COOPERATIVE CALCIUM AND ZINC SIGNALING UNDERLIES OXIDANT-INDUCED NEURONAL INJURY

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Intracellular zinc release triggered by oxidative injury leads to cellular K^+ efflux and neuronal apoptosis. Low intracellular K^+ enables protease and nuclease activation during cell death processes. Our laboratory has demonstrated that the reduction in cytosolic K^+ occurs via zinc-dependent phosphorylation of Kv2.1 by Src kinase and p38 MAPK, leading to the insertion of new Kv2.1 channels into the plasma membrane of dying neurons. As calcium dyshomeostasis is also observed in many models of neuronal injury, I tested the hypothesis that zinc- and Kv2.1-mediated apoptosis also depended on increased intracellular calcium. I observed that oxidant exposure triggered release of calcium from the endoplasmic reticulum, which led to the activation of the calcium/calmodulin-dependent protein kinase II (CaMKII). Molecular or pharmacological inhibition of CaMKII prevented both the oxidant-induced K^+ current enhancement and, importantly, subsequent cell death. Further, I found that CaMKII regulated apoptosis by its direct association with the SNARE protein syntaxin, with syntaxin mediating the plasma membrane insertion of Kv2.1 via enhanced association with the phosphorylated channel. As such, Kv2.1/syntaxin binding was enhanced under conditions that promoted apoptosis, and inhibiting CaMKII prevented this interaction. Moreover, expression of a Kv2.1-derived peptide containing the channel's c-terminal syntaxin binding site (C1a) was also sufficient to disrupt Kv2.1/syntaxin binding, preventing both the oxidant-induced K^+ current enhancement and downstream apoptosis. Together, these findings support a novel role for calcium and CaMKII in

an established zinc-mediated injury cascade. Further, they suggest that the metals work in concert to elicit the pro-apoptotic Kv2.1 channel activity necessary for oxidative stress-induced apoptosis. Finally, disruption of the pro-apoptotic syntaxin/Kv2.1 interaction may lead to novel therapeutic approaches for neurological disorders associated with oxidant-induced signaling.

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PREFACE

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1.0 INTRODUCTION

1.1 OXIDATIVE STRESS: FOCUS ON AGING

Brain aging is marked by gradual, general cellular dysfunction occurring as a result of structural, chemical, and genetic alterations that manifest themselves as cognitive decline, albeit with great variability among individuals. While these changes are a normal and unavoidable part of the life cycle of neurons, aging itself is also a risk factor for a number of late-life neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). Although the molecular underpinnings of age-related neuropathology have not been completely resolved, one recurring explanation for the alterations observed with age, which has been revisited repeatedly for nearly 50 years, is the free radical theory of aging. This theory posits that the deleterious cellular changes that occur during aging and cognitive decline can be attributed in part to a continuous deregulation of intracellular reactive oxygen species (ROS) production over time (Harman, 1965), a phenomenon usually referred to as oxidative stress. Although this theory has been modified slightly in more recent years (Beckman and Ames, 1998), oxidative modifications caused by chronic ROS production remain recognized as a critical constituent of numerous neuropathological processes, and therefore represent a vitally important topic in the field of neurodegeneration research.

In the brain, ROS are produced as a physiological consequence of the normal oxidative processes related to cellular signaling, metabolism, and homeostasis (Lander, 1997; D'Autreaux and Toledano, 2007). Further, in addition to their presence merely as a passive byproduct of these processes, ROS also play an important and active role in a number of physiological cellular functions including gene expression, long-term potentiation, and the immune response (e.g. Sen and Packer, 1996; Knapp and Klann, 2002). As such, ROS are an integral component of a neuron's intracellular milieu. However, while ROS are important for normal cellular processing under certain circumstances, they are more widely recognized for their deleterious role in the initiation and propagation of neuronal injury. Namely, unregulated, excess production of these reactive intermediates during oxidative stress can have strong toxic effects on proteins, lipids, and nucleic acids. Moreover, ROS are also capable of triggering injurious signaling cascades that ultimately result in the demise of neurons by apoptosis or other forms of cell death (Beckman and Crow, 1993). Oxidative stress-induced cellular dysfunction can also exacerbate ROS production downstream of the initial insult, thereby maintaining the oxidative stress state in a self-propagating injury cycle that can lead to neuronal death if left unchecked (Beckman and Ames, 1998; Finkel and Holbrook, 2000).

1.2 ROS GENERATION AND MAINTENANCE OF PROPER OXIDATIVE HOMEOSTASIS

Under normal circumstances, ROS are produced primarily as a limited byproduct of oxidative phosphorylation during the formation of ATP, which occurs via a set of redox reactions in mitochondria (Chance et al., 1979). Mitochondrial dysfunction, however, is commonly

associated with neural injury cascades, and thus, in addition to their role in physiological ROS generation, mitochondria are regarded as one of principal producers of oxidative intermediates in pathological conditions. ROS themselves can also contribute to mitochondrial dysfunction either indirectly through the initiation of toxic signaling cascades that target mitochondria, or through direct damage to mitochondrial DNA (Richter et al., 1988; Esposito et al., 1999; Melov et al., 1999; Wallace, 2005). In addition to mitochondria, other intracellular generators of reactive metabolites contribute to oxidative stress during aging, including NADPH oxidases, nitric oxide synthases (NOSs), lipoxygenases (LOXs), and peroxisomes. Extraneuronal sources of ROS such as microglia, a non-neuronal, supporting cell involved in CNS immune responses, as well as exogenous stimuli such as UV light, ionizing radiation, and environmental toxins, also contribute to age-related neuronal dysfunction.

The brain, despite representing only 2% of the body weight, receives 15% of the cardiac blood output and accounts for 20% of the body's total oxygen consumption. The pronounced oxidative metabolism present in the brain results in a large generation of ROS during normal function. Neurons contain a system of enzymes and antioxidants to detoxify ROS after they are produced, as well as mechanisms to repair oxidant-induced damage once it has occurred; still, neurons become highly vulnerable to ROS-mediated damage when they are not able to adapt to ROS overproduction during times of stress. Therefore, oxidative stress occurs either from an overproduction of ROS, a deficiency in the antioxidant response, or both. Oxidative stress can thus be further defined as a condition in which the number of ROS produced surpasses a threshold over which they can no longer be adequately neutralized (Halliwell, 1992). Further, the deleterious consequences of oxidative stress tend to be exacerbated in the aged brain due to the

combination of increased oxidant production (Gabbita et al., 1997), along with decreased ability to detoxify ROS and repair oxidatively stressed tissue (Barnett and King, 1995).

While it is not entirely surprising that cells become less able to combat the increased concentrations of injurious oxidative intermediates as they age, oxidative stress does not occur in isolation, and thus is not solely responsible for the toxic cellular processes observed during age-related neuropathology. Of course, many interrelated dysfunctional cellular processes coincide to lead to the immense cell loss observed during AD and other neurodegenerative disorders. One such factor, which appears to be inextricably linked to oxidative stress damage in neurons, is zinc dyshomeostasis. Indeed, much like uncontrolled ROS production can have profoundly detrimental effects on neurons during aging, dyshomeostasis of intracellular zinc is also a crucial determinant of the fate of neurons in the aged brain.

1.3 ZINC IN THE BRAIN

Zinc is a ubiquitous trace element found throughout the body, including the brain, with particular abundance in the auditory brainstem, olfactory bulb, amygdala, hippocampus, and cortex (Frederickson et al., 1988; Weiss et al., 2000; Frederickson et al., 2000; Sekler et al., 2002). The cation plays a pivotal role in a multitude of cellular processes including neurotransmission, enzymatic activity, gene regulation, and structural maintenance and stabilization of proteins (Vallee and Falchuk, 1993; Choi and Koh, 1998; Frederickson et al., 2005). Due to its widespread function within neurons, intracellular zinc concentrations are tightly regulated, as proper homeostasis of the metal is critical in the maintenance of normal cellular processing. Indeed, zinc binds with high affinity to a very large number of proteins: roughly 3000 human

genes, or 10% of the genome, have been identified as encoding for zinc-binding proteins (Andreini et al., 2006). While the majority (80-90%) of the zinc present in the brain is found bound to metal-binding proteins, the remaining fraction is packaged within synaptic vesicles of a large sub-population of excitatory neurons. This synaptic or vesicular zinc is released in an activity-dependent manner, and can modulate the activation of several neurotransmitter receptors, including NMDA, AMPA, $GABA_A$ and glycine receptors (for review see Smart et al., 2004; Paoletti et al., 2009; Sensi et al., 2011), as well as voltage-dependent ion channels (e.g. Grauert et al., 2013). In addition, synaptically released zinc interacts with a specific zinc-sensing metabotropic receptor (mZnR/GPR39) to modulate synaptic activity through its effect both on the outward chloride transporter KCC2, and on the synthesis of 2-arachidonoylglycerol, an endocannabinoid that modulates probability of presynaptic neurotransmitter release (Besser et al., 2009; Chorin et al., 2011; Saadi et al., 2012; Perez-Rosello et al., 2013).

1.4 ZINC TOXICITY

It is well established that zinc exposure is toxic to neurons both *in vitro* (Yokoyama et al., 1986; Choi et al., 1988) and *in vivo* (Lees et al., 1990; Cuajungco et al., 1996). The overall concentration of the metal within the brain is ~150 μM, although the vast majority of intracellular zinc is normally rendered immobile through buffering by cytosolic metal-binding proteins and sequestration into organelles (Sensi et al., 2011). However, when neurons are damaged, as occurs during oxidative stress, bound intracellular zinc can be released into the cytosol, where it then triggers a number of detrimental signaling processes including those that lead to further ROS production, marking the start of a positive feedback loop involving

intracellular zinc release and ROS generation. Synaptic zinc has also been associated with neuronal dysfunction by its transfer from over-active presynaptic zinc-containing neurons to postsynaptic cells via calcium-permeable channels, including, but not limited to a sub-class of AMPA receptors (Weiss et al., 1993; Koh et al., 1996; Sensi et al., 2000). While proper zinc homeostasis is critical at all stages of life, the delicate balance required to keep zinc levels in check appears to be particularly precarious in the aged brain. This is likely due to the fact that, as mentioned earlier, relative ROS levels increase as we age, and intracellular zinc fluxes appear to be very susceptible to perturbation by ROS. Indeed, zinc has been proposed as being a critical link between oxidative stress and aging (Frazzini et al., 2006). To begin to understand the mutual regulation between intracellular zinc release and ROS generation, it is first necessary to review what is currently known about how zinc is maintained within neurons and, more importantly, how the metal is liberated from metal-binding proteins during oxidative injury.

1.5 ZINC DYSHOMEOSTASIS AND NEURONAL INJURY

While the largest concentration of zinc in the brain is bound to intracellular metal-binding proteins, there is a second pool, localized to synaptic vesicles of glutamatergic neurons, which constitutes 10-20% of the total concentration of the metal in neurons (Frederickson, 1989). In early studies, it was thought that cytoplasmic influx of synaptically released zinc, referred to as "translocation," was the primary source of toxic intracellular zinc increases during neuronal injury. This idea, however, was inconsistent with later studies of mice that lacked the gene encoding ZnT3, the transporter responsible for loading zinc in synaptic vesicles (Cole et al., 1999). Despite the fact that these animals were devoid of vesicular zinc, increased intracellular

concentrations of the metal and subsequent cell death still occurred, even in the apparent absence of zinc translocation from pre- to post-synaptic neurons (Lee et al., 2000). Thus, these findings strongly suggested that other sources of zinc release could also be contributing to the increased intracellular levels of the metal observed during neuronal injury. Since then, it has become increasingly clear that increased cytosolic zinc resulting from liberation from intracellular stores can be highly toxic during oxidative and other types of neuronal injury.

1.6 INTRACELLULAR SOURCES OF ZINC RELEASE

One of the main intracellular zinc binding proteins within neurons is metallothionein III (MT III). MT III is one member of a family of thiol-rich metal-binding proteins, and is the primary isoform found in neurons (Hidalgo et al., 2001). Close to a third of the 61-68 amino acids that constitute MT III are cysteine residues, cumulatively capable of binding up to seven zinc ions via their thiol side chains. Other MT isoforms have been shown to bind the seven zinc ions with varying affinities, supporting a role for MTs in the dynamic regulation of zinc levels dictated by the needs of the cell at any given time (Krezel and Maret, 2007). In this capacity, MT III acts as an intracellular regulator of zinc homeostasis via coordinated binding and release of the metal. Due to the very low redox potential of its thiols (-366 mV), MT III is readily oxidized, even by relatively mild oxidants. This oxidation results in the liberation of the bound zinc ions (Maret and Vallee, 1998); thus, while zinc itself is redox-inactive, its association with MT III makes it extremely susceptible to changes in cellular redox state. The release of zinc from MT III by oxidants causes a substantial increase in intracellular zinc concentration, and this single event has been established as a powerful inducer of neuronal injury (Aizenman et al., 2000).

Once zinc has been liberated from MT III, it can have numerous adverse effects on neuronal function. As mentioned previously, mitochondria are the primary producers of ROS in neurons, and zinc plays a critical role in the regulation of mitochondrial dysfunction and ROS generation during neuronal injury. Following intracellular liberation of the cation, mitochondria have been shown to take up cytoplasmic free zinc through both the calcium uniporter as well as through an independent import mechanism that has yet to be identified (Sensi et al., 2003; Malaiyandi et al., 2005). Once sequestered in the organelle, zinc can inhibit the electron transport chain, thereby reducing mitochondrial membrane potential, which subsequently leads to an increase in ROS generation (Sensi et al., 1999; Dineley et al., 2005; Dietz et al., 2008; Medvedeva et al., 2009). Interestingly, MT III has also been shown to translocate to mitochondria and release zinc ions within the mitochondrial intramembranous space, suggesting a dynamic regulation of zinc homeostasis by the combined actions of MT III and mitochondria (Ye et al., 2001). In addition to the MT III-mediated zinc effects on mitochondria, the organelle itself is shown to contain an independent store of zinc, which can be released during injury (Sensi et al., 2003). In that study, co-treatment with the thiol oxidant 2,2'-dithiodipyridine (DTDP) and the mitochondrial protonophore FCCP resulted in a greater increase in cytosolic zinc than was observed by treatment with either drug alone, which the authors attributed to the existence of distinct stores of the metal that can each be liberated by unique injurious stimuli. Thus, it appears that mitochondria and MT III work in tandem to dynamically regulate intracellular availability of the cation. However, while it is known that both MT III and mitochondria can modulate zinc levels, additional work is still needed to reveal the specific contributions of each pool to the propagation of pro-death cascades following various forms of neuronal injury.

1.7 ZINC-MEDIATED ROS GENERATION *IN VITRO*

In addition to triggering ROS production from mitochondria, zinc has also been found to be involved in injurious oxidant generation from a number of extra-mitochondrial sources. Interestingly, ROS generation and intracellular zinc release appear to be common constituents of a number of toxic signaling pathways in neurons. One of the better-studied zinc-mediated apoptosis cascades involves exogenous ROS-triggered zinc liberation and subsequent generation of endogenous oxidative intermediates. Specifically, cytosolic accumulation of MT III-liberated zinc can be caused by exposure to the oxidant DTDP or peroxynitrite ($ONOO^-$), a physiological oxidant generated by the reaction between free radicals nitric oxide (NO) and superoxide (Beckman et al., 1990). The increase in intracellular zinc then promotes production of superoxide from the enzyme 12-lipoxygenase (12-LOX), loss of mitochondrial membrane potential, and activation of Src kinase and p38 MAPK, the latter by upstream MAPKKK apoptosis signal-regulating kinase 1 (ASK-1; Aizenman et al., 2000; McLaughlin et al., 2001; Zhang et al., 2004a; Aras and Aizenman, 2005). Once activated, Src and p38 directly phosphorylate the voltage gated, delayed rectifier Kv2.1 channel at residues Y124 and S800, respectively, to trigger the insertion of new channels into the plasma membrane, leading to enhanced K^+ efflux and consequent reduction in intracellular K^+, caspase activation, and finally, apoptotic cell death (Aizenman et al., 2000; McLaughlin et al., 2001; Redman et al., 2007; Redman et al., 2009). The oxidant-induced, zinc-initiated signaling cascade is accompanied by concomitant intracellular calcium release from the endoplasmic reticulum (ER), and downstream CaMKII activation, which is necessary for the exocytotic introduction of Kv2.1 channels into the plasma membrane (McCord and Aizenman, 2013). Exposure to NO or activated microglia-derived $ONOO^-$ can also initiate this zinc- and Kv2.1-dependent apoptosis cascade (Bossy-Wetzel et al., 2004; Knoch et

al., 2008), and NO exposure has also been shown to lead to cytosolic zinc accumulation in hippocampal neurons *in vivo* (Cuajungco and Lees, 1998).

Independently, NO, in combination with depletion of the antioxidant glutathione, was shown to activate 12-LOX, which resulted in cell death of neuronal cultures (Canals et al., 2003). While this study didn't investigate the role of intracellular zinc release in this process, based on the injurious stimulus used and the downstream effects observed, these findings lend further support to the notion that various stimuli could trigger a common zinc-mediated injury cascade. Additionally, a number of studies have reported cell death following zinc-dependent activation of NADPH oxidase and nitric oxide synthase (NOS), which are the enzymes responsible for generating superoxide and NO, respectively (Noh and Koh, 2000; Kim and Koh, 2002; Kumar et al., 2012). While these reports focused on the effect of exogenous zinc exposure, considering the close relationship between intracellular zinc release and $ONOO^-$, as well the ability of exogenously applied zinc to enter neurons, it is not unreasonable to assume that intracellular zinc stores may also play a role in NADPH oxidase and NOS co-activation. Taken together, it appears that many of the components involved in injurious mitochondrial and extra-mitochondrial ROS production, as well as the downstream processes triggered by ROS, all seem to share a common association with intracellular zinc release.

1.8 ZINC IN HUMAN NEURODEGENERATION: SPOTLIGHT ON ALZHEIMER'S DISEASE

The experimental findings summarized thus far illustrate the concept that intracellular zinc release is a common toxic event in certain forms of oxidant-induced neuronal apoptosis. As

oxidative stress is a major contributor to brain aging and age-related pathology, it is feasible that zinc dyshomeostasis may also be involved in disorders associated with aging neurons (Mocchegiani et al., 2005). While the exact role of intracellular zinc in the pathophysiology of neurodegenerative disorders is not entirely clear, there is a growing body of work implicating the metal in age-related neurodegeneration. Oxidative stress-induced cell death is common between Alzheimer's disease, Parkinson's disease, and ALS, as well as many other neurological disorders (Behl et al., 1994; Mecocci et al., 1994; Smith et al., 1998; Dexter et al., 1989; Olanow, 1993; Wiedau-Pazos et al., 1996). However, because the literature on the role of metals in AD is extensive, this review will focus only on zinc deregulation during AD in an attempt to paint a more cohesive picture demonstrating the fact that this metal, when unregulated, can wreak havoc on the health of neurons in an aging brain.

AD is characterized by several pathological hallmarks including amyloid plaque deposits, aggregation of neurofibrillary tangles (NFTs) composed of the protein tau in a hyperphosphorylated form, and synaptic loss and neuronal deterioration, predominantly through apoptosis (Hanger et al., 2009). Amyloid plaques are comprised primarily of beta amyloid (Aβ), a ~40 amino acid long peptide generated through cleavage of the amyloid precursor protein (APP; Kang et al., 1987). Accumulation of Aβ during AD has been shown to cause neuronal apoptosis both *in vitro* and *in vivo* (Kowall et al., 1992; Loo et al., 1993). Additionally, AD and oxidative stress appear to go hand in hand, with ROS production being both a cause and consequence of Aβ aggregation (Markesbery, 1997; Butterfield et al., 2001).

The toxic role of zinc dyshomeostasis has become an important topic in the study of AD pathology (Bush and Tanzi, 2008; Greenough et al., 2013). However, the majority of this work has focused on how synaptically released zinc contributes to AD-related neuronal dysfunction

and death, and studies to characterize the effects of changes in endogenous intracellular zinc levels remain sparse. Still, the few reports that have examined this source of zinc support a toxic role for increased intraneuronal zinc in AD. One of the first studies to consider intracellular zinc deregulation in AD brains found that not only did the metal localize to extracellular amyloid plaque deposits, but that cytosolic zinc levels were increased as well, particularly in neurons exhibiting intracellular neurofibrillary tangles (Suh et al., 2000). More recently, the effect of oxidative stress on intracellular zinc mobilization was determined in neurons derived from 3xTg-AD mice, a triple transgenic AD mouse model that exhibits both Aβ and tau pathology. Importantly, this study found that intracellular zinc levels were substantially higher in 3xTg-AD neurons than in control cells following exposure to DTDP (Sensi et al., 2008). Thus, while studies of cytosolic zinc changes in AD are still in their relative infancy, these results suggest that intracellular zinc liberation could be critical for the progression of AD pathology, and that these effects appear to be mediated by the metal's interaction with ROS.

Zinc plays an important role in Aβ aggregation, as the peptide has been shown to bind to the metal (Bush et al., 1994a). Additionally, not only does zinc exposure induce the aggregation of amyloid plaques (Bush et al., 1994b; Esler et al., 1996), but the plaques themselves are rich in zinc, as well as copper. It is thought that Aβ is primarily responsible for inducing a state of oxidative stress during AD through direct production of oxidants (Markesbery, 1997; Huang et al., 1999; Butterfield et al., 2001), as well as through activation of microglia and subsequent generation of $ONOO^-$ (Goodwin et al., 1995; Meda et al., 1995; McDonald et al., 1997; Sturchler-Pierrat, 1997; Weldon et al., 1998). As mentioned earlier, $ONOO^-$ production originating from both neurons and microglia appears to be a key trigger of zinc-dependent neuronal apoptosis (Zhang et al., 2004a; Knoch et al., 2008). Further, hydrogen peroxide, an

oxidant produced by Aβ directly (Huang et al., 1999), can cause the release of zinc from MT III, subsequently causing aggregation of Aβ. Although it was not specified if MT III was localized intra- or extracellularly, Aβ aggregates induced by endogenous zinc release were morphologically distinct from those induced by exogenous zinc application, suggesting a unique role for different zinc pools during AD (Durand et al., 2010). Interestingly, chelation of the cation facilitates the dissolution of these toxic deposits both *in vitro* (Huang et al., 1997) and in post-mortem AD brain tissue (Cherny et al., 1999). Unlike humans, aged mice and rats do not express Aβ in an aggregated form, and as such, do not exhibit the related neuropathology. Intriguingly, one notable difference between human and rodent Aβ is the peptide's ability to bind zinc, with human Aβ exhibiting a much higher affinity for the metal (Huang et al., 2004). Thus, it is tempting to speculate that zinc could be directly responsible for the characteristic toxic aggregation of Aβ observed in AD patients, although more work is needed to definitively confirm this.

While it is known that both exogenous and synaptically released zinc induce Aβ aggregation (Bush et al., 1994b; Deshpande et al., 2009), it appears that increased intracellular zinc may also play a role in this process, although conclusive work demonstrating this has yet to be undertaken. Aβ was originally identified as solely being localized extracellularly; however, other studies have shown that Aβ is also found in the cytoplasm of neurons (Turner et al., 1996; Wild-Bode et al., 1997; Gouras et al., 2000). Interestingly, a number of studies have shown that intracellular Aβ ($A\beta_i$) formation precedes the appearance of extracellular Aβ, lending support to the hypothesis that an intracellular pool of the peptide is a prerequisite for extracellular plaque formation (Walsh et al., 2000; Wirths et al., 2001). However, further work is required to clarify if $A\beta_i$ plays a causative role in the formation of extracellular plaques, and if intracellular zinc

release promotes the induction of $A\beta_i$. Nonetheless, in support of a role for cytosolic zinc, $A\beta_i$ accumulation has been shown to correlate with microglial activation, increased NO production, and p38 activation (Rodrigo et al., 2004; Takuma et al., 2009), all of which are events that have previously been linked to pro-apoptotic intracellular zinc release (McLaughlin et al., 2001; Bossy-Wetzel et al., 2004; Knoch et al., 2008). Further, $A\beta_i$ accumulation can be triggered by exposure to oxidants (Ohyagi et al., 2000), and its accumulation has been shown to be localized to mitochondria, which also contain zinc (Rodrigo et al., 2004), and to the ER (Hartmann et al., 1997), an organelle recently shown to be involved in zinc- and Kv2.1-dependent neuronal apoptosis (McCord and Aizenman, 2013). Zinc has been shown to be localized to the ER (Stork and Li, 2010; Taylor et al., 2012) and released following $OONO^-$ exposure (Lin et al., 2013), further bolstering a potential role for the cation in $A\beta_i$ accumulation within the ER. Taken together, it is reasonable to hypothesize that intracellular zinc release could be an important factor in the accumulation of Aβ within neurons observed during AD.

Despite the presence of zinc in Aβ and evidence for its critical role in the aggregation of this toxic peptide, not all findings support a deleterious role for zinc in AD. In fact, exposure to low micromolar concentrations of the metal has been shown to destabilize Aβ aggregation and be protective against Aβ-induced toxicity (Garai et al., 2007). However, while low levels of zinc may protect neurons from Aβ-mediated damage, exposure to higher concentrations of the metal are toxic under otherwise equivalent conditions (Lovell et al., 1999). Like the effect of exogenous zinc on AD-related toxicity, the concentration of endogenous zinc within AD brains is also a contentious issue, although discrepancies in these studies could very well be a result of differences in the conditions under which the measurements were taken. Nonetheless, it appears that although some studies have reported decreased zinc levels in certain regions of AD brains

(Danscher et al., 1997; Panayi et al., 2002), the overall trend supports an increase in cerebral zinc during AD (Thompson et al., 1988; Deibel et al., 1996; Danscher et al., 1997; Religa et al., 2006). Indeed, zinc chelation has proven to be neuroprotective against Aβ-mediated toxicity (Lee et al., 2004). Moreover, high concentrations of the metal have been localized to amyloid plaques and neuropil derived from AD brains (Constantinidis, 1990; Suh et al., 2000; Lovell et al., 1998), further supporting a role for increased zinc in brain areas relevant to AD pathology. Aβ-localized zinc has also been shown to contribute to AD-related damage via its effect on toxic iron accumulation (Duce et al., 2010). During AD, increases in intracellular iron can exacerbate oxidative stress and contribute to tau aggregation (Bartzokis et al., 1994; Smith et al., 1997; Yamamoto et al., 2002). Recently, APP was shown to possess ferroxidase activity that contributed to iron export and a reduction in oxidative stress in a mouse model of AD. APP ferroxidase activity was inhibited by zinc, and was negatively correlated with increased Aβ accumulation, suggesting that the zinc originated from within amyloid plaques (Duce et al., 2010). Aβ pathology can also influence the activity of certain kinases that have been closely associated with zinc deregulation and neuronal injury. Namely, numerous studies have reported increased phosphorylation of p38 in AD brains (Hensley et al., 1999; Zhu et al., 2000; Zhu et al., 2001; Pei et al., 2001; Sun et al., 2003), which is also required for zinc-mediated neuronal apoptosis (McLaughlin et al., 2001). Additionally, ASK-1, the upstream MAPKKK of p38, has been linked to AD-related toxicity. Specifically, Aβ-mediated ROS production led to activation of ASK-1 and downstream cell death in PC12 cells and cortical neurons (Kadowaki et al., 2005). Thus, many of the pathological changes that take place during AD appear to be very similar to those observed in injury related to oxidant-induced zinc liberation and downstream apoptosis.

Similar to Aβ, zinc can also directly bind to tau to facilitate aggregation of the protein into NFTs (Mo et al., 2009). Like the bimodal regulation of Aβ-induced toxicity, modulation of tau by exogenous zinc also appears to be concentration-dependent, with lower concentrations of the metal causing a decrease in phospho-tau, while higher levels cause an increase (Boom et al., 2009). Although these findings were based on exogenously applied zinc, the authors concluded that because tau accumulates intracellularly, the observed effect was likely due to translocation of exogenous zinc into the cytosol. Thus, while the effect of endogenous intracellular zinc release was not investigated, these studies nonetheless support a potential role for intracellular liberation of the cation in the regulation of tau during AD. Further support for this idea comes from evidence for accumulation of zinc predominantly within neurons that display NFTs (Suh et al., 2000). As mentioned previously, hyperphosphorylation of tau is required for its aggregation into NFTs, and many of the kinases involved in the zinc- and Kv2.1-mediated apoptosis cascade described previously also phosphorylate tau. Specifically, tau can be directly phosphorylated by both p38 (Reynolds et al., 1997) and CaMKII (Litersky et al., 1996). Further, exogenous zinc application can trigger Src kinase-dependent inactivation of PP2A, the primary phosphatase responsible for dephosphorylating tau (Liu et al., 2005; Liu et al., 2008). As mentioned earlier, zinc-dependent Src activity is also responsible for phosphorylating Kv2.1 channels prior to their insertion into the plasma membrane during to apoptosis (Redman et al., 2009).

1.9 ZINC AND AUTOPHAGIC DYSFUNCTION

Autophagy is a catabolic system used within cells to clear dysfunctional or unused proteins and macromolecules before they cause damage to neurons. Degradation of malfunctioning cellular

components during autophagy occurs in lysosomes, which are acidic organelles containing hydrolase enzymes that facilitate the decomposition process. Autophagy is important in the clearance of protein aggregates (Johansen and Lamark, 2011), and autophagic deregulation has come to be regarded as a key occurrence in AD-related pathology (Cuervo, 2008; Nixon and Yang, 2011). It has been suggested that cell death can result from oxidative stress-induced accumulation of Aβ within lysosomes, leading to lysosomal membrane permeabilization (LMP) and subsequent release of Aβ and other toxic molecules into the cytosol (Zheng et al., 2009). In addition to toxicity caused by LMP, reduced autophagy, which has been observed in AD, can also lead to cell death due to a buildup of damaged molecules that would otherwise be degraded. In this scenario, decreased autophagy can be toxic to neurons independently of the effects of LMP. While this area of AD research is still relatively new, zinc also appears to play an important role in lysosomal dysfunction triggered by oxidative stress. Namely, oxidant exposure has been shown to cause an accumulation of zinc within lysosome-derived vesicles, as well as within the cytosol itself, leading to apoptosis of hippocampal neurons. Further, apoptosis was prevented by the zinc chelator TPEN, demonstrating that the increase in cytosolic zinc was responsible for the observed toxicity (Hwang et al., 2008).

Zinc deregulation during autophagic dysfunction has also been shown to have clinical significance. Administration of the prototype AD drug clioquinol can cause a reduction of tau and Aβ, as well as an improvement in cognitive performance (Regland et al., 2001; Ritchie et al., 2003). The effect of clioquinol on Aβ aggregation was initially thought to occur because of the drug's ability to chelate zinc (Cherny et al., 2001). However, more recent work revealed that clioquinol actually functions by acting as an ionophore to increase influx of zinc into neurons from the extracellular space, which can then induce autophagy in neurons and astrocytes (Park et

al., 2011). Specifically, this study found that zinc localized to autophagic machinery (autophagic vacuoles, autolysosomes, and lysosomes), and was necessary for the clioquinol-induced clearance of accumulated huntingtin protein, which aggregates in Huntington's disease. Thus, it appears that clioquinol may be important in degrading the Aβ and tau aggregates observed in AD, in part through its effect on extra- and intracellular zinc levels. However, this same study also showed that clioquinol caused zinc-dependent cell death in cortical neurons, although this experiment was not performed in the presence of Aβ or under other conditions that would mimic zinc levels or the overall cellular environment manifested in AD, and it is thus difficult to deduce the effect of clioquinol on neuronal viability during AD from this study alone.

Both abnormally increased and decreased autophagy can be detrimental to neurons depending on the circumstances in which it takes place. The studies summarized here underscore the complex nature of the role of zinc in regulating autophagic dysfunction. Indeed, it appears that changes in intracellular zinc levels can dictate if autophagy will adopt a pro-death or pro-survival function (Lee and Koh, 2010). As zinc dyshomeostasis can be observed both extracellularly and intracellulary during AD, much work remains to acquire a better understanding of the delicate balance of zinc that underlies normal cellular function, and at exactly what point metal-regulatory processes go awry to propagate the pathological effects observed during neurodegeneration.

1.10 MT III AND NEURODEGENERATION

As mentioned previously, MT III is one of the primary zinc-binding proteins within neurons, and therefore plays an integral role in maintaining homeostasis of the metal. In contrast to MT I and

MT II, which are normally induced by increased levels of free metals within cells, MT III is constitutively expressed. Knockdown of MT III can lead to increased oxidant-triggered intracellular zinc levels, while overexpression of the protein substantially reduces the amount of zinc detected within neurons following oxidant exposure (Aras et al., 2009). Interestingly, numerous independent studies have reported downregulation of MT III during AD (Uchida et al., 1991; Tsuji et al., 1992; Yu et al., 2001). While these studies did not directly measure the effect of reduced MT III expression on intracellular zinc levels, they suggest that increased intracellular zinc concentration due to a reduction in MT III expression could be relevant to the pathological effects observed during the progression of the disease. Others, looking at the molecular consequences of changes in MT III during AD have shown that exogenous MT III exposure prevents the accumulation of Aβ and rescues neurons from Aβ-induced cell death (Irie et al., 2001), supporting a beneficial role of MT III through the sequestration of extracellular zinc. Along these lines, MT III has been suggested to be secreted by cells in the brain, albeit by not well-defined pathways (Chung and West, 2004; Manso et al., 2011). Seemingly paradoxically, a few studies have reported increased MT III expression in AD (Zambenedetti et al., 1998; Carrasco et al., 1999), although investigations describing this phenomenon are much less common than those reporting decreased MT III. Further, MT III can act as both an acceptor and a donor of zinc, and thus changes in MT III expression could have different effects depending on the cellular environment and redox state within neurons. Knockdown of MT III in astrocytes has also been shown to lead to a decrease in the degradative capacity of lysosomes (Lee et al., 2010), consistent with the reduced autophagy observed during AD. However, the lack of MT III also corresponded with decreased oxidant-induced zinc release, revealing the need for further studies to fully understand the role of MT III and its association with zinc in autophagic dysfunction.

Nonetheless, the fact that changes in MT III expression are almost universally observed in AD suggests that deregulation of MT III is likely contributing to the zinc dyshomeostasis observed.

1.11 ZINC AND ITS RELATION TO CALCIUM DEREGULATION

Like zinc, intracellular calcium dyshomeostasis also appears to play a crucial role in AD-related pathology (LaFerla, 2002; Berridge et al., 2010; Berridge 2013). Free intracellular calcium is normally in the nanomolar range within neurons; however, cellular injury can cause deregulation of the cation, resulting in a marked increase in the intracellular calcium concentration that can lead to cellular dysfunction. Further, simultaneous increases in intracellular zinc and calcium have been observed in a number of injurious signaling cascades related to ischemic, excitotoxic, and oxidative injury (Sensi et al., 2002; Medvedeva et al., 2009; Vander Jagt et al., 2009). However, details regarding the downstream signaling pathways activated by these two metals during oxidative injury are just beginning to be clarified (McCord and Aizenman, 2013). Similarly, although evidence has implicated calcium dyshomeostasis as a trigger for AD pathology (Khachaturian, 1989), if and how calcium and zinc cooperate to regulate pathological signaling during AD is still unclear. While there is some indication of a correlation between intracellular zinc and calcium levels in AD brains (Ishihara et al., 2002), direct evidence linking the two metals to pathological processes specifically associated with this disorder does not yet exist. Still, despite the lack of definitive proof, intracellular calcium and zinc release do appear to mediate many of the same processes during AD.

Just as Aβ can induce zinc liberation from internal, metal-binding stores via ROS production, the peptide can also trigger calcium release from the ER. This increase in calcium

leads to ROS generation, mitochondrial dysfunction, caspase activation, and apoptosis (Ferreiro et al., 2006). As discussed earlier, all of these cellular changes have also been reported during oxidant-induced, zinc- and calcium-mediated neuronal apoptosis. Additionally, like zinc, cellular alterations induced by increased intracellular calcium contribute to intraneuronal Aβ accumulation and neurotoxicity (Pierrot et al., 2006; Demuro et al., 2013). Specifically, a depolarization-induced increase in cytosolic calcium can trigger phosphorylation of APP and tau, leading to subsequent $A\beta_i$ accumulation and cell death (Pierrot et al., 2006). These calcium-dependent phosphorylation events were mediated by GSK-3β, a kinase known for its role in the phosphorylation of both tau and APP (Aplin et al., 1996) that has previously been shown to be activated downstream of ER calcium release (Hartigan and Johnson, 1999). Zinc can also induce GSK-3β phosphorylation, an event that corresponds to zinc-mediated activation of p38 MAPK (An et al., 2005). In another study, accumulation of $A\beta_i$ was shown to trigger IP3-mediated calcium release from the ER that was necessary for $A\beta_i$-induced toxicity (Demuro et al., 2013).

Like zinc, changes in intracellular calcium levels are extremely sensitive to oxidative stress. DTDP, a common inducer of intracellular zinc release (Aizenman et al., 2000; McLaughlin et al., 2001), has also been shown to trigger calcium release from the sarcoplasmic reticulum following oxidation of ryanodine receptors (RyRs) in cardiomyocytes (Zaidi et al., 1989). Further, DTDP-induced ER calcium release has recently been observed during zinc-dependent apoptosis in cortical neurons (McCord and Aizenman, 2013). Additionally, RyRs can also be nitrosylated by nitric oxide, leading to calcium release (Xu et al., 1998; Kakizawa et al., 2013). Deregulation of both the ER and mitochondria and the resultant effects on intraneuronal calcium levels appear to be important determinants of the progression of AD-related pathological processes (Green et al., 2008; Mattson, 2010; Adam-Vizi et al., 2010). Both oxidative stress and

mitochondrial dysfunction have been shown to occur early in the pathogenesis of AD (Nunomura et al., 2001; Moreira et al., 2006), and mitochondria and the ER can physically interact to regulate intracellular calcium levels in response to changes in redox state (Csordas and Hajnoczky, 2009; Hayashi et al., 2009). As mentioned previously, intracellular zinc can also regulate mitochondria and ROS production (Dineley et al., 2003; Sensi et al., 2003). Further, increased intracellular calcium triggered by glutamate exposure has been shown to contribute to mitochondrial ROS production and subsequent release of zinc from intracellular stores (Dineley et al., 2008). While these studies were not specific to any one disease, it is conceivable that similar injurious parallel processes are taking place during AD and other neurodegenerative disorders. In fact, a signaling cascade has been proposed to account for the seemingly coordinated pathways activated by zinc and calcium during AD (Corona et al., 2011). Taken together, and considering the interrelated roles of calcium and zinc in other injury models, work to reveal potential parallel processing by the metals awaits as an exciting new opportunity to enhance our understanding of the cellular signaling events underlying AD neuropathology.

1.12 ROLE OF KV2.1 CHANNELS IN HEALTH AND DISEASE

1.12.1 Physiological functions of Kv2.1

As mentioned previously, a necessary downstream event in oxidant-induced, zinc-mediated apoptosis is increased K^+ efflux through the delayed-rectifier Kv2.1 channel. Kv2.1 is one member of the voltage-gated potassium (Kv) channel family, a class of ion channels comprised of 12 subtypes (Kv1-12) that are critical in the regulation of neuronal excitability (Pongs, 1999;

Gutman et al., 2005; Johnston et al., 2010). Kv channels consist of four α subunits, each composed of six transmembrane domains, as well as a cytoplasmic N- and C-terminus. Kv2.1, one of two members of the delayed rectifying Kv2 α subunit-containing family, is characterized by its slow gating kinetics, activation at relatively depolarized potentials (Frech, et al., 1989; Du et al., 2000), and localization to the soma, proximal dendrites, and axon initial segments of neurons (Trimmer, 1991; Scannevin et al., 1996; Sarmiere et al., 2008).

While Kv2.1 does play a role in controlling membrane potential, studies over the past 15 years have found that the channel has unique properties in this regard that are not typical of most Kv channels. Kv2.1 is responsible for conducting the majority of current encompassing the delayed rectifier K^+ current, I_K (Murakoshi and Trimmer, 1999). However, unlike many other Kv channels, which can influence membrane potential even after a single action potential, Kv2.1 does not normally regulate neuronal function as related to single action potentials, nor does it normally affect passive electrical properties of neurons such as resting membrane potential and input resistance (Du et al., 2000; Guan et al., 2013). Instead, Kv2.1 channel-mediated currents only become significant in conditions that induce repetitive action potential firing (Du et al., 2000; Mohapatra et al., 2009), and thus serve to diminish the neuronal responses to periods of heightened activity. As such, Kv2.1 is regarded as a regulator of homeostatic plasticity, a term applied to a neuron's ability to maintain an optimal level of intrinsic activity despite continuous fluctuations in synaptic input (Surmeier and Foehring, 2004).

1.12.2 Kv2.1 phosphorylation regulates channel activity

One of the primary determinants of Kv2.1 function is the phosphorylation status of the channel. Kv2.1 is highly phosphorylated under normal conditions (Park et al., 2006), and the large

number of phosphates near the channel's voltage sensor is thought to influence the voltage-dependence of activation such that Kv2.1 only becomes activated at relatively depolarized potentials (Perozo and Bezanilla, 1990; Du et al., 2000). Calcineurin-dependent dephosphorylation of several C-terminal residues results in a leftward shift in the voltage-dependence of channel activation as a means to regulate neuronal excitability during periods of high-frequency firing (Murakoshi et al., 1997; Du et al., 2000). Interestingly, exposure to injurious excitatory stimuli such as glutamate or kainic acid also leads to Kv2.1 dephosphorylation and subsequent hyperpolarization of the channel's activation voltage, again in order to prevent neural overexcitation (Misonou et al., 2004; Park et al., 2006). Moreover, calcineurin-mediated dephosphorylation of Kv2.1 also affects channel localization. As mentioned, a large proportion of Kv2.1 channels are normally maintained in dense clusters at neuronal plasma membranes (Trimmer, 1991). Activity-dependent dephosphorylation of these channels triggers their dispersal into a more uniform localization, although at present, the purpose of channel declustering is unclear. Interestingly, Kv2.1 phosphorylation state, voltage-dependence of activation, and channel localization all appear to be mediated by an association between the channel's N and C termini, as disruption of this interaction influences each of these parameters (Mohapatra et al., 2008).

Calcineurin-independent phosphorylation of two distinct Kv2.1 channel residues, N-terminal Y124 and C-terminal S800, by zinc-mediated activation of Src kinase and p38 MAPK, can also influence channel activity, although phosphorylation of these sites is thought to occur solely during apoptosis, and functions to enhance K^+ efflux upstream of cell death (Redman et al., 227, 2009). In this case, whether the N and C termini physically interact has yet to be

documented, although it does appear that the phosphorylation status of residues Y124 and S800 may be interdependent (Figure 6B; Redman et al., 2009; unpublished observations).

1.12.3 Kv2.1 as a facilitator of exocytosis

In addition to their role as regulators of neuronal excitability, Kv2.1 channels have also been shown to be an important constituent of the cellular machinery responsible for large dense core vesicle (LDCV)-mediated exocytosis. The role of Kv2.1 during exocytosis appears to be dynamic and context-dependent, as the channel has been shown to function in two independent capacities in this regard. First, Kv2.1 can suppress depolarization-induced release via its more traditional role in the hyperpolarization of the membrane potential (MacDonald et al., 2002; MacDonald and Wheeler, 2003). Conversely, Kv2.1 has also been shown to facilitate exocytosis via an interaction with syntaxin. However, these studies, carried out in the same laboratory, revealed a discrepancy in the mechanism underlying Kv2.1 modulation by syntaxin, suggesting dynamic regulation of the channel. Namely, an initial report found that the binding of syntaxin to Kv2.1 promoted exocytosis by inhibiting surface delivery of the channel, which reduced K^+ efflux, thereby minimizing hyperpolarization of the membrane potential (Leung et al., 2003). Later studies, however, found that Kv2.1/syntaxin binding regulates exocytosis entirely independently of the channel's ability to conduct K^+ (Singer-Lahat et al., 2007; Feinshreiber et al., 2010; Dai et al., 2012). Specifically, Kv2.1/syntaxin binding facilitated secretion by enhancing vesicle recruitment to the plasma membrane, and this effect was observed even with a non-conducting Kv2.1 channel mutant (Feinshreiber et al., 2010). Thus, depending on the environment, Kv2.1 can either inhibit or enhance vesicle release, the latter scenario occurring through either a channel pore-dependent or pore-independent mechanism, both of which require

a physical interaction with syntaxin. Presumably, these disparities can be attributed to both the known activity dependence of vesicle release, and to the dynamic and context-dependent functioning of Kv2.1. Taken together, these findings suggest that the modulation of physiological Kv2.1 activity is a highly nuanced process, the details of which are still under investigation.

1.12.4 Kv2.1-mediated K^+ efflux and neuronal injury

While Kv2.1 does regulate various physiological processes in neurons, the channel is also involved in a number of neuropathological processes, including those observed in AD. Low intracellular K^+ is a requisite step in many apoptosis pathways, as it facilitates protease and nuclease activation, cytochrome c release from mitochondria, and apoptosis-related cellular volume decrease (Hughes et al., 1997; Yu et al., 1997; Bortner et al., 1997; Maeno et al., 2000; Cain et al., 2001). Intracellular zinc release and downstream Kv2.1-mediated K^+ efflux can both be triggered by exposure to DTDP, NO, and activated microglia (Pal et al., 2003; Bossy-Wetzel et al., 2004; Knoch et al., 2008). Further, oxidant-induced K^+ current enhancement is prevented by the zinc chelator TPEN, demonstrating the dependence of apoptotic Kv2.1 activity on zinc (McLaughlin et al., 2001).

In addition to its role in zinc-mediated apoptosis, altered Kv2.1 activity has also been shown to be involved in the toxic cellular processes related to AD. For a number of years, studies have demonstrated an enhancement of voltage-gated, delayed rectifier K^+ currents following exposure of neurons and astrocytes to Aβ (Jalonen et al., 1997; Yu et al., 1998; Colom et al., 1998). More recently, though, changes specifically within the Kv2.1 channel have been identified in animal models of AD. Namely, upregulation of Kv2.1 mRNA and protein has been

reported in rats injected with Aβ; this change in Kv2.1 expression was accompanied by impaired performance on spatial memory tasks (Pan et al., 2004). It is well known that deficits in acetylcholine are intimately related to AD-associated cognitive decline, thus forming the basis of acetylcholinesterase (AChE)-based therapy in the disease (Bartus et al., 1982; Francis et al., 1999). Interestingly, the acetylcholinesterase inhibitor galantamine, used in the treatment of AD, has been shown to cause a reduction in basal delayed rectifier K^+ currents in hippocampal neurons (Pan et al., 2003; Vicente et al., 2010), likely arising from Kv2.1 channels (Zhang et al., 2004b).

Hydrogen peroxide exposure has been shown to directly oxidize Kv2.1 channels, leading to channel oligomerization and downstream apoptosis. Importantly, an oxidation-resistant Kv2.1 channel cysteine mutant (C73A) that prevented oligomerization also attenuated toxicity induced by Aβ exposure (Cotella et al., 2012). Interestingly, enhanced oligomerization of Kv2.1 was also observed in a mouse model of AD, although how this change in Kv2.1 structure contributed to AD-related cognition decline was not determined (Cotella et al., 2012). In contrast to reports of enhanced Kv2.1-mediated K^+ currents during apoptosis, this study found that oxidant exposure actually decreased K^+ currents, and the Kv2.1C73A mutation blocked this effect. However, this discrepancy could be explained by the fact that it takes ~3 hours to observe the Kv2.1-mediated K^+ current enhancement after oxidant exposure (McLaughlin et al., 2001), and this group only examined currents immediately following the oxidative insult. Further, the toxic effects of Kv2.1 channel oligomerization may have occurred independently of the change in K^+ currents, as a second oligomerization-impaired channel mutant (C73S) was found to be non-conducting, yet still rescued cells from oxidant-induced apoptosis (Cotella et al., 2012). Thus, it is not yet clear how alterations in Kv2.1-mediated K^+ currents may influence Aβ-mediated toxicity.

1.13 CONCLUSIONS

Oxidative stress can generally be considered both a cause and an effect of the neuropathological changes seen in AD and other age-related neurodegenerative disorders. In addition, links between increased intracellular zinc, oxidative stress, and age-related neurodegeneration have been established in numerous studies, and zinc dyshomeostasis appears to be a common constituent of a multitude of pathological neuronal processes. Nonetheless, a direct causative role of intracellular zinc release in human neurodegenerative disorders has yet to be firmly established. It is known that zinc homeostasis is critical for proper brain function, and even minor disturbances to this delicate balance can trigger an accumulation of zinc, which can have extremely adverse effects on the fate of neurons in AD and related disorders. As such, modulation of intracellular zinc levels could be a particularly important target in order to protect against AD-related injurious cellular pathways. However, while chelation of zinc may be an effective neuroprotective strategy *in vitro*, the potential therapeutic benefits of zinc chelation become much more complex when studying changes in zinc levels *in vivo*, in particular as zinc can also act as a neuromodulator or neurotransmitter, and it has a wide range of additional essential functions in neurons, as well as throughout the organism, including, but not limited to regulation of gene expression. This matter is thus complicated by the fact that both increased and decreased intracellular zinc can be neurotoxic, presumably depending on specific cellular conditions. Still, the fact that drugs like the zinc ionophore clioquinol have been effective in abrogating some of the pathological consequences of AD in clinical trials (Regland et al., 2001; Ritchie et al., 2003), presumably through its effect on modulating zinc levels, indicates that the metal plays a pivotal role in the progression of AD, and that strategies targeting zinc could hold the key to finding better treatments for this currently incurable disease. Still, future work is

required to determine exactly how clioquinol and its association with zinc improve the outcome of AD progression. Further, investigation into the potential off-target effects of this drug is crucial, as increased intracellular zinc, as illustrated throughout this review, is lethal to neurons in a variety of settings.

1.14 THESIS GOALS

The goal of my dissertation was to elucidate upstream signaling cascades responsible for the introduction of Kv2.1 K^+ channels into the plasma membrane and consequent loss of intracellular K^+ that has come to be regarded as a requisite event in neuronal apoptosis. Both intracellular zinc release and enhanced K^+ efflux are common constituents of numerous apoptosis pathways, and our laboratory has worked to uncover several the intermediate cellular signals linking these two injurious processes. Although calcium dysregulation has also been implicated in neuronal apoptosis, whether signals activated by this metal converge on cellular K^+ efflux had not been described. My research project was directed at uncovering potential cooperative convergence of calcium and zinc in Kv2.1-dependent neuronal apoptosis. I first determined whether calcium-dependent activation of CaMKII facilitated the apoptotic K^+ current enhancement and downstream apoptosis, and the molecular signaling process activated by this kinase. These findings are detailed in Chapter 2, and have formed the basis of a manuscript that was recently published (McCord and Aizenman, 2013). The studies presented in Chapter 2 also uncovered a role for the SNARE protein syntaxin in the modulation of Kv2.1 activity during apoptosis. Thus, in subsequent experiments I examined in more detail how syntaxin regulated Kv2.1, and how interfering with this interaction affected viability of oxidatively stressed

neurons. The results are presented in Chapter 3, and are currently being compiled into a manuscript for publication. The findings comprising my thesis establish how calcium initiates an injurious signaling cascade that works cooperatively with a known zinc-initiated cascade to mediate the apoptotic membrane insertion of Kv2.1 channels. As such, this dissertation serves to integrate a previously unrecognized cellular signaling cascade into an established zinc- and Kv2.1-dependent apoptosis pathway.

2.0 CONVERGENT CALCIUM AND ZINC SIGNALING REGULATES APOPTOTIC KV2.1 POTASSIUM CURRENTS

2.1 ABSTRACT

A simultaneous increase in cytosolic zinc and calcium accompanies the initiation of neuronal cell death signaling cascades. However, the molecular convergence points of cellular processes activated by these cations are poorly understood. Here, we show that calcium-mediated activation of the calcium/calmodulin-dependent protein kinase II (CaMKII) is required for a cell death-enabling process previously shown to also depend on zinc. We have reported that oxidant-induced intraneuronal zinc liberation triggers a syntaxin-dependent incorporation of Kv2.1 channels into the plasma membrane. This can be detected as a marked enhancement of delayed rectifier K^+ currents in voltage clamp measurements observed at least three hours following a short exposure to an apoptogenic stimulus. This is the process responsible for the cytoplasmic loss of K^+ that enables protease and nuclease activation during apoptosis. In the present study, we demonstrate that an oxidative stimulus also promotes intracellular calcium release and downstream activation of CaMKII, which, in turn, modulates the ability of syntaxin to interact with Kv2.1. Pharmacological or molecular inhibition of CaMKII prevents the K^+ current enhancement observed following oxidative injury and, importantly, significantly increases neuronal viability. These findings reveal a previously unrecognized cooperative convergence of

calcium- and zinc-mediated injurious signaling pathways, providing a potential new target for therapeutic intervention in neurodegenerative conditions associated with oxidative stress.

2.2 INTRODUCTION

Calcium has long been recognized as a critical component of neuronal cell death pathways triggered by oxidative, ischemic, and other forms of injury (Choi, 1995). Indeed, calcium deregulation has been associated with a variety of detrimental processes in neurons, including mitochondrial dysfunction (Sims and Anderson, 2002), generation of reactive oxygen species (Gleichmann and Mattson, 2011), and activation of apoptotic signaling cascades (Elmore, 2007). More recently, zinc, a metal crucial for proper cellular functioning (Maret, 2013), has been found to be closely linked to many of the injurious conditions in which calcium had been thought to play a prominent role (Calderone et al., 2004; Bonanni et al., 2006; Lee and Koh, 2010; Shuttleworth and Weiss, 2011; Sensi et al., 2011). In fact, it has been suggested that a number of deleterious properties initially attributed to calcium may have significant zinc-mediated components (Stork and Li, 2006; Vander Jagt et al., 2009). Although it is virtually impossible to chelate, or remove calcium without disrupting zinc levels (Smith, 2009), the introduction of techniques to monitor calcium and zinc simultaneously in cells (Devinney et al., 2005) has made it increasingly apparent that both cations have important yet possibly distinct roles in neuronal cell death (Vander Jagt et al., 2009; Sensi et al., 2002; Dietz et al., 2009; Medvedeva et al., 2009; Martin et al., 2011). However, the relationship between the cell death signaling pathways activated by the cations is unclear, and possible molecular points of convergence between these signaling cascades have yet to be identified.

Injurious oxidative and nitrosative stimuli lead to the liberation of intracellular zinc from metal binding proteins (Aizenman et al., 2000). The released zinc, in turn, triggers p38 MAPK- and Src-dependent Kv2.1 channel insertion into the plasma membrane, resulting in a prominent increase in delayed rectifier K^+ currents in dying neurons, with no change in activation voltage, approximately 3 hours following a brief exposure to the stimulus (Yu et al., 1997; McLaughlin et al., 2001; Pal et al., 2003; Pal et al., 2006; Redman et al., 2007; Redman et al., 2009; Shepherd et al., 2012). The increase in Kv2.1 channels present in the membrane mediates a pronounced loss of intracellular K^+, likely accompanied by chloride (Wei et al., 2004; Bortner and Cidlowski, 2007), that facilitates apoptosome assembly and caspase activation (Yu et al., 1997; Bortner et al., 1997; Hughes et al., 1997; Montague et al., 1999; Hughes and Cidlowski, 1999; Cain et al., 2001; Thompson et al., 2001). Indeed, K^+ efflux appears to be a requisite event for the completion of many apoptotic programs, including oxidant-induced, zinc-mediated neuronal death (McLaughlin et al., 2001).

Calcium has been suggested to regulate the p38 MAPK signaling cascade via CaMKII-mediated activation of the MAP3K apoptosis signaling kinase-1 (ASK-1; Takeda et al., 2004). As ASK-1 is also required for p38-dependent manifestation of the zinc-triggered, Kv2.1-mediated enhancement of K^+ currents (Aras and Aizenman, 2005), we hypothesized that the p38 activation cascade may provide a point of downstream convergence between calcium and zinc signals following oxidative injury. Here, we report that calcium and zinc signals do in fact converge on a cellular event critical for the K^+ current enhancement, and that CaMKII is required for this process. However, CaMKII does not act upstream of p38 activation as originally hypothesized, but instead interacts with the N-ethylmaleimide-sensitive factor attachment protein

receptor (SNARE) protein syntaxin, which we previously showed to be necessary for the insertion of Kv2.1-encoded K^+ channels following an apoptotic stimulus (Pal et al., 2006).

2.3 MATERIALS AND METHODS

Cell Culture and Transfection Procedures. For electrophysiological experiments, mixed cortical neuronal/glial cultures were prepared from embryonic day 16 (E16) Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA). Cortices were dissociated with trypsin, and the resultant cell suspension, adjusted to 670,000 cells per well, was plated on glass coverslips in 6-well plates as described previously (Hartnett et al., 1997). Non-neuronal cell proliferation was inhibited after two weeks in culture with 1–2 μM cytosine arabinoside, and cultures utilized at three to four weeks *in vitro*. For biochemical experiments, neuronal-enriched cultures (Rosenberg and Aizenman, 1989) were prepared from E18 rats and seeded onto culture plates at a density of 470,000 cells per well. Cytosine arabinoside was applied five days after plating to reduce the glial cell population, and cultures were used at 12–14 days *in vitro*. For neuronal transfection, cells were treated for five hours in serum free medium with 2 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA), 50 μL Optimem (GIBCO, Grand Island, NY), and 1.5 μg DNA per well. For Chinese hamster ovary (CHO) cell transfections, cells were plated on coverslips in 24-well plates at a density of 5.6×10^4 cells per well for electrophysiological measurements or in 100 mm dishes at a density of 1.7×10^6 cells per dish for biochemical studies. Cells were treated for 3-4 hours in serum free medium with a total of 1.2 μL Lipofectamine and 0.28 μg DNA per well for electrophysiology, or 55.1 μL Lipofectamine and

7.334 μg DNA per dish for biochemistry. Following transfection, cells were maintained in F12 medium containing fetal bovine serum at 37°C, 5% CO_2 for 24 hours prior to experimentation.

Drug Treatments and Antibodies. The apoptotic stimulus for electrophysiological and biochemical experiments was 10 minute exposure to 30 μM 2,2'-dithiodipyridine (DTDP) at 37°C, 5% CO_2. For electrophysiology, the DTDP-containing solution was removed prior to three-hour incubation in fresh medium containing 10 μM butoxy-carbonyl-aspartate-fluoromethyl ketone (BAF), a cysteine protease inhibitor that maintains cell viability without affecting K^+ currents. Myristoylated CaMKIINtide (1 μM; EMD, Gibbstown, NJ) was used to inhibit CaMKII; thapsigargin (1 μM; Sigma Aldrich, St. Louis, MO) was used to deplete endoplasmic reticulum calcium stores. *N,N,N',N'*-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN, 3 μM) and BAPTA-AM (100 μM) were used to chelate zinc and calcium, respectively. Antibodies were purchased from the following suppliers: rabbit anti-p38, anti-phospho-p38, anti-CaMKIIα, and anti-phospho-CaMKII from Cell Signaling Technologies (Danvers, MA); rabbit anti-syntaxin from Abcam (Cambridge, MA); rabbit anti-oxidized-CaMKII were either a gift from M. Anderson (University of Iowa Carver College of Medicine) or purchased from Millipore (Temecula, CA); mouse anti-Kv2.1 from NeuroMab (Davis, CA); mouse anti-phospho-tyrosine from BD Biosciences (San Jose, CA); mouse anti-CaMKII from Santa Cruz Biotechnology (Dallas, TX). Immunoaffinity-purified serum directed at phosphorylated S800 of Kv2.1 was prepared in our laboratory (Redman et al., 2007); rabbit anti-phospho-Kv2.1S603 antibodies were a gift from J. Trimmer (University of California Davis).

Electrophysiological Measurements. Current recordings were performed on eGFP-positive co-transfected neurons or CHO cells using the whole-cell patch clamp configuration technique as described previously (McLaughlin et al., 2001). The intracellular (electrode)

solution contained (in mM): 100 K-gluconate, 11 EGTA, 10 KCl, 1 $MgCl_2$, 1 $CaCl_2$ x $2H_2O$, 10 HEPES; pH adjusted to 7.2 with concentrated KOH; 2.2 ATP and 0.33 GTP were added and the osmolarity was adjusted to 280 mOsm with sucrose. The extracellular solution contained (in mM): 115 NaCl, 2.5 KCl, 2.0 $MgCl_2$, 10 HEPES, 10 D-glucose; 0.25 μM tetrodotoxin; pH adjusted to 7.2. Measurements were obtained under whole-cell voltage clamp with an Axopatch-1D amplifier and pClamp software (Molecular Devices, Sunnyvale, CA), using 2-3 MΩ recording electrodes. Electrodes were pulled from 1.5 mm borosilicate glass (Warner Instruments, Hamden, CT) with a model P-97 mechanical pipette puller (Sutter Instruments, Novato, CA). Series resistance was partially compensated (80%) in all cases. Currents were filtered at 2 kHz and digitized at 10 kHz with a Digidata 1440A Digitizer (Molecular Devices). K^+ currents were evoked with incremental 10 mV voltage steps to +80 mV from a holding potential of –80 mV. To determine current density values, steady-state current amplitudes were measured 180 msec after the initiation of the +10 mV step and normalized to cell capacitance.

Viability Assays. Viability in transfected neurons was assessed using a luciferase reporter assay (Boeckman and Aizenman, 1996; Aras et al., 2008). Twenty-four hours after transfection with a luciferase reporter plasmid and any other plasmids of interest, cultures were treated overnight with either vehicle or microglia (50,000 cells/mL) (Cheepsunthorn et al., 2001) plated directly on top of neurons and activated by interferon-γ (IFN-γ) and lipopolysaccharide (LPS) (Knoch et al., 2008). Cells were then assayed for luciferase-mediated luminescence using a SteadyLite luciferase assay system (PerkinElmer, Waltham, MA). The luciferase reagent was added directly to cells and luminescence measured after 10 minutes using a Wallac 1420 96-well microplate reader (PerkinElmer Life Sciences, Waltham, MA). Cell viability is proportional to

luciferase luminescence, confirmed by cell counts in GFP co-transfected sister coverslips (Aras et al., 2008).

Electrophoresis and Immunoblotting. Protein samples from equal amounts of cell lysate were separated on 7.5% or 10% sodium dodecyl sulphate (SDS) polyacrylamide gels by electrophoresis using the Mini Protean 3 System (Bio-Rad, Hercules, CA). Separated protein bands were transferred onto 0.2 μm nitrocellulose membranes (Bio-Rad), blocked with 1% Bovine Serum Albumin (BSA) in PBS with 0.05% Tween 20 (PBST) at room temperature for 1 hour, and probed with appropriate primary antibodies diluted in PBST. Blots were then incubated with infrared fluorescent goat secondary antibodies at room temperature for 1 hour, visualized using the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE), and quantified using infrared fluorimetry.

Immunoprecipitation. Protein was harvested by washing cell culture dishes twice with ice cold PBS followed by a five-minute incubation with 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) buffer. Protein A/G agarose bead slurry (Santa Cruz, Dallas, TX) was added to the samples and rocked at 4°C for 1 hour in order to pre-clear non-specific protein binding. Samples were then incubated overnight at 4°C with the appropriate immunoprecipitating antibodies. The next day, following another incubation with the bead slurry, the protein samples were prepared/denatured by the addition of sample preparation buffer (625 mM Tris, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% β-mercaptoethanol) and heated at 95°C for 5 min prior to SDS-PAGE gel electrophoresis and immunoblotting.

Intraneuronal zinc and calcium Imaging. To assess changes in intracellular free zinc, the zinc-sensitive fluorescent reporter FluoZin-3 (Molecular Probes, Carlsbad, CA) was utilized. FluoZin-3 responds robustly to small changes in zinc, is insensitive to high calcium and

magnesium, and is unaffected by low intracellular pH or oxidants (Devinney et al., 2005). Further, zinc signals from surrounding astrocytes are minimal under these conditions, as these cells appear to buffer intracellular zinc more effectively than neurons (Dineley et al., 2000). Intracellular calcium levels were measured using the ratiometric, fluorescent calcium dye Fura-2 (Teflabs, Austin, TX). Coverslips were loaded with either FluoZin-3 AM or Fura-2 AM (5 μM, 30 minutes) and immediately transferred to a recording chamber (Warner, Hamden, CT) mounted on an inverted epifluorescence microscope. Using a computer controlled monochromator (Polychrome II, TILL photonics, Martinsried, Germany) and CCD camera (IMAGO, TILL photonics), images were acquired every 10 seconds by exciting cells with 485 nm light (measured at 520 nm emission) for zinc imaging, or alternately with 340 and 380 nm light (measured at 510 nm emission) for calcium imaging. After background subtraction, fluorescence was expressed as the difference between the average baseline fluorescence just before treatment and the average peak fluorescence during treatment (ΔF) for 10-20 neurons per coverslip.

Data analysis. Data are expressed as mean ± SEM. Statistical analysis was performed using Student's *t* test or ANOVA with post hoc comparisons, as indicated in figure legends. An α of $p<0.05$ was considered statistically significant.

2.4 RESULTS

2.4.1 Injurious oxidant exposure leads to CaMKII activation in neurons

Exposure to the thiol oxidant 2,2'-dithiodipyridine (DTDP) liberates intracellular zinc and enables neuronal injury via a p38- and Kv2.1-dependent process (Aizenman et al., 2000; McLaughlin et al., 2001). However, DTDP has also been shown to induce intracellular calcium release in cardiomyocytes through cysteine oxidation of sarcoplasmic reticulum ryanodine receptors (Zaidi et al., 1989). Thus, the first step in uncovering a possible role for calcium in zinc-dependent oxidative injury was to determine if DTDP caused a similar increase in calcium in neurons. First, we confirmed the effects of DTDP on intraneuronal zinc release using the specific fluorescent zinc indicator FluoZin-3 AM (Gee et al., 2002). As expected (Aizenman et al., 2000), we observed a pronounced increase in fluorescent signal in cultured rat cortical neurons following 30 μM DTDP exposure, which was abolished by concurrent treatment with the zinc chelator *N,N,N',N'*-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN; 3 μM). TPEN was also sufficient to decrease basal FluoZin-3 fluorescence, presumably by effectively competing with the fluorescent indicator for the cation (Figure 1A).

To monitor DTDP-induced changes in intraneuronal calcium, we used the fluorescent calcium indicator Fura-2 AM. Cortical neurons were exposed to 30 μM DTDP in the presence of 3 μM TPEN to chelate any liberated zinc that would otherwise interfere with a measureable calcium signal, as Fura-2 also detects free zinc (Grynkiewicz et al., 1985). Exposure to DTDP + TPEN led to a significant increase in Fura-2 fluorescence, demonstrating that, similar to cardiomyocytes, the oxidizing agent also induces a calcium response in neurons. To identify the source of the released calcium, we depleted endoplasmic reticulum (ER) calcium stores with the

ER calcium-ATPase inhibitor thapsigargin (TG; 1 μM) prior to exposure to DTDP + TPEN. Under these conditions, exposure to the thiol-oxidizing agent did not generate a change in fluorescence, confirming that the released calcium originates from the ER (Figure 1B). Of note, ryanodine receptors, which as mentioned earlier can be gated by DTDP (Zaidi et al., 1989), spatially co-localize with Kv2.1 channel clusters in neurons (Antonucci et al., 2001).

To determine if DTDP-induced calcium release led to activation of CaMKII, immunoblotting was performed on protein samples obtained from neuronal cultures treated with 30 μM DTDP alone or in combination with either 3 μM TPEN or the acetoxymethyl ester of the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (BAPTA-AM; 100 μM). DTDP exposure caused a significant increase in T286-phosphorylated (active) CaMKII, which was unaffected by zinc chelation but blocked by BAPTA-AM (Figure 1C). These results indicate that DTDP-liberated calcium, but not zinc, is responsible for CaMKII phosphorylation. In addition to calcium-dependent activation of CaMKII, sustained activation of the kinase can be achieved via the oxidation of its M281/282 residues (Erickson et al., 2008). However, we observed no difference in immunoblots obtained from CaMKII-immunoprecipitated protein samples from vehicle- and DTDP-treated neurons using an antibody directed toward the oxidized forms of M281/282 (Figure 1D; 100 μM H_2O_2 serving as a positive control, Figure 2). Therefore, it is unlikely that oxidative CaMKII activation appreciably contributes to the signaling cascades triggered by coordinated intracellular release of calcium and zinc. Lastly, we found that activation of CaMKII, in and of itself, does not influence zinc release, as treatment with the myristoylated (myr) inhibitory CaMKII peptide CaMKIINtide (1 μM) prior to DTDP exposure had no effect on FluoZin-3 fluorescence (Figure 1A). Taken together, these

results demonstrate that a thiol oxidative insult, previously shown to initiate a zinc-mediated cell death pathway in neurons, can also trigger calcium-dependent activation of CaMKII.

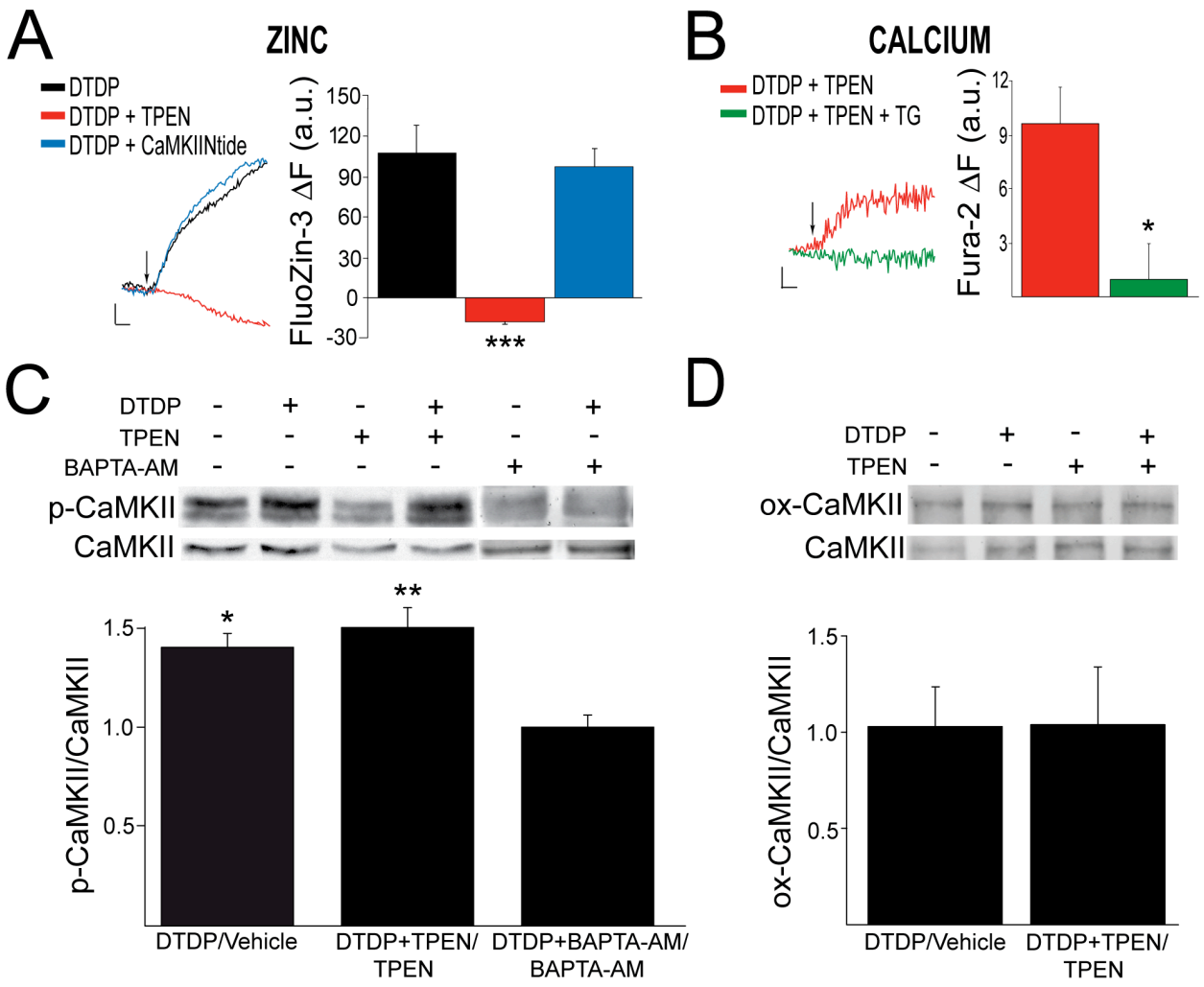

Figure 1: Oxidant exposure leads to intracellular zinc and calcium release and CaMKII activation

Figure 1. *(A)* Neurons loaded with the zinc indicator FluoZin-3 AM were exposed to DTDP (30 μM) with or without TPEN (3 μM). A subset of cells was exposed to myr-CaMKIINtide (1 μM) prior to DTDP treatment. *Left:* representative responses averaged from ~15 cells from a single coverslip. Arrow indicates start of drug application (calibration: 15 absolute units or a.u., 2 min; FluoZin-3 emission measured at 520 nm). *Right:* quantified results, with ΔF equal to the difference between the average baseline fluorescence just before DTDP treatment and the average maximal fluorescence during treatment (mean ± SEM, n=5-7; ***$p<0.001$, paired t-test). *(B)* Neurons loaded with the calcium indicator Fura-2 AM were exposed to DTDP (30 μM) in the presence of TPEN (3 μM); some coverslips were pre-treated with thapsigargin (TG; 1 μM, 30 min) to deplete ER calcium. *Left:* representative responses averaged from ~15 cells in a single coverslip (calibration: 10 a.u., 2 min; Fura-2 emission measured at 510 nm). *Right:* quantified results (n=5; *$p<0.05$, paired t-test). *(C)* Representative immunoblot and quantified results from neuronal samples obtained under control conditions or after 10 min exposure to 30 μM DTDP in the absence or presence of either 3 μM TPEN or 100 μM BAPTA-AM. Blots were probed with antibodies specific for p-CaMKII or CaMKII. Data are expressed as a ratio of mean ± SEM signal of phospho to total CaMKII (n=5; **$p<0.01$, *$p<0.05$; ANOVA/Bonferroni). Neither TPEN nor BAPTA-AM alone significantly decreased CaMKII protein levels, when averaged across all experiments. *(D)* Representative immunoprecipitation and quantification of blots derived from neuronal samples obtained under control conditions or after 10 min exposure to 30 μM DTDP with or without 3 μM TPEN. Following CaMKII immunoprecipitation, blots were probed with antibodies against oxidized M281/282 CaMKII or total CaMKII. Data are expressed as a ratio of mean ± SEM signal of oxidized to total CaMKII (n=3).

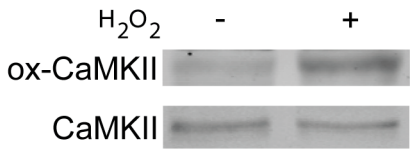

Figure 2: CaMKII methionine oxidation by hydrogen peroxide

Figure 2. Representative immunoblot from protein samples were obtained from neuronal-enriched cultures under control conditions or after 10-minute exposure to 100 μM H_2O_2. Blots were probed with antibodies specific for oxidized M281/282 CaMKII or total CaMKII.

2.4.2 CaMKII acts independently of p38 activation

ASK-1, an upstream kinase in the p38 MAPK signaling pathway, has been reported to be activated by CaMKII in a calcium-dependent fashion (Takeda et al., 2004). Since zinc-triggered activation of p38 also requires ASK-1 (McLaughlin et al., 2001; Aras and Aizenman, 2005), we hypothesized that a convergence of calcium and zinc signaling processes may occur at the level of p38 phosphorylation. Immunoblots were obtained from protein samples of cortical neurons previously exposed to DTDP (30 μM) alone or in the presence of either myr-CaMKIINtide (1 μM) or TPEN (3 μM). As expected (McLaughlin et al., 2001), TPEN significantly prevented the effect of DTDP on p38 phosphorylation. In contrast, myr-CaMKIINtide was unable to block this effect (Figure 3A). Thus, although DTDP-triggered calcium release leads to CaMKII activation in neurons, the kinase does not appear to be involved in the phosphorylation of p38 following zinc liberation.

We next investigated if activity of the kinase was in fact required for an oxidant-induced increase in K^+ currents. To explore this we utilized Chinese hamster ovary (CHO) cells, a recombinant expression system that lacks endogenous voltage-gated K^+ channels (Pal et al., 2003; Yu and Kerchner, 1998), yet has all the cell signaling components required to produce an apoptotic current surge in Kv2.1-transfected cells (Pal et al., 2003). CHO cells expressing Kv2.1 with or without a kinase-inactive CaMKIIK42R mutant were subjected to a 10-minute exposure to 30 μM DTDP followed by 3-hour maintenance in butoxy-carbonyl-aspartate-fluoromethyl ketone (BAF; 10 μM), a broad-spectrum protease inhibitor used during recording for its ability to prevent apoptosis downstream of the K^+ current enhancement (McLaughlin et al., 2001). We found that the DTDP-induced increase in K^+ current densities seen in Kv2.1-expressing CHO

cells was absent in cells co-expressing CaMKIIK42R (Figure 3B), strongly suggesting that CaMKII activity is necessary for manifestation of the apoptotic K^+ current enhancement.

Kv2.1-expressing CHO cells were also used to confirm that the involvement of CaMKII in the K^+ current enhancement occurs independently of p38 activation. Our group has shown that p38 directly phosphorylates Kv2.1 at residue S800, which is required for the apoptotic K^+ current enhancement to commence (Redman et al., 2007). Moreover, CHO cells expressing a pseudo-phosphorylated mutant, Kv2.1S800E, exhibit an innate, p38-independent increase in K^+ current densities that mimics the enhanced currents observed in apoptogen-exposed, wild type Kv2.1-expressing cells (Redman et al., 2007). Here, we observed that the increased current densities seen in Kv2.1S800E-expressing CHO cells were absent in cells that also expressed CaMKIIK42R (Figure 3C). These observations support our biochemical results obtained in neurons (Figure 3A) and strongly argue that although CaMKII is necessary for the increase in K^+ currents, its site of action is distinct from the events leading to phosphorylation of Kv2.1 residue S800 by p38.

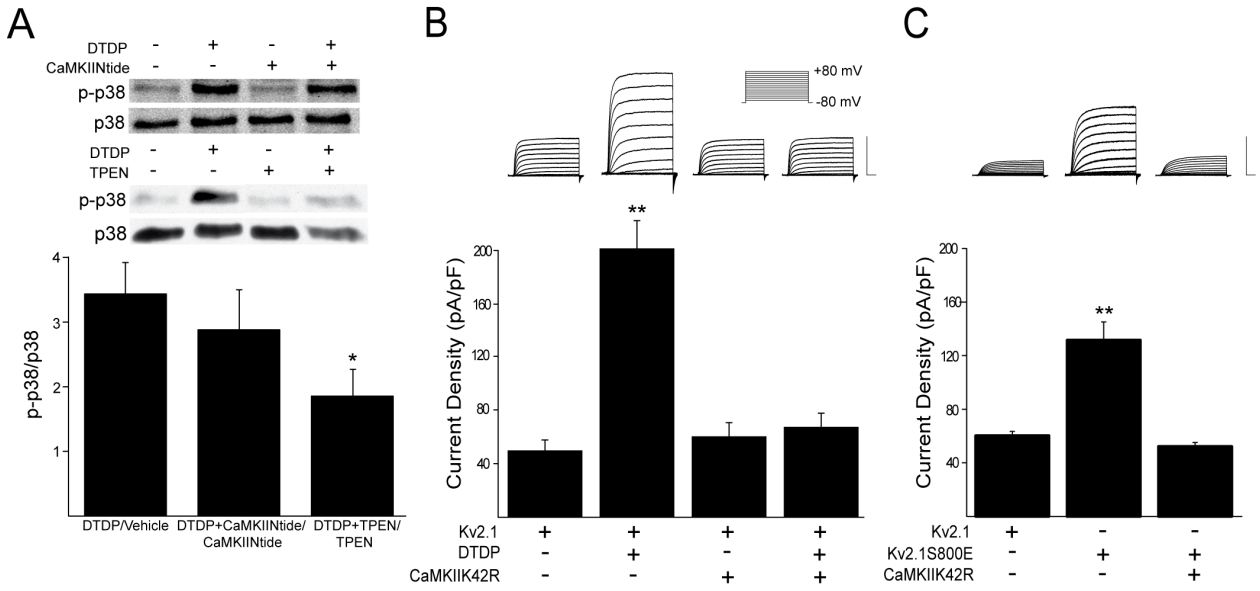

Figure 3: CaMKII modulates Kv2.1-mediated K^+ currents independently of p38 activation

Figure 3. *(A)* Zinc chelation, but not CaMKII inhibition, prevents DTDP-induced p38 activation. Representative immunoblot and quantified results from neuronal samples obtained under control conditions or after 10 min exposure to 30 μM DTDP in the absence or presence of 1 μM CaMKIINtide or 3 μM TPEN. Blots were probed with antibodies specific for p-p38 or p38. Data are expressed as a ratio of mean ± SEM signal of phospho to total p38 (n=7 for control and n=6 for CaMKIINtide and TPEN groups; *$p<0.05$; ANOVA/Bonferroni). Note that there was no significant effect of either treatment on basal p38 protein levels across all experiments. *(B)* Representative whole-cell K^+ currents and pooled mean ± SEM current densities recorded from Kv2.1-expressing CHO cells treated (10 min) with either vehicle (n=15) or 30 μM DTDP (n=10), and CHO cells co-expressing Kv2.1 and CaMKIIK42R treated with vehicle (n=9) or 30 μM DTDP (n=11). Recordings were performed 3 hr following exposure. *(C)* CaMKIIK42R blocks the K^+ current enhancement induced by the Kv2.1S800E mutation. Representative whole-cell K^+ currents and corresponding mean ± SEM current densities recorded from CHO cells expressing either Kv2.1 (n=9), Kv2.1S800E (n=26), or Kv2.1S800E + CaMKIIK42R (n=10). Currents were evoked by a series voltage steps from -80 mV to +80 mV in 10 mV increments. To determine current density values, steady-state current amplitudes were measured 180 msec after the initiation of the +30 mV step and normalized to cell capacitance. Representative traces shown were selected as they match the current densities shown on the bar graphs. Calibration: 5 nA, 25 msec; **$p<0.01$; ANOVA/Dunnett.

2.4.3 CaMKII interacts with syntaxin

CaMKII has previously been shown to regulate exocytosis via a specific interaction with syntaxin (Ohyama et al., 2002; Nomura et al., 2003; Hirling and Scheller, 1996; Bonanno et al., 2005). In addition, syntaxin is known to bind to the most proximal region of the Kv2.1 C terminus, termed C1a, during calcium-facilitated exocytosis in pancreatic and other non-neuronal cells (Leung et al., 2003; Singer-Lahat et al., 2007, 2008). Importantly, we previously showed that syntaxin is also required for the membrane insertion of Kv2.1 channels during apoptosis (Pal et al., 2006). Based on these observations, and because CaMKII is also necessary for the K^+ current increase, we hypothesized that an interaction between CaMKII and syntaxin would be detected in our system. Syntaxin immunoprecipitates of protein samples obtained from CHO cells transfected with a syntaxin-expressing plasmid, with or without Kv2.1, were probed for CaMKII. Results revealed that endogenous CaMKII co-immunoprecipitates with syntaxin, and that this interaction is enhanced by Kv2.1 co-expression (Figure 4A). In these experiments we also confirmed the previously described association of syntaxin and Kv2.1 (Figure 4A; Leung et al., 2003; Singer-Lahat et al., 2007, 2008). The increased CaMKII signal in Kv2.1-expressing cells could be the result of a direct interaction of the kinase with the K^+ channel. Although we detected a measurable, albeit weak Kv2.1 signal in immunoblots obtained from CaMKII-immunoprecipitated protein samples, this interaction was disrupted in cells overexpressing the C1a region of Kv2.1 (Figure 4B). As mentioned previously, C1a contains the channel's syntaxin-binding site, and can displace native Kv2.1 from syntaxin (Singer-Lahat et al., 2007). Thus, we suggest that syntaxin provides the link between CaMKII and Kv2.1. Nonetheless, we tested whether CaMKII overexpression in CHO cells could lead to phosphorylation of Kv2.1, as a low-stringency scan of possible phosphorylation sites of the channel sequence (Obenauer et al., 2003)

revealed up to 7 putative serine targets for CaMKII. In contrast to a pronounced shift in the molecular weight of the channel following phosphorylation by cyclin-dependent kinase 5 (CDK5), a known multiple-residue kinase of Kv2.1 (Cerda and Trimmer, 2011), CaMKII had no effect (Figure 5). Furthermore, the specific actions of CDK5 on Kv2.1 residue S603 phosphorylation were confirmed with the use of a phospho-specific antibody (Cerda and Trimmer, 2011), again with CaMKII generating no signal (Figure 5). This result is altogether not surprising, however, given that calcium-dependent changes in Kv2.1 phosphorylation status have been primarily linked to calcineurin phosphatase activity and dephosphorylation of the channel (Mohapatra et al., 2007).

Next, we tested whether phosphorylation of the Kv2.1 residues involved in the apoptotic K^+ current enhancement led to an increased association of the channel and syntaxin. We also investigated whether interfering with CaMKII function disrupted this interaction. In addition to p38-dependent phosphorylation of Kv2.1 S800, an N-terminal Src kinase-targeted tyrosine (Y124; Tiran et al., 2003) is critical for the expression of the apoptotic current enhancement (Redman et al., 2009). We have recently found that overexpression of Src in CHO cells is sufficient to trigger maximal phosphorylation of both Y124 and S800, the latter likely occurring via activation of endogenous p38 (Figure 6; Mohapatra et al., 2007). Here, we found that Src overexpression in CHO cells significantly enhanced the ability of syntaxin to bind Kv2.1, and inclusion of the kinase inactive CaMKIIK42R mutant completely abolished this effect (Figure 4C). These results indicate that changes in the phosphorylation profile of Kv2.1 that normally lead to the apoptotic K^+ current enhancement are accompanied by an increased interaction of the channel with syntaxin, and that a functional CaMKII is necessary for this process.

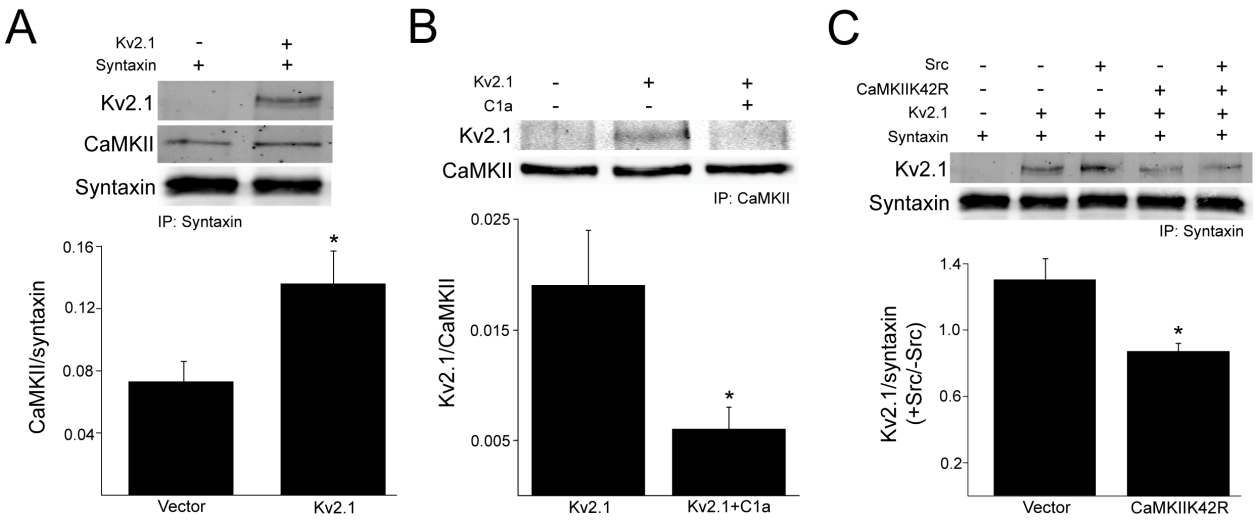

Figure 4: CaMKII regulates Kv2.1/syntaxin binding

Figure 4. *(A)* Co-immunoprecipitation of CaMKII and syntaxin is enhanced in the presence of Kv2.1. Representative co-immunoprecipitation and quantification of blots derived from CHO cells transiently expressing syntaxin with or without Kv2.1. Following syntaxin immunoprecipitation, blots were probed with antibodies against Kv2.1, CaMKII, or syntaxin (mean ± SEM, n=4; *$p<0.05$; paired t-test). *(B)* Co-immunoprecipitation of Kv2.1 and CaMKII is disrupted by overexpression of Kv2.1 C1a region, which contains the syntaxin binding site. Representative co-immunoprecipitation and quantified results from CHO cells transfected with Kv2.1 or Kv2.1 + C1a. Following CaMKII immunoprecipitation, blots were probed with antibodies against Kv2.1 or CaMKII (n=4; *$p<0.05$; paired t-test). *(C)* CaMKII inhibition prevents the increase in Src-mediated Kv2.1/syntaxin binding. Representative co-immunoprecipitation and quantified results of protein samples from syntaxin-expressing CHO cells co-transfected with either Kv2.1, Kv2.1 + Src, Kv2.1 + CaMKIIK42R, or Kv2.1 + CaMKIIK42R + Src. Syntaxin was immunoprecipitated and blots were probed with antibodies against Kv2.1 or syntaxin (n=5; *$p<0.05$; paired t-test).

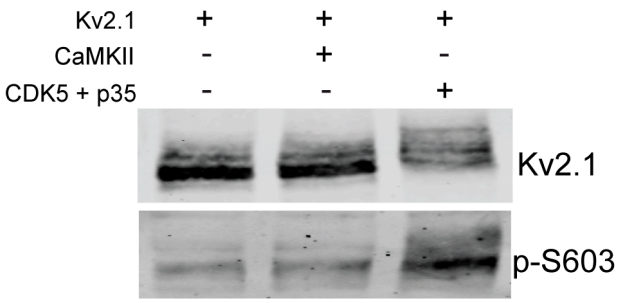

Figure 5: CaMKII does not cause phosphorylation-induced shift in molecular weight of Kv2.1

Figure 5. Immunoblot from Kv2.1-expressing CHO cells co-transfected with CaMKII or CDK5 + p35 demonstrates that unlike CDK5, CaMKII does not elicit a phosphorylation-induced molecular weight shift of Kv2.1. Membranes were probed with antibodies specific for Kv2.1 or p-Kv2.1S603, a known target of CDK5 used to confirm the effect of the kinase on Kv2.1 (Cerda and Trimmer, 2011).

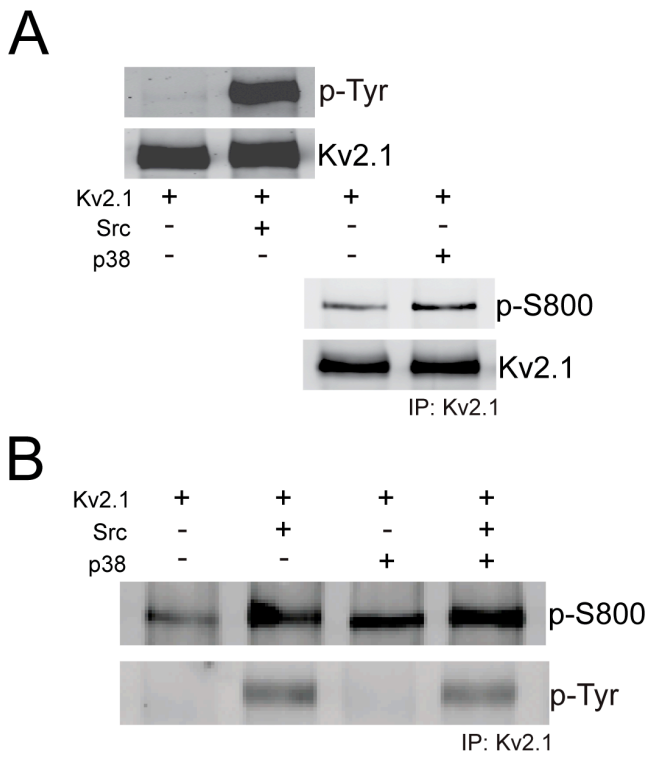

Figure 6: Src expression induces the phosphorylation of Kv2.1 by Src and endogenous p38

Figure 6. *(A)* Cells were co-transfected with Kv2.1 and either Src or p38, and Kv2.1 was immunoprecipitated. Membranes were probed with antibodies specific for p-Kv2.1S800, p-tyrosine, or Kv2.1 to serve as a loading control. *(B)* Cells were co-transfected with Kv2.1 and either Src, p38, or both. Immunoprecipitation of Kv2.1 was followed by probing with antibodies specific for p-S800 or p-tyrosine.

2.4.4 CaMKII is required for oxidant-induced K^+ current enhancement and cell death in neurons

Lastly, we evaluated whether disruption of CaMKII activity could prevent the endogenous apoptotic K^+ current increase and be neuroprotective in oxidant-exposed cortical neurons. We observed that both myr-CaMKIINtide (1 μM) and transfection with CaMKIIK42R blocked the increased K^+ current densities normally seen in neurons 3 hours after DTDP exposure (30 μM, 10 min; Figure 7A, B). In a separate set of studies, we confirmed that the zinc chelator TPEN (3 μM) also effectively inhibited the increase in K^+ currents induced by DTDP exposure (Figure 8). Next, to determine if inhibition of CaMKII was neuroprotective, we transfected neurons with a plasmid encoding CaMKIIK42R (or empty vector) in addition to a luciferase-expressing plasmid used to assess viability in transfected cells (Aras et al., 2008). Neurons were later exposed to activated rat microglia (Cheepsunthorn et al., 2001), which we previously showed to be a powerful inducer of zinc-dependent, Kv2.1-mediated injury via peroxynitrite production (Knoch et al., 2008). We observed that neurons expressing CaMKIIK42R were significantly protected from microglia toxicity when compared to vector-expressing cells (Figure 7C). Moreover, neurons expressing this mutant were also protected from DTDP toxicity (60 μM, 10 min; Figure 9). From these data we conclude that a CaMKII activity is necessary for both the K^+ current enhancement and subsequent lethal injury in neurons.

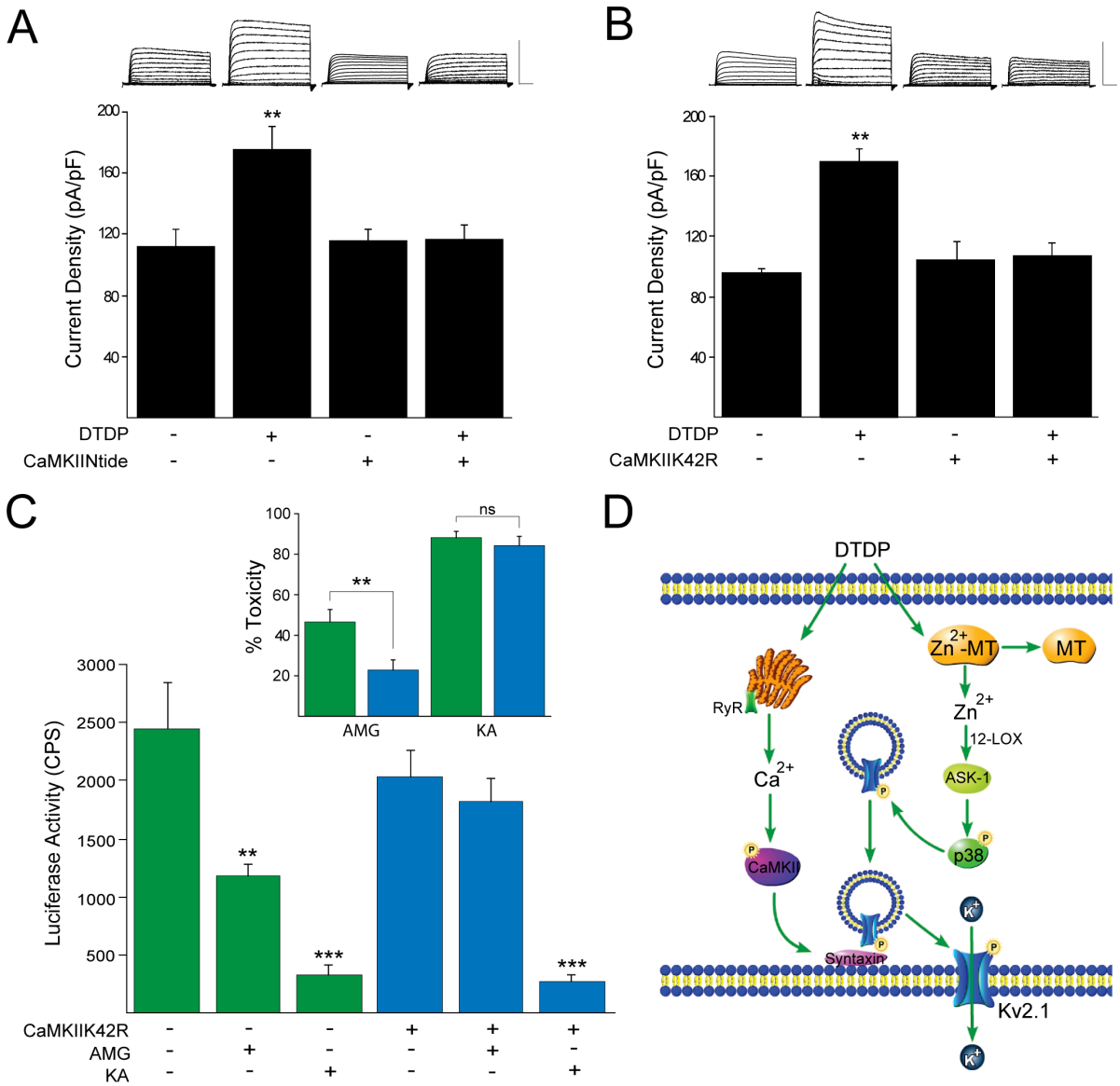

Figure 7: CaMKII inhibition blocks apoptotic K^+ current increase and cell death in neurons

Figure 7: *(A)* Representative whole-cell K^+ currents and corresponding mean ± SEM current densities recorded under control conditions (n=15) or 3 hr after exposure (10 min) to 30 μM DTDP (n=13), 1 μM CaMKIINtide (n=12), or 30 μM DTDP + 1 μM CaMKIINtide (n=14). *(B)* Representative whole-cell K^+ currents and corresponding mean ± SEM current densities recorded from untransfected cells without (n=7), or with 30 μM DTDP (n=8), and CaMKIIK42R-expressing cells without (n=10), or with 30 μM DTDP (n=9). Currents were evoked by a series of voltage steps from -80 mV to +80 mV in 10 mV increments. To determine current density values, steady-state current amplitudes were measured 180 msec after the initiation of the +10 mV step and normalized to cell capacitance. Calibration: 10 nA, 25 ms; **p<0.01; ANOVA/Dunnett. *(C)* CaMKIIK42R-expressing neurons are resistant to an overnight exposure to activated microglia (AMG) when compared to vector-expressing cells. Kainic acid (KA; 1 mM overnight) was used as an index for non-apoptotic, total neuronal kill (mean ± SEM of a single representative experiment performed in quadruplicate. **p<0.01, ***p<0.001, when compared to untreated cells from each group; ANOVA/Bonferroni). *(Inset)* Percent toxicity from either AMG or KA exposure for vector-expressing (green) or CaMKIIK42R-expressing (blue) neurons (mean ± SEM, n=7; **p<0.01; paired t-test). *(D)* Pathway illustrating the convergence of zinc- and calcium-mediated signaling pathways during oxidative neuronal injury. Oxidant exposure leads to simultaneous release of calcium and zinc, with consequent calcium-dependent activation of CaMKII, and zinc-dependent phosphorylation of p38 via a 12-LOX-associated activation of ASK-1 (Zhang et al., 2004; 2007). These parallel pathways then converge to facilitate the association of syntaxin with Kv2.1.

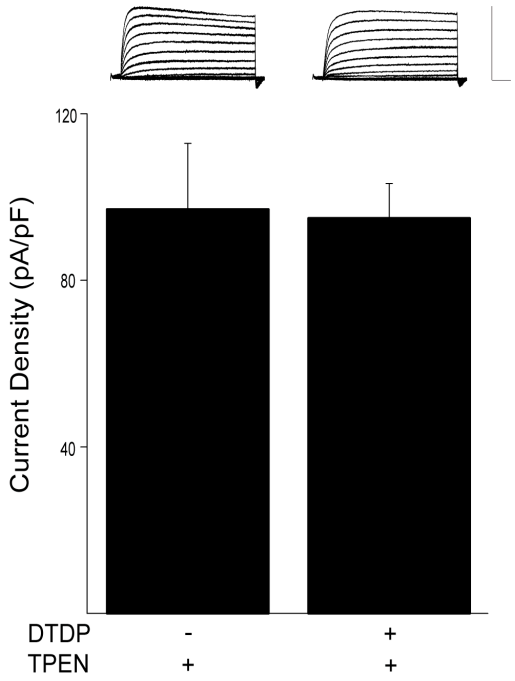

Figure 8: Zinc chelation prevents the DTDP-induced enhancement of K^+ currents in neurons

Figure 8. Representative whole-cell K^+ currents and corresponding mean ± SEM current densities (at +10 mV) recorded after exposure to 3 μM TPEN (n=7) or 3 μM TPEN + 30 μM DTDP (n=9). Currents were evoked by a series of voltage steps from -80 mV to +80 mV in 10-mV increments. Calibration: 10 nA, 25 ms.

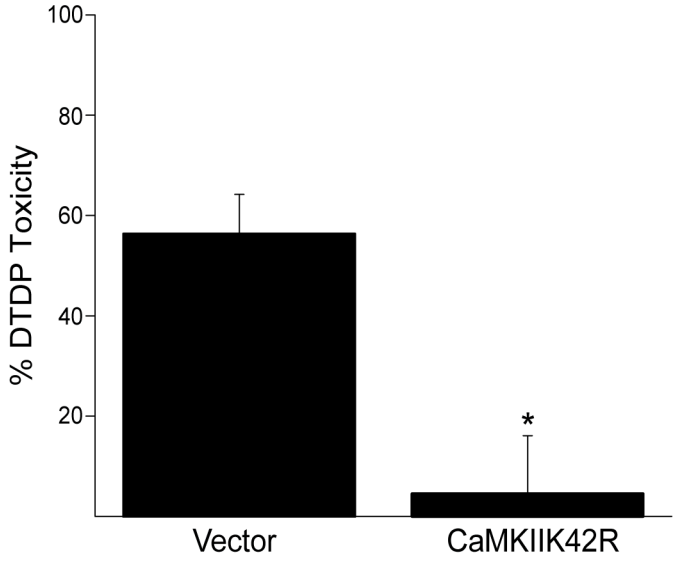

Figure 9: CaMKII inhibition prevents DTDP-induced toxicity in neurons

Figure 9. Neurons transfected with either CaMKIIK42R or an empty vector were exposed to DTDP (60 μM, 10 min) and viability was assessed 24 h following exposure by using a luciferase expression assay (Aras et al., 2008). Mean ± SEM, n=3; *p<0.05; paired t-test.

2.5 DISCUSSION

Defining the specific contributions of calcium and zinc to neuronal injury pathways has been hindered by the fact that most, if not all, calcium-binding agents and indicators also effectively interact with zinc (Grynkiewicz et al., 1985; Stork and Li, 2006; Smith, 2009; Haase et al., 2009). While zinc probes that do not appreciably interfere with calcium-mediated signals exist (Arslan et al., 1985; Tomat and Lippard, 2010; Radford and Lippard, 2013), and simultaneous changes in calcium and zinc have been detected in injured neurons (Sensi et al., 2002; Dietz et al., 2009; Medvedeva et al., 2009; Martin et al., 2011), identifying the specific and separate downstream signaling cascades activated by each cation has remained elusive. Here, the demonstration that intraneuronal calcium rises independently of zinc following an oxidative stimulus, and that calcium-dependent CaMKII activity is necessary for the syntaxin-mediated regulation of apoptotic Kv2.1-mediated K^+ currents, provides a glimpse into the molecular underpinning of what are likely to be widespread dual cation regulatory processes in cells.

CaMKII has been previously implicated in numerous neuronal cell death processes, as inhibition of the kinase is neuroprotective in both *in vitro* and *in vivo* injury models (Lin et al., 2004; Vest et al., 2010; Chen et al., 2011; Liu et al., 2012). Nonetheless, as is the case for many other signaling kinase systems, CaMKII activity is associated with the regulation of both neuroprotective and neurodestructive cellular signaling pathways (Ashpole and Hudmon, 2011; Coultrap et al., 2011). Further, CaMKII is a ubiquitous protein found in high concentrations throughout the brain (Erondu and Kennedy, 1985). As such, inhibiting kinase activity is likely to be a poor candidate approach for translational therapeutic research studies. We show here, however, that CaMKII activity is necessary for the syntaxin-dependent, apoptosis-enabling enhancement of Kv2.1-mediated K^+ current densities following an oxidative insult. CaMKII

likely mediates this process via the direct interaction between the kinase and syntaxin (Figure 4A), a mechanism that may be amenable to manipulation as a novel therapeutic strategy.

Exactly how CaMKII participates in the syntaxin-dependent, apoptotic exocytotic insertion of Kv2.1 channels into the plasma membrane has yet to be resolved. During exocytosis, syntaxin transitions from a closed to an open conformation, which can only occur after the linker domain of the protein is released from its SNARE-binding H3 domain (Dulubova et al., 1999; Misura et al., 2000). Interestingly, not only has CaMKII been shown to bind to the linker domain of syntaxin during neuronal exocytosis, but it is also the only known protein to bind to this region (Ohyama et al., 2002). Based on our co-immunoprecipitation results, we suggest that the enhanced binding of syntaxin to the dual-phosphorylated Kv2.1 is facilitated by the direct interaction of functional CaMKII with syntaxin. Moreover, this process may be promoted by the recruitment of additional CaMKII to the Kv2.1/syntaxin complex, as our results also suggest.

Most Kv2.1 channels are typically found in clusters on the somato-dendritic plasma membrane (Trimmer, 1991). Although the clustered channels may conduct current poorly (O'Connell et al., 2010), these aggregates appear to be the site of surface delivery of additional Kv2.1 protein (Deutsch et al., 2012). Therefore, it will be important to establish in future work whether stimuli that affect channel clustering (Misonou et al., 2005; Mohapatra et al., 2009) can regulate the SNARE-dependent insertion of new channels during apoptosis, as was recently evaluated for a stromal cell-derived chemokine (Shepherd et al., 2012). To aid in the resolution of these issues, the results presented in this study establish calcium and CaMKII as a critical regulator of the injurious, Kv2.1-dependent K^+ current increase in neurons. Importantly, we also identify an unprecedented cooperative convergence of zinc- and calcium-mediated signaling pathways during oxidative neuronal cell death.

3.0 SYNTAXIN-BINDING DOMAIN OF KV2.1 IS ESSENTIAL FOR THE EXPRESSION OF APOPTOTIC POTASSIUM CURRENTS

3.1 ABSTRACT

Intracellular signaling cascades triggered by oxidative injury can lead to upregulation of Kv2.1 K^+ channels at the plasma membrane of dying neurons. Membrane incorporation of new channels is necessary for enhanced K^+ efflux and the consequent reduction of intracellular K^+, which facilitates apoptosis. We showed previously that the observed increase in K^+ currents is a SNARE-mediated process, and that the SNARE protein syntaxin binds directly to Kv2.1 channels. In the present study, we tested whether disrupting the interaction of Kv2.1 and syntaxin promoted the survival of cortical neurons following injury. Syntaxin is known to bind to Kv2.1 in a domain comprised of amino acids 411-522 of the channel's cytoplasmic C-terminus (C1a). Here, we show that this domain is required for the apoptotic K^+ current enhancement. Moreover, expression of an isolated Kv2.1-derived C1a peptide is sufficient to suppress the injury-induced increase in currents by interfering with Kv2.1/syntaxin binding. By sub-dividing the C1a peptide, we were able to localize the syntaxin binding site on Kv2.1 to the most plasma membrane-distal residues of C1a. Importantly, expression of this peptide segment in neurons prevented the apoptotic K^+ current enhancement and cell death following an oxidative insult, without largely impairing baseline K^+ currents or normal electrical profiles of neurons. These

results establish that binding of syntaxin to Kv2.1 is crucial for the manifestation of oxidant-induced apoptosis, and thereby suggest a potential new direction for therapeutic intervention in the treatment of neurodegenerative disorders.

3.2 INTRODUCTION

Potassium is the most abundant intracellular cation in neurons. It is essential for maintaining the resting membrane potential and also for regulating cellular volume (Pasantes-Morales et al., 1993; Yu, 2003). During injury, K^+ efflux and intracellular K^+ loss critically contribute to the apoptotic volume decrease (Bortner et al., 1997; Maeno et al., 2000), a hallmark morphological feature of programmed cell death (Kerr et al., 1972). In addition, reduced cytosolic K^+ enables apoptosis by providing a permissive environment for activation of caspases and nucleases (Hughes et al., 1997; Yu et al., 1997). Kv2.1, a voltage-dependent delayed-rectifier K^+ channel normally involved in the regulation of high-frequency repetitive firing (Pongs, 1999; Du et al., 2000; Guan et al., 2013), acts as the primary conduit for K^+ efflux during apoptotic cell death in neocortical and hippocampal neurons (Pal et al., 2003; Shen et al., 2009). This process occurs via the N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent exocytotic incorporation of new Kv2.1-encoded channels into the plasma membrane of dying neurons, measurable as a large enhancement of voltage-dependent K^+ currents (McLaughlin et al., 2001; Pal et al., 2003, 2006). Importantly, inhibiting any of the upstream signaling events leading to the insertion of Kv2.1, or blocking Kv2.1 channel function itself, is neuroprotective (Yu et al., 1997; McLaughlin et al., 2001; Pal et al., 2003; Aras and Aizenman, 2011).

The SNARE protein syntaxin was previously shown to interact with the most proximal region of the Kv2.1 C-terminus, termed C1a, in a process regulating dense-core vesicle-mediated exocytosis in PC12 cells and dorsal root ganglion (DRG) neurons (Singer-Lahat et al., 2007, 2008; Feinshreiber et al., 2010). Further, introduction of an isolated Kv2.1-derived C1a peptide disrupted Kv2.1/syntaxin binding and inhibited the physiological consequences of this interaction (Singer-Lahat et al., 2007). We recently demonstrated that the association between Kv2.1 and syntaxin is enhanced under conditions that lead to apoptosis (McCord and Aizenman, 2013), suggesting that interfering with this interaction may provide a novel neuroprotective therapeutic strategy. In this study we evaluate *(1)* whether the syntaxin-binding domain of Kv2.1 is necessary for the apoptotic K^+ current enhancement, and *(2)* if introduction of C1a or sub-domains of the peptide can prevent the increase in K^+ currents and be neuroprotective *in vitro*.

3.3 MATERIALS AND METHODS

Cell Culture and Transfection Procedures. For electrophysiological experiments, mixed cortical neuronal/glial cultures were prepared from embryonic day 16 (E16) Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA). Cortices were dissociated with trypsin, and the resultant cell suspension, adjusted to 670,000 cells per well, was plated on glass coverslips in 6-well plates as described previously (Hartnett et al., 1997). Non-neuronal cell proliferation was inhibited after two weeks in culture with 1–2 μM cytosine arabinoside, and cultures utilized at three to four weeks *in vitro*. For neuronal transfection, cells were treated for five hours in serum-free medium with 2 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA), 50 μL Optimem (GIBCO, Grand Island, NY), and 1.5 μg DNA per well. For Chinese hamster ovary

(CHO) cell transfections, cells were plated on coverslips in 24-well plates at a density of 5.6 x 10^4 cells per well for electrophysiological measurements or in 100 mm dishes at a density of 1.7 x 10^6 cells per dish for biochemical studies. Cells were treated for 3-4 hours in serum-free medium with a total of 1.2 μL Lipofectamine and 0.28 μg DNA per well for electrophysiology, or 55.1 μL Lipofectamine and 7.33 μg DNA per dish for biochemistry. Following transfection, cells were maintained in F12 medium containing fetal bovine serum at 37°C, 5% CO_2 for 24 hours prior to experimentation.

Drug Treatments and Antibodies. The apoptotic stimulus for electrophysiological experiments was 10 minute exposure to 30 μM 2,2'-dithiodipyridine (DTDP) at 37°C, 5% CO_2. The DTDP-containing solution was removed prior to three-hour incubation in fresh medium containing 10 μM butoxy-carbonyl-aspartate-fluoromethyl ketone (BAF), a cysteine protease inhibitor that maintains cell viability without affecting K^+ currents. Antibodies were purchased from the following suppliers: rabbit anti-syntaxin from Abcam (Cambridge, MA); mouse anti-syntaxin from Millipore (Temecula, CA); mouse anti-Kv2.1 from NeuroMab (Davis, CA).

Electrophysiological Measurements. Current recordings were performed on eGFP-positive co-transfected neurons or CHO cells using the whole-cell patch clamp configuration technique as described previously (McLaughlin et al., 2001). The intracellular (electrode) solution contained (in mM): 100 K-gluconate, 11 EGTA, 10 KCl, 1 $MgCl_2$, 1 $CaCl_2$ x $2H_2O$, 10 HEPES; pH adjusted to 7.2 with concentrated KOH; 2.2 ATP and 0.33 GTP were added and the osmolarity was adjusted to 280 mOsm with sucrose. The extracellular solution contained (in mM): 115 NaCl, 2.5 KCl, 2.0 $MgCl_2$, 10 HEPES, 10 D-glucose; 0.25 μM tetrodotoxin; pH adjusted to 7.2. Measurements were obtained under whole-cell voltage clamp with an Axopatch-1D amplifier and pClamp software (Molecular Devices, Sunnyvale, CA), using 2-3 MΩ

recording electrodes. Electrodes were pulled from 1.5 mm borosilicate glass (Warner Instruments, Hamden, CT) with a model P-97 mechanical pipette puller (Sutter Instruments, Novato, CA). Series resistance was partially compensated (80%) in all cases. Currents were filtered at 2 kHz and digitized at 10 kHz with a Digidata 1440A Digitizer (Molecular Devices). K^+ currents were evoked with incremental 10 mV voltage steps to +80 mV from a holding potential of –80 mV. To determine current density values, steady-state current amplitudes were measured 180 msec after the initiation of the +10 mV step and normalized to cell capacitance. For current clamp experiments, the intracellular (electrode) solution contained (in mM): 94 K-gluconate, 30 KCl, 10 phosphocreatine di-tris salt, 10 HEPES, 0.2 EGTA, 4 ATP, 0.3 GTP; pH adjusted to 7.3 with concentrated KOH. The extracellular solution contained (in mM): 146 NaCl, 7.8 glucose, 20 HEPES, 4.7 KCl, 0.6 $MgSO_4$, 1.6 $NaHCO_3$, 0.13 NaH_2PO_4, 2.5 $CaCl_2$; pH adjusted to 7.3 with concentrated KOH. Measurements were performed at room temperature with an Axoclamp 2B amplifier and G-clamp 2.2 software (Kullmann et al., 2004). Voltage responses were induced with 1 s current steps ranging from − 120 pA to 120 pA. Virtual excitatory synapses were implemented under dynamic clamp according to $g_{syn}(t)=k*t*\exp(-t / tau)$ with tau=1.5 ms and E_{rev}=0 mV. By repeatedly adjusting the scaling factor k, an automated binary search algorithm determined threshold synaptic conductance (thresh-g_{syn}), the conductance required to reach firing threshold (Kullmann et al., 2004).

Immunoprecipitation. Protein was harvested by washing cell culture dishes with ice cold PBS followed by a short incubation with 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) buffer. Protein A/G agarose bead slurry (Santa Cruz, Dallas, TX) was added to the samples and rocked at 4°C for 1 hour in order to preclear non-specific protein binding. Samples were then incubated overnight at 4°C with the appropriate

immunoprecipitating antibodies. Following another incubation with the bead slurry, the protein samples were prepared/denatured by the addition of sample preparation buffer (625 mM Tris, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% β-mercaptoethanol) and heated at 95°C for 5 min prior to SDS-PAGE gel electrophoresis and immunoblotting.

Electrophoresis and Immunoblotting. Protein samples from equal amounts of cell lysate were separated on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels by electrophoresis using the Mini Protean 3 System (Bio-Rad, Hercules, CA). Separated protein bands were transferred onto 0.2 μm nitrocellulose membranes (Bio-Rad), blocked with 1% Bovine Serum Albumin (BSA) in PBS with 0.05% Tween 20 (PBST) at room temperature for 1 hour, and probed with appropriate primary antibodies diluted in PBST. Blots were then incubated with infrared fluorescent goat secondary antibodies at room temperature for 1 hour, visualized using the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE), and quantified using infrared fluorimetry.

Viability Assays. Viability of transfected neurons was assessed by cell counts of GFP+ cells. Twenty-four hours after transfection with eGFP in the absence or presence of $C1a_{441-522}$, cultures were treated overnight with either vehicle or microglia (50,000 cells/mL) (Cheepsunthorn et al., 2001) plated directly on top of neurons and activated by interferon-γ (IFN-γ) and lipopolysaccharide (LPS) (Knoch et al., 2008). The following day, cells were preserved using 4% paraformaldehyde as reported previously (Aras et al., 2008). GFP+ cells were counted in 20 random areas per coverslip using a 20X objective. Coverslips were chosen at random by the person performing the cell counts. Experiments were performed in three separate culture dates, with three coverslips per condition, per experiment.

3.4 RESULTS

3.4.1 Syntaxin-interacting domain of Kv2.1 is required for apoptotic K^+ enhancement

The most proximal ~110 amino acids of the C-terminus of Kv2.1, corresponding to amino acids 411-522 of the rat protein sequence, contain the channel's syntaxin binding site (C1a; Tsuk et al., 2005). Previous studies in DRG neurons and *Xenopus* oocytes demonstrated that overexpression of a Kv2.1 channel mutant lacking its C1a domain (Kv2.1ΔC1a) results in a marked reduction of co-immunoprecipitation between syntaxin and Kv2.1, as well as an inhibition of Kv2.1-mediated exocytosis (Singer-Lahat et al., 2007; Feinshreiber et al., 2010). As syntaxin is also involved in the apoptotic regulation of Kv2.1-mediated K^+ currents (Pal et al., 2006), we first investigated whether the C1a region of Kv2.1 was required for this process. To explore this possibility, we utilized Chinese hamster ovary (CHO) cells, a recombinant expression system that lacks endogenous voltage-gated K^+ channels (Yu and Kerchner, 1998), yet has all the cell signaling components required to produce an apoptotic current enhancement in Kv2.1-transfected cells (Pal et al., 2003). CHO cells expressing either wild-type (WT) Kv2.1, Kv2.1ΔC1a, or a 1:1 ratio of Kv2.1 and Kv2.1ΔC1a were subjected to a 10-minute exposure to 30 μM 2,2'-dithiodipyridine (DTDP), a thiol oxidant that initiates a zinc- and calcium-dependent signaling cascade responsible for the enhancement of Kv2.1 K^+ currents observed during apoptosis (McLaughlin et al., 2001; McCord and Aizenman, 2013). DTDP treatment was followed by 3-hour maintenance in butoxy-carbonyl-aspartate-fluoromethyl ketone (BAF; 10 μM), a broad-spectrum protease inhibitor used during recording for its ability to prevent apoptosis downstream of the K^+ current enhancement (McLaughlin et al., 2001). We found that the DTDP-induced increase in K^+ currents observed in Kv2.1-expressing CHO cells was absent in cells expressing Kv2.1ΔC1a,

suggesting that syntaxin binding to Kv2.1 is an integral component of the machinery mediating insertion of new channels into the plasma membrane during oxidative injury. Further, cells transfected with a 1:1 ratio of Kv2.1 and Kv2.1ΔC1a plasmids were also refractory to the actions of DTDP, demonstrating that even when WT Kv2.1 is present, expression of the mutant channel is sufficient to block the effect of the oxidant on the apoptotic increase in Kv2.1-mediated K^+ currents (Figure 10A). Importantly, cells expressing the mutant channel exhibited K^+ currents that appeared indistinguishable from WT Kv2.1-mediated currents (Figure 10B), indicating that normal trafficking and expression of this channel is not dependent on the cytoplasmic C1a region, as shown previously (Singer-Lahat et al., 2007).

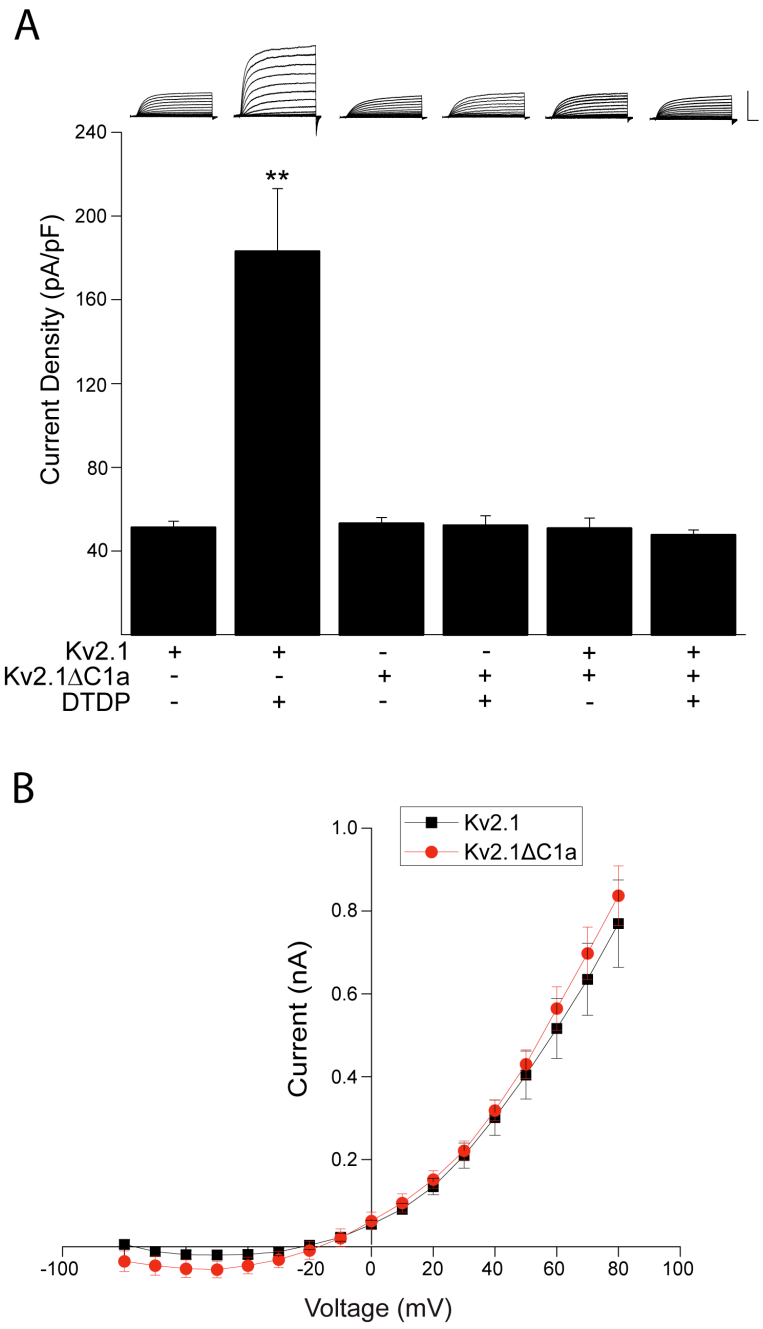

Figure 10: The syntaxin-binding C1a domain of Kv2.1 is required for expression of the apoptotic K^+ current enhancement

Figure 10. *(A)* Representative whole-cell K^+ currents and pooled mean ± SEM current densities recorded from Kv2.1-expressing CHO cells without (n=19) or with (n=11) 30 μM DTDP, Kv2.1ΔC1a-expressing CHO cells without (n=10) or with (n=10) 30 μM DTDP, or CHO cells expressing a 1:1 ratio of Kv2.1 and Kv2.1ΔC1a without (n=9) or with (n=16) 30 μM DTDP. Results show that the increase in K^+ currents triggered by DTDP is suppressed in CHO cells expressing Kv2.1ΔC1a. Currents were evoked by a series voltage steps from -80 mV to +80 mV in 10 mV increments. To determine current density values, steady-state current amplitudes were measured 180 msec after the initiation of the +10 mV step and normalized to cell capacitance. Scale bars: 3 nA, 25 msec; **p<0.01, ANOVA/Dunnett. *(B)* Mean ± SEM IV curves of CHO cells expressing Kv2.1 or Kv2.1ΔC1a show that upon depolarization, Kv2.1 mutants lacking their C1a domain exhibit similar K^+ currents as WT Kv2.1 channels.

3.4.2 Kv2.1-derived syntaxin-binding peptide blocks oxidant-induced increase in K^+ currents

Incubation with a Kv2.1-derived GST-fusion C1a peptide prevents the binding of syntaxin to endogenous Kv2.1 in PC12 cells (Singer-Lahat et al., 2008). Thus, we investigated whether expression of C1a in cortical neurons was sufficient to prevent the increase in Kv2.1 K^+ currents triggered by exposure to an oxidative insult. We found that unlike cells expressing an empty vector, those transfected with a C1a-expressing plasmid failed to exhibit the enhanced K^+ current densities normally elicited by DTDP (Figure 11A). Notably, C1a had no effect on non-apoptotic, basal delayed-rectifier K^+ currents (Figure 11B), again suggesting that the interaction of syntaxin with the C1a region of native Kv2.1 channels is important for their apoptotic regulation, but may not be critical for their steady state expression.

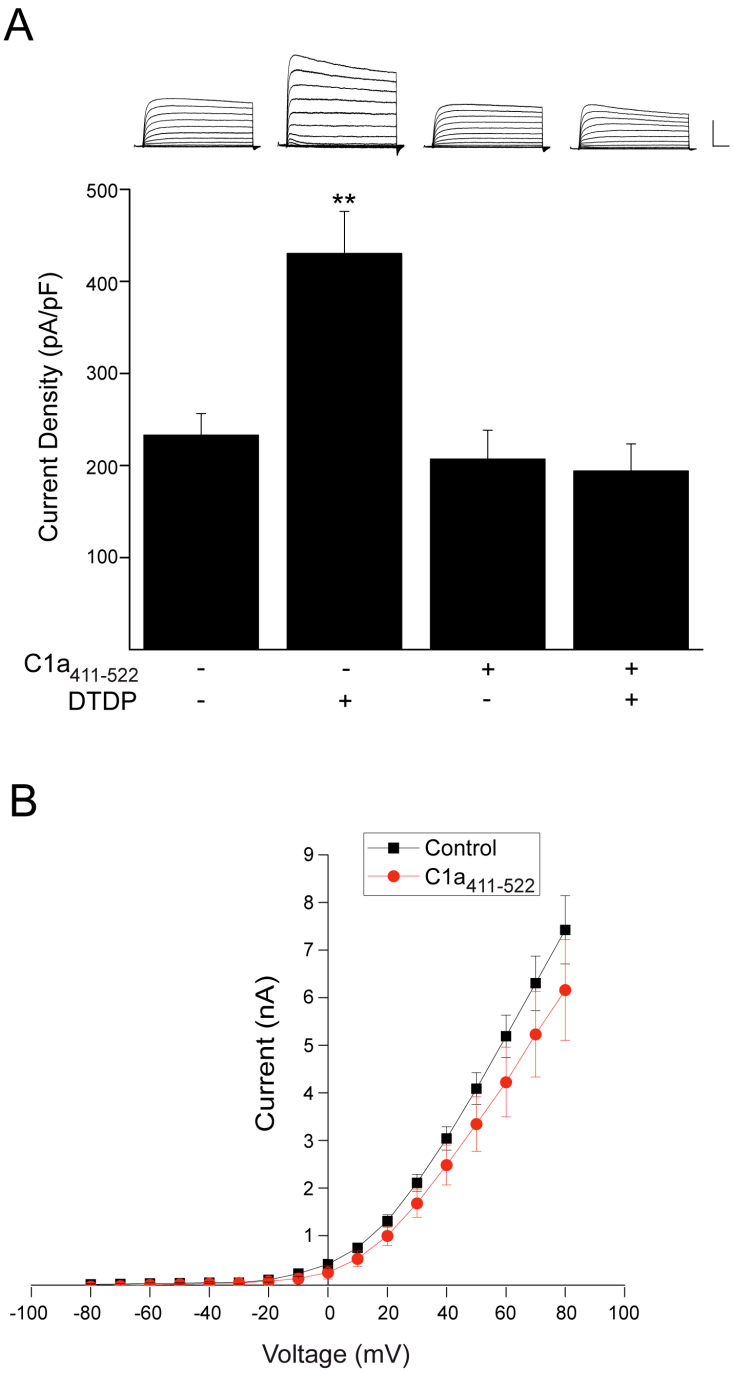

Figure 11: C1a expression blocks the increase in oxidant-induced K^+ currents normally observed following DTDP exposure

Figure 11. *(A)* Representative whole-cell K^+ currents and pooled mean ± SEM current densities recorded from untransfected neurons without (n=10) or with (n=8) 30 μM DTDP, and from C1a-expressing neurons without (n=8) or with (n=9) 30 μM DTDP. Currents were evoked by a series voltage steps from -80 mV to +80 mV in 10 mV increments. To determine current density values, steady-state current amplitudes were measured 180 msec after the initiation of the +10 mV step and normalized to cell capacitance. Scale bars: 5 nA, 25 msec; **$p<0.01$, ANOVA/Dunnett. *(B)* Mean ± SEM IV curves of untransfected and C1a-expressing neurons demonstrate that the peptide does not significantly alter K^+ currents when compared to control cells.

3.4.3 Refining the syntaxin-interacting region within C1a

After establishing that C1a effectively precluded the oxidant-induced K^+ current increase in neurons, we sought to identify the sub-domains within C1a that could retain the ability to disrupt Kv2.1/syntaxin binding. We first divided C1a into overlapping proximal and distal segments, hereafter referred to as $C1a_{411\text{-}472}$ and $C1a_{441\text{-}522}$, respectively. We then performed co-immunoprecipitation experiments to compare the ability of C1a and the smaller segments to displace Kv2.1 from syntaxin. Syntaxin immunoprecipitates of protein samples obtained from CHO cells transfected with a syntaxin-expressing plasmid, and co-transfected with Kv2.1 together with C1a, $C1a_{411\text{-}472}$, or $C1a_{441\text{-}522}$, were probed for Kv2.1. We found that both C1a and the distal segment, $C1a_{441\text{-}522}$, inhibited Kv2.1/syntaxin binding, while the proximal segment, $C1a_{411\text{-}472}$, did not (Figure 12A). These results reveal that syntaxin binds to Kv2.1 in the more distal portion of the C1a domain of the channel's C-terminus. In subsequent experiments, we found that similar to $C1a_{411\text{-}472}$, the overlapping middle segment, $C1a_{441\text{-}472}$, had no effect on syntaxin's ability to bind to Kv2.1 (mean ± SEM ratio of Kv2.1 to syntaxin: baseline=0.24±0.08, $C1a_{441\text{-}472}$=0.25±0.1; n=3), suggesting that the syntaxin binding site on Kv2.1 is most likely found within residues 472-522 of C1a.

We previously showed that in CHO cells, overexpression of Src kinase, which induces phosphorylation of two Kv2.1 residues required for apoptosis, enhances the binding of Kv2.1 to syntaxin (McCord and Aizenman, 2013). Therefore, we evaluated whether $C1a_{441\text{-}522}$ also retained the ability to displace Kv2.1 from syntaxin under these conditions, which was indeed the case (Figure 12B).

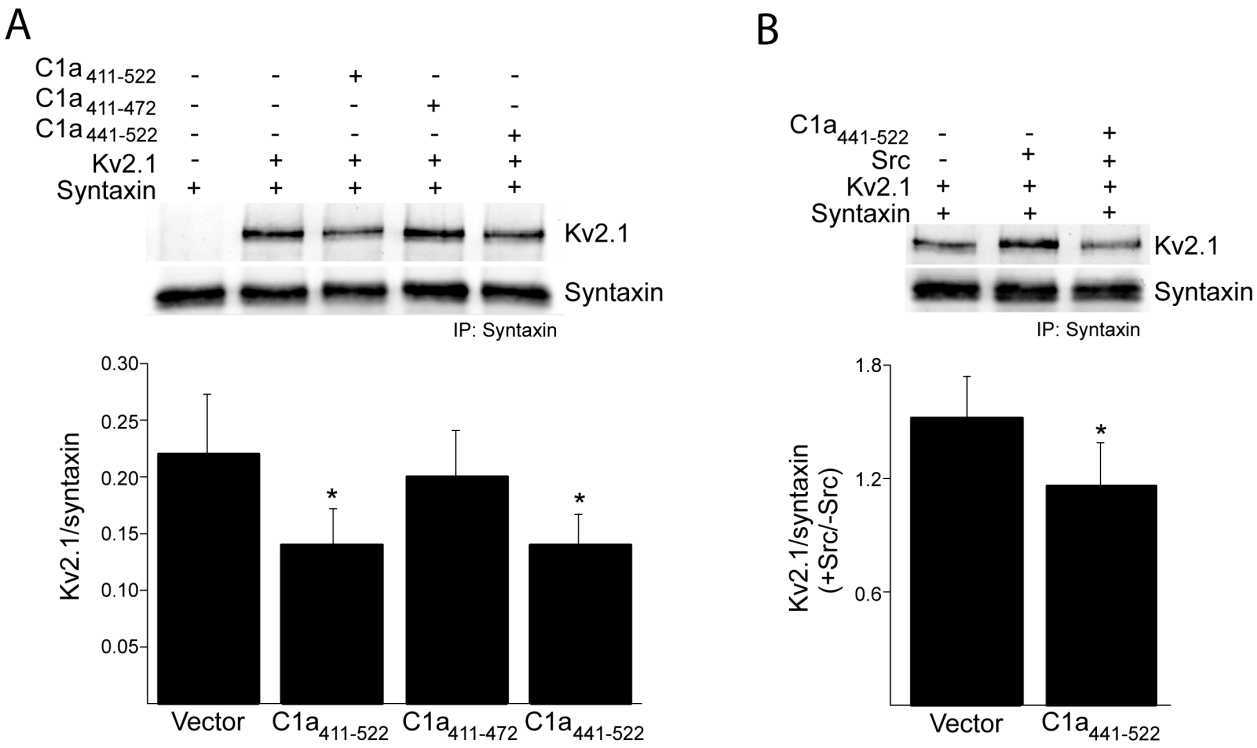

Figure 12: Kv2.1/syntaxin binding is disrupted by C1a peptides

Figure 12. *(A)* Representative co-immunoprecipitation and quantified results from syntaxin-expressing CHO cells co-transfected with Kv2.1, in addition to C1a, $C1a_{411-472}$, or $C1a_{441-522}$. Following syntaxin immunoprecipitation, blots were probed with antibodies against Kv2.1 or syntaxin (n=6; *p<0.05, ANOVA/Bonferroni). *(B)* $C1a_{441-522}$ prevents Kv2.1/syntaxin binding in the presence of Src kinase, which was previously shown to enhance this interaction (McCord and Aizenman, 2013). Representative co-immunoprecipitation and quantified results from syntaxin-expressing CHO cells co-transfected with Kv2.1 and Src in the absence or presence of $C1a_{441-522}$. Following syntaxin immunoprecipitation, blots were probed with antibodies against Kv2.1 or syntaxin (n=6; *p<0.05, paired t-test).

3.4.4 $C1a_{441-522}$ blocks K^+ current enhancement in CHO cells and is neuroprotective

Once we had established that both C1a and $C1a_{441-522}$ were able to disrupt binding of syntaxin to Kv2.1, we evaluated whether expression of C1a and $C1a_{441-522}$ was also able to prevent the apoptotic K^+ current enhancement. To investigate this, we used CHO cells expressing a pseudo-phosphorylated Kv2.1 channel mutant, Kv2.1S800E, which exhibits an innate, phosphorylation-independent increase in K^+ current densities that mimics the enhanced currents observed in apoptogen-exposed, WT Kv2.1-expressing cells (Redman et al., 2007). CHO cells were transfected with WT Kv2.1 or Kv2.1S800E in the absence or presence of either C1a or $C1a_{441-522}$. Similar to what was observed in DTDP-treated, C1a-expressing neurons, co-transfection with C1a suppressed the enhanced K^+ currents observed in Kv2.1S800E-expressing CHO cells. Importantly, $C1a_{441-522}$ also blocked the increase in currents produced by the S800E channel mutant (Figure 13), further indicating that syntaxin binds to Kv2.1 within the most distal region of C1a.

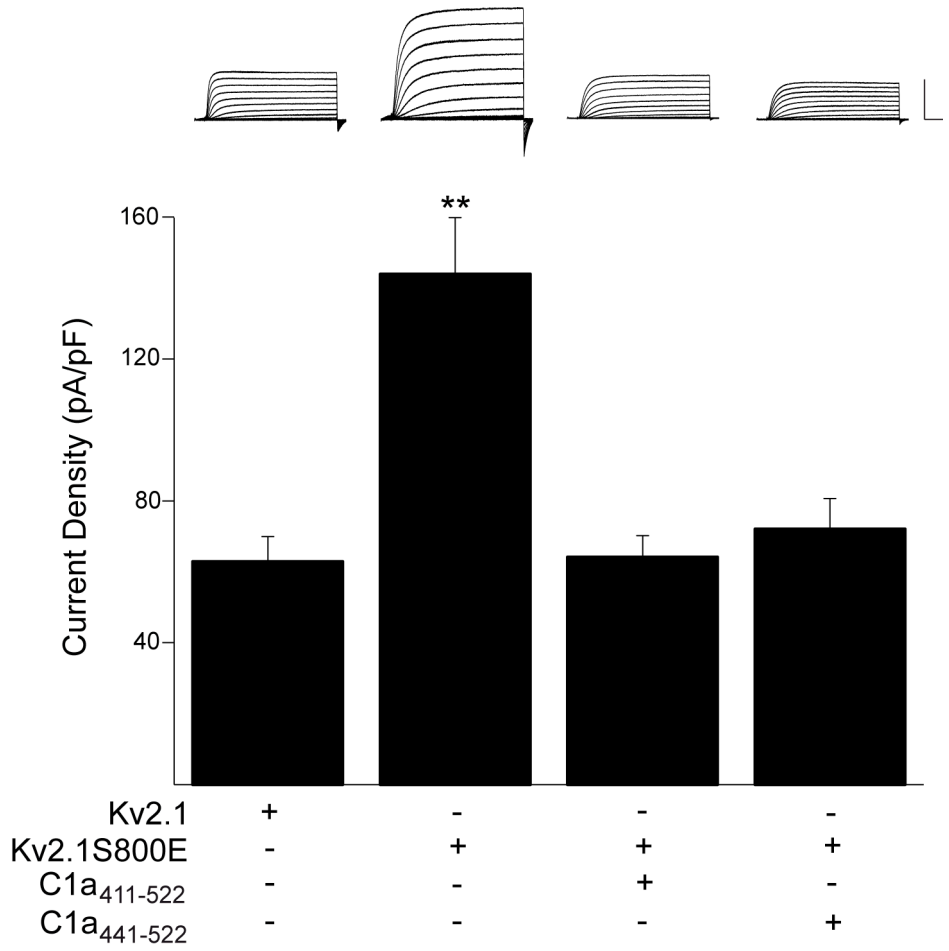

Figure 13: C1a and C1a$_{441-522}$ block K$^+$ current enhancement in CHO cells

Figure 13. Representative whole-cell K^+ currents and pooled mean ± SEM current densities recorded from CHO cells expressing Kv2.1 (n=14), Kv2.1S800E (n=11), Kv2.1S800E + C1a (n=16) or Kv2.1S800E + C1a$_{441-522}$ (n=14). Currents were evoked by a series voltage steps from -80 mV to +80 mV in 10 mV increments. To determine current density values, steady-state current amplitudes were measured 180 msec after the initiation of the +10 mV step and normalized to cell capacitance. Scale bars: 5 nA, 25 msec; **p<0.01, ANOVA/Dunnett.

We next evaluated whether $C1a_{441-522}$ was sufficient to protect neurons against oxidative injury. Cells expressing $C1a_{441-522}$ or an empty vector were exposed to activated microglia, a known inducer of Kv2.1-dependent neuronal cell death via peroxynitrite production and intraneuronal zinc liberation (Knoch et al., 2008). While microglia reduced viability of vector-expressing cells by nearly 40%, there was no detrimental effect on neurons expressing $C1a_{441-522}$ (Figure 14). Finally, in a series of current-clamp experiments, we found that vector- and $C1a_{441-522}$-expressing neurons showed no differences in resting membrane potential, input resistance, or threshold synaptic conductance (Figure 15A). We did, however, observe a small but significant increase in firing frequency as a function of depolarizing current in $C1a_{441-522}$-expressing neurons, suggesting that $C1a_{441-522}$ may have a minimal effect on a neuron's propensity for firing action potentials (Figure 15B). Overall, however, the expression of $C1a_{441-522}$ was relatively innocuous to neurons. Taken together, these findings suggest that targeting the interaction between Kv2.1 and syntaxin may offer a novel therapeutic strategy in the prevention of neurodegeneration that, at least under relatively acute conditions, does not substantially affect neuronal electrophysiological properties.

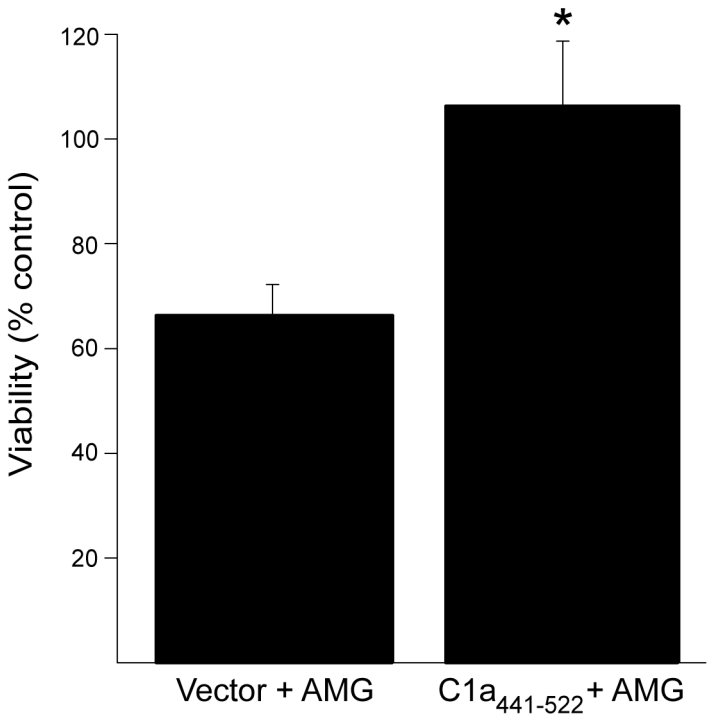

Figure 14: C1a$_{441-522}$ protects against apoptotic cell death in cortical neurons

Figure 14. Cell counts reveal that unlike vector-expressing neurons, those expressing C1a$_{441-522}$ are resistant to overnight exposure to activated microglia, a known inducer of Kv2.1-dependent neuronal cell death (Knoch et al., 2008). Mean ± SEM counts of GFP+ cells from three independent experiments; *$p<0.05$, paired t-test.

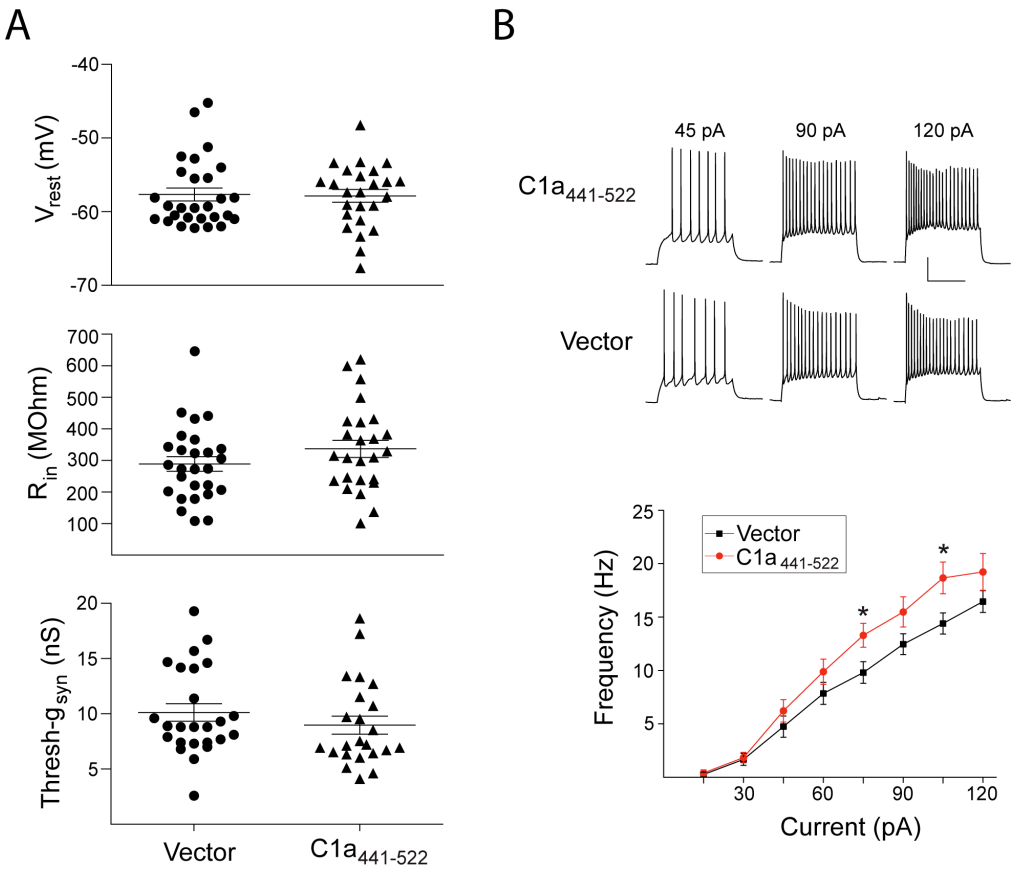

Figure 15: C1a$_{441-522}$ has minimal effect on electrical properties of neurons

Figure 15. *(A)* Resting membrane potential (Vm) and input resistance (R_{in}) were measured under current clamp, and threshold synaptic conductance (Thresh-g_{syn}) was measured under dynamic clamp in neurons expressing an empty vector or C1a$_{441-522}$ (mean ± SEM; n=23-28 cells per group). *(B)* Repetitive firing was recorded in vector- or C1a$_{441-522}$-expressing neurons using 1 sec depolarization current steps of increasing amplitude from 15-120 pA. Representative traces of neurons from each group at three depolarizing currents, and mean ± SEM frequency-current relationships at eight separate currents (*$p<0.05$, t-test between vector- and C1a$_{441-522}$-expressing neurons; n=23-28 cells per group. Scale bars: 20 mV, 500 msec). Experiments completed by Paul Kullmann, PhD.

3.5 DISCUSSION

The findings presented here characterize a cytoplasmic domain within the delayed-rectifier Kv2.1-encoded K^+ channel that is critical for the expression of neuronal apoptosis. We show that Kv2.1 enables cell death through its direct interaction with syntaxin, which promotes the enhancement of K^+ currents and cytoplasmic K^+ loss normally observed following apoptotic injury (Bortner et al., 1997; Yu et al., 1997; Pal et al., 2003). Importantly, disrupting Kv2.1/syntaxin binding using a plasmid encoding the cytoplasmic Kv2.1 domain $C1a_{441\text{-}522}$ prevented the K^+ current enhancement and was neuroprotective without substantially affecting basal K^+ currents or the electrical behavior of cortical neurons. These results suggest that the processes underlying the membrane insertion of Kv2.1 during apoptosis (Pal et al., 2006) may be distinct from channel trafficking under non-injurious conditions.

Kv2.1 channels have historically been recognized as regulators of neuronal membrane potential due to their ability to conduct K^+, especially during periods of high-frequency firing (Du et al., 2000; Guan et al., 2013). However, it is now becoming evident that these channels actually play a much more widespread role in cells than initially thought. In various cell types including PC12s, pancreatic β-cells, and DRG neurons, Kv2.1 has been shown to facilitate exocytosis in a process that occurs independently of the channel's ability to conduct K^+ (Singer-Lahat et al., 2007; Feinshreiber et al., 2010; Dai et al., 2012). Further, a direct association between syntaxin and the C1a domain of the channel regulated Kv2.1 in its exocytosis-associated function, and disrupting this interaction using the C1a peptide inhibited release (Singer-Lahat et al., 2007, 2008). Together with our findings, these results reveal that Kv2.1/syntaxin binding is a dynamic, highly regulated process, the consequence of which is dictated by the cellular context and function for which the channels are required.

Other lines of evidence also support a context-dependent role of Kv2.1. In neurons and transfected HEK293 cells, Kv2.1 channels residing in the plasma membrane have been shown to localize either to dense, ~2 micron-sized clusters, or to a non-clustered formation spread more diffusely across the cell surface (Trimmer, 1991; Tamkun et al., 2007; O'Connell et al., 2010). Interestingly, in addition to having distinct localizations, these two populations of channels also have unique functions. Specifically, it was shown that channels in a clustered pattern may not actually conduct K^+, and that it is the unclustered population that is responsible for the measureable K^+ currents observed in electrophysiological recordings (O'Connell et al., 2010). More recently, the ability to conduct of Kv2.1 to K^+ was shown to negatively correlate with plasma membrane channel density regardless of channel localization, although this study was performed in a recombinant expression system, and this correlation has not yet been confirmed in neurons (Fox et al., 2013). Instead, the clustered channels act as a platform for exo- and endocytosis of new Kv2.1 channels. Moreover, it was reported that in addition to Kv2.1, another member of the Kv channel family, Kv1.4, was also exocytosed within Kv2.1 clusters, suggesting that these regions may act as specialized membrane insertion centers for a number of proteins (Deutsch et al., 2012). While these findings lend further support for a role of Kv2.1 in exocytosis, work to determine the specific contribution of SNAREs in this setting has yet to be undertaken.

Given the multifaceted nature of Kv2.1 channels in various cellular settings, the question now arises as to which of these different functions underlie the pro-apoptotic insertion of channels during oxidative injury. It appears that in this capacity, Kv2.1 may function both as a facilitator of channel exocytosis and as an ion conductor. Namely, since clustered channels were shown to be a platform for insertion (Deutsch et al., 2012), it is possible that during apoptosis,

pre-existing, clustered channels are responsible for promoting the exocytosis of new Kv2.1 channels, with the newly inserted channels then generating the enhanced K^+ currents observed during cell death. Thus, if syntaxin binds to the clustered population of channels regulating exocytosis, it is possible that during apoptosis, in addition to preventing the insertion of new Kv2.1 channels, disrupting Kv2.1/syntaxin binding with $C1a_{441-522}$ could also potentially hinder exocytosis of other proteins such as Kv1.4. We did observe a small but significant increase in firing frequency as a function of current in $C1a_{441-522}$-transfected neurons, suggesting that $C1a_{441-522}$ may, to a minimal extent, affect normal trafficking of Kv2.1 or other Kv channels involved in regulating excitability. However, the fact that $C1a_{441-522}$ had no effect on basal K^+ currents, resting membrane potential, input resistance, or threshold synaptic conductance, and that $C1a_{441-522}$-expressing neurons remained viable, indicates that the peptide does not adversely affect normal Kv2.1-mediated processes. Moreover, previous studies have demonstrated that in addition to the C1a domain, secondary syntaxin binding sites may also exist on Kv2.1 (Leung et al., 2003), suggesting that SNARE-dependent processes may be in place even without C1a. While further studies are required to fully elucidate the role of Kv2.1/syntaxin binding in oxidative neuronal injury, the ability of $C1a_{441-522}$ to render neurons completely viable in the face of a lethal injurious stimulus offers a promising new approach to the treatment of neurological conditions associated with apoptotic injury.

4.0 GENERAL DISCUSSION

Although a number of studies have reported a simultaneous increase in intracellular zinc and calcium during neuronal injury, whether the metals work in concert to mediate cell death had heretofore remained unclear (Sensi et al., 2002; Vander Jagt et al., 2009; Medvedeva et al., 2009). As dyshomeostasis of either calcium or zinc can have extremely adverse effects on neurons (Choi, 1995; Sensi et al., 2011), advancing our understanding of how these two cations cooperatively regulate neuronal injury could lead to the development of strategies to halt the toxic signaling cascades triggered by the metals before neurons succumb to apoptosis. The findings presented in this dissertation establish a role for calcium and its downstream target CaMKII in a previously characterized zinc- and Kv2.1-dependent apoptosis pathway (Aras and Aizenman, 2011). Namely, CaMKII was shown to be indispensible for the SNARE-mediated plasma membrane insertion of Kv2.1; however, several questions remain regarding the precise details underlying the action of the kinase, which will be addressed below. Additionally, this work also serves to enhance our knowledge of the mechanism responsible for the plasma membrane insertion of Kv2.1 channels requisite for neuronal apoptosis. As injurious K^+ efflux during apoptosis in cortical neurons occurs primarily through Kv2.1 (Pal et al., 2003), identifying techniques to selectively inhibit the pro-apoptotic function of these channels could provide a novel opportunity for the prevention of cell death during disorders related to oxidative stress. However, as will be discussed here, the physiological and pathophysiological roles of

neuronal Kv2.1 appear to be much more complex than initially realized. This discussion will, in part, attempt to unify studies describing the disparate putative cellular roles of Kv2.1, primarily during neuronal injury, in an attempt to convey the notion that Kv2.1 exists along a spectrum of specific functional roles dictated by the particular status of the channel (e.g. localization, phosphorylation, etc.), temporal profile of injury and overall cellular environment, as well as the precise upstream signals that converge on the channel.

4.1 ROLE OF CAMKII IN OXIDATIVE NEURONAL INJURY

CaMKII is a ubiquitously expressed protein kinase represented by four isoforms: α, β, γ, and δ. α and β, the isoforms constituting neuronal CaMKII, represent up to 2% of the total protein in certain brain areas such as the hippocampus, and up to 1% in the forebrain (Erondu and Kennedy, 1985). CaMKII exists as holoenzymes containing ~8-14 subunits, each comprised of a catalytic, regulatory, and association domain. The kinase is normally rendered inactive through inhibition of the catalytic domain by the regulatory domain, which obstructs both the ATP- and substrate-binding sites; activation of CaMKII occurs as the result of calcium/calmodulin-dependent displacement of the regulatory domain from the catalytic domain, and subsequent intersubunit autophosphorylation of the Thr286 residue.

Although CaMKII has previously been implicated in oxidant-induced neuronal injury, the results presented in Chapter 2 were the first to demonstrate a role for the kinase in zinc- and Kv2.1-dependent apoptosis. Still, while this work established CaMKII as a regulator of Kv2.1 activity through its direct interaction with syntaxin, several questions remain regarding the details of the kinase's involvement in the cell death-enabling pathway triggered by oxidants.

Previous studies carried out in cardiac tissue have found increased CaMKIIδ expression during arrthymias and heart failure (Hoch et al., 1998; Ai et al., 2005; Kushnir et al., 2010; Respress et al., 2013), a consequence of which appears to be direct phosphorylation of RyRs by CaMKII, leading to calcium release and subsequent injurious calcium dyshomeostasis. Based on these findings, it is possible that during zinc-mediated oxidative neuronal injury, in addition to interacting with syntaxin downstream of ER calcium release, CaMKII could also contribute to the ER calcium release process itself. If CaMKII does mediate the initial release of calcium from the ER, it is not clear if a distinct source of calcium would activate the kinase, or if once activated, CaMKII would then target ER RyRs to trigger further calcium release. However, since all of the calcium released during this process originated from the ER (Figure 1B), it is more likely that CaMKII activation would occur exclusively downstream of the initial ER calcium release. Still, although CaMKII-mediated phosphorylation of RyRs has been observed in the heart, it has not been established if this same process occurs in the brain. Further, while activation of CaMKII by methionine oxidation appears to be involved in the kinase's regulation of RyRs (Ho et al., 2013), and cardiac function in general (Erickson et al., 2008; Purohit et al., 2013), this mechanism of activation of CaMKII does not occur during oxidative neuronal injury (Figure 1D). Indeed, oxidation-dependent CaMKII activation has only been observed in heart cells, and it has yet to be established if a similar process occurs with neuronal CaMKII.

Another question that has yet to be answered is precisely how CaMKII modulates syntaxin, and how inhibition of the kinase exerts its protective effect. CaMKII physically interacts with syntaxin (Figure 4A), and transfection with a kinase-inactive CaMKIIK42R mutant prevents Kv2.1/syntaxin binding (Figure 4C), the apoptotic enhancement of Kv2.1-mediated K^+ currents, and downstream cell death (Figure 7). Thus, because the kinase-inactive

mutant lacks the ability to phosphorylate its substrate, it is likely that prior to apoptosis, CaMKII regulates syntaxin via phosphorylation, although the functional consequences of this phosphorylation event are not yet clear. Previously, it was hypothesized that binding of CaMKII to syntaxin could induce a conformational change to facilitate the conversion of syntaxin from a closed to an open form required for subsequent SNARE binding (Ohyama et al., 2002; Nomura et al., 2003). However, more recent evidence has shown that SNARE complex formation can occur in the absence of CaMKII/syntaxin binding, although vesicle recycling and vesicle density were reduced when this interaction was attenuated (Watanabe et al., 2013). Thus, it is possible that inhibition of Kv2.1/syntaxin binding by expression of CaMKIIK42R prevents the Kv2.1-mediated K^+ current enhancement by affecting the availability of Kv2.1-containing vesicles at the plasma membrane. Clearly, further research is needed to fully elucidate the molecular mechanism by which CaMKII regulates syntaxin.

While the kinase-inactive CaMKIIK42R mutant would be unable to phosphorylate syntaxin, it is not clear if the mutation also prevents CaMKII from physically interacting with syntaxin, or if this process remains intact. Based on previous reports, though, it appears as though it is binding of calcium/calmodulin, and not ATP, that dictates CaMKII trafficking and interaction with other proteins. Specifically, CaMKII containing the K42R mutation was found to retain the ability to translocate to the post-synaptic density, while disruption of calmodulin binding prevented this process (Shen and Meyer, 1999). Further, in hippocampal neurons, CaMKII was shown to interact with NR2B subunit of the NMDA receptor in the even absence of ATP, as long as calcium/calmodulin was present (Bayer et al., 2001). Therefore, it is a definite possibility that in our studies, CaMKII expressing the K42R mutation still interacts with syntaxin despite its inability to phosphorylate its target.

Another question that has yet to be addressed is how CaMKII is inactivated during Kv2.1-mediated apoptosis. Dephosphorylation of CaMKII residue Thr286 inactivates the kinase, and dephosphorylation of this site has been found to be mediated primarily by protein phosphatase 2A (PP2A; Strack et al., 1997). Interestingly, zinc has been shown to regulate Src-dependent inactivation of neuronal PP2A (Sun, et al., 2012; Xiong et al., 2013). Thus, while CaMKII activation is not affected by zinc (Figure 1C), it is possible that the increase in intracellular zinc observed during apoptotic injury serves to prolong CaMKII activity by inhibiting PP2A, thereby preventing the inactivation of the kinase. Biochemical experiments determining the effect of the zinc chelator TPEN on oxidant-induced CaMKII Thr286 phosphorylation at multiple time points may clarify the effect of the metal on CaMKII inactivation.

4.2 EFFECT OF KV2.1 PHOSPHORYLATION DURING APOPTOSIS

Dual phosphorylation of Kv2.1 residues Y124 and S800 is necessary to elicit the apoptotic increase in K^+ currents and downstream cell death (Redman et al., 2009). Findings in Chapter 3 demonstrated that Kv2.1 phosphorylation also promotes the binding of syntaxin to Kv2.1 prior to apoptosis. Questions remain regarding the specific function of phosphorylation of residues Y124 and S800, and on which population of channels this phosphorylation occurs. Because Kv2.1 can bind to syntaxin under basal conditions (Singer-Lahat, 2007), it is unlikely that phosphorylation of S800 is directly responsible for this interaction, although basal S800 phosphorylation can also been observed (Fig. 6). Channels expressing a non-phosphorylatable S800A mutation do not exhibit apoptotic Kv2.1-mediated K^+ currents, yet are still expressed at the plasma membrane

and maintain basal K^+ currents, suggesting that phosphorylation of this site is not essential for normal trafficking of Kv2.1 (Redman et al., 2007). Thus, it is possible that p38 is localized to vesicles containing those channels that will be responsible for generating the increased K^+ currents observed during apoptosis, and that phosphorylation of those channels by p38 triggers their delivery to SNARE proteins at the cell surface, which then facilitates the incorporation of channels into the plasma membrane. If this were the case, the enhanced binding of Kv2.1 and syntaxin induced by phosphorylation of Kv2.1 would likely be the result of an increased presence of channels in the vicinity of plasma membrane-bound syntaxin. Presumably then, channels lacking the C1a domain (Kv2.1ΔC1a) and exposed to DTDP (Figure 10) are still delivered to the plasma membrane, but cannot undergo exocytosis due to an inability to bind to syntaxin. Further, since the apoptotic K^+ current enhancement was also absent cells expressing both WT Kv2.1 and Kv2.1ΔC1a plasmids, it appears that each of the channel's four subunits must contain an intact syntaxin binding site to be involved in the apoptotic current enhancement. However, if the Kv2.1ΔC1a mutation prevents the K^+ current enhancement directly through the inability to bind syntaxin, or if it does so by altering the conformation of the channel and its relation to adjacent subunits remains to be established.

While the work presented in this dissertation firmly demonstrate that Kv2.1/syntaxin binding mediates oxidative neuronal apoptosis, precisely how this association contributes to cell death is not yet entirely clear. The channel directly interacts with syntaxin in non-injurious conditions as well, and it is not known if the consequences of this interaction are different in distinct cellular settings. Still, these findings suggest that Kv2.1 is a multifaceted ion channel that is dynamically regulated during both health and disease, with the phosphorylation status of the channel being an important determinant of its function. Further, and as will be discussed below,

it appears that even subtle differences in the cellular environment in which Kv2.1 channels are found could result in variable responses from the channel that play an integral role in determining the fate of neurons.

4.3 ROLE OF KV2.1 DURING PERIODS OF PATHOLOGICAL HYPEREXCITABILITY

Kv2.1 is normally maintained in a highly phosphorylated state (Park et al., 2006), localized in part to dense somato-dendritic clusters within the plasma membrane (Trimmer, 1991). Most of the channel's phosphorylation sites are located within the unusually long C-terminus, which accounts for more than half (443/853) of the channel's total amino acid residues. Intriguingly, Kv2.1 has a larger C-terminus than any other Kv channel, and it is the region of the channel that is most distinct from its closest relative, Kv2.2. This distinctive property of Kv2.1 likely contributes to its unique functional role amongst Kv channels. Indeed, phosphorylation of Kv2.1, especially in the C-terminus, has been shown to exert significant effects on overall channel function (Antonucci et al., 2001; Mohapatra et al., 2007; Redman et al., 2009).

Conditions of injurious neuronal hyperexcitability, such as epileptic seizures, are marked by excessive glutamate and calcium release that can cause excitotoxicity in affected neurons. Similar to its effect on physiological high-frequency firing, Kv2.1 has also been shown to contribute to the suppression of neuronal excitability under pathological conditions such as ischemia, or following exposure to glutamate or kainic acid. The mechanism underlying channel modulation within this context involves an increase in intracellular calcium and consequent calcineurin phosphatase-dependent dephosphorylation of Kv2.1 at several C-terminal amino acid

residues. This change in phosphorylation state triggers a hyperpolarizing shift in the channel's voltage-dependence of activation (with no effect on maximal K^+ current densities), and is accompanied by a lateral dispersal of channels from clusters into a more diffuse localization. These modifications contribute to an increase in Kv2.1 activity, which manifests as a dampened response to injurious over-excitation, and thus serves as a putative innate protective mechanism within neurons (Misonou et al., 2004; Misonou et al., 2005; Mohapatra et al., 2009).

Interestingly, while the general mechanism described above underlies Kv2.1 activity during over-excitation, certain details of the signaling cascade vary in a stimulus-specific manner. Two separate studies conducted by James Trimmer and colleagues demonstrated that both exposure to glutamate or kainic acid, and induction of hypoxia/ischemia triggered Kv2.1 dephosphorylation by calcineurin, a shift in activation voltage, and channel dispersal, which together suppressed excitatory activity. Moreover, in both studies, these effects were rapid and reversible, suggesting that this is a transient response to short-term changes in overall neural activity (Misonou et al., 2004; Misonou et al., 2005). Curiously, though, while both exposure to glutamate or kainite, and induction of hypoxia/ischemia led to the aforementioned cellular alterations, there were also several modest distinctions between the mechanisms underlying these effects. For example, while Kv2.1 modulation by glutamate or kainate relies on the influx of extracellular calcium into neurons (Misonou et al., 2004), hypoxia/ischemia-induced channel regulation largely depends on calcium release from intracellular stores (most likely mitochondria), and is therefore unaffected by chelation of extracellular calcium. Further, Kv2.1 channel modulation by hypoxia/ischemia is, remarkably, not dependent on glutamate (Misonou et al., 2005). Thus, while it appears that Kv2.1 functions primarily as a suppressor of neuronal

firing during periods of hyperexcitability, precisely how this effect is achieved appears to be quite a nuanced process that depends, at least in part, on the nature of the injurious stimulus.

4.4 KV2.1-MEDIATED K^+ EFFLUX DURING OXIDANT-INDUCED NEURONAL APOPTOSIS

Our understanding of Kv2.1 function is further complicated by the fact that enhanced Kv2.1-mediated K^+ efflux can also facilitate neurotoxicity in models of oxidant-induced apoptosis. As described earlier in the dissertation, oxidative injury triggers the incorporation of new Kv2.1 channels into the plasma membrane, leading to an increase in K^+ currents and consequent reduction in intracellular K^+ that precedes apoptosis. This insertion event is dependent upon the phosphorylation of channel residues Y124 and S800 by Src kinase and p38 MAPK, respectively, and unlike hyperexcitability, occurs in the absence of changes in channel gating kinetics (Yu et al., 1997; Pal et al., 2003; Pal et al., 2006). Interestingly, while several channel residues have been shown to be targets of calcineurin dephosphorylation during hyperexcitability, S800 is not one of them, and does not appear to play a role in altering channel gating and localization under these conditions (Park et al., 2006). In fact, at present, the sole known function of phosphorylation of residue S800 is to facilitate the pro-apoptotic introduction of Kv2.1 into the plasma membrane, suggesting that this event likely contributes to the functionally distinct role of Kv2.1 during apoptosis. Further, the injury-induced increase in I_k is specific to apoptosis, and is not observed in neurons undergoing other forms of cell death, such as necrosis (Yu et al., 1997; Pal et al., 2003). Thus, Kv2.1 appears to play divergent roles during conditions related to hyperexcitability and those involving oxidative apoptosis, a distinction that is dictated in part by

the specific phosphorylation state of the channel. However, what other factors contribute to the distinct functions assumed by Kv2.1 during different types of neuronal injury is less clear, as similar upstream signaling events have been observed during both oxidative injury and hyperexcitability. For example, increased intracellular zinc, which is a hallmark of Kv2.1-dependent apoptosis and is required for the phosphorylation of Y124 and S800 (Aizenman et al., 2000; Redman et al., 2007; Redman et al., 2009), has also been shown to mediate ischemia-induced channel dephosphorylation and alterations in gating kinetics (Aras et al., 2009). Moreover, intracellular calcium release was recently shown to be a component of oxidant-induced, Kv2.1-mediated apoptosis (McCord and Aizenman, 2013).

The uncertainty surrounding the regulation of Kv2.1 was further magnified following a recent report demonstrating that the effect of the chemokine stromal cell-derived factor-1 (SDF-1α) on Kv2.1 depended on exposure time, with short-term exposure inducing changes in Kv2.1 reminiscent of its function during hyperexcitability, while prolonged exposure led to changes in channel activity similar to those observed during oxidative apoptosis. Specifically, acute exposure (30 minutes) of cultured hippocampal neurons to SDF-1α led to calcineurin-dependent Kv2.1 dephosphorylation, cluster dispersal, and a leftward shift in the voltage-dependence of channel activation, all as a means to suppress neuronal activity in order to prevent non-apoptotic cell death. Conversely, prolonged exposure (three hours) to SDF-1α led to apoptosis via p38-dependent Kv2.1 plasma membrane insertion and enhancement of K^+ currents (Shepherd et al., 2012). These findings suggest that Kv2.1 activity initially serves to reduce the extent of non-apoptotic neuronal cell death; however, presumably after reaching a currently unidentified cellular injury threshold, Kv2.1 function shifts to serving in a pro-apoptotic capacity. This

phenomenon could be relevant to disorders such as cerebral ischemia, whereby neurons undergo either apoptosis or necrosis, depending on the spatial and temporal profile of the initial injury.

Taken together, it appears that Kv2.1 channels exist along a spectrum of possible activities, and how the channel functions is dictated by the type of injury sustained, duration of stimulus exposure, the phosphorylation state of the channel, presumably in addition to other factors that have yet to be established. What is particularly interesting about this differential regulation of the channel is that seemingly minor distinctions—e.g. exposure to the same stimulus for 30 minutes versus three hours (Shepherd et al., 2012)—can result in markedly different responses from Kv2.1, suggesting that the channel can sense relatively subtle changes in cellular state and respond accordingly. As such, a comprehensive understanding of what molecular processes underlie Kv2.1 function in various cellular settings will likely by essential for the development of successful therapeutic strategies aimed at this channel.

4.5 KV2.1 MAINTAINS SEVERAL DISTINCT ACTIVITY PROFILES

Kv2.1 channels thus seem to maintain several activity profiles that are determined by the needs of the cell or specific cellular environment at a given time. Recent studies have shown that in addition to the population of Kv2.1 localized to clusters, a substantial proportion of channels exists in a more diffuse localization, even under basal conditions (Tamkun et al., 2007). Interestingly, the clustered population was found to be non-conducting, and instead acted as a platform for exocytosis, while the dispersed channels appeared to be responsible for the delayed rectifier K^+ currents observed in neurons. Thus, it appears that Kv2.1 channels normally maintain a dual role as either ion conductors or facilitators of exocytosis, possibly primarily depending on

their localization (Profile 1). It is not know what molecular processes underlie the delineation between these two functions, but the phosphorylation state of the channel could perhaps play an important role.

During periods of high-frequency firing, both physiological and pathophysiological, the clustered population of channels disperses and activation kinetics shift following dephosphorylation (Profile 2). Although it is not known if channel dispersal is a cause or effect of the change in gating kinetics, declustering may occur to convert some of these channels from secretors to K^+ conductors to accommodate the change in synaptic activity and prevent over-excitation. Therefore, channel declustering could signify the shift in the functional balance toward ion conduction as a means to maintain cellular homeostasis. Further, it is possible that channel declustering also prevents over-excitation by displacing Kv2.1 in order to disable the exocytotic machinery and therefore inhibit neurotransmission; however, whether Kv2.1/syntaxin binding mediates synaptic vesicle release (versus LDCV release) has yet to be determined directly. The mechanism underlying channel dispersal is still unclear, although the phosphorylation state of Kv2.1 appears to be an important determinant of function, as variable dephosphorylation leads to a graded shift in voltage-dependence of channel activation (Park et al., 2006). This model, however, is complicated by the fact that declustering and concomitant changes in channel activation profiles are not reflected by an overall increase in current densities. Thus, exactly what population of channels is in a conducting mode or not prior to declustering remains to be determined.

During oxidative injury, Kv2.1 channels adopt a third profile that has been characterized by our laboratory, and is marked by the delivery of new channels to the plasma membrane that results in increased K^+ efflux prior to apoptosis (Profile 3). The effect of oxidative injury on

channel localization (clustered or dispersed) is not known, but as speculated in Chapter 3, clusters likely remain intact to facilitate the incorporation on new channels into the membrane. Kv2.1-mediated K^+ efflux and downstream apoptosis has been observed following serum deprivation and after exposure to various injurious stimuli including oxidants, activated microglia, and staurosporine (Pal et al., 2003; Knoch et al, 2008; Zhou et al., 2012). Therefore, adoption of this profile by Kv2.1, which depends on the phosphorylation of channel residues Y124 and S800, appears to be common to numerous forms of neuronal injury.

Recent studies have suggested a fourth possible activity profile for Kv2.1 channels that could be relevant to Alzheimer's disease and other neurodegenerative disorders. In this case, oxidation of Kv2.1 causes channel oligomerization through the formation of intersubunit disulfide bridges between two N- and C-terminal cysteine residues, C73 and C710. Oxidant-induced oligomerization triggered ROS production and apoptosis, with these effects being blocked in CHO cells expressing a channel mutant (Kv2.1C73A) that is incapable of oligomerizing. Interestingly, aged wild-type mice exhibited enhanced Kv2.1 oligomerization, and this effect was exacerbated in aged 3xTg-AD transgenic mice. Further, exposure to Aβ led to Kv2.1 oligomerization and subsequent apoptosis that was absent in Kv2.1C73A-expressing cells (Cotella et al., 2010). The mechanism underlying Kv2.1 oligomerization-induced toxicity was later revealed to involve oxidant-induced accumulation of Kv2.1 at the plasma membrane, presumably due to an inability of channels internalize properly. The apoptotic effect of channel oligomerization was found to be mediated by Src and JNK kinases, and was thought to stem from disruption of lipid rafts, with cholesterol supplementation of the rafts conferring an anti-apoptotic effect (Wu et al., 2013). Of note, Kv2.1 channels were previously found to be localized to lipid rafts (Martens et al., 2000; Martens et al., 2001), and disruption of the rafts by prolonged

cholesterol depletion triggered a hyperpolarizing shift in the voltage-dependence of channel inactivation as well as an increase in channel cluster size (O'Connell and Tamkun, 2005).

Although Kv2.1 oligomerization does appear to be a consequence of some forms of oxidative stress and an important factor in the propagation of Aβ-mediated toxicity, it is too soon to conclude that this channel profile is entirely distinct from that adopted during previously described forms of oxidative apoptosis (Profile 3), which places phosphorylation-dependent Kv2.1 channel upregulation at the plasma membrane as a critical upstream event in apoptosis (Pal et al., 2003; Redman et al., 2009). Although the oligomerization model (Profile 4) argues that the toxic effect of Kv2.1 actually stems from oligomerization-induced disruption of lipid rafts and subsequent Src- and JNK-mediated apoptosis, it is likely that phosphorylation-dependent and oligomerization-dependent apoptosis are not mutually exclusive, and therefore both explanations could be considered to be true. Which activity profile is adopted seems to depend on the time course of the injurious sequelae, with oligomerization occurring at earlier time points, while channel insertion and apoptotic current expression, allowing for optimal caspase activity, occurs much later.

4.6 LOCATING THE MOLECULAR SWITCH DURING KV2.1-MEDIATED APOPTOSIS

Because the requirements for anti-death versus pro-death Kv2.1 function appear to be discrete, it is likely that a specific set of biochemical changes underlie this transition. Presumably, then, a clinically effective Kv2.1 channel modulator would need to target events specific to the adoption of one channel profile or another. Otherwise, nonspecific Kv2.1 channel modulators used during

oxidative injury could have the unwanted side effect of excitotoxicity, while those used during disorders of hyperexcitability could result in increased apoptosis. Further, because it appears that the activity profile of Kv2.1 channels is determined by a delicate molecular balance within cells, use of inhibitors derived from endogenous Kv2.1-regulating mechanisms would likely be the best approach to achieving the desired specificity that is lacking in current Kv2.1 channel blockers.

The findings presented here, in combination with previous work in our laboratory, have begun to unravel the molecular mechanisms underlying Kv2.1 channel activity in oxidant-induced apoptosis. During periods of hyperexcitability, Kv2.1 exerts a protective effect via dephosphorylation and consequent hyperpolarization of voltage-dependence of activation, leading to decreased firing frequency. These changes presumably occur within Kv2.1 channels embedded in the plasma membrane at the time of injury, as glutamate exposure triggered these modifications without an effect on the number of channels present at the cell surface (Misonou et al., 2004). Conversely, the pro-death function of Kv2.1 seen during oxidative injury occurs as a result of new channels being delivered to the plasma membrane (Pal et al., 2006). Thus, while some of the upstream components of these signaling pathways are similar, the pool of channels exerting the effect on cell survival may in fact be distinct. Therefore, one potential approach to minimizing apoptotic K^+ efflux without inadvertently inducing excitotoxicity would be to impede the delivery of Kv2.1 channels to the plasma membrane by targeting a component of the delivery/exocytosis machinery.

The work presented in Chapter 3 established that the SNARE protein syntaxin binds to the C1a domain of Kv2.1 to facilitate the pro-apoptotic plasma membrane insertion of the channel. Interruption of this interaction using C1a-derived peptides prevented the K^+ current

enhancement and downstream apoptosis without substantially affecting basal K^+ currents or passive electrical properties of neurons. Thus, while syntaxin and Kv2.1 do interact during exocytosis in non-apoptotic conditions, acutely disrupting this association does not appear to substantially hinder normal Kv2.1 trafficking. Further, CHO cells transiently expressing a Kv2.1 channel mutant lacking its syntaxin binding site (Kv2.1ΔC1a), and neurons expressing the C1a peptide itself exhibited normal basal K^+ currents and current-voltage relationships one day post-transfection. These findings bode well for Kv2.1/syntaxin binding as a potential target for therapeutic intervention, although future experiments will be needed to clarify if and how disrupting Kv2.1/syntaxin binding affects channel trafficking over time. It is possible that syntaxin is abundant enough within neurons that even in the presence of C1a, an unbound proportion of the protein would remain able to interact with Kv2.1 to facilitate exocytosis. Moreover, because Kv2.1 has been shown to be both a suppressor and promoter of exocytosis in different cellular settings (MacDonald et al., 2002; Singer-Lahat et al., 2007), it is not apparent how inhibiting the channel during apoptosis would affect release under these conditions. Further, it is not known if syntaxin has different affinities for Kv2.1 during apoptosis versus exocytosis, although we showed that Kv2.1/syntaxin binding is enhanced when the channel is phosphorylated at residues Y124 and S800, suggesting that the interaction is promoted during apoptosis. Based on these experiments, it is not clear if channel phosphorylation per se enhances Kv2.1/syntaxin binding, or if the increase in binding is simply due to a phosphorylation-induced increase in channels at plasma membrane prior to apoptosis, which would bring Kv2.1 in close enough proximity to syntaxin for the interaction to take place. Again, future experiments are required to fully elucidate this mechanism.

4.7 KV2.1 CHANNEL BLOCKERS AS A THERAPEUTIC STRATEGY

Until now, numerous K^+ channel blockers have had success in preventing apoptotic CNS injury both *in vitro* and *in vivo* (Huang et al., 2001; Wei et al., 2003; Zaks-Makhina et al., 2004; Hu et al., 2006). However, these compounds have not progressed past the level of animal models. One reason for this is the lack of specificity of most currently available K^+ channel blockers. For example, while the compounds TEA, 4-AP, and a number of natural K^+ channel blockers such as hanatoxin (derived from spider venom) inhibit Kv2.1, they also act on other Kv channels as well (Swartz and MacKinnon, 1995; Madeja et al, 2000; Zhang et al., 2003). This is not surprising considering the structural similarities between certain Kv channels, particularly in the transmembrane domain, which is the target of many K^+ channel blockers. Additionally, Kv channel modulators can also affect non-Kv channel targets such as Na^+, K^+-ATPases, NMDA receptors, and calcium channels (Eckstein-Ludwig et al., 1998; Wang et al., 2003). As mentioned previously, the C-terminus of Kv2.1 has been shown to mediate a number of neuropathological conditions, mostly through its dynamic phosphorylation. This domain is relatively unique to the Kv2.1 channel, as it displays much less striking sequence homology to its closest relative, Kv2.2, than does the rest of the channel. Therefore, disrupting the interaction of Kv2.1 and syntaxin that takes place in the channel's C-terminus, as I have done in my dissertation research, could be a promising new direction to take in the development of Kv2.1 channel modulators to protect against oxidant-induced apoptosis.

BIBLIOGRAPHY

Adam-Vizi V, Starkov AA (2010). "Calcium and mitochondrial reactive oxygen species generation: how to read the facts." J Alzheimers Dis **20 Suppl 2**: S413-426.

Ai X, Curran JW, Shannon TR, Bers DM, Pogwizd SM (2005). "Ca2+/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca2+ leak in heart failure." Circ Res **97**(12): 1314-1322.

Aizenman E, Stout AK, Hartnett KA, Dineley KE, McLaughlin B, Reynolds, IJ (2000). "Induction of neuronal apoptosis by thiol oxidation: putative role of intracellular zinc release." J Neurochem **75**(5): 1878-1888.

An W, Bjorkdahl C, Liu R, Cowburn RF, Winbald B, Pei JJ (2005). "Mechanism of zinc-induced phosphorylation of p70 S6 kinase and glycogen synthase kinase 3beta in SH-SY5Y neuroblastoma cells." J Neurochem **92**(5): 1104-1115.

Andreini CL, Banci L, Bertini I, Rosato A (2006). "Counting the zinc-proteins encoded in the human genome." J Proteome Res **5**(1): 196-201.

Antonucci DE, Lim ST, Vassanelli S, Trimmer JS (2001). "Dynamic localization and clustering of dendritic Kv2.1 voltage-dependent potassium channels in developing hippocampal neurons." Neuroscience **108**(1): 69-81.

Aplin AE, Gibb GM, Jacobsen JS, Gallo JM, Anderton BH (1996). "In vitro phosphorylation of the cytoplasmic domain of the amyloid precursor protein by glycogen synthase kinase-3beta." J Neurochem **67**(2): 699-707.

Aras MA, Aizenman E (2011). "Redox regulation of intracellular zinc: molecular signaling in the life and death of neurons." Antioxid Redox Signal **15**(8): 2249-2263.

Aras MA, Hara H, Hartnett KA, Kandler K, Aizenman E (2009). "Protein kinase C regulation of neuronal zinc signaling mediates survival during preconditioning." J Neurochem **110**(1): 106-117.

Aras MA, Hartnett KA, Aizenman E (2008). "Assessment of cell viability in primary neuronal cultures." Curr Protoc Neurosci **Chapter 7**: Unit 7 18.

Aras MA, Aizenman E (2005). "Obligatory role of ASK1 in the apoptotic surge of K+ currents." Neurosci Lett **387**(3): 136-140.

Arslan P, Di Virgilio F, Beltrame M, Tsien RY, Pozzan T (1985). "Cytosolic Ca2+ homeostasis in Ehrlich and Yoshida carcinomas. A new, membrane-permeant chelator of heavy metals reveals that these ascites tumor cell lines have normal cytosolic free Ca2+." J Biol Chem **260**(5): 2719-2727.

Ashpole NM, Hudmon A (2011). "Excitotoxic neuroprotection and vulnerability with CaMKII inhibition." Mol Cell Neurosci **46**(4): 720-730.

Barnett YA, King CM (1995). "An investigation of antioxidant status, DNA repair capacity and mutation as a function of age in humans." Mutat Res **338**(1-6): 115-128.

Bartus RT, Dean RL, 3rd, Beer B, Lippa AS (1982). "The cholinergic hypothesis of geriatric memory dysfunction." Science **217**(4558): 408-414.

Bartzokis GD, Sultzer D, Mintz J, Holt LE, Marx P, Phelan CK, Marder SR (1994). "In vivo evaluation of brain iron in Alzheimer's disease and normal subjects using MRI." Biol Psychiatry **35**(7): 480-487.

Bayer KU, De Koninck P, Leonard AS, Hell JW, Schulman H (2001). "Interaction with the NMDA receptor locks CaMKII in an active conformation." Nature **411**(6839): 801-805.

Beckman KB, Ames BN (1998). "The free radical theory of aging matures." Physiol Rev **78**(2): 547-581.

Beckman JS, Crow JP (1993). "Pathological implications of nitric oxide, superoxide and peroxynitrite formation." Biochem Soc Trans **21**(2): 330-334.

Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA (1990). "Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide." Proc Natl Acad Sci U S A **87**(4): 1620-1624.

Behl C, Davis JB, Lesley R, Schubert D (1994). "Hydrogen peroxide mediates amyloid beta protein toxicity." Cell **77**(6): 817-827.

Berridge MJ (2013). "Dysregulation of neural calcium signaling in Alzheimer disease, bipolar disorder and schizophrenia." Prion **7**(1): 2-13.

Berridge MJ (2010). "Calcium hypothesis of Alzheimer's disease." Pflugers Arch **459**(3): 441-449.

Besser L, Chorin E, Sekler I, Silverman WF, Atkin S, Russell JT, Hershfinkel M (2009). "Synaptically released zinc triggers metabotropic signaling via a zinc-sensing receptor in the hippocampus." J Neurosci **29**(9): 2890-2901.

Bonanni LM, Chachar M, Jover-Mengual T, Li H, Jones A, Yokota H, Ofengeim D, Flannery RJ, Miyawaki T, Cho CH, Polster BM, Pypaert M, Hardwick JM, Sensi SL, Zukin RS, Jonas EA (2006). "Zinc-dependent multi-conductance channel activity in mitochondria isolated from ischemic brain." J Neurosci **26**(25): 6851-6862.

Bonanno G, Giambelli R, Raiteri L, Tiraboschi E, Zappettini S, Musazzi L, Raiteri M, Racagni G, Popoli M (2005). "Chronic antidepressants reduce depolarization-evoked glutamate release and protein interactions favoring formation of SNARE complex in hippocampus." J Neurosci **25**(13): 3270-3279.

Boom AM, Authelet M, Dedecker R, Frederick C, Van Heurck R, Daubie V, Leroy K, Pochet R, Brion JP (2009). "Bimodal modulation of tau protein phosphorylation and conformation by extracellular Zn2+ in human-tau transfected cells." Biochim Biophys Acta **1793**(6): 1058-1067.

Bortner CD, Cidlowski JA (2007). "Cell shrinkage and monovalent cation fluxes: role in apoptosis." Arch Biochem Biophys **462**(2): 176-188.

Bortner CD, Hughes FM, Jr., Cidlowski JA (1997). "A primary role for K+ and Na+ efflux in the activation of apoptosis." J Biol Chem **272**(51): 32436-32442.

Bossy-Wetzel E, Talantova MV, Lee WD, Scholzke MN, Harrop A, Mathews E, Gotz T, Han J, Ellisman MH, Perkins GA, Lipton SA (2004). "Crosstalk between nitric oxide and zinc pathways to neuronal cell death involving mitochondrial dysfunction and p38-activated K+ channels." Neuron **41**(3): 351-365.

Bush AI, Tanzi RE (2008). "Therapeutics for Alzheimer's disease based on the metal hypothesis." Neurotherapeutics **5**(3): 421-432.

Bush AI, Pettingell, WH, Jr., Paradis MD, Tanzi RE (1994a). "Modulation of A beta adhesiveness and secretase site cleavage by zinc." J Biol Chem **269**(16): 12152-12158.

Bush AI, Pettingell WH, Multhaup G, d Paradis M, Vonsattel JP, Gusella JF, Beyreuther K, Masters CL, Tanzi RE (1994b). "Rapid induction of Alzheimer A beta amyloid formation by zinc." Science **265**(5177): 1464-1467.

Butterfield D, Drake J, Pocernich C, Castegna A (2001). "Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide." Trends Mol Med **7**(12): 548-554.

Cain K, Langlais C, Sun XM, Brown DG, Cohen GM (2001). "Physiological concentrations of K+ inhibit cytochrome c- dependent formation of the apoptosome." J Biol Chem **276**(45): 41985-41990.

Calderone A, Jover T, Mashiko T, Noh KM, Tanaka H, Bennett MV, Zukin RS (2004). "Late

calcium EDTA rescues hippocampal CA1 neurons from global ischemia-induced death." J Neurosci **24**(44): 9903-9913.

Canals S, Casarejos MJ, de Bernardo S, Rodriguez-Martin E, Mena MA (2003). "Nitric oxide triggers the toxicity due to glutathione depletion in midbrain cultures through 12-lipoxygenase." J Biol Chem **278**(24): 21542-21549.

Carrasco JM, Giralt M, Molinero A, Penkowa M, Moos T, Hidalgo J (1999). "Metallothionein (MT)-III: generation of polyclonal antibodies, comparison with MT-I+II in the freeze lesioned rat brain and in a bioassay with astrocytes, and analysis of Alzheimer's disease brains." J Neurotrauma **16**(11): 1115-1129.

Cerda O, Trimmer JS (2011). "Activity-dependent phosphorylation of neuronal Kv2.1 potassium channels by CDK5." J Biol Chem **286**(33): 28738-28748.

Chance BH, Sies H, Boveris A (1979). "Hydroperoxide metabolism in mammalian organs." Physiol Rev **59**(3): 527-605.

Cheepsunthorn P, Radov L, Menzies S, Reid J, Connor JR (2001). "Characterization of a novel brain-derived microglial cell line isolated from neonatal rat brain." Glia **35**(1): 53-62.

Chen S, Xu Y, Xu B, Guo M, Zhang Z, Liu L, Ma H, Chen Z, Luo Y, Huang S, Chen L (2011). "CaMKII is involved in cadmium activation of MAPK and mTOR pathways leading to neuronal cell death." J Neurochem **119**(5): 1108-1118.

Cherny RA, Atwood CS, Xilinas ME, Gray DN, Jones WD, McLean CA, Barnham KJ, Volitakis I, Fraser FW, Kim Y, Huang X, Goldstein LE, Koir RD, Lim JT, Beyreuther K, Zheng H, Tanzi RE, Masters CL, Bush AI (2001). "Treatment with a copper-zinc chelator markedly and rapidly inhibits beta-amyloid accumulation in Alzheimer's disease transgenic mice." Neuron **30**(3): 665-676.

Cherny RA, Legg JT, McLean CA, Fairlie DP, Huang X, Atwood CS, Beyreuther K, Tanzi RE, Masters CL, Bush AI (1999). "Aqueous dissolution of Alzheimer's disease Abeta amyloid deposits by biometal depletion." J Biol Chem **274**(33): 23223-23228.

Choi DW, Koh JY (1998). "Zinc and brain injury." Annu Rev Neurosci **21**: 347-375.

Choi DW (1995). "Calcium: still center-stage in hypoxic-ischemic neuronal death. "Trends Neurosci **18**(2): 58-60.

Choi DW, Yokoyama M, Koh J (1988). "Zinc neurotoxicity in cortical cell culture." Neuroscience **24**(1): 67-79.

Chorin E, Vinograd O, Fleidervish I, Gilad D, Herrmann S, Sekler I, Aizenman E, Hershfinkel M (2011). "Upregulation of KCC2 activity by zinc-mediated neurotransmission via the mZnR/GPR39 receptor." J Neurosci **31**(36): 12916-12926.

Chung RS, West AK (2004). "A role for extracellular metallothioneins in CNS injury and repair." Neuroscience **123**(3): 595-599.

Cole TB, Wenzel HJ, Kafer KE, Schwartzkroin PA, Palmiter RD (1999). "Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene." Proc Natl Acad Sci U S A **96**(4): 1716-1721.

Colom LV, Diaz ME, Beers DR, Neely A, Xie WJ, Appel SH (1998). "Role of potassium channels in amyloid-induced cell death." J Neurochem **70**(5): 1925-1934.

Constantinidis J. (1990). "[Alzheimer's disease and the zinc theory]." Encephale **16**(4): 231-239.

Corona C, Pensalfini A, Frazzini V, Sensi SL (2011). "New therapeutic targets in Alzheimer's disease: brain deregulation of calcium and zinc." Cell Death Dis **2**: e176.

Cotella D, Hernandez-Enriquez B, Wu X, Li R, Pan Z, Levelille J, Link CD, Oddo S, Sesti F (2012). "Toxic role of K+ channel oxidation in mammalian brain." J Neurosci **32**(12): 4133-4144.

Coultrap SJ, Vest RS, Ashpole NM, Hudmon A, Bayer KU (2011). "CaMKII in cerebral ischemia." Acta Pharmacol Sin **32**(7): 861-872.

Csordas G, Hajnoczky G (2009). "SR/ER-mitochondrial local communication: calcium and ROS." Biochim Biophys Acta **1787**(11): 1352-1362.

Cuajungco MP, Lees GJ (1998). "Nitric oxide generators produce accumulation of chelatable zinc in hippocampal neuronal perikarya." Brain Res **799**(1): 118-129.

Cuervo, A. M. (2008). "Autophagy and aging: keeping that old broom working." Trends Genet **24**(12): 604-612.

Cuajungco MP, Lees GJ (1996). "Prevention of zinc neurotoxicity in vivo by N,N,N',N'-tetrakis (2-pyridylmethyl) ethylene-diamine (TPEN)." Neuroreport **7**(7): 1301-1304.

Dai XQ, Manning Fox JE, Chikvashvili D, Casimir M, Plummer G, Hajmrie C, Spigelman AF, Kin T, Singer-Lahat D, Kang Y, Shapiro AM, Gaisano HY, Lotan I, MacDonald PE (2012). "The voltage-dependent potassium channel subunit Kv2.1 regulates insulin secretion from rodent and human islets independently of its electrical function." Diabetologia **55**(6): 1709-1720.

Danscher, G., K. B. Jensen, et al. (1997). "Increased amount of zinc in the hippocampus and amygdala of Alzheimer's diseased brains: a proton-induced X-ray emission spectroscopic analysis of cryostat sections from autopsy material." J Neurosci Methods **76**(1): 53-59.

D'Autreaux B, Toledano MB (2007). "ROS as signalling molecules: mechanisms that generate

specificity in ROS homeostasis." Nat Rev Mol Cell Biol **8**(10): 813-824.

Deibel MA, Ehmann WD, Markesbery WR (1996). "Copper, iron, and zinc imbalances in severely degenerated brain regions in Alzheimer's disease: possible relation to oxidative stress." J Neurol Sci **143**(1-2): 137-142.

Demuro A, Parker I (2013). "Cytotoxicity of intracellular abeta42 amyloid oligomers involves Ca2+ release from the endoplasmic reticulum by stimulated production of inositol trisphosphate." J Neurosci **33**(9): 3824-3833.

Deshpande A, Kawai, et al. (2009). "A role for synaptic zinc in activity-dependent Abeta oligomer formation and accumulation at excitatory synapses." J Neurosci **29**(13): 4004-4015.

Deutsch E, Weigel AV, Akin EJ, Hansen G, Haberkorn CJ, Loftus R, Krapf D, Tamkun MM (2012). "Kv2.1 cell surface clusters are insertion platforms for ion channel delivery to the plasma membrane." Mol Biol Cell **23**(15): 2917-2929.

Devinney MJ, 2nd, Reynolds IJ, Dineley KE (2005). "Simultaneous detection of intracellular free calcium and zinc using fura-2FF and FluoZin-3." Cell Calcium **37**(3): 225-232.

Dexter DT, Carter Cj, Wells FR, Javoy-Agid F, Agid Y, Lees A, Jenner P, Marsden CD (1989). "Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease." J Neurochem **52**(2): 381-389.

Dietz RM, Weiss JH, Shuttleworth CW (2009). "Contributions of Ca2+ and Zn2+ to spreading depression-like events and neuronal injury." J Neurochem **109 Suppl 1**: 145-152.

Dietz RM, Weiss JH, Shuttleworth CW (2008). "Zn2+ influx is critical for some forms of spreading depression in brain slices." J Neurosci **28**(32): 8014-8024.

Dineley KE, Devinney MJ, 2nd, Zeak JA, Rintoul GL, Reynolds IJ (2008). "Glutamate mobilizes [Zn2+] through Ca2+ -dependent reactive oxygen species accumulation." J Neurochem **106**(5): 2184-2193.

Dineley KE, Richards LL, Votyakova TV, Reynolds IJ (2005). "Zinc causes loss of membrane potential and elevates reactive oxygen species in rat brain mitochondria." Mitochondrion **5**(1): 55-65.

Dineley KE, Votyakova TV, Reynolds IJ (2003). "Zinc inhibition of cellular energy production: implications for mitochondria and neurodegeneration." J Neurochem **85**(3): 563-570.

Du J, Haak LL, Phillips-Tansey E, Russell JT, McBain CJ (2000). "Frequency-dependent regulation of rat hippocampal somato-dendritic excitability by the K+ channel subunit Kv2.1." J Physiol **522 Pt 1**: 19-31.

Duce JA, Tsatsanis A, Cater MA, James SA, Robb E, Wikhe K, Leong SL, Perez K, Johanssen T, Greenough MA, Cho HH, Galatis D, Moir RD, Masters CL, McLean C, Tanzi RE, Cappai R, Barnham KJ, Ciccotosto GD, Rogers JT, Bush AI (2010). "Iron-export ferroxidase activity of beta-amyloid precursor protein is inhibited by zinc in Alzheimer's disease." Cell **142**(6): 857-867.

Dulubova I, Sugita S, Hill, S, Hosaka M, Fernandez I, Sudhof TC, Rizo J (1999). "A conformational switch in syntaxin during exocytosis: role of munc18." EMBO J **18**(16): 4372-4382.

Durand J, Meloni G, Talmard C, Vasak M, Faller P(2010). "Zinc release of Zn(7)-metallothionein-3 induces fibrillar type amyloid-beta aggregates." Metallomics **2**(11): 741-744.

Eckstein-Ludwig U, Rettinger J, Vasilets LA, Schwarz W (1998). "Voltage-dependent inhibition of the Na+,K+ pump by tetraethylammonium." Biochim Biophys Acta **1372**(2): 289-300.

Elmore S (2007). "Apoptosis: a review of programmed cell death." Toxicol Pathol **35**(4): 495-516.

Erickson JR, Joiner ML, Guan X, Kutschke W, Yang J, Oddis CV, Bartlett RK, Lowe JS, O'Donnell SE, Aykin-Burns N, Zimmerman MC, Zimmerman K, Ham AJ, Weiss RM, Spitz DR, Shea MA, Colbran RJ, Mohler PJ, Anderson ME (2008). "A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation." Cell **133**(3): 462-474.

Erondu NE, Kennedy MB (1985). "Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain." J Neurosci **5**(12): 3270-3277.

Esler WP, Stimson ER, Jennings JM, Ghilardi JR, Mantyh PW, Maggio JE (1996). "Zinc-induced aggregation of human and rat beta-amyloid peptides in vitro." J Neurochem **66**(2): 723-732.

Esposito LA, Melov S, Panov A, Cottrell BA, Wallace DC (1999). "Mitochondrial disease in mouse results in increased oxidative stress." Proc Natl Acad Sci U S A **96**(9): 4820-4825.

Feinshreiber L, Singer-Lahat D, Friedrich R, Matti U, Sheinin A, Yizhar O, Nachman R, Chikvashvili D, Rettig J, Ashery U, Lotan I (2010). "Non-conducting function of the Kv2.1 channel enables it to recruit vesicles for release in neuroendocrine and nerve cells." J Cell Sci **123**(Pt 11): 1940-1947.

Ferreiro E, Resende R, Costa R, Oliveira CR, Pereira CM (2006). "An endoplasmic-reticulum-specific apoptotic pathway is involved in prion and amyloid-beta peptides neurotoxicity." Neurobiol Dis **23**(3): 669-678.

Finkel T, Holbrook NJ (2000). "Oxidants, oxidative stress and the biology of ageing." Nature

408(6809): 239-247.

Fox PD, Loftus RJ, Tamkun MM (2013). "Regulation of Kv2.1 K(+) conductance by cell surface channel density." J Neurosci **33**(3): 1259-1270.

Francis PT, Palmer AM, Snape M, Wilcock GK (1999). "The cholinergic hypothesis of Alzheimer's disease: a review of progress." J Neurol Neurosurg Psychiatry **66**(2): 137-147.

Frazzini V, Rockabrand E, Mocchegiani E, Sensi SL (2006). "Oxidative stress and brain aging: is zinc the link?" Biogerontology **7**(5-6): 307-314.

Frech GC, VanDongen AM, Schuster G, Brown AM, Joho RH (1989). "A novel potassium channel with delayed rectifier properties isolated from rat brain by expression cloning." Nature **340**(6235): 642-645.

Frederickson CJ, Koh JY, Bush AI (2005). "The neurobiology of zinc in health and disease." Nat Rev Neurosci **6**(6): 449-462.

Frederickson CJ, Suh SW, Silva D, Thompson RB (2000). "Importance of zinc in the central nervous system: the zinc-containing neuron." J Nutr **130**(5S Suppl): 1471S-1483S.

Frederickson CJ (1989). "Neurobiology of zinc and zinc-containing neurons." Int Rev Neurobiol **31**: 145-238.

Frederickson CJ, Howell GA, Haigh MD, Danscher G (1988). "Zinc-containing fiber systems in the cochlear nuclei of the rat and mouse." Hear Res **36**(2-3): 203-211.

Gabbita SP, Butterfield DA, Hensley K, Shaw W, Carney JM (1997). "Aging and caloric restriction affect mitochondrial respiration and lipid membrane status: an electron paramagnetic resonance investigation." Free Radic Biol Med **23**(2): 191-201.

Garai K, Sahoo B, Kaushalya SK, Desai R, Maiti S (2007). "Zinc lowers amyloid-beta toxicity by selectively recipitating aggregation intermediates." Biochemistry **46**(37): 10655-10663.

Gee KR, Zhou ZL, Qian WJ, Kennedy R (2002). "Detection and imaging of zinc secretion from pancreatic beta-cells using a new fluorescent zinc indicator." J Am Chem Soc **124**(5): 776-778.

Gleichmann M, Mattson MP (2011). "Neuronal calcium homeostasis and dysregulation." Antioxid Redox Signal **14**(7): 1261-1273.

Goodwin JL,Uemura E, Cunnick JE (1995). "Microglial release of nitric oxide by the synergistic action of beta-amyloid and IFN-gamma." Brain Res **692**(1-2): 207-214.

Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V, Buxbaum JD, Xu H, Greengard P, Relkin NR (2000). "Intraneuronal Abeta42

accumulation in human rain." Am J Pathol **156**(1): 15-20.

Grauert A, Engel DA, Ruiz AJ (2013). "Endogenous zinc depresses GABAergic transmission via T-type Ca2+ channels and broadens the time-window for integration of glutamatergic inputs in dentate granule cells." J Physiol.

Green KN, Demuro A, Akbari Y, Hitt BD, Smith IF, Parker I, LaFerla FM (2008). "SERCA pump activity is physiologically regulated by presenilin and regulates amyloid beta production." J Cell Biol **181**(7): 1107-1116.

Greenough MA, Camakaris J, Bush AI (2013). "Metal dyshomeostasis and oxidative stress in Alzheimer's disease." Neurochem Int **62**(5): 540-555.

Grynkiewicz G, Poenie M, Tsien RY (1985). "A new generation of Ca2+ indicators with greatly improved fluorescence properties." J Biol Chem **260**(6): 3440-3450.

Guan D, Armstrong WE, Foehring RC (2013). "Kv2 channels regulate firing rate in pyramidal neurons from rat sensorimotor cortex." J Physiol.

Gutman GA, Chandy KG, Grissmer S, Lazdunski M, McKinnon D, Pardo LA, Robertson GA, Rudy B, Sanguinetti MC, Stuhmer W, Wang X (2005). "International Union of Pharmacology. LIII. Nomenclature and molecular relationships of voltage-gated potassium channels." Pharmacol Rev **57**(4): 473-508.

Haase H, Hebel S, Engelhardt G, Rink L (2009). "Zinc ions cause the thimerosal-induced signal of fluorescent calcium probes in lymphocytes." Cell Calcium **45**(2): 185-191.

Halliwell B (1992). "Reactive oxygen species and the central nervous system." J Neurochem **59**(5): 1609-1623.

Hanger DP, Anderton BH, Noble W (2009). "Tau phosphorylation: the therapeutic challenge for neurodegenerative disease." Trends Mol Med **15**(3): 112-119.

Harman D (1965). "The Free Radical Theory of Aging: Effect of Age on Serum Copper Levels." J Gerontol **20**: 151-153.

Hartigan JA, Johnson GV (1999). "Transient increases in intracellular calcium result in prolonged site-selective increases in Tau phosphorylation through a glycogen synthase kinase 3beta-dependent pathway." J Biol Chem **274**(30): 21395-21401.

Hartmann T, Bieger SC, Bruhl B, Tienari PJ, Ida N, Allsop D, Roberts GW, Masters CL, Dotti CG, Unsicker K, Beyreuther K (1997). "Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides." Nat Med **3**(9): 1016-1020.

Hartnett KA, Stout AK, Rajdev S, Rosenberg PA, Reynolds IJ, Aizenman E (1997). "NMDA

receptor-mediated neurotoxicity: a paradoxical requirement for extracellular Mg2+ in Na+/Ca2+-free solutions in rat cortical neurons in vitro." J Neurochem **68**(5): 1836-1845.

Hayashi T, Rizzuto R, Hajnoczky G, Su TP (2009). "MAM: more than just a housekeeper." Trends Cell Biol **19**(2): 81-88.

Hensley K, Floyd RA, Zheng NY, Nael R, Robinson KA, Nguyen X, Pye QN, Stewart CA, Geddes J, Markesbery WR, Patel E, Johnson GV, Bing G (1999). "p38 kinase is activated in the Alzheimer's disease brain." J Neurochem **72**(5): 2053-2058.

Hidalgo J, Aschner M, Zatta P, Vasak M (2001). "Roles of the metallothionein family of proteins in the central nervous system." Brain Res Bull **55**(2): 133-145.

Hirling H, Scheller RH (1996). "Phosphorylation of synaptic vesicle proteins: modulation of the alpha SNAP interaction with the core complex." Proc Natl Acad Sci U S A **93**(21): 11945-11949.

Ho HT, Liu B, Snyder JS, Lou Q, Brundage EA, Velez-Cortes F, Wang H, Ziolo MT, Anderson ME, Sen CK, Wehrens XH, Fedorov VV, Biesiadecki BJ, Hund TJ, Gyorke S (2013). "Ryanodine receptor phosphorylation by oxidized CaMKII contributes to the cardiotoxic effects of cardiac glycosides." Cardiovasc Res.

Hoch B, Haase H, Schulze W, Hagemann D, Morano I, Krause EG, Karczewski P (1998). "Differentiation-dependent expression of cardiac delta-CaMKII isoforms." J Cell Biochem **68**(2): 259-268.

Hu CL, Liu Z, Zeng XM, Liu ZQ, Chen XH, Zhang ZH, Mei YA (2006). "4-aminopyridine, a Kv channel antagonist, prevents apoptosis of rat cerebellar granule neurons." Neuropharmacology **51**(4): 737-746.

Huang J, Yao Y, Lin J, Ye YH, Sun WY, Tang Dagger WX (2004). "The solution structure of rat Abeta-(1-28) and its interaction with zinc ion: insights into the scarcity of amyloid deposition in aged rat brain." J Biol Inorg Chem **9**(5): 627-635.

Huang H, Gao TM, Gong L, Zhuang Z, Li X (2001). "Potassium channel blocker TEA prevents CA1 hippocampal injury following transient forebrain ischemia in adult rats." Neurosci Lett **305**(2): 83-86.

Huang X, Atwood CS, Hartshorn MA, Multhaup G, Goldstein LE, Scarpa RC, Cuajungco MP, Gray DN, Lim J, Moir RD, Tanzi RE, Bush AI (1999). "The A beta peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction." Biochemistry **38**(24): 7609-7616.

Huang X, Atwood CS, Moir RD, Hartshorn MA, Vonsattel JP, Tanzi RE, Bush AI (1997). "Zinc-induced Alzheimer's Abeta1-40 aggregation is mediated by conformational factors." J Biol Chem **272**(42): 26464-26470.

Hughes FM, Jr., Cidlowski JA (1999). "Potassium is a critical regulator of apoptotic enzymes in vitro and in vivo." Adv Enzyme Regul **39**: 157-171.

Hughes FM, Jr, Bortner CD, Purdy GD, Cidlowski JA (1997). "Intracellular K+ suppresses the activation of apoptosis in lymphocytes." J Biol Chem **272**(48): 30567-30576.

Hwang JJ, Lee SJ, Kim TY, Cho JH, Koh JY (2008). "Zinc and 4-hydroxy-2-nonenal mediate lysosomal membrane permeabilization induced by H2O2 in cultured hippocampal neurons." J Neurosci **28**(12): 3114-3122.

Irie Y, Keung WM (2001). "Metallothionein-III antagonizes the neurotoxic an neurotrophic effects of amyloid beta peptides." Biochem Biophys Res Commun **282**(2): 416-420.

Ishihara R, Ide-Ektessabi A, Ikeda K, Mizuno Y, Fujisawa S, Takeuchi T, Ohta T (2002). "Investigation of cellular metallic elements in single neurons of human brain tissues." Neuroreport **13**(14): 1817-1820.

Jalonen TO, Charniga CJ, Wielt DB (1997). "beta-Amyloid peptide-induced morphological changes coincide with increased K+ and Cl- channel activity in rat cortical astrocytes." Brain Res **746**(1-2): 85-97.

Johansen T, Lamark T (2011). "Selective autophagy mediated by autophagic adapter proteins." Autophagy **7**(3): 279-296.

Johnston J, Forsythe ID, Kopp-Scheinpflug C (2010). "Going native: voltage-gated potassium channels controlling neuronal excitability." J Physiol **588**(Pt 17): 3187-3200.

Kadowaki H, Nishitoh H, Urano F, Sadamitsu C, Matsuzawa A, Takeda K, Masutani H, Yodoi J, Urano Y, Nagano T, Ichijo H (2005). "Amyloid beta induces neuronal cell death through ROS-mediated ASK1 activation." Cell Death Differ **12**(1): 19-24.

Kakizawa S, Yamazawa T, Iino M (2013). "Nitric oxide-induced calcium release: activation of type 1 ryanodine receptor by endogenous nitric oxide." Channels (Austin) **7**(1): 1-5.

Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Muller-Hill B (1987). "The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor." Nature **325**(6106): 733-736.

Kerr JF, Wyllie AH, Currie AR (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer **26**(4): 239-257.

Khachaturian ZS (1989). "Calcium, membranes, aging, and Alzheimer's disease. Introduction and overview." Ann N Y Acad Sci **568**: 1-4.

Kim YH, Koh JY (2002). "The role of NADPH oxidase and neuronal nitric oxide synthase in

zinc-induced poly(ADP-ribose) polymerase activation and cell death in cortical culture." Exp Neurol **177**(2): 407-418.

Knapp LT, Klann E (2002). "Potentiation of hippocampal synaptic transmission by superoxide requires the oxidative activation of protein kinase C." J Neurosci **22**(3): 674-683.

Knoch ME, Hartnett KA, Hara H, Kandler K, Aizenman E (2008). "Microglia induce neurotoxicity via intraneuronal Zn(2+) release and a K(+) current surge." Glia **56**(1): 89-96.

Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, Choi DW (1996). "The role of zinc in selective neuronal death after transient global cerebral ischemia." Science **272**(5264): 1013-1016.

Kowall NW, McKee AC, Yankner BA, Beal MF (1992). "In vivo neurotoxicity of beta-amyloid [beta(1-40)] and the beta(25-35) fragment." Neurobiol Aging **13**(5): 537-542.

Krezel A, Maret W (2007). "Dual nanomolar and picomolar Zn(II) binding properties of metallothionein." J Am Chem Soc **129**(35): 10911-10921.

Kullmann PH, Wheeler DW, Beacom J, Horn JP (2004). "Implementation of a fast 16-Bit dynamic clamp using LabVIEW-RT." J Neurophysiol **91**(1): 542-554.

Kumar A, Singh BK, Ahmad I, Shukla S, Patel DK, Srivastava G, Kumar V, Pandey HP, Singh C (2012). "Involvement of NADPH oxidase and glutathione in zinc-induced dopaminergic neurodegeneration in rats: similarity with paraquat neurotoxicity." Brain Res **1438**: 48-64.

Kushnir A, Shan J, Betzenhauser MJ, Reiken S, Marks, AR (2010). "Role of CaMKIIdelta phosphorylation of the cardiac ryanodine receptor in the force frequency relationship and heart failure." Proc Natl Acad Sci U S A **107**(22): 10274-10279.

LaFerla FM (2002). "Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease." Nat Rev Neurosci **3**(11): 862-872.

Lander HM (1997). "An essential role for free radicals and derived species in signal transduction." FASEB J **11**(2): 118-124.

Lee SJ, Koh JY(2010). "Roles of zinc and metallothionein-3 in oxidative stress-induced lysosomal dysfunction, cell death, and autophagy in neurons and astrocytes." Mol Brain **3**(1): 30.

Lee SJ, Park MH, Kim HJ, Koh JY (2010). "Metallothionein-3 regulates lysosomal function in cultured astrocytes under both normal and oxidative conditions." Glia **58**(10): 1186-1196.

Lee JY, Friedman JE, Angel I, Kozak A, Koh JY (2004). "The lipophilic metal chelator DP-109 reduces amyloid pathology in brains of human beta-amyloid precursor protein transgenic

mice." Neurobiol Aging **25**(10): 1315-1321.

Lee JY, Cole TB, Palmiter RD, Koh JY (2000). "Accumulation of zinc in degenerating hippocampal neurons of ZnT3-null mice after seizures: evidence against synaptic vesicle origin." J Neurosci **20**(11): RC79.

Lees GJ, Lehmann A, Sandberg M, Hamberger A (1990). "The neurotoxicity of zinc in the rat hippocampus." Neurosci Lett **120**(2): 155-158.

Leung YM, Kang Y, Gao X, Xia F, Xie H, Sheu L, Tsuk S, Lotan I, Tsushima RG, Gaisano HY (2003). "Syntaxin 1A binds to the cytoplasmic C terminus of Kv2.1 to regulate channel gating and trafficking." J Biol Chem **278**(19): 17532-17538.

Lin W, Buccella D, Lippard SJ (2013). "Visualization of peroxynitrite-induced changes of labile Zn2+ in the endoplasmic reticulum with benzoresorufin-based fluorescent probes." J Am Chem Soc **135**(36): 13512-13520.

Lin KF, Chang RC, Suen KC, So KF, Hugon J (2004). "Modulation of calcium/calmodulin kinase-II provides partial neuroprotection against beta-amyloid peptide toxicity." Eur J Neurosci **19**(8): 2047-2055.

Litersky JM, Johnson GV, Jakes R, Goedert M, Lee M, Seubert P (1996). "Tau protein is phosphorylated by cyclic AMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase II within its microtubule-binding domains at Ser-262 and Ser-356." Biochem J **316 (Pt 2)**: 655-660.

Liu Z, Xu J, Shen X, Lv C, Xu T, Pei D (2012). "CaMKII antisense oligodeoxynucleotides protect against ischemia-induced neuronal death in the rat hippocampus." J Neurol Sci **314**(1-2): 104-110.

Liu R, Zhou XW, Tanila H, Bjorkdahl C, Wang JZ, Guan ZZ, Cao Y, Gustafsson JA, Winblad B, Pei JJ (2008). "Phosphorylated PP2A (tyrosine 307) is associated with Alzheimer neurofibrillary pathology." J Cell Mol Med **12**(1): 241-257.

Liu F, Grundke-Iqbal I, Iqbal K, Gong CX (2005). "Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation." Eur J Neurosci **22**(8): 1942-1950.

Loo DT, Copani A, Pike CJ, Whittemore ER, Walencewicz AJ, Cotman CW (1993). "Apoptosis is induced by beta-amyloid in cultured central nervous system neurons." Proc Natl Acad Sci U S A **90**(17): 7951-7955.

Lovell MA, Xie C, Markesbery WR(1999). "Protection against amyloid beta peptide toxicity by zinc." Brain Res **823**(1-2): 88-95.

Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, Markesbery WR (1998). "Copper, iron

and zinc in Alzheimer's disease senile plaques." J Neurol Sci **158**(1): 47-52.

MacDonald PE, Wheeler MB (2003). "Voltage-dependent K(+) channels in pancreatic beta cells: role, regulation and potential as therapeutic targets." Diabetologia **46**(8): 1046-1062.

MacDonald PE, Sewing S, Wang J, Joseph JW, Smukler SR, Sakellaropoulos G, Saleh MC, Chan CB, Tsushima RG, Salapatek AM, Wheeler MB (2002). "Inhibition of Kv2.1 voltage-dependent K+ channels in pancreatic beta-cells enhances glucose-dependent insulin secretion." J Biol Chem **277**(47): 44938-44945.

Madeja M, Muller V, Musshoff U, Speckmann EJ (2000). "Sensitivity of native and cloned hippocampal delayed-rectifier potassium channels to verapamil." Neuropharmacology **39**(2): 202-210.

Maeno E, Ishizaki Y, Kanaseki T, Hazama A, Okada Y (2000). "Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis." Proc Natl Acad Sci U S A **97**(17): 9487-9492.

Malaiyandi LM, Vergun O, Dineley KE, Reynolds IJ (2005). "Direct visualization of mitochondrial zinc accumulation reveals uniporter-dependent and -independent transport mechanisms." J Neurochem **93**(5): 1242-1250.

Manso Y, Adlard PA, Carrasco J, Vasak M, Hidalgo J (2011). "Metallothionein and brain inflammation." J Biol Inorg Chem **16**(7): 1103-1113.

Maret W (2013). "Zinc biochemistry: from a single zinc enzyme to a key element of life." Adv Nutr **4**(1): 82-91.

Maret W, Vallee BL (1998). "Thiolate ligands in metallothionein confer redox activity on zinc clusters." Proc Natl Acad Sci U S A **95**(7): 3478-3482.

Markesbery WR (1997). "Oxidative stress hypothesis in Alzheimer's disease." Free Radic Biol Med **23**(1): 134-147.

Martens JR, Sakamoto N, Sullivan SA, Grobaski TD, Tamkun MM (2001). "Isoform-specific localization of voltage-gated K+ channels to distinct lipid raft populations. Targeting of Kv1.5 to caveolae." J Biol Chem **276**(11): 8409-8414.

Martens JR, Navarro-Polanco R, Coppock EA, Nishiyama A, Parshley L, Grobaski TD, Tamkun MM (2000). "Differential targeting of Shaker-like potassium channels to lipid rafts." J Biol Chem 275(11): 7443-6.

Martin LJ, Adams NA, Pan Y, Price A, Wong M (2011). "The mitochondrial permeability transition pore regulates nitric oxide-mediated apoptosis of neurons induced by target deprivation." J Neurosci **31**(1): 359-370.

Mattson MP (2010). "ER calcium and Alzheimer's disease: in a state of flux." Sci Signal **3**(114): pe10.

McCord MC, Aizenman E (2013). "Convergent Ca2+ and Zn2+ signaling regulates apoptotic Kv2.1 K+ currents." Proc Natl Acad Sci U S A **110**(34): 13988-13993.

McDonald DR, Brunden KR, Landreth GE (1997). "Amyloid fibrils activate tyrosine kinase-dependent signaling and superoxide production in microglia." J Neurosci **17**(7): 2284-2294.

McLaughlin B, Pal S, Tran MP, Parsons AA, Barone FC, Erhardt JA, Aizenman E (2001). "p38 activation is required upstream of potassium current enhancement and caspase cleavage in thiol oxidant-induced neuronal apoptosis." J Neurosci **21**(10): 3303-3311.

Mecocci P, MacGarvey U, Beal MF (1994). "Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease." Ann Neurol **36**(5): 747-751.

Meda L, Cassatella MA, Szendrei GI, Otvos L, Jr, Baron P, Villalba M, Ferrari D, Rossi F (1995). "Activation of microglial cells by beta-amyloid protein and interferon-gamma." Nature **374**(6523): 647-650.

Medvedeva YV, Lin B, Shuttleworth CW, Weiss JH (2009). "Intracellular Zn2+ accumulation contributes to synaptic failure, mitochondrial depolarization, and cell death in an acute slice oxygen-glucose deprivation model of ischemia." J Neurosci **29**(4): 1105-1114.

Melov S, Coskun P, Patel M, Tuinstra R, Cottrell B, Jun AS, Zastawny TH, Dizdaroglu M, Goodman SI, Huang TT, Miziorko H, Epstein CJ, Wallace DC (1999). "Mitochondrial disease in superoxide dismutase 2 mutant mice." Proc Natl Acad Sci U S A **96**(3): 846-851.

Misonou H, Mohapatra DP, Menegola M, Trimmer JS (2005). "Calcium- and metabolic state-dependent modulation of the voltage-dependent Kv2.1 channel regulates neuronal excitability in response to ischemia." J Neurosci **25**(48): 11184-11193.

Misonou H, Mohapatra DP, Park EW, Leung V, Zhen D, Misonou K, Anderson AE, Trimmer JS (2004). "Regulation of ion channel localization and phosphorylation by neuronal activity." Nat Neurosci **7**(7): 711-718.

Misura KM, Scheller RH, Weis WI (2000). "Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex." Nature **404**(6776): 355-362.

Mo ZY, Zhu YZ, Zhu HL, Fan JB, Chen J, Liang Y (2009). "Low micromolar zinc accelerates the fibrillization of human tau via bridging of Cys-291 and Cys-322." J Biol Chem **284**(50): 34648-34657.

Mocchegiani E, Bertoni-Freddari C, Marcellini F, Malavolta M (2005). "Brain, aging and neurodegeneration: role of zinc ion availability." Prog Neurobiol **75**(6): 367-390.

Mohapatra DP, Misonou H, Pan SJ, Held JE, Surmeier DJ, Trimmer JS (2009). "Regulation of intrinsic excitability in hippocampal neurons by activity-dependent modulation of the KV2.1 potassium channel." Channels (Austin) **3**(1): 46-56.

Mohapatra DP, Siino DF, Trimmer JS (2008). "Interdomain cytoplasmic interactions govern the intracellular trafficking, gating, and modulation of the Kv2.1 channel." J Neurosci **28**(19): 4982-4994.

Mohapatra DP, Park KS, Trimmer JS (2007). "Dynamic regulation of the voltage-gated Kv2.1 potassium channel by multisite phosphorylation." Biochem Soc Trans **35**(Pt 5): 1064-1068.

Montague JW, Bortner CD, Hughes FM, Jr., Cidlowski JA (1999). "A necessary role for reduced intracellular potassium during the DNA degradation phase of apoptosis." Steroids **64**(9): 563-569.

Moreira PI, Cardoso SM, Santos MS, Oliveira CR (2006). "The key role of mitochondria in Alzheimer's disease." J Alzheimers Dis **9**(2): 101-110.

Murakoshi H, Trimmer JS (1999). "Identification of the Kv2.1 K+ channel as a major component of the delayed rectifier K+ current in rat hippocampal neurons." J Neurosci **19**(5): 1728-1735.

Murakoshi H, Shi G, Scannevin RH, Trimmer JS (1997). "Phosphorylation of the Kv2.1 K+ channel alters voltage-dependent activation." Mol Pharmacol **52**(5): 821-828.

Nixon RA, Yang DS (2011). "Autophagy failure in Alzheimer's disease--locating the primary defect." Neurobiol Dis **43**(1): 38-45.

Noh KM, Koh JY (2000). "Induction and activation by zinc of NADPH oxidase in cultured cortical neurons and astrocytes." J Neurosci **20**(23): RC111.

Nomura K, Ohyama A, Komiya Y, Igarashi M (2003). "Minimal residues in linker domain of syntaxin 1A required for binding affinity to Ca2+/calmodulin-dependent protein kinase II." J Neurosci Res **72**(2): 198-202.

Nunomura A, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, Jones PK, Ghanbari H, Wataya T, Shimohama S, Chiba S, Atwood CS, Petersen RB, Smith MA (2001). "Oxidative damage is the earliest event in Alzheimer disease." J Neuropathol Exp Neurol **60**(8): 759-767.

Obenauer JC, Cantley LC, Yaffe MB (2003). "Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs." Nucleic Acids Res **31**(13): 3635-3641.

O'Connell KM, Loftus MR, Tamkun MM (2010). "Localization-dependent activity of the Kv2.1 delayed-rectifier K+ channel." Proc Natl Acad Sci U S A **107**(27): 12351-12356.

O'Connell KM, Tamkun MM (2005). "Targeting of voltage-gated potassium channel isoforms to distinct cell surface microdomains." J Cell Sci **118**(Pt 10): 2155-2166.

Ohyagi Y, Yamada T, Nishioka K, Clarke NJ, Tomlinson AJ, Naylor S, Nakabeppu Y, Kira J, Younkin SG (2000). "Selective increase in cellular A beta 42 is related to apoptosis but not necrosis." Neuroreport **11**(1): 167-171.

Ohyama A, Hosaka K, Komiya Y, Akagawa K, Yamauchi E, Taniguchi H, Sasagawa N, Kumakura K, Mochida S, Yamauchi T, Igarashi M (2002). "Regulation of exocytosis through Ca2+/ATP-dependent binding of autophosphorylated Ca2+/calmodulin-activated protein kinase II to syntaxin 1A." J Neurosci **22**(9): 3342-3351.

Olanow CW (1993). "A radical hypothesis for neurodegeneration." Trends Neurosci **16**(11): 439-
444.

Pal SK, Takimoto K, Aizenman E, Levitan ES (2006). "Apoptotic surface delivery of K+ channels." Cell Death Differ **13**(4): 661-667.

Pal S, Hartnett KA, Nerbonne JM, Levitan ES, Aizenman E (2003). "Mediation of neuronal apoptosis by Kv2.1-encoded potassium channels." J Neurosci **23**(12): 4798-4802.

Pan Y, Xu X, Tong X, Wang X (2004). "Messenger RNA and protein expression analysis of voltage-gated potassium channels in the brain of Abeta(25-35)-treated rats." J Neurosci Res **77**(1): 94-99.

Pan YP, Xu XH, Wang XL (2003). "Galantamine blocks delayed rectifier, but not transient outward potassium current in rat dissociated hippocampal pyramidal neurons." Neurosci Lett **336**(1): 37-40.

Panayi AE, Spyrou NM, Iversen BS, White MA, Part P (2002). "Determination of cadmium and zinc in Alzheimer's brain tissue using inductively coupled plasma mass spectrometry." J Neurol Sci **195**(1): 1-10.

Paoletti P, Vergnano AM, Barbour B, Casado M (2009). "Zinc at glutamatergic synapses." Neuroscience **158**(1): 126-136.

Park MH, Lee SJ, Byun HR, Kim Y, Oh YJ, Koh JY, Hwang JJ (2011). "Clioquinol induces autophagy in cultured astrocytes and neurons by acting as a zinc ionophore." Neurobiol Dis **42**(3): 242-251.

Park KS, Mohapatra DP, Misonou H, Trimmer JS (2006). "Graded regulation of the Kv2.1 potassium channel by variable phosphorylation." Science **313**(5789): 976-979.

Pasantes-Morales H, Maar TE, Moran J (1993). "Cell volume regulation in cultured cerebellar granule neurons." J Neurosci Res **34**(2): 219-224.

Pei JJ, Braak E, Braak H, Grundke-Iqbal I. Iqbal K, Winblad B, Cowburn RF (2001). "Localization of active forms of C-jun kinase (JNK) and p38 kinase in Alzheimer's disease brains at different stages of neurofibrillary degeneration." J Alzheimers Dis **3**(1): 41-48.

Perez-Rosello T, Anderson CT, Schopfer FJ, Zhao Y, Gilad D, Salvatore SR, Freeman BA, Hershfinkel M, Aizenman E, Tzounopoulos T (2013). "Synaptic Zn2+ inhibits neurotransmitter release by promoting endocannabinoid synthesis." J Neurosci **33**(22): 9259-9272.

Perozo E, Bezanilla F (1990). "Phosphorylation affects voltage gating of the delayed rectifier K+ channel by electrostatic interactions." Neuron **5**(5): 685-690.

Pierrot N, Santos SF, Feyt C, Morel M, Brion JP, Octave JN (2006). "Calcium-mediated transient phosphorylation of tau and amyloid precursor protein followed by intraneuronal amyloid-beta accumulation." J Biol Chem **281**(52): 39907-39914.

Pongs O (1999). "Voltage-gated potassium channels: from hyperexcitability to excitement." FEBS Lett **452**(1-2): 31-35.

Purohit A, Rokita AG, Guan X, Chen B, Koval OM, Voigt N, Neef S, Sowa T, Gao Z, Luczak ED, Stefansdottir H, Behunin AC, Li N, El-Accaoui RN, Yang B, Swaminathan PD, Weiss RM, Wehrens XH, Song LS, Dobrev D, Maier LS, Anderson ME (2013). "Oxidized Ca(2+)/calmodulin-dependent protein kinase II triggers atrial fibrillation." Circulation **128**(16): 1748-1757.

Radford RJ, Lippard SJ (2013). "Chelators for investigating zinc metalloneurochemistry." Curr Opin Chem Biol **17**(2): 129-136.

Redman PT, Hartnett KA, Aras MA, Levitan ES, Aizenman E (2009). "Regulation of apoptotic potassium currents by coordinated zinc-dependent signalling." J Physiol **587**(Pt 18): 4393-4404.

Redman PT, He K, Hartnett KA, Jefferson BS, Hu L, Rosenberg PA, Levitan ES, Aizenman E (2007). "Apoptotic surge of potassium currents is mediated by p38 phosphorylation of Kv2.1." Proc Natl Acad Sci U S A **104**(9): 3568-3573.

Regland B, Lehmann W, Abedini I, Blennow K, Jonsson M, Karlsson I, Sjogren M, Wallin A, Xilinas M, Gottfries CG (2001). "Treatment of Alzheimer's disease with clioquinol." Dement Geriatr Cogn Disord **12**(6): 408-414.

Religa D, Strozyk D, Cherny RA, Volitakis I, Haroutunian V, Winblad B, Naslund J, Bush AI (2006). "Elevated cortical zinc in Alzheimer disease." Neurology **67**(1): 69-75.

Respress JL, van Oort RJ, Li N, Rolim N, Dixit SS, deAlmeida A, Voigt N, Lawrence WS, Skapura DG, Skardal K, Wisloff U, Wieland T, Ai X, Pogwizd SM, Dobrev D, Wehrens XH (2012). "Role of RyR2 phosphorylation at S2814 during heart failure progression." Circ Res **110**(11): 1474-1483.

Reynolds CH, Nebreda AR, Gibb GM, Utton MA, Anderton BH (1997). "Reactivating kinase/p38 phosphorylates tau protein in vitro." J Neurochem **69**(1): 191-198.

Richter C, Park JW, Ames BN (1988). "Normal oxidative damage to mitochondrial and nuclear DNA is extensive." Proc Natl Acad Sci U S A **85**(17): 6465-6467.

Ritchie CW, Bush AI, Mackinnon A, Macfarlane S, Mastwyk M, MacGregor L, Kiers L, Cherny R, Li QX, Tammer A, Carrington D, Mavros C, Volitakis I, Xilinas M, Ames D, Davis S, Beyreuther K, Tanzi RE, Masters CL (2003). "Metal-protein attenuation with iodochlorhydroxyquin (clioquinol) targeting Abeta amyloid deposition and toxicity in Alzheimer disease: a pilot phase 2 clinical trial." Arch Neurol **60**(12): 1685-1691.

Rodrigo J, Fernandez-Vizarra P, Castro-Blanco S, Bentura ML, Nieto M, Gomez-Isla T, Martinez-Murillo R, MartInez A, Serrano J, Fernandez AP (2004). "Nitric oxide in the cerebral cortex of amyloid-precursor protein (SW) Tg2576 transgenic mice." Neuroscience **128**(1): 73-89.

Saadi RA, He K, Hartnett KA, Kandler K, Hershfinkel M, Aizenman E (2012). "SNARE-dependent upregulation of potassium chloride co-transporter 2 activity after metabotropic zinc receptor activation in rat cortical neurons in vitro." Neuroscience **210**: 38-46.

Sarmiere PD, Weigle CM, Tamkun MM (2008). "The Kv2.1 K+ channel targets to the axon initial segment of hippocampal and cortical neurons in culture and in situ." BMC Neurosci **9**: 112.

Scannevin RH, Murakoshi H, Rhodes KJ, Trimmer JS (1996). "Identification of a cytoplasmic domain important in the polarized expression and clustering of the Kv2.1 K+ channel." J Cell Biol **135**(6 Pt 1): 1619-1632.

Sekler I, Moran A, Hershfinkel M, Dori A, Margulis A, Birenzweig N, Nitzan Y, Silverman WF (2002). "Distribution of the zinc transporter ZnT-1 in comparison with chelatable zinc in the mouse brain." J Comp Neurol **447**(3): 201-209.

Sen CK, Packer L (1996). "Antioxidant and redox regulation of gene transcription." FASEB J **10**(7): 709-720.

Sensi SL, Paoletti P, Koh JY, Aizenman E, Bush AI, Hershfinkel M (2011). "The neurophysiology and pathology of brain zinc." J Neurosci **31**(45): 16076-16085.

Sensi SL, Rapposelli IG, Frazzini V, Mascetra N (2008). "Altered oxidant-mediated

intraneuronal zinc mobilization in a triple transgenic mouse model of Alzheimer's disease." Exp Gerontol **43**(5): 488-492.

Sensi SL, Ton-That D, Sullivan PG, Jonas EA, Gee KR, Kaczmarek LK, Weiss JH (2003). "Modulation of mitochondrial function by endogenous Zn2+ pools." Proc Natl Acad Sci U S A **100**(10): 6157-6162.

Sensi SL, Ton-That D, Weiss JH (2002). "Mitochondrial sequestration and Ca(2+)-dependent release of cytosolic Zn(2+) loads in cortical neurons." Neurobiol Dis **10**(2): 100-108.

Sensi SL, Yin HZ, Weiss JH (2000). "AMPA/kainate receptor-triggered Zn2+ entry into cortical neurons induces mitochondrial Zn2+ uptake and persistent mitochondrial dysfunction." Eur J Neurosci **12**(10): 3813-3818.

Sensi SL, Yin HZ, Carriedo SG, Rao SS, Weiss JH (1999). "Preferential Zn2+ influx through Ca2+-permeable AMPA/kainate channels triggers prolonged mitochondrial superoxide production." Proc Natl Acad Sci U S A **96**(5): 2414-2419.

Shen QJ, Zhao YM, Cao DX, Wang XL (2009). "Contribution of Kv channel subunits to glutamate-induced apoptosis in cultured rat hippocampal neurons." J Neurosci Res **87**(14): 3153-3160.

Shen K, Meyer T (1999). "Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation." Science **284**(5411): 162-166.

Shepherd AJ, Loo L, Gupte RP, Mickle AD, Mohapatra DP (2012). "Distinct modifications in Kv2.1 channel via chemokine receptor CXCR4 regulate neuronal survival-death dynamics." J Neurosci **32**(49): 17725-17739.

Shuttleworth CW, Weiss JH (2011). "Zinc: new clues to diverse roles in brain ischemia." Trends Pharmacol Sci **32**(8): 480-486.

Sims NR, Anderson MF (2002). "Mitochondrial contributions to tissue damage in stroke." Neurochem Int **40**(6): 511-526.

Singer-Lahat D, Chikvashvili D, Lotan I (2008). "Direct interaction of endogenous Kv channels with syntaxin enhances exocytosis by neuroendocrine cells." PLoS One **3**(1): e1381.

Singer-Lahat D, Sheinin A, Chikvashvili D, Tsuk S, Greitzer D, Friedrich R, Feinshreiber L, Ashery U, Benveniste M, Levitan ES, Lotan I (2007). "K+ channel facilitation of exocytosis by dynamic interaction with syntaxin." J Neurosci **27**(7): 1651-1658.

Smart TG, Hosie AM, Miller PS (2004). "Zn2+ ions: modulators of excitatory and inhibitory synaptic activity." Neuroscientist **10**(5): 432-442.

Smith RM (2009) NIST crtitically selected stability constants of metal complexes. In NIST

Scientific and Technical Databases.

Smith MA, Hirai K, Hsiao K, Pappolla MA, Harris PL, Siedlak SL, Tabaton M, Perry G (1998). "Amyloid-beta deposition in Alzheimer transgenic mice is associated with oxidative stress." J Neurochem **70**(5): 2212-2215.

Smith MA, Richey Harris PL, Sayre LM, Beckman JS, Perry G (1997). "Iron accumulation in Alzheimer disease is a source of redox-generated free radicals." Proc Natl Acad Sci U S A **94**(18): 9866-9868.

Stork CJ, Li YV (2010). "Zinc release from thapsigargin/IP3-sensitive stores in cultured cortical neurons." J Mol Signal **5**: 5.

Stork CJ, Li YV (2006). "Intracellular zinc elevation measured with a "calcium-specific" indicator during ischemia and reperfusion in rat hippocampus: a question on calcium overload." J Neurosci **26**(41): 10430-10437.

Strack S, Barban MA, Waszinski BE, Colbran RJ (1997). "Differential inactivation of postsynaptic density-associated and soluble Ca2+/calmodulin-dependent protein kinase II by protein phosphatases 1 and 2A." J Neurochem **68**(5): 2119-2128.

Sturchler-Pierrat C, Abramowski D, Duke M, Wiederhold KH, Mistl C, Rothacher S, Ledermann B, Burki K, Frey P, Paganetti PA, Waridel C, Calhoun ME, Jucker M, Probst A, Staufenbiel M, Sommer B (1997). "Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology." Proc Natl Acad Sci U S A **94**(24): 13287-13292.

Suh SW, Jensen KB, Jensen MS, Silva DS, Kesslak PJ, Danscher G, Frederickson CJ (2000). "Histochemically-reactive zinc in amyloid plaques, angiopathy, and degenerating neurons of Alzheimer's diseased brains." Brain Res **852**(2): 274-278.

Sun XY, Wei YP, Xiong Y, Wang XC, Xie AJ, Wang XL, Yang Y, Wang Q, Lu YM, Liu R, Wang JZ (2012). "Synaptic released zinc promotes tau hyperphosphorylation by inhibition of protein phosphatase 2A (PP2A)." J Biol Chem **287**(14): 11174-11182.

Sun A, Liu M, Nguyen XV, Bing G (2003). "P38 MAP kinase is activated at early stages in Alzheimer's disease brain." Exp Neurol **183**(2): 394-405.

Surmeier DJ, Foehring R (2004). "A mechanism for homeostatic plasticity." Nat Neurosci **7**(7): 691-692.

Swartz, KJ, MacKinnon R (1995). "An inhibitor of the Kv2.1 potassium channel isolated from the venom of a Chilean tarantula." Neuron **15**(4): 941-949.

Takeda K, Matsuzawa A, Nishitoh H, Tobiume K, Kishida S, Ninomiya-Tsuji J, Matsumoto K, Ichijo H (2004). "Involvement of ASK1 in Ca2+-induced p38 MAP kinase activation."

EMBO Rep **5**(2): 161-166.

Takuma K, Fang F, Zhang W, Yan S, Fukuzaki E, Du H, Sosunov A, McKhann G, Funatsu Y, Nakamichi N, Nagai T, Mizoguchi H, Ibi D, Hori O, Ogawa S, Stern DM, Yamada K, Yan SS (2009). "RAGE-mediated signaling contributes to intraneuronal transport of amyloid-beta and neuronal dysfunction." Proc Natl Acad Sci U S A **106**(47): 20021-20026.

Tamkun MM, O'Connell KM, Rolig AS (2007). "A cytoskeletal-based perimeter fence selectively corrals a sub-population of cell surface Kv2.1 channels." J Cell Sci **120**(Pt 14): 2413-2423.

Taylor KM, Hiscox S, Nicholson RI, Hogstrand C, Kille P (2012). "Protein kinase CK2 triggers cytosolic zinc signaling pathways by phosphorylation of zinc channel ZIP7." Sci Signal **5**(210): ra11.

Thompson GJ, Langlais C, Cain K, Conley EC, Cohen GM (2001). "Elevated extracellular [K+] inhibits death-receptor- and chemical-mediated apoptosis prior to caspase activation and cytochrome c release." Biochem J **357**(Pt 1): 137-145.

Thompson CM, Markesbery WR, Ehmann WD, Mao YX, Vance DE (1988). "Regional brain trace-element studies in Alzheimer's disease." Neurotoxicology **9**(1): 1-7.

Tiran Z, Peretz A, Attali B, Elson A (2003). "Phosphorylation-dependent regulation of Kv2.1 Channel activity at tyrosine 124 by Src and by protein-tyrosine phosphatase epsilon." J Biol Chem **278**(19): 17509-17514.

Tomat E, Lippard SJ (2010). "Ratiometric and intensity-based zinc sensors built on rhodol and rhodamine platforms." Inorg Chem **49**(20): 9113-9115.

Trimmer JS (1991). "Immunological identification and characterization of a delayed rectifier K+ channel polypeptide in rat brain." Proc Natl Acad Sci U S A **88**(23): 10764-10768.

Tsuji S, Kobayashi H, Uchida Y, Ihara Y, Miyatake T (1992). "Molecular cloning of human growth inhibitory factor cDNA and its down-regulation in Alzheimer's disease." EMBO J **11**(13): 4843-4850.

Tsuk S, Michaelevski I, Bentley GN, Joho RH, Chikvashvili D, Lotan I (2005). "Kv2.1 channel activation and inactivation is influenced by physical interactions of both syntaxin 1A and the syntaxin 1A/soluble N-ethylmaleimide-sensitive factor-25 (t-SNARE) complex with the C terminus of the channel." Mol Pharmacol **67**(2): 480-488.

Turner RS, Suzuki N, Chyung AS, Younkin SG, Lee VM (1996). "Amyloids beta40 and beta42 are generated intracellularly in cultured human neurons and their secretion increases with maturation." J Biol Chem **271**(15): 8966-8970.

Uchida Y, Takio K, Titani K, Ihara Y, Tomonaga M (1991). "The growth inhibitory factor that is

deficient in the Alzheimer's disease brain is a 68 amino acid metallothionein-like protein." Neuron **7**(2): 337-347.

Vallee BL, Falchuk KH (1993). "The biochemical basis of zinc physiology." Physiol Rev **73**(1): 79-118.

Vander Jagt TA, Connor JA, Weiss JH, Shuttleworth CW (2009). "Intracellular Zn2+ increases contribute to the progression of excitotoxic Ca2+ increases in apical dendrites of CA1 pyramidal neurons." Neuroscience **159**(1): 104-114.

Vest RS, O'Leary H, Coultrap SJ, Kindy MS, Bayer KU (2010). "Effective post-insult neuroprotection by a novel Ca(2+)/ calmodulin-dependent protein kinase II (CaMKII) inhibitor." J Biol Chem **285**(27): 20675-20682.

Vicente MI, Costa PF, Lima PA (2010). "Galantamine inhibits slowly inactivating K+ currents with a dual dose-response relationship in differentiated N1E-115 cells and in CA1 neurones." Eur J Pharmacol **634**(1-3): 16-25.

Wallace DC (2005). "A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine." Annu Rev Genet **39**: 359-407.

Walsh DM, Tseng BP, Rydel RE, Podlisny MB, Selkoe DJ (2000). "The oligomerization of amyloid beta-protein begins intracellularly in cells derived from human brain." Biochemistry **39**(35): 10831-10839.

Wang XQ, Xiao AY, Yang A, LaRose L, Wei L, Yu SP (2003). "Block of Na+,K+-ATPase and induction of hybrid death by 4-aminopyridine in cultured cortical neurons." J Pharmacol Exp Ther **305**(2): 502-506.

Watanabe Y, Katayama N, Takeuchi K, Togano T, Itoh R, Sato M, Yamazaki M, Abe M, Sato T, Oda K, Yokoyama M, Takao K, Fukaya M, Miyakawa T, Watanabe M, Sakimura K, Manabe T, Igarashi M (2013). "Point Mutation in Syntaxin-1A Causes Abnormal Vesicle Recycling, Behaviors, and Short Term Plasticity." J Biol Chem **288**(48): 34906-34919.

Wei L, Xiao AY, Jin C, Yang A, Lu ZY, Yu SP (2004). "Effects of chloride and potassium channel blockers on apoptotic cell shrinkage and apoptosis in cortical neurons." Pflugers Arch **448**(3): 325-334.

Wei L, Yu SP, Gottron F, Snider BJ, Zipfel GJ, Choi DW (2003). "Potassium channel blockers attenuate hypoxia- and ischemia-induced neuronal death in vitro and in vivo." Stroke **34**(5): 1281-1286.

Weiss JH, Sensi SL, Koh JY (2000). "Zn(2+): a novel ionic mediator of neural injury in brain disease." Trends Pharmacol Sci **21**(10): 395-401.

Weiss JH, Hartley DM, Koh JY, Choi DW (1993). "AMPA receptor activation potentiates zinc

neurotoxicity." Neuron **10**(1): 43-49.

Weldon DT, Rogers SD, Ghilardi JR, Finke MP, Cleary JP, O'Hare E, Esler WP, Maggio JE, Mantyh PW (1998). "Fibrillar beta-amyloid induces microglial phagocytosis, expression of inducible nitric oxide synthase, and loss of a select population of neurons in the rat CNS in vivo." J Neurosci **18**(6): 2161-2173.

Wiedau-Pazos M, Goto JJ, Rabizadeh S, Gralla EB, Roe JA, Lee MK, Valentine JS, Bredesen DE (1996). "Altered reactivity of superoxide dismutase in familial amyotrophic lateral sclerosis." Science **271**(5248): 515-518.

Wild-Bode C, Yamazaki T, Capell A, Leimer U, Steiner H, Ihara Y, Haass C (1997). "Intracellular generation and accumulation of amyloid beta-peptide terminating at amino acid 42." J Biol Chem **272**(26): 16085-16088.

Wirths O, Multhaup G, Czech C, Blanchard V, Moussaoui S, Tremp G, Pradier L, Beyreuther K, Bayer TA (2001). "Intraneuronal Abeta accumulation precedes plaque formation in beta-amyloid precursor protein and presenilin-1 double-transgenic mice." Neurosci Lett **306**(1-2): 116-120.

Wu X, Hernandez-Enriquez B, Banas M, Xu R, Sesti F (2013). "Molecular mechanisms underlying the apoptotic effect of KCNB1 K+ channel oxidation." J Biol Chem **288**(6): 4128-4134.

Xu L, Eu JP, Meissner G, Stamler JS (1998). "Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation." Science **279**(5348): 234-237.

Yamamoto A, Shin RW, Hasegawa K, Naiki H, Sato H, Yoshimasu F, Kitamoto T (2002). "Iron (III) induces aggregation of hyperphosphorylated tau and its reduction to iron (II) reverses the aggregation: implications in the formation of neurofibrillary tangles of Alzheimer's disease." J Neurochem **82**(5): 1137-1147.

Ye B, Maret W, Vallee BL (2001). "Zinc metallothionein imported into liver mitochondria modulates respiration." Proc Natl Acad Sci U S A **98**(5): 2317-2322.

Yokoyama M, Koh J, Choi DW (1986). "Brief exposure to zinc is toxic to cortical neurons." Neurosci Lett **71**(3): 351-355.

Yu SP (2003). "Regulation and critical role of potassium homeostasis in apoptosis. "Prog Neurobiol **70**(4): 363-386.

Yu WH, Lukiw WJ, Bergeron C, Niznik HB, Fraser PE (2001). "Metallothionein III is reduced in Alzheimer's disease." Brain Res **894**(1): 37-45.

Yu SP, Kerchner GA (1998). "Endogenous voltage-gated potassium channels in human embryonic kidney (HEK293) cells." J Neurosci Res **52**(5): 612-617.

Yu SP, Farhangrazi ZS, Ying HS, Yeh CH, Choi DW (1998). "Enhancement of outward potassium current may participate in beta-amyloid peptide-induced cortical neuronal death." Neurobiol Dis **5**(2): 81-88.

Yu SP, Yeh CH, Sensi SL, Gwag BJ, Canzoniero LM, Farhangrazi ZS, Ying HS, Tian M, Dugan LL, Choi DW (1997). "Mediation of neuronal apoptosis by enhancement of outward potassium current." Science **278**(5335): 114-117.

Zaidi NF, Lagenaur CF, Abramson JJ, Pessah I, Salama G (1989). "Reactive disulfides trigger Ca2+ release from sarcoplasmic reticulum via an oxidation reaction." J Biol Chem **264**(36): 21725-21736.

Zaks-Makhina E, Kim Y, Aizenman E, Levitan ES (2004). "Novel neuroprotective K+ channel inhibitor identified by high-throughput screening in yeast." Mol Pharmacol **65**(1): 214-219.

Zambenedetti P, Giordano R, Zatta P (1998). "Metallothioneins are highly expressed in astrocytes and microcapillaries in Alzheimer's disease." J Chem Neuroanat **15**(1): 21-26.

Zhang Y, Aizenman E, DeFranco DB, Rosenberg PA (2007). "Intracellular zinc release, 12-lipoxygenase activation and MAPK dependent neuronal and oligodendroglial death." Mol Med **13**(7-8): 350-355.

Zhang Y, Wang H, Li J, Jimenez DA, Levitan ES, Aizenman E, Rosenberg PA (2004). "Peroxynitrite-induced neuronal apoptosis is mediated by intracellular zinc release and 12-lipoxygenase activation." J Neurosci **24**(47): 10616-10627.

Zhang HX, Zhang W, Jin HW, Wang XL (2004). "Galantamine blocks cloned Kv2.1, but not Kv1.5 potassium channels." Brain Res Mol Brain Res **131**(1-2): 136-140.

Zhang ZH, Lee YT, Rhodes K, Wang K, Argentieri TM, Wang Q (2003). "Inhibitory effects of pimozide on cloned and native voltage-gated potassium channels." Brain Res Mol Brain Res **115**(1): 29-38.

Zheng L, Kagedal K, Dehvari N, Benedikz E, Cowburn R, Marcusson J, Terman A (2009). "Oxidative stress induces macroautophagy of amyloid beta-protein and ensuing apoptosis." Free Radic Biol Med **46**(3): 422-429.

Zhou MH, Yang G, Jiao S, Hu CL, Mei YA (2012). "Cholesterol enhances neuron susceptibility to apoptotic stimuli via cAMP/PKA/CREB-dependent up-regulation of Kv2.1." J Neurochem **120**(4): 502-514.

Zhu X, Rottkamp CA, Hartzler A, Sun Z, Takeda A, Boux H, Shimohama S, Perry G, Smith MA (2001). "Activation of MKK6, an upstream activator of p38, in Alzheimer's disease." J Neurochem **79**(2): 311-318.

Zhu X, Rottkamp CA, Boux H, Takeda A, Perry G, Smith MA (2000). "Activation of p38 kinase links tau phosphorylation, oxidative stress, and cell cycle-related events in Alzheimer disease." J Neuropathol Exp Neurol **59**(10): 880-888.