enhanced calcineurin/NFAT signaling correlates positively with Aβ load and cognitive impairment in AD patients (Abdul et al. 2009). Other studies have also shown increased calcineurin expression and activity in human AD and AD model mice brains, respectively (Norris et al. 2005, Kuchibhotla et al. 2008, D'Amelio et al. 2011). *In vitro* experiments accordingly suggest a critical role for calcineurin activation in Aβ-mediated neurotoxicity. In primary rat hippocampal cultures, exposure to toxic Aβ oligomers produces calpain-mediated calcineurin cleavage, enhanced calcineurin activity, and neuronal atrophy that is abrogated by FK506 (Mohmmad Abdul et al. 2011). In rat brain slices, Aβ oligomers provoke calcineurin activity, and a calcineurin activity-dependent decrease in levels of phosphorylated BAD, indicating that calcineurin-mediated apoptosis may contribute to AD pathogenesis (Reese et al. 2008).

The RCAN1.1 isoform is implicated in an injurious role particularly in AD-related neuronal cell damage. Protein and mRNA levels of the RCAN1.1 isoform are markedly increased in the brains of AD patients (Fuentes et al. 2000, Ermak et al. 2001, Sun et al. 2011). Increased concentrations of Aβ1-42 peptide, a major component of amyloid plaques, induce higher RCAN1.1 mRNA levels *in vitro* (Ermak et al. 2001). Transient over-expression of RCAN1.1 induces caspase-dependent apoptosis in neurons (Sun et al. 2011, Ermak et al. 2012), and renders neurons more susceptible to Aβ-induced apoptotic cell death (Sun et al. 2011). Our

results showing increased cell death in RCAN1.1-over-expressing neurons (Figure 9) are in agreement with these findings. Further, we demonstrated that RCAN1.1-induced neurotoxicity could be partially attenuated by inhibition of calcineurin activity, suggesting that calcineurin over-activation is a key component of RCAN1.1-mediated cell death (Figure 9). A previous study, showing that in angiogenesis, RCAN1.1 facilitates calcineurin activation in contrast to RCAN1.4 (Qin et al. 2006), supports our proposed mechanism. Based on these results and the likely role of calcineurin dysregulation in AD, we suggest that chronically increased levels of RCAN1.1 may facilitate AD-related neurodegeneration via over-activation of calcineurin.

Despite these investigations, additional studies will be required to further clarify the role of RCAN1.1 in AD neuronal cell death. In contrast to our findings and the published studies described above, Ermak and colleagues have explored the hypothesis that RCAN1.1 promotes AD-related neurodegeneration via either inhibition of calcineurin activity, activation of glycogen synthase kinase-3β (GSK-3β), or both, resulting in hyper-phosphorylation of tau. In support of this hypothesis, RCAN1.1 over-expression stimulates GSK-3β expression. Furthermore, aged mice that over-express RCAN1.1 exhibit enhanced levels of phosphorylated tau in their hippocampi (Ermak et al. 2006, Ermak et al. 2011). Whether RCAN1.1 increases kinase activity of GSK-3β, and whether RCAN1.1 inhibition of calcineurin activity specifically contributes to generation of hyper-phosphorylated tau and its associated neuropathology, require further investigation.

Reports in non-neuronal cells also demonstrate conflicting conclusions on the role of RCAN1.1 in modulating cell viability. In glucocorticoid-treated, cultured human leukemic cells

undergoing apoptosis, RCAN1.1 mRNA and protein expression are up-regulated. In agreement with our results, RCAN1.1 is thought to promote the cell death-signaling pathway, but via inhibition of calcineurin activity (Hirakawa et al. 2009) rather than over-activation (Figure 9). Another study that explored RCAN1 function in the heart of RCAN1-lacking mice demonstrated a calcineurin-facilitative role for RCAN1, similar to our findings. However, immune cells from RCAN1 knockout mice undergo apoptotic cell death that is worsened by loss of calcineurin expression, indicating that reduced calcineurin activity promotes a cell injurious pathway in these cells (Sanna et al. 2006). Because these mice were lacking both RCAN1.1 and RCAN1.4 protein expression, it is difficult to compare this result to our study. Future isoform-specific investigations, as we have done, will elucidate the specific role of RCAN1.1 and RCAN1.4 in regulating cell viability in neuronal and non-neuronal systems.

4.3.4 Additional mechanisms of RCAN1.1-mediated neuronal damage

Although we focused on the effects of RCAN1 over-expression on neuronal cell viability, RCAN1.1 may also contribute to neuronal damage by disrupting mitochondrial processes and synaptic function through cell signaling pathways that may be dependent or independent of calcineurin activity regulation. Chronic over-expression of RCAN1.1 in a neural cell line reduces mitochondrial mass and triggers mitochondrial membrane depolarization, leading to autophagy (Ermak et al. 2012). Whether these effects are mediated by regulation of calcineurin phosphatase activity is unknown. Adrenal chromaffin cells from RCAN1.1-over-expressing cells display compromised vesicle recycling via a mechanism that may be at least partially dependent on inhibition of calcineurin activity (Keating et al. 2008, Zanin et al. 2013). In agreement with an effect of increased RCAN1.1 protein levels on synaptic function, transgenic mice over-

expressing RCAN1.1 exhibit reduced hippocampal volume and decreased spine density, along with impaired LTP and deficits in short and long-term memory. Interestingly, hippocampal calcineurin activity is comparable in wild-type and RCAN1.1-transgenic mice. In contrast, RCAN1.1-over-expressing mice exhibit lowered levels of activated CAMKII and ERK, two proteins that promote LTP and signaling pathways associated with memory (Martin et al. 2012). These findings suggest a calcineurin-independent mechanism of impaired synaptic transmission in these animals.

4.4 CONCLUDING REMARKS

Our understanding of excitotoxic neuronal cell death in stroke has certainly progressed significantly over the past few decades. Almost sixty years have passed since a link between glutamate and stroke was first proposed. Landmark studies that established the role of glutamate/NMDA receptor signaling in promoting ischemic cell death were published over three decades ago. Indeed, the elucidation of cell signaling pathways in neuronal ischemia has led to the identification of numerous potential molecular targets for neuroprotection. Despite these advances, an effective therapy for stroke remains elusive. Drugs that demonstrate considerable neuroprotective efficacy *in vitro* and in animal stroke models have largely failed in human clinical trials. Thus, thoroughly characterizing specific neuronal cell death and cell adaptive-signaling pathways in ischemia is crucially important. The findings presented in this dissertation further our understanding of Kv2.1 channel regulation in ischemic preconditioning, and establish distinct roles of calcineurin activation in both excitotoxic neuroprotection and neuronal cell death. These studies may provide new direction for therapeutic approaches in stroke.

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