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Ubiquitin Ligase Trim32 and Chloride-sensitive WNK1 as Regulators of Potassium Channels in the Brain

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ABSTRACT

The voltage-gated potassium channel Kv1.2 impacts membrane potential and therefore excitability of neurons. Expression of Kv1.2 at the plasma membrane (PM) is critical for channel function, and altering Kv1.2 at the PM is one way to affect membrane excitability. Such is the case in the cerebellum, a portion of the brain with dense Kv1.2 expression, where modulation of Kv1.2 at the PM can impact electrical activity of neurons and ultimately cerebellum-dependent learning. Modulation of Kv1.2 at the PM can occur through endocytic trafficking of the channel; however mechanisms behind this process in the brain remain to be defined.

The goal of this dissertation was to identify and characterize modalities endogenous to the brain that influence the presence of Kv1.2 at the neuronal plasma membrane. Mass spectrometry (MS) was used to first identify interacting proteins and post-translational modifications (PTM) of Kv1.2 from cerebellar tissue, and the roles of these interactions and modifications on Kv1.2 function were evaluated in two studies:

The first study investigated Trim32, a protein enzyme that catalyzes ubiquitylation, a PTM involved in protein degradation, but also in non-degradative events such as endocytic trafficking. Trim32 was demonstrated to associate and localize with Kv1.2 in cerebellar neurons by MS, immunoblotting (IB), and immunofluorescence (IF), and also demonstrated the ability to ubiquitylate Kv1.2 \textit{in vitro} through purified recombinant proteins. Utilizing cultured cells through a combination of mutagenesis, biochemistry, and quantitative MS, a working model of Kv1.2 modulation was developed in which Trim32 influences Kv1.2 surface expression by two mechanisms that both involve cross-talk of ubiquitylation and phosphorylation sites of Kv1.2.

The second study investigated WNK1, a chloride-sensitive kinase which regulates cellular homeostasis. Using MS, IB, and IF, WNK1 was demonstrated to associate and localize with Kv1.2 in cerebellar neurons by MS, immunoblotting (IB), and immunofluorescence (IF), and also demonstrated the ability to ubiquitylate Kv1.2 \textit{in vitro} through purified recombinant proteins. Utilizing cultured cells through a combination of mutagenesis, biochemistry, and quantitative MS, a working model whereby WNK1 modulates surface Kv1.2. Activation of the downstream target SPAK kinase, also identified by MS to associate with Kv1.2 in the brain, by WNK1 was additionally found to influence the manner of WNK1 modulation of Kv1.2.

In addition to providing new models of Kv1.2 modulation in the brain, these studies propose novel biological roles for Trim32 and WNK1 that may ultimately impact neuronal excitability.
CITATIONS

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LIST OF ABBREVIATIONS

([Ca^{2+}]_i – Intracellular calcium
AC – Adenyl cyclase
ADHD – Attention Deficit Hyperactive Disorder
ADP – Adenosine-5’-diphosphate
AMP – Adenosine-5’-monophosphate
AP – Associated protein or action potential
APC – Anaphase-promoting complex/cyclosome
ASD – Autism Spectrum Disorders
ATP – Adenosine-5’-triphosphate
BC – Basket cell
BK – Renal large-conductance Ca (2+)-activated K(+) 
CDC34 – Cell division cycle 34
C terminus – Carboxy terminus
DNA – Deoxyribonucleic acid
DUB – De-ubiquitinating enzyme
E1 – Ubiquitin-activating enzyme
E2 – Ubiquitin-conjugating enzyme
E3 – Ubiquitin ligase
EBC – Eye-blink conditioning
EGFR – Epidermal growth factor receptor
ENaC – Epithelial sodium channel
FBS – Fetal bovine serum
GABA – γ-amino butyric acid
GFP – Green fluorescent protein
GST – Glutathione-S-transferase
HA – Hemagglutinin
HEK – Human embryonic kidney
HECT – Homologous to the E6-AP Carboxyl Terminus
HEPES – 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC – High through-put liquid chromatography
ITSN1 – Intersectin1
Kv – Voltage-gated potassium channel
Kir – Inward-rectifying potassium channel
LC-MS/MS – Liquid chromatography tandem mass spectrometry
LUBAC – Linear ubiquitin assembly complex
M1 – Methionine 1
MiRNA – MicroRNA
MS – Mass spectrometry
MVB – Multivesicular body
NCC – Sodium chloride cotransporter
NaV – Voltage-gated sodium channel
NHL – Asparagine histidine lysine
NKCC – Sodium potassium two chloride cotransporter
N terminus – Amino terminus
OSR1 – Oxidative stress-responsive 1
PBS – Phosphate-buffered saline
PC – Purkinje cell
PKA – Protein Kinase A
PO₄³⁻ – Phosphate
PHA2 – Pseudohypoaldosteronism type 2
PHD – Plant homeodomain
CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

Proteins are essential to life, and how proteins contribute to cell function is a fundamental question in science (Eisenberg, Marcotte, Xenarios, & Yeates, 2000). Around the turn of the 21st century, large-scale genomic sequencing facilitated the development of computational methods to identify shared functional properties among proteins, often by inference from sequence and structural homology (Eisenberg et al., 2000). However, these methods can be limited by the fact that an individual protein’s function is often critically dependent on the larger protein complex in which it is found as well as on its post-translational modification state (Gingras, Gstaiger, Raught, & Aebersold, 2007; Spirin & Mirny, 2003; Stoilova-McPhie, Ali, & Laezza, 2013). Mass spectrometry (MS) is a tool that can provide information about both of these key parameters. Indeed, MS is now perhaps the best method for identifying the specific components of a protein complex, as well as the specific sites and types of post-translational modification, that actually exist in living organisms (Aebersold & Mann, 2003). As with any tool, protein peptide identification by MS is not without shortcomings, drawbacks, and pitfalls, particularly at the level of analysis, (Lubec and Afjehi-Sadat, 2010) and careful considerations must be made during sample preparation and analysis. Given this strength of MS, this approach was used as a starting point and foundation for the data presented in Chapters 2 and 3. Both chapters present evidence for unique regulation and trafficking of voltage-gated ion channels; Chapter 2 presents data
for regulation by ubiquitylation and the ubiquitin ligase Trim32 while Chapter 3 presents data for regulation by atypical WNK1 and SPAK kinases. The following literature review is intended to provide additional background for the major topics discussed in the chapters that follow.

**Protein Post-Translational Modification**

*Overview of Protein Post-Translational Modification*

Protein function is often regulated by some type of post-translational modification. Extracellular and intrinsic signals are modulated and fine-tuned by PTMs, which regulate protein functioning, trafficking, and survival. PTMs also can assist with general biological processes such as cell signaling, proliferation, migration, and interaction with other cells (Doerig, Rayner, Scherf, & Tobin, 2015). PTMs can occur at any point in the life cycle of the protein.

Shortly after translation, PTMs can assist with the proper folding, stability, and biosynthetic trafficking of proteins (Dunham et al., 2012). Once properly folded and localized, PTMs can continuously influence the catalytic and overall biological activity of the protein. Once a protein reaches the end of its life cycle, PMTs serve as tags to mark its eventual destruction. Ultimately, PTMs impact a protein throughout the entirety of its lifespan.

The human proteome consists of enzymes such as kinases, phosphatases, transferases, ligases, and others which perform over 200 types of PTMs by adding, removing, or transferring modification groups. Examples of PTM modification groups include: addition of methyl and acetyl groups to a lysine for histone code development on
chromatin, palmityl groups to a cysteine to help drive protein association with membranes, N-acetylglucosamine to a serine/threonine to control enzyme activity and gene expression in glucose homeostasis, among many others (Pawson & Nash, 2003). The following sections will focus on two of the most common PTMs, phosphorylation and ubiquitylation, which influence the dynamic trafficking of mature proteins at the plasma membrane.

**Protein Phosphorylation**

Phosphorylation is the most prevalent and understood post-translational modification. Approximately one-third of proteins in the human proteome are estimated to be a substrate for phosphorylation at some point in the protein lifespan (P. Cohen, 2000). Protein phosphorylation is the process of attaching the chemical phosphate group (PO$_4^{3-}$) to a protein. In Eukaryotic cells, phosphorylation occurs primarily on a serine, threonine, or tyrosine but in some rare cases also histidine and aspartate (Thomason & Kay, 2000). Attachment of the phosphate group alters the proteins electrostatic properties and often induces a conformational change that alters activity and/or recruit of downstream proteins. The effects of phosphorylation on protein function are varied, and multiple modifications within a single protein can lead to complex outcomes (Barford, Das, & Egloff, 1998; Pawson & Nash, 2003; Pellicena & Kuriyan, 2006).

**Protein Kinases**

A protein kinase is responsible for transferring the phosphate group to a protein substrate. Protein kinases are involvement in coordination of nearly every cellular process, as the activity, localization, and overall function of many proteins is directed by
kinase phosphorylation. Protein kinases deliver a single phosphoryl group from ATP to the \( \gamma \) position to the hydroxyl group (-OH) oxygen on a serine, threonine, or tyrosine side chain of a protein. This process can be rapidly reversed by protein phosphatases which remove the phosphate group and release it back into solution. Together, protein kinases and phosphatases provide a back and forth mechanism that dynamically regulates the homeostasis of a protein’s phosphorylation state (collectively reviewed in (Cheng, Qi, Paudel, & Zhu, 2011)).

Approximately 2% of human genes encode for protein kinases. Of the 518 protein kinases, 478 belong to a single superfamily with related catalytic domain sequence (Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002). Various kinase classification methods have been developed, most of which separate them into groups and families based on similarity of sequence and biochemical functionality (Martin, Anamika, & Srinivasan, 2010).

Protein kinases are highly conserved throughout evolution. Prior to the discovery of atypical kinases, one of the many conserved subdomains encompassing the catalytic core, subdomain II, contains a lysine residue that was, at the time, present in all protein kinase catalytic domains. Mutation of this lysine can render a kinase catalytically inactive, which is also proximally close to the ATP molecule and suggested to be involved in the stabilization and binding of ATP and the phosphate transfer process. This invariant lysine was described as crucial for binding ATP (Carrera, Alexandrov, & Roberts, 1993). At the turn of the century, however, a new group of kinases were discovered that appeared to catalyze phosphorylation without this crucial lysine.
Atypical WNK Protein Kinases

WNK (With-No-Lysine) kinases are a four member group (WNK1-4) of atypical serine/threonine protein kinases. Their atypical subcategorization results from the unusual placement of the conserved, catalytic lysine of subdomain II that is replaced by a cysteine residue (Figure 1). The group that first characterized these kinases originally believed the catalytic lysine was simply missing and called them “With-No-Lysine” (B. Xu et al., 2000). However, within the same initial study, the group also determined lysine 233 (K233) in human WNK1, which is found within kinase subdomain I and conserved across all WNK homologs, is critical for catalytic activity, and mutation of this lysine to methionine disrupted kinase activity (B. Xu et al., 2000). The crystal structure of WNK1 later showed that the folding structure of active WNK1 actually positions K233 to a location analogous to the lysine normally conserved within subdomain II, and that inactive WNK1 actually adopts a conformation that pulls the lysine out of catalytic position (Min, Lee, Cobb, & Goldsmith, 2004). WNK1 remains the best characterized of the four isoforms. The functional role of WNK kinases within human renal function has been the focal point of WNK research and often acts as a model for WNK signaling in other systems.
Figure 1: Structure of WNK1. Top: Highlights the kinase domain and length of WNK1. Bottom: Ribbon representation of the crystal structure of the WNK1 highlighting the unusual placement of the catalytic lysine, K233, which is found within β strand 2 rather than strand 3 of other kinases. The red circle highlights K233 and the typical lysine location where, instead, a cysteine is found (C250).

Image adapted with permission from work published by Min et al. Crystal Structure of the Kinase Domain of WNK1, a Kinase that Causes a Hereditary Form of Hypertension. Structure. 2004 July; 12(7) 1303-1311.

WNK Signaling in the Kidney

WNK kinases maintain cellular homeostasis by sensing changes in intracellular chloride, cell volume, and extracellular osmolarity, and respond by adjusting the transport of sodium (Na\(^+\)), potassium (K\(^+\)), and chloride (Cl\(^-\)) through regulation of reciprocal cation-chloride co-transporters (CCC) (reviewed collectively in (Alessi et al., 2014)). In
the distal convoluted tubule of the kidney, WNK kinases are highly expressed where they sit atop of a signaling pathway in the regulation of CCCs.

WNK kinases influence cation-co-transporters through activation of immediately downstream SPAK/OSR1 kinases (Figure 2). SPAK and OSR1 associate with both WNKs and CCCs by docking their conserved carboxyl-terminal domain (CCT) with RFXV/I motifs on WNKs and CCCs. Association of WNKs with SPAK/OSR1 allows WNKs to phosphorylate and activate SPAK/OSR1, while association of SPAK/OSR1 with CCCs allows SPAK/ORS1 to phosphorylate and traffic CCCs. Members of the WNK family bind SPAK/ORS1 differently, however, and can have different impacts on cellular signaling. For example, WNK1 phosphorylates and activates SPAK/OSR1 up to 10 fold more than WNK4 (Bazua-Valenti & Gamba, 2015), and its SPAK induced activation of NCC can be antagonized by WNK4 (Chavez-Canales et al., 2014). Aside from regulating cation transport through SPAK/OSR1 induced CCC trafficking, WNK kinases can traffic other proteins, such as ion channels, without the aid of SPAK/OSR1 activation.
Figure 1:2 Proposed mechanism by which the WNK-SPAK/OSR1 signaling pathway regulates salt re-absorption and blood pressure. Osmotic stress can trigger WNK1 to induce trafficking of cation co-transporters through activation of downstream SPAK/OSR1. This process occurs by a series of phosphorylations: osmotic stress induces autophosphorylation and activation of WNK1, and activated WNK1 phosphorylates SPAK/OSR1 which, in turn, phosphorylates and trafficks cation-cotransporters. WNK4 can oppose this process.

Image obtained with permission from work originally published by Ciaran Richardson, and Dario R. Alessi. J Cell Sci. The regulation of salt transport and blood pressure by the WNK-SPAK/OSR1 signalling pathway. 2008;121:3293-3304
Protein Trafficking by WNK kinases Beyond Cation-co-transporters

WNK-SPAK regulation of CCCs in the kidney is rather well established, however the WNK-SPAK kinase network has been shown to influence other protein trafficking. WNK kinases activate endocytosis of renal outer medullary potassium (ROMK) channels, inward-rectifier potassium ($K_{ir}$) which transport $K^+$ out of cells (Zagorska et al., 2007). SiRNA knockdown of endogenous WNK1 in cultured cells increases ROMK1 surface expression, and it was demonstrated that a complex of WNK1-WNK4 binds the endocytic scaffolding protein intersectin to induce clathrin-dependent endocytosis of ROMK1 independent of SPAK/OSR1 (He, Wang, Huang, & Huang, 2007; C. L. Huang, Yang, & Lin, 2008; Lin, Yue, Zhang, & Wang, 2014). Additionally, WNK4 inhibits renal large-conductance Ca(2+)-activated K(+) (BK) by reducing their surface and whole cell expression through enhanced ubiquitination mediated lysosomal degradation (Z. Wang et al., 2013; Yue, Zhang, Lin, Sun, & Wang, 2013), while in contrast, WNK1 enhances BK channel function by reducing lysosomal degradation (Y. Liu et al., 2015). WNK1 also activates the serum and glucocorticoid-regulated kinase 1 (SGK1) to enhance activation of the epithelial sodium channel (ENaC) (B. E. Xu, Stippec, Chu, et al., 2005; B. E. Xu, Stippec, Lazrak, Huang, & Cobb, 2005). Together, these examples demonstrate that in addition to CCCs, WNK kinases can also traffic ion channels.

WNK Expression and Connection to Hypertension

WNK1 is expressed ubiquitously in mice as early as embryonic days E8-9 with enrichment in the cardiovascular system. During development, WNK1 knockout mice have cardiac deficits and impaired angiogenesis as early as E10.5 and failed vascular
system remodeling leads to death *in utero* by E13.5 (Delaloy et al., 2006; J. Xie et al., 2009). Interestingly, the angiogenic and cardiac defects were nearly identical in global and endothelial-specific OSR1 knockout mice, and knockin of endothelial-specific constitutively active OSR1 rescued the defects (J. Xie, Yoon, Yang, Lin, & Huang, 2013), demonstrating that the WNK-SPAK/OSR1 signaling pathway is crucial for proper development.

As demonstrated by the transgenic mouse models, altered expression of kinases within the WNK signaling pathway can have severe physiological consequences, and likewise can impact human health. Mutations in the WNK1 and WNK4 genes cause a type of hereditary hypertension known as pseudohypoaldosteronism type II (PHAII) (reviewed in (Bazua-Valenti & Gamba, 2015)). Patients with PHAII have disrupted regulation of ion transport, resulting in hypertension, hyperkalemia, and hyperchloremic metabolic acidosis. Overall, conditions that result in increased expression of WNK kinases seem to result in PHAII (Susa et al., 2014).

Clinical features of PHAII can be explained by impaired ubiquitylation of WNK kinases (Shibata, Zhang, Puthumana, Stone, & Lifton, 2013). WNK4 reduces surface expression of ROMK, and ubiquitylation of WNK4 signals kinase degradation and results in increased ROMK at the surface. A mouse model for PHAII where mice have a single-copy BAC transgene including the human genomic locus containing the $\text{WNK4}^{Q562E}$ allele present hypertension and hyperkalemia similar to humans with the same mutation. These mice were found to have significantly higher levels of WNK4 in the distal nephron in the kidney compared to mice expressing $\text{WNK4}^{WT}$, and the increased WNK4 was
reported to be from impaired degradation. Increased WNK4 will result in a relative elimination of ROMK, a trait that contributes to hyperkalemia in PHAII (Lalioti et al., 2006). Similarly to genetic mutations impairing WNK4 degradation, genetic mutations that disrupt the catalytic activity of the ubiquitin ligases that catalyze WNK4 polyubiquitylation cause PHAII. In fact, within a study of 52 families with PHAII, ~80% of the disease was caused by ubiquitin ligase mutation. Therefore, although PHAII is attributed to the change in expression and activity of kinases, ubiquitin ligases are the primary culprits behind the disease (Herrera & Coffman, 2013).

Protein Ubiquitylation

Following phosphorylation, recent advances in proteomics have identified ubiquitylation (or ubiquitination) as one of the next most abundant and versatile PTMs in mammalian tissue (W. Kim et al., 2011). In a similar historical context to phosphorylation (reviewed in (P. Cohen, 2002)), several decades following the discovery of ubiquitin passed before its functional role breached confinement of a single signaling mechanism in the scientific literature, however ubiquitylation is now accepted as a major signaling event involved with a myriad of biological events (Chen & Sun, 2009; Komander, 2009).

Protein ubiquitylation involves the attachment of the ubiquitin protein to a protein substrate. Discovered by Hershko and colleagues in 1978, the small ubiquitin protein was originally reported as a required component for the proteasome to breakdown proteins in cell extracts (Ciehanover, Hod, & Hershko, 1978). Aaron Ciechanover, Avram Hershko and Irwin Rose were later awarded the Nobel Prize in Chemistry in 2004 for this
discovery (Giles, 2004). Work particularly within the lab of Varshavsky and colleagues in living mammalian cells throughout the 1980s caused a radical change in the general understanding of intracellular circuits, as they identified the ubiquitin system was essential for the majority of protein degradation in living cells, required for cell viability, and performed major roles in the cell cycle, DNA repair, protein synthesis, transcriptional regulation, and other processes (reviewed in (Varshavsky, 2006)). These discoveries sparked a massive expansion of the ubiquitin field in the 1990s. Now, ubiquitylation has been demonstrated to assist orchestrating a plethora of physiological functions.

Ubiquitin-protein ligation requires three enzymes (Hershko, Heller, Elias, & Ciechanover, 1983) in a three step, sequential action: First, a ubiquitin-activating enzyme (or E1) utilizes ATP to activate the C-terminal glycine residue of ubiquitin (release of PP\textsubscript{i} and AMP). Next, activated ubiquitin is then transferred to an active-site cysteine residue of a ubiquitin-conjugating enzyme (or E2). Finally, ubiquitin is transferred to a ubiquitin-ligating enzyme (or E3) which ligates the C-terminal glycine residue of ubiquitin in an amide isopeptide-linkage to the ε-amino group of an internal lysine or α-amino group of the N-terminal methionine residue in the target protein (Hershko & Ciechanover, 1998).

The covalent bond between substrate lysine and ubiquitin glycine has been fruitful for modern proteomic identification of ubiquitylation, as ubiquitylated proteins digested with trypsin (a serine protease which cleaves the carboxy-terminal end of lysine and arginine amino acids) will result in peptides still containing -K-ε-GG motif following cleavage of bound ubiquitin from -K-ε-GG(cut)-R-ubiquitin. In addition to the diagnostic advantages of this 114.0429 Da motif, anti-K-ε-GG antibodies have allowed advanced
enrichment of ubiquitylated proteins prior to digest and greatly expanded the detected sites among the literature yielding over 10,000 unique sites on over 4,000 proteins (W. Kim et al., 2011; Udeshi et al., 2013; Wagner et al., 2012). This approach has led to similar growth in depth of global phosphorylation and acetylation studies (Choudhary et al., 2009; Huttlin et al., 2010; S. C. Kim et al., 2006; Olsen et al., 2006).

Ubiquitylation, like phosphorylation, is a reversible process and undergoes counter regulation by the activity of isopeptidases known as deubiquitinating enzymes (DUBs) (Clague, Coulson, & Urbe, 2012) that function analogously to the removal of phosphate by protein phosphatases. An estimated 100 deubiquitinating enzymes within mammalian cells reverse ubiquitylation by rapidly removing ubiquitin from substrates at various signaling checkpoints and recycle ubiquitin for further modification (Reyes-Turcu, Ventii, & Wilkinson, 2009). Activity coordination between the E3 ligases and deubiquitinating proteins are then recognized by a plethora of effector proteins with ubiquitin-binding domains (UBD) which bind non-covalently through the various surface patches of ubiquitin for various further processing (Clague, Liu, & Urbe, 2012; Hershko & Ciechanover, 1998; Rahighi & Dikic, 2012). DUBs enable tight regulation of the ubiquitin signaling events (Hershko & Ciechanover, 1998).

**Polyubiquitylation**

Ubiquitin binds a substrate classically through covalent attachment of it’s C-terminal carboxylate group to a target lysine. Ubiquitin, an 8.5kDa, 76 amino acid long protein, can also be a substrate for ubiquitin binding, as seven amino acids in the sequence of ubiquitin are a lysine.
Ubiquitylation has many different forms. The seven lysines K6, K11, K27, K29, K33, K48, and K63 as well as an amino-terminal methionine are all potential substrates for ubiquitylation, and successive addition of ubiquitin by E1, E2, and E3 enzymes, collectively referred to as polyubiquitylation, result in ubiquitin chains varying in length, topology, and proximal moiety (the lysine of chain origin). All seven lysines reside on different surfaces of ubiquitins tertiary structure (Figure 3).

Each of the polyubiquitin moieties form unique overall structure among the continuous ubiquitin-ubiquitin linkages. Polyubiquitylation chains form endogenously in homogenous chains (e.g. all K48 linkages) with heterogeneous chains being currently speculative. All linkage types are thought to exist within all cells at various abundances (Pickart & Fushman, 2004; Y. Wang, Tang, Wang, & Wang, 2014). Approximately half of the populated linkage types are K48 and K63.

**Lysine 48 Polyubiquitylation Chains and Degradation**

In 1989, a ubiquitin chain with distinct topology of isopeptide bonds on K48 was discovered, and the poly-ubiquitylation chain was proposed to bind a substrate to the proteasome (later shown to be the 26S proteasome) and be essential for its degradation (Chau et al., 1989). Polyubiquitylation at K48 is well characterized as a molecular tag for degradation by the 26S proteasome. Destruction is carried out by proteolysis of a protein tagged by a lysine chain of at least four (Thrower, Hoffman, Rechsteiner, & Pickart, 2000) tightly packed, compact globular conformation which the proteasome recognizes, removes the ubiquitin for recycling, and ubiquitylated peptides are permitted to enter the barrel-shape structure where the central proteolytic core chops the protein into smaller
peptides of 3-25 amino acids (Hershko & Ciechanover, 1998). These oligopeptides are then released into the cytoplasm or nucleoplasm and can be further digested to single amino acids by various soluble peptidases (reviewed in (Korolchuk, Menzies, & Rubinsztein, 2010)).

Intriguingly, ubiquitylation can also target substrates for degradation by autophagy-lysosome destruction, a degradation signaling pathway often thought to provide bulk, nonspecific degradation (including entire organelles) (Ciechanover, 2005). Autophagy is a vesicular trafficking pathway limited to the cytoplasm and capable of degrading a wider spectrum of substrates than a proteasome. Although little mechanistic similarities occur, these two pathways of degradation share the ability to degrade soluble, unfolded polypeptide chains (Ding & Yin, 2008). In the 1990s it was discovered that lysosomes can degrade proteasomal subunits (Cuervo, Palmer, Rivett, & Knecht, 1995), causing autophagy-lysosome degradation to be hypothesized as a means of controlling cellular concentration of proteasomes (Korolchuk et al., 2010).

During selective autophagy, like the ubiquitin-proteasome system, it is ubiquitylation that tags a target for degradation. Unlike the UPS, however, substrates recognized for autophagy-lysosome degradation are signaled through K63 polyubiquitylation chains (Welchman, Gordon, & Mayer, 2005).

Lysine 63 Polyubiquitylation Chains

Ubiquitin-conjugates with K63 chains were originally reported in the mid-1990s (Galan & Haguenauer-Tsapis, 1997; Spence, Sadis, Haas, & Finley, 1995). K63 polyubiquitin chains, although also capable of lysosomal and proteasomal degradation
signaling, (Saeki et al., 2009), are well defined to contribute to a variety of cellular events including DNA repair, signal transduction, receptor endocytosis, and kinase activation (reviewed in (Chen & Sun, 2009)). K63 branches were demonstrated to facilitate and stimulate endocytosis (Galan & Haguenauer-Tsapis, 1997). Although K63 can catalyze degradation, essential roles for K63 in cell signaling are considered its more primary function.

Atypical Polyubiquitylation

In contrast to K48 and K63, the cellular roles of atypical polyubiquitylation e.g. K6, K11, K27, K29, K3, and M1 have yet to be clearly elucidated (reviewed in (Kulathu & Komander, 2012)). K29 branches have been detected in yeast and mammals, and can be generated in vitro, but little is known regarding any bio functional signaling (Kristariyanto et al., 2015). K11 branches have been shown to provide signals for control of the cell-cycle and innate immune response (Castaneda, Kashyap, Nakasone, Krueger, & Fushman, 2013; Matsumoto et al., 2010; Rape, 2010; Wickliffe, Williamson, Meyer, Kelly, & Rape, 2011; Wu et al., 2010). In addition to internal lysines, the amino-terminal methionine of ubiquitin can also be a substrate for ubiquitylation. These M1 linkages, assembled by the linear ubiquitin assembly complex (LUBAC) are involved with diverse signaling processes that are not yet clearly defined (reviewed in (Rieser, Cordier, & Walczak, 2013)).
Figure 1:3 Ubiquitin and its lysine residues. The ribbon model of ubiquitin structure highlights the seven lysines residing on different surfaces of its tertiary structure. The linking point in linear chains, M1, is spatially close to K63. Red numbers in parenthesis indicate relative abundance detected in *S. cerevisiae*.


**Monoubiquitylation**

Although polyubiquitylation remains the forefront for ubiquitylation research, monoubiquitylation is also biologically significant for DNA repair, viral budding, and endosomal sorting. Monoubiquitylation, like K63 polyubiquitylation, can also signal endocytosis of membrane proteins. Monoubiquitylation can also work in conjunction with other PTMs to direct the localization of proteins to particular cellular compartments (Hicke, 2001; Mosesson & Yarden, 2006). Recent MS studies designed to identify the types of ubiquitin chains on ubiquitylated proteins have revealed that monoubiquitin is
more common than previously assumed. Using anti-K-ε-GG antibodies, one study extended the ubiquitin-modified proteome (ubiquinome) by identifying ~19,000 ubiquitin binding sites of ~5,000 proteins (originally determined to be 1,075 proteins with 100 sites) while also reporting that the majority are subject to non-degradation signaling ubiquitylation (W. Kim et al., 2011; Peng et al., 2003). During the same year, another study demonstrated that ~50% of conjugated ubiquitin is bound to substrates as mono or multiubiquitin (Ziv et al., 2011).

Outside of the nucleus, monoubiquitylation serves to direct endocytosed, modified proteins to specific subcellular locations (reviewed in (Haglund, Di Fiore, & Dikic, 2003)) and endocytic regulators themselves also undergo monoubiquitylation that work to couple substrate recruitment to the ubiquitylation reaction (coupled monoubiquitylation) through use of their ubiquitin binding domains (Polo et al., 2002; Woelk et al., 2006). However, a growing body of literature shows that monoubiquitylation can also be directly involved in the endocytic trafficking of membrane proteins such as ROMK1, which has been reported to decrease surface but not total expression following channel monoubiquitylation (Lin et al., 2005). Altogether, ubiquitylation can modulate membrane proteins in a variety of ways, including endocytic trafficking. Consequently, like protein kinases, the enzymes responsible for catalyzing ubiquitylation have important roles in regulating protein function.

E3 Ubiquitin Ligase Structure and Function

Ubiquitylation is catalyzed by the sequential action of three enzymes collectively referred to as E1, E2, and E3 in a three step process where the reaction catalyzed by each
enzyme involves the transfer of a covalent bond with ubiquitin to either the next enzyme or, finally, the substrate. E1 ubiquitin-activating enzymes utilize ATP-Mg$_{2+}$ to charge and form a thioester bond with ubiquitin. Charged ubiquitin is then subsequently passed along to a second E2-conjugating enzyme and finally the carboxy-terminus of ubiquitin forms an isopeptide bond with the lysine of a protein substrate. During this process, E3-ubiquitin ligases provide the substrate recognition for ubiquitylation. E3s recruit E2s, recognize the substrate, and directly catalyze or assist the transfer of ubiquitin from E2 to the substrate. The human genome encodes one E1, about 40 E2, and around six hundred E3 enzymes (W. Li et al., 2008). In humans, the over 600 E3 ligases are categorized into 4 groups based on their structural motif: HECT-type, RING-finger-type, U-box-type or PHD-finger-type, with HECT and RING being the two major types of E3’s in eukaryotes (reviewed in (Hou & Deng, 2015; W. Li et al., 2008; Nakayama & Nakayama, 2006)). Although four structural categories exist, E3 ubiquitin ligases are generally classified into two families, HECT-type or RING-type, based on their function. HECT-type E3s have a conserved cysteine residue that accepts ubiquitin from E2~Ub forming an E3~Ub thioester. Ubiquitin is then transferred from this covalent E3 intermediate to substrate in contrast to RING-type E3s which catalyze the direct transfer of ubiquitin from E2~Ub to substrate (Figure 4).

**HECT-type Ubiquitin Ligases**

Homologous to the E6-AP Carboxyl Terminus (HECT)-type ubiquitin ligases consist of a small class of E3 ligases which share C-terminal HECT domains that are functionally similar to the cellular protein E6-AP (or UBE3A), the E6 protein of the
cancer-associate human papillomavirus type 16 and 18 which targets p53 for degradation by ubiquitylation. HECT E3s covalently bind ubiquitin by a cysteine residue in the catalytic domain and shuttle it onto the target substrate (Huibregtse, Scheffner, Beaudenon, & Howley, 1995). The HECT domain is composed of an N-terminal lobe for E2 binding and a smaller C-terminal lobe that contains the catalytic cysteine for ubiquitin accepting. A flexible hinge region lies between the N- and C-terminal lobes that bridges the lobes and allows E2 and E3 catalytic sites to be brought close together during shuttling of the ubiquitin (Maspero et al., 2011). Only 28 human HECT-type ubiquitin ligases exist and the vast majority of ligases belong to the RING type.

**RING-type Ubiquitin Ligases**

Most E3 ligases belong to the RING family. Really Interesting New Gene (RING) based E3 ligases, originally described in 1991 (Freemont, Hanson, & Trowsdale, 1991) are encoded by over 600 human genes (human kinases are limited to 518 genes) and comprise one of the largest enzyme families. RING-type E3 ligases share a common 40-60 amino acid RING domain which bind two zinc atoms to zinc-binding “fingers” (reviewed in (Callis, 2014; Deshaies & Joazeiro, 2009)). RING domains bind E2s and promote a direct transfer of ubiquitin to substrate. Unlike HECT domain E3 ligases, RING domain containing ligases do not involve an E3-linked ubiquitin thioester intermediate. Bi-substrate enzyme RINGs bind the reactants E2~Ub and substrate, in no precededented order, produce products E2 and substrate~Ub with a Michaelis complex intermediate. For further ubiquitylation, however, the E2 must be released to allow a fresh E2~Ub to bind, as E2s cannot be recharged due to the overlapping binding surfaces
of E1s and E3s. Polyubiquitylation by RING E3s starts with a slow, chain initiation step followed by a fast, chain elongation step. Unlike conventional bi-substrate enzymes, which bind both reactants in close proximity to facilitate the reaction, E3s bind a substrate and E2~Ub up to 50 Angstroms apart, suggesting conformational changes likely occur to bring the two reactants closer together for facilitation of the reaction. Ubiquitylation by RING E3s, like nearly every biological process, is regulated by phosphorylation, through effects on the substrate, E2, or E3 itself. Substrate phosphorylation is the most common. Many Cullin-RING ligase substrates such as Skp-1Culin-F-box (SCF) must be modified, typically by phosphorylation, in order to bind an E3 and undergo ubiquitylation. Others, such as Anaphase-promoting complex/cyclosome (APC/C)-Cdc20 are activated by phosphorylation via direct phosphorylation to the E3 allowing tighter binding of the Cdc20 adaptor. Additionally, binding of APC/ to adaptor Cdh1 during mitosis is inhibitory as phosphorylated Cdh1 is unable to bind APC/C. Altogether, phosphorylation is a critical determinant of ubiquitylation promotion.
Figure 1: Cartoon highlighting ligation of ubiquitin to a substrate protein by HECT or RING E3 ligases. A. HECT E3 ligases form a covalent bond with ubiquitin and a cysteine within their catalytic domain prior to shuttling ubiquitin to the substrate. B. Ring E3 ligases bind E2s and promote a direct transfer of ubiquitin to substrate.


Ubiquitin Ligase Tripartite Motif-containing Protein 32

Tripartite motif-containing protein 32 (Trim32) is part of the Trim/RBCC protein super family that contributes to various biological signaling including growth, apoptosis, cell cycle regulation, muscular physiology, viral and innate immune response, and cancer (Hatakeyama, 2011; Ikeda & Inoue, 2012). Trim proteins contain the tripartite/RBCC motif composed of a RING domain, one or two B-box domains, and a coiled-coil domain (Figure 5) (Meroni, 2012). Trim32 is further subcategorized as a Trim-NHL protein due to six C-terminal asparagine, histidine, and lysine (NHL) amino acid repeats that function as protein-protein binding domains (Schwamborn, Berezikov, & Knoblich, 2009). Trim-NHL proteins have been shown to regulate expression of microRNAs (miRNA), small
noncoding RNAs that regulate gene expression post-transcriptionally, and thus ultimately provide translational control of certain proteins (Loedige & Filipowicz, 2009; Wulczyn, Cuevas, Franzoni, & Rybak, 2011). Trim32 specifically interacts with AGO1 (via its NHL domain) and activates miRNA *let-7a* to promote differentiation in mouse progenitor cells (Schwamborn et al., 2009).

**Figure 1:5 Schematic of Trim32 structure and sites of disease-associated mutation.** RING domain provides E3 ligase function of Trim32. NHL repeats are locations of protein interaction and microRNA activation. P130S mutation within the BBOX domain causes BBS, while various mutations with the NHL repeats cause LGMD2A. Image adapted with permission from work originally published by Matthew Locke et al. *Hum. Mol. Genet.* TRIM32 is an E3 ubiquitin ligase for dysbindin 2009; 18:2344-2358.

*Trim32 Ubiquitin Ligase Functionality*

The RING domain of Trim32 provides ubiquitin ligase catalysis. Several proteins have been reported as substrates for Trim32 catalyzed ubiquitylation: Trim32 can ubiquitylate actin *in vitro* and decrease endogenous actin in HEK293 cells (Kudryashova, Kudryashov, Kramerova, & Spencer, 2005), ubiquitylate Abl-interactor 2 (Abi2) *in vitro* and promote degradation in HEK293T (Kano, Miyajima, Fukuda, & Hatakeyama, 2008), bind and promote polyubiquitylation and potential degradation of dysbindin in HEK293 cells (Locke, Tinsley, Benson, & Blake, 2009) and catalyze ubiquitylation of Piasy *in vitro* to promote its destabilization and degradation (Albor et al., 2006). All of these examples show Trim32 is able to catalyze ubiquitylation of particular substrates, either
directly by using purified recombinant proteins in vitro where Trim32 is the only E3 ligase present, or overexpression in heterologous systems, in both cases monitoring electrophoretic shifts by Western blot. Tools that can demonstrate ubiquitylation of a substrate by Trim32 or any E3 ligase directly in tissue are currently limited.

*Trim32 Contributes to the Pathology of Human Disease*

The ubiquitin system has been implicated in the pathogenesis of many diseases and genetic disorders. Human neurological disorders appear to be particularly vulnerable to mutations in ubiquitin ligases (Kessler, 2013). Mutation of the Trim32 gene causes several disease states. Various point mutations within the NHL repeats D487N (third NHL repeat), R394H (first NHL repeat), T520TfsX13 (fourth NHL repeat), and D588del (fifth NHL repeat) are reported to cause LGMD2H (Locke et al., 2009). In addition to LGMD2H, D487N mutation also causes the more severely debilitating sarcotubular myopathy (Schoser et al., 2005).

*Trim32 in the Adult Brain*

Trim32 has been studied primarily with respect to pathologies arising from its effects in non-neuronal tissue. Although Trim32 is primarily known to cause disease states with phenotypes localized to skeletal muscle, Kudryashova et al. in 2009 observed that brain Trim32 exceeds muscle Trim32 by over 100 fold and concluded that Trim32 must have a novel role in the nervous system (Kudryashova, Wu, Havton, & Spencer, 2009). A mutation within the B-box domain (P130S) causes an unrelated disease Bardet-Biedl Syndrome (Chiang et al., 2006) that has both physical and neurological deficits. Additionally, increased expression of Trim32 has been reported in the occipital lobe of
Alzheimer’s disease patients (Yokota et al., 2006), while Trim32 null mice have abnormal neurofilament accumulation (Kudryashova et al., 2009)- a symptom of various neurodegenerative diseases (Q. Liu et al., 2004). Finally, Trim32 genetic disruption has been identified as a risk factor for Autism Spectrum Disorders (ASDs), Attention Deficit Hyperactive Disorder (ADHD), and other neurodevelopmental disease (Lionel et al., 2014).

**Voltage-gated Potassium Channel 1.2**

*Membrane Potential of Excitable Cells*

Electrical activity of biological organisms is driven, for the most part, by ions. Living cells are enclosed within a plasma membrane (PM), a semi-permeable lipid bilayer structure that separates the extra- and intra-cellular space and prevents ions from freely passing into the cell. The unequal separation of charged ions and other molecules across the PM generates gradients of both chemical concentration and electrical potential, collectively referred to as the electrochemical gradient, and a driving force is produced for the ions to cross the PM. In electrical cells, the difference in electrical potential across the PM of a quiescent cell, called the resting membrane potential, is driven primarily by Na\(^+\) and K\(^+\) ions. At a resting membrane potential, a cell is in a steady state of continuous passive influx of Na\(^+\) and efflux of K\(^+\) through resting channels that are counterbalanced by Na\(^+\)-K\(^+\) pumps utilizing ATP to pump the ions against their respective gradients. The unequal positive outward flux caused by Na\(^+\)-K\(^+\) pump (3 Na\(^+\) out, 2 K\(^+\) in/ ATP)
generates a general hyperpolarization of the PM as the interior is negative relative to the exterior.

Ion channels embedded within the PM gate the passage of ions across the membrane. Various factors can trigger the opening of ion channels that allow ions to pass across the PM down their concentration gradients. In the case of K\(^+\), where greater amounts of K\(^+\) reside in the cytoplasm at rest, opening of these channels results in a loss of positive charge within the cell which increases the negativity of the membrane potential and hyperpolarizes the cell making it less excitable. Similarly, opening of Na\(^+\) channels causes an opposite influx of Na\(^+\), increased positive charge, and depolarization to the membrane. As Na\(^+\) influx is a major force for depolarization and generation of action potentials, K\(^+\) efflux counterbalances and limits depolarization.

Overview of Voltage-gated Potassium Channels

Neurons communicate with each other through action potentials (AP) that involve major alterations in membrane potential. Changes to membrane potential can trigger opening of certain ion channels, and if a membrane reaches a certain threshold of depolarization, an action potential is generated whereby voltage-gated sodium (Nav) channels open to allow the rapid influx of Na\(^+\) ions followed quickly by voltage-gated potassium channels (Kv) opening to allow efflux of K\(^+\) and repolarization of the membrane. Potassium channel opening at the PM drive the membrane potential towards that of the equilibrium potential of potassium which, in most neurons, causes a hyperpolarization that oppose excitation. At resting membrane potential, the majority of Kv channels are closed until a depolarization threshold is reached, which triggers the
channels to open and repolarize the membrane potential. Thus, some Kv channels return depolarized cells to resting state following an AP. Some Kv channels, however, are open at sub-threshold voltages and provide major contribution to the resting membrane potential.

General Categorization and Structure of Voltage-gated Potassium Channels

Kv channels are divided into subfamilies originally described by genes *Drosophila* as Shaker, Shab, Shaw, and Shal and correspond to the alpha subunit classifications of Kv1, Kv2, Kv3, and Kv4, respectfully in vertebrates (Salkoff et al., 1992). Channels encoded by Shaker, Shal, Shab, and Shaw express currents with different biophysical properties. Unlike *Drosophila*, which has only one member per subfamily, the mammalian genome contains subfamily genes of Kv channels such as Kv1 which ranges from Kv1.1 to Kv1.12. Structurally, Kv channels are transmembrane channels that tetramerize to form the functional channel. Kv channels may be homotetramers, and also, in the case of Kv1, Kv7, and Kv10 families, heterotetramers formed by different alpha subunits within the same family (with only a few exceptions of different family member tetramerizing) with their own unique channel properties (reviewed in: (Gutman et al., 2005)). Additionally, within the same cell, different Kv channels can localize to different cellular components for specific functionality (Southan & Robertson, 1998). Several Kv1 channels activate at subthreshold voltage in many excitable cells where they can contribute to the regulation of cell excitability (Bekkers & Delaney, 2001; Dodson, Barker, & Forsythe, 2002; Shen, Hernandez-Lopez, Tkatch, Held, & Surmeier, 2004). Kv1.2 is one such channel.
Overview of Kv1.2 Structure and Function

Kv1.2 provides a major contribution to the resting membrane potential of certain excitable cells. Kv1.2 channels can be activated at subthreshold potentials, with reported half activation values ranging from -40mV to +30mV (Rezazadeh, Kurata, Claydon, Kehl, & Fedida, 2007) and are therefore considered low-voltage-activated channels that can open at resting potential or by small depolarization (Al-Sabi, Kaza, Dolly, & Wang, 2013). These channels are also rapidly activated.

As with all Kv channel alpha subunits, several functional domains exist within Kv1.2: the T1 tetramerization domain within the N-terminus, six transmembrane domains (S1-S6), including the S4 voltage-sensing domain, and a potassium selective pore between S5 and S6, extracellular domains, and a C-terminus. Both the N and C termini provide multiple sites for PTM (Mathie, Wooltorton, & Watkins, 1998).

Post-translational Modification Regulates Kv1.2

Like many proteins, Kv1.2 is subject to post-translational modification. N-linked glycosylation on the first extracellular loop of Kv1.2 is involved in the trafficking of newly translated Kv1.2 (Shi & Trimmer, 1999; Zhu et al., 2003). Fully maturated Kv1.2 at the PM is subject to phosphorylation. Tyrosine phosphorylation of Kv1.2 suppresses channel current and causes down regulation (X. Y. Huang, Morielli, & Peralta, 1993). Current suppression by tyrosine phosphorylation has been shown to occur by channel endocytosis (Nesti, Everill, & Morielli, 2004). Additionally, serine phosphorylation of Kv1.2 have also been reported and suggested to impact Kv1.2 surface expression and trafficking of Kv1.2 (J. W. Yang, Vacher, Park, Clark, & Trimmer, 2007; Connnors et al.,
2007). It should be noted that post-translational modifications of Kv1.2 could also be changing channel properties, such as gating, which could in turn alter channel function. Indeed, this has been shown to occur with many ion channels including Kv1.2 (Huang et al., 1993), but remains an entirely separate topic of investigation outside the scope of this work.

**Kv1.2 in the Brain**

Kv1.2 is abundantly expressed in the nervous system and can be found residing throughout different critical compartment of a neuron (Dodson et al., 2002; Glazebrook et al., 2002; Goldberg et al., 2008; Kole, Letzkus, & Stuart, 2007). In dendrites, where outside information is conveyed into a neuron, Kv1.2 is localized in cerebellar Purkinje cells (Khavandgar, Walter, Sageser, & Khodakhah, 2005) and hippocampal pyramidal cells (Sheng, Tsaur, Jan, & Jan, 1994) to oppose dendritic depolarization. Summated dendritic input can depolarize a neuron to a threshold capable of generating an action potential, and Kv1.2 in the neuronal soma such as sensory neurons (Andrews & Kunze, 2001) and striatal medium spiny neurons (Shen et al., 2004) can oppose this process as well. Kv1.2 can also be found Axon Initial Segment that contribute to the generation and shaping of an AP (Lorincz & Nusser, 2008), as well flanking domains called juxtaparanode where they modulate axonal excitability by preventing recurrent nodal excitation (Rasband et al., 1998; Vabnick et al., 1999). Kv1.2 also is found at axonal branch points in some neurons, particularly inhibitory interneuron basket cells (Tan & Llano, 1999; Zhang, Messing, & Chiu, 1999). Altogether, Kv1.2 is abundantly expressed
within the mammalian central nervous system where it can provide regulation of neuronal excitation.

**Exploring Kv1.2 Regulation in the Brain**

Kv1.2 regulates neuronal excitability throughout the brain. As with cultured cells, modulation of Kv1.2 expression in neurons regulate its influence on excitability. For example, regulation of Kv1.2 in the cerebellum has important consequences for cerebellar function, affecting not only PC dendritic excitability but cerebellum-dependent associative learning as well (Williams, Fuchs, Green, & Morielli, 2012). Kv1.2 function can be modulated by endocytic trafficking triggered by protein-protein interaction in cultured cells (Hattan, Nesti, Cachero, & Morielli, 2002; Williams, Markey, Doczi, & Morielli, 2007), however the mechanisms governing Kv1.2 trafficking in the brain remain largely unknown.

Given the criticality of Kv1.2 in the central nervous system, defining modalities for Kv1.2 modulation endogenous to the brain may provide valuable insight toward understanding fundamental neuronal processes. Studies performed in isolated culture systems have generated the majority of known regulatory mechanisms of Kv1.2 in the past, however advancements in proteomics allow investigation of proteins in the native tissue context more plausible. By starting with an unbiased proteomic approach, novel protein interactions and post-translational modifications of Kv1.2 can be readily detected from tissue. Such proteomic output can then be further tested in model-systems to determine mechanisms of Kv1.2 modulation in the brain.
Summary

The preceding literature review provided background information intended to supplement the data presented in the manuscripts of Chapters 2 & 3. For both projects, the conceptual framework was based off initial MS experiments which detected both PTMs and protein associations with Kv1.2 purified from cerebellar extracts that were previously unidentified. In Chapter 2, cross-talk between protein ubiquitylation and phosphorylation was examined in regard to regulation of Kv1.2 by the ubiquitin ligase Trim32. In Chapter 3, regulation of Kv1.2 by the atypical WNK and closely related SPAK kinases was investigated. Both studies present potential mechanisms of novel ion channel and perhaps neuronal excitability regulation in the cerebellum and further interpretation beyond the manuscripts is explored in the Comprehensive Discussion of Chapter 4.
References for Chapter 1


CHAPTER 2: UBIQUITIN LIGASE TRIM32 DYNAMICALLY MODULATES THE VOLTAGE-GATED ION CHANNEL KV1.2

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Abstract

Trim32 is ubiquitously expressed throughout the nervous system, however functional roles for the ubiquitin ligase in the developed brain are not well understood. Here, using mass spectrometry, we report that Trim32 associates with Kv1.2, an ion channel critically involved with establishing and maintaining membrane excitability in neurons. Using immunofluorescence we show that Trim32 co-localizes with Kv1.2 to regions within the cerebellum. In vitro ubiquitylation assays demonstrated that Trim32 is catalytically equipped to ubiquitylate Kv1.2. Using co-expression of Kv1.2 and Trim32 in HEK293 cells we show that Trim32 regulates the surface expression of the channel in cultured cells by two mechanisms, one ubiquitin-dependent and one not. Finally, using a combination of quantitative mass spectrometry, mutagenesis, and flow cytometry, we show that the effect of Trim32 on Kv1.2 by both mechanisms involve cross-talk with Kv1.2 phosphorylation.

Introduction

Protein ubiquitylation is the covalent attachment of the small protein ubiquitin to a lysine residue within a target protein, either as single units (monoubiquitylation for one or multiubiquitylation for more than one) or as ubiquitin chains (polyubiquitylation). Polyubiquitin chains arise by sequential addition of ubiquitin proteins onto a lysine within ubiquitin, and the specific lysine targeted produces different chains with distinct biological effects. The most understood role of ubiquitylation involves the generation of polyubiquitin chains linked at lysine 48 (K48). This type of polyubiquitylation has a prominent role in targeting proteins for degradation by the 26S proteasome (Ozkaynak,
Finley, & Varshavsky, 1984). Because the polyubiquitylation-proteasome protein degradation pathway is by far the most extensively studied, it is not surprising that most work investigating the role of ubiquitylation in the brain has focused on this pathway (Artinian et al., 2008; Jarome, Werner, Kwapis, & Helmstetter, 2011; Lopez-Salon et al., 2001; Reis, Jarome, & Helmstetter, 2013; Rodriguez-Ortiz, Balderas, Saucedo-Alquicira, Cruz-Castaneda, & Bermudez-Rattoni, 2011). However, recent large-scale proteomic assessments of the mammalian ubiquitinome have expanded our understanding of both the magnitude and diversity of ubiquitylation within the brain (W. Kim et al., 2011; Wagner et al., 2012). Increasingly, evidence indicates that proteasome-independent effects, mediated by other types of poly, multi, or monoubiquitylation, can function to regulate membrane proteins in a way that is conceptually similar to phosphorylation (Haglund et al., 2003; Hicke, 2001; Mosesson & Yarden, 2006). One example is a study identifying monoubiquitylation of the Inositol 1,4,5-Trisphosphate Receptor (IP$_3$R) which found eleven sights all within the regulatory domains. The authors proposed location-specific monoubiquitylation as an essential mechanism for non-proteolytic regulation of the IP$_3$R (Sliter, Aguiar, Gygi, & Wojcikiewicz, 2011; Sliter et al., 2008).

Enzymatic transfer of ubiquitin to a protein involves the activity of three enzymes (termed E1-E3), with the E3 (or ubiquitin) ligase conferring substrate specificity (Hershko et al., 1983). One E3 ligase, Trim32, is highly expressed in mammalian brain and skeletal muscle (Kudryashova et al., 2009). Most of what is known about Trim32 concerns its role in muscle function and disease. Mutations within Trim32 causes Limb-Girdle muscular dystrophy type 2A (LGMD2H) (Frosk et al., 2002) and the more severely debilitating sarcotubular myopathy (STM) (Schoser et al., 2005). Most reported
substrates of Trim32 are proteins involved in hereditary skeletal muscle disorders such as actin and myosin (Kudryashova et al., 2005), desmin (S. Cohen, Zhai, Gygi, & Goldberg, 2012), ABL2 (Kano et al., 2008) and PIASY (Albor et al., 2006). In contrast, despite its high neuronal expression, Trim32’s molecular targets and roles in the brain are far less understood. Two recent studies indicate that Trim32 is involved in complex emotional behavior and may have a role in depression and anxiety disorders (Hillje et al., 2015; Ruan et al., 2014). Genetic studies have identified links between Trim32 and a range of neurological disorders, including Autism Spectrum Disorders (ASD) and Attention Deficit Hyperactive Disorder (ADHD) (Lionel et al., 2014). Intriguingly, ASD and ADHD linked to Trim32 dysfunction are comorbid with epilepsy, a condition that involves neuronal hyper-excitability (Lo-Castro & Curatolo, 2014). Mood disorders, ASD and seizure disorder are associated with defects in neurodevelopment, a process dependent on Trim32 as a neural stem cell fate determinant (Hillje et al., 2015; Hillje, Worlitzer, Palm, & Schwamborn, 2011; Schwamborn et al., 2009). Thus, current models focus on neurodevelopment as a primary mechanism for Trim32 effects in the brain. However recent studies have identified potassium channelopathies as potential contributors to ASD as well (reviewed in (Schmunk & Gargus, 2013)).

Voltage-gated ion channels are major determinants of neuronal excitability. The voltage-gated potassium channel Kv1.2 is expressed widely throughout the nervous system, but it is expressed to the highest density within the cortex of the cerebellum (McNamara, Averill, Wilkin, Dolly, & Priestley, 1996; McNamara, Muniz, Wilkin, & Dolly, 1993; Veh et al., 1995). Its expression there is largely concentrated within presynaptic terminals that provide inhibitory input to Purkinje cells (Khavandgar et al., 1996; McNamara, Averill, Wilkin, Dolly, & Priestley, 1996; McNamara, Muniz, Wilkin, & Dolly, 1993; Veh et al., 1995).
2005; Sheng et al., 1994; Southan & Robertson, 1998) and within Purkinje cell dendrites where it influences responses to excitatory input. Because Purkinje cells form the core computational unit within the cerebellar cortex, and because they also provide its sole output to cerebellar nuclei and ultimately the rest of the brain, Kv1.2, through its effect on Purkinje cells, has a uniquely central impact on cerebellar function. This was recently underlined by the finding that mutations within Kv1.2 cause a specific type of inherited cerebellar ataxia (G. Xie et al., 2010).

In this study we identify a new role for Trim32 in the cerebellum as a modulator of the potassium channel Kv1.2. Using mass spectrometry (MS), we identified Trim32 as part of the cerebellar Kv1.2 protein interactome and identified sites of ubiquitylation within native cerebellar Kv1.2. These in vivo findings provided the foundation for in vitro studies to determine the mechanisms by which Trim32 regulates Kv1.2. We found that Trim32 mediates mono-ubiquitylation of Kv1.2 which in turn modulates the channels’ endocytic trafficking. Further, we used a quantitative MS approach to show that Trim32 not only affects channel ubiquitylation, but also influences phosphorylation at two C-terminal serines (S434 and S440) that lay adjacent to the channel’s primary ubiquitylation site (K437), and that phosphorylation at these sites have differential, ubiquitylation dependent effects on Kv1.2 trafficking. Therefore, our findings indicate that Kv1.2 function is determined by inter-dependent effects of ubiquitylation and phosphorylation. These findings indicate that Trim32 mediated regulation of Kv1.2 constitutes a potential novel pathway for the acute regulation of neuronal excitability in the brain.
Results

The Ubiquitin Ligase Trim32 Co-purifies with Kv1.2 from Rat Cerebellar Extracts

The cell surface level of Kv1.2 is governed by a homeostatic balance between channel endocytosis from and recycling back to the plasma membrane, a process controlled by serine/threonine as well as tyrosine phosphorylation of the channel (Connors, Ballif, & Morielli, 2008; Nesti et al., 2004; Stirling, Williams, & Morielli, 2009). Mechanistic specificity for the regulation of ion channels is conferred to a large extent by the composition of an associated macromolecular complex of proteins. Such interactions determine the channel’s sub-cellular localization, but also confer bias towards regulation by specific signaling pathways (Swayne, Altier, & Zamponi, 2014).

To identify novel interactions of Kv1.2 within the brain, we examined the interactome of Kv1.2 using an unbiased proteomic approach: Kv1.2 was purified from tissue homogenates that were separated into non-nuclear membrane fractions prior to lysis. Kv1.2 and associated proteins were separated by SDS-PAGE electrophoresis, and separated gel bands were subjected to an in-gel digestion with trypsin to produce tryptic peptides that were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Figure 1A shows a representative gel stained with Coomassie Blue to indicate proteins isolated within the appropriate molecular weight range for both Kv1 channels and Trim32 following Kv1.2 IP. Cerebellum from two rats (6 week old) were lysed in the presence of protease inhibitors only (HALT cocktail of 100mM AEBSF*HCl, 80µM Aprotinin, 5mM Bestatin, E-64, 1.5mM EDTA, 2mM Leupeptin, and 1mM Pepstatin A = MS trial 1), protease inhibitors plus DUB and proteasome inhibitors (HALT protease
cocktail, 5mM N-Ethylmaleimide, and 10µM MG-132 = MS trial 2), and protease, DUB, proteasome, and phosphatase inhibitors (HALT protease cocktail, 5mM N-Ethylmaleimide, and 10µM MG-132, 1mM NaF, 1mM NaOrthovanadate, and 20mM BAPTA= MS trial 3) yielding 20, 17, and 37 total detected peptides of Kv1.2, respectively. In all conditions, the ubiquitin ligase Trim32 was detected as a novel interacting protein by 3 or more unique tryptic peptides that were never present in parallel, nonspecific IP controls (Figure 1B). This interaction was also detected by Western blot (WB) analysis, either through IP of Kv1.2 (Figure 1C, left) or Trim32 (Figure 1C, right), both of which demonstrated Co-IP of the interacting partner in the lysis conditions of MS trial 2 and 3.

Detection of Trim32 from our homogenates was low in the absence of DUB and proteasome inhibitors, and likewise co-IP was not detected by IP of either Kv1.2 or Trim32 (Supplementary information; Appendix A Figure A:2), suggesting that preservation of the association of Trim32 with Kv1.2 may be impacted by enzymes that alter ubiquitylation.

**Trim32 Localizes with Kv1.2 in the Cerebellum**

In the brain, Kv1.2 is especially abundant in the cerebellar cortex. The channel is expressed post-synaptically in the dendrites of Purkinje cells (PC), the major computational entity within the cerebellum, and pre-synaptically in the axon terminals of basket cell (BC) interneurons which provide strong inhibitory synaptic input to PCs (Southan & Robertson, 1998, 2000). Residing within these two key cerebellar locations, Kv1.2 is able to affect both the inhibition and excitation to PCs, the sole output of the
cerebellar cortex, and thus influence cerebellar processing to the rest of the brain. Our immunofluorescence (IF) analysis revealed that Trim32 is ubiquitous throughout the cerebellum (Figure 2). Co-staining with Parvalbumin, a marker for PCs and molecular layer interneurons, showed that Trim32 is clearly present in BC soma, cellular origin of inhibitory complexes called Pinceau which form a basket-like structure around the axon initiation segment of PCs (Figure 2A and B). Imaging at higher magnification showed co-localization in the Pinceau more clearly (Figure 2C). In our experiments, Trim32 did not always overlap with Kv1.2 due to the extensive expression of Trim32 in locations such as the granule layer and PC somas where Kv1.2 is absent. As a multifunctional protein enzyme, Trim32 was not expected to exclusively localized with Kv1.2 and these images are intended to simply provide evidence that Trim32 and Kv1.2 are simple able to be localized in the cerebellum. Analysis was performed using a Deltavision Restoration Microscopy System on fixed, permeabilized 50μM thick cerebellar slices and results were replicated in two separate experiments.

**Trim32 can Directly Ubiquitylate Kv1.2 in Vitro**

After establishing that Trim32 interacts and localizes with Kv1.2 in the cerebellum, we next investigated its functional role on the channel. To determine whether Trim32 can directly ubiquitylate Kv1.2, an *in vitro* ubiquitylation assay using recombinant purified GST-fused Trim32 and Kv1.2 variants was performed (Figure 3). Kv1.2, in the presence of only E1, E2, Trim32 (E3), ATP, and ubiquitin, is ubiquitylated at either it’s C or N terminus (GST-Kv1.2C/GST-Kv1.2N) by Trim32 as determined by stepwise ~10kDa molecular weight increases of Kv1.2 (Figure 3B). The reaction was
initiated by the addition of ATP to activate ubiquitin. As a control, the reaction was also prevented in an identical experiment by withholding ATP alone and, as expected, no ubiquitylation was detected. Trim32 self ubiquitylation was additionally used as a ubiquitylation confirmation (data not shown). To confirm the electrophoretic band shifts were not due to ubiquitylation of GST alone, the reaction was repeated with GST cleaved Kv1.2C/N and higher molecular weight bandshifts of Kv1.2 were still observed (Figure 3C), indicating that ubiquitylation is occurring on Kv1.2.

**Kv1.2 is Ubiquitylated in the Cerebellum**

Ubiquitylation of Kv1.2 at two N-terminal lysines (K49 and K63) has recently been reported by a single, large scale LC-MS/MS study in the mouse brain (Wagner et al., 2012). No sites of Kv1.2 ubiquitylation have been reported in the cerebellum specifically. In the same LC-MS/MS experiments that detected the Trim32 interaction (Figure 1A), we also detected ubiquitylation of Kv1.2 at C-terminal lysine K437 (Figure 4). In particular, two peptides of lysine 437 were detected in each of the three MS trials (Figure 4C). The previously reported N-terminal lysines were also both detected in one of three MS trails (Supplementary Information; Appendix C Figure C:2). Given that Trim32 was the only ubiquitin ligase detected by mass spectrometry from cerebellar homogenates following Kv1.2 purification, we hypothesize that Trim32 could ubiquitylate Kv1.2 there.
Trim32 Increases Kv1.2 Surface Expression in HEK293 Cells through a Mechanism Dependent on Channel Ubiquitylation.

 Trafficking of Kv1.2 modulates its expression at the surface and therefore overall impact on membrane potential (Nesti et al., 2004; Stirling et al., 2009). Recently, we have shown that both BC axon termini and Purkinje cell dendrites are locations of AC-dependent Kv1.2 trafficking (Williams et al., 2012). To date, no modulators of Trim32 signaling have been reported, limiting in vivo manipulation. However, heterologous systems have been valuable tools for investigating Kv1.2 trafficking mechanisms, and allow the overexpression of mutated constructs that may prevent or cripple the process of ubiquitylation. We therefore next utilized HEK293 cells to determine if Trim32 can regulate Kv1.2.

 Kv1.2 function is regulated by its endocytosis from and recycling back to the plasma membrane (Nesti et al., 2004; Stirling et al., 2009). To measure changes in surface Kv1.2 levels in HEK293 cells we used a flow cytometry assay as previously described (Nesti et al., 2004). Figures using this assay depict normalized count as the objective measure in which we interpret as surface Kv1.2. Over-expression of ubiquitin can drive ubiquitylation of proteins and so we used this method to investigate the effect of ubiquitin on Kv1.2 trafficking. We used co-expression with wild-type ubiquitin (WT-Ub) or with a form of ubiquitin that can only induce monoubiquitylation (lysines within ubiquitin mutated to arginine) of substrates (mUb). WT-Ub caused an overall decrease of surface channel (−37%, t(df) = 6.65(10), p < 0.0001, n = 6 each) while mUb caused an increase (+32%, t(d) = 8.27(10), p < 0.0001, n = 6 each) (Figure 5A). We next
investigated the influence of Trim32 on Kv1.2 surface expression (Figure 5B). Like mUb, co-expression of wild-type Trim32 caused a significant increase of surface channel (+15%, t(df) = 4.56(16), \( p < 0.001 \), \( n = 6 \) (+vector), \( n = 12 \) (+Trim32WT)) (Figure 5B, left graph). In cerebellar tissue, K437 is the most prevalent site of ubiquitylation detected by multiple LC-MS/MS experiments. We therefore used site directed mutagenesis to assess the role of this lysine on Trim32 mediated channel regulation. We found that mutation of K437 to alanine (K437A) caused baseline surface Kv1.2 to decrease compared to the wild-type channel (-32%, 7.52(16), \( p < 0.0001 \), \( n = 6 \) (Kv1.2WT), \( n = 12 \) (Kv1.2K437A). Strikingly, this point mutation eliminated the surface Kv1.2 increase seen with Trim32 co-expression +0.004%, \( t(df) = 0.17(22), p = 0.87 \), \( n = 12 \) each). Taken together, overexpression of Trim32 appears to cause a significant increase of surface Kv1.2 through a mechanism that may involve mono-ubiquitylation at lysine 437, the most prevalent site of ubiquitylation detected in cerebellar tissue.

To determine whether over-expression of Trim32 or mUb involves modification of other lysine residues within Kv1.2, we assessed their effect on mutant forms of Kv1.2 that have all C and N terminal lysines replaced with arginine (Kv1.2KR). Kv1.2KR dramatically attenuated the Trim32 and mUb effects on Kv1.2 trafficking: 2-way ANOVA significant main effect of co-expressions (column); \( F(2, 105) = 52.50, p < 0.0001 \), and a significant main effect of Kv1.2 lysines (row), \( F(1, 102) = 125.2, p < 0.0001 \), \( n = 12 \) (Kv1.2WT+ vector), \( n = 24 \) (Kv1.2WT + Trim32), \( n = 18 \) (Kv1.2WT + mUb), \( n = 12 \) Kv1.2KR, \( n = 24 \) (Kv1.2KR + Trim32), \( n = 12 \) (Kv1.2KR + mUb). To test whether the effect of Trim32 on Kv1.2 was dependent on the ubiquitin ligase activity of Trim32 we used a version of
Trim32 lacking its catalytic RING domain (Trim32ΔRING). In duplicate experiments we found that this version of Trim32 had no effect on Kv1.2 (Figure 5D): 1-way ANOVA, F(3, 44) = 188.2, \( p < 0.0001 \); Kv1.2WT + Trim32WT-Flag (+100%, q(df) = 13.82(44), \( p < 0.0001 \), \( n = 12 \) each), Kv1.2WT + Trim32WT-GFP (+133%, q(df) = 18.40(44), \( p < 0.0001 \), \( n = 12 \) each), Kv1.2WT + Trim32ΔRING (-5.32%, q(df) = 0.7341(44), \( p > 0.05 \), \( n = 12 \) each. Collectively, these results suggest that Trim32 is able to increase Kv1.2 at the cell surface through channel monoubiquitylation.

As indicated in Figure 3, attachment of the small protein ubiquitin to a substrate adds a molecular weight increase (~8.5kDa without FLAG tag) that can be detected through electrophoretic bandshifts via immunoblot analysis. We therefore used WB analysis to determine whether overexpression of either Trim32 or mUb causes detectable changes in the electrophoretic mobility of Kv1.2 expressed in HEK cells (Figure 5E).

Overexpression of Kv1.2 in HEK cells produces two main bands representing different glycosylation states of the channels development (lower = high mannose and higher = mature). In the presence of either Trim32WT or mUb, two clear bands of approximately 8.5kDa increased molecular weight from the high mannose Kv1.2 appear that is only faintly detected in the Kv1.2 only (+ empty vector) transfection (Figure 5B). Total Kv1.2 had no significant change in three replicate experiments (data not shown). Taken together with the flow cytometry data, Trim32 is able to cause detectable monoubiquitylation that causes a functional change in Kv1.2 expressed at the cell surface that is independent of degradation.
Trim32 Decreases Kv1.2 Surface Expression Independent of Ubiquitylation of Kv1.2

During the course of this study we observed that the confluence of plated cells dramatically impacted the effect of Trim32 on Kv1.2 surface expression. In other contexts, Trim32 has been shown to have ubiquitin-independent effects, the most understood being the binding of proteins to its NHL repeats for activation of microRNAs (Loedige & Filipowicz, 2009; Wulczyn et al., 2011). We therefore asked if the confluence-dependent effects that we observed on Kv1.2 might involve ubiquitin-independent mechanisms. In the following experiments, we utilized cells grown to higher confluence (plated at higher density and grown one additional day) simply as a representation of a different cellular condition that may demonstrate different signaling patterns than that of the lower confluence conditions of the previous experiments. We found that in highly confluent cells, Trim32 caused a significant decrease of surface Kv1.2 (-32%, \( t(df) = 8.30(60), p < 0.0001, n = 12 \) (Kv1.2WT + vector), \( n = 6 \) (Kv1.2 + Trim32)) (Figure 6A, left). MUb, however, continued to cause a significant increase in surface Kv1.2 (+18%, \( t(df) = 4.72(60) \). As expected, replacing Kv1.2WT with Kv1.2KR diminished the Kv1.2 surface increase by mUb (+3.7%, \( t(df) = 1.20(60), p > 0.05, n = 12 \) (Kv1.2KR + vector), \( n = 12 \) (Kv1.2 + mUb)) however, unlike cells plated at low confluence, the effect of Trim32 was unchanged in Kv1.2KR channels (-37%, \( t(df) = 11.86(60), p < 0.0001, n = 12 \) each; mean difference between rows (Kv1.2WT vs Kv1.2KR) = 5.29%, \( p > 0.05 \); two-way ANOVA significant lysine effect, \( F(1, 60) = 11.24, p < 0.0025 \), significant co-expression factor \( F(2, 60) = 196.2, p < 0.0001, n = 12 \) (Kv1.2WT + vector), \( n = 6 \) (Kv1.2 + Trim32), \( n = 12 \) (Kv1.2WT + mUb), \( n = \)
12(Kv1.2KR + vector), n = 12 (Kv1.2KR + Trim32), n = 12 (Kv1.2KR + mUb). This effect was enhanced by replacing Trim32WT with Trim32ΔRING (mean difference between Kv1.2WT+ GFP-Trim32WT vs Kv1.2WT + GFP-Trim32ΔRING = -39%, \( p < 0.0001 \)), lending strong support for this being a ubiquitin-independent effect of Trim32 on Kv1.2 (Figure 6A, right).

Recent studies have shown that ubiquitylation and phosphorylation can have substantial cross talk. Phosphorylation can promote or inhibit ubiquitylation leading to or altering various signaling events (reviewed in (Hunter, 2007)). Additionally, ubiquitin ligases can be activated by phosphorylation, and similarly, kinases can be deactivated by ubiquitylation (Persaud et al., 2014; Snyder, 2009). We wondered if the presence of Trim32 can influence the phosphorylation state of Kv1.2 during high confluence conditions. To investigate this, we used the quantitative LC-MS/MS method of Stable Isotope Labeling of Cells in Culture (SILAC) to detect quantitative changes of phosphorylation following co-expression with Trim32. Kv1.2 was expressed alone (+empty vector) in cells cultured in media containing arginine and lysine amino acids with light isotopes (K\( ^{12} \)C\( _6 \) & \( ^{14} \)N\( _2 \) and R\( ^{12} \)C\( _6 \) & \( ^{14} \)N\( _4 \)), while Kv1.2 was co-expressed with Trim32 in cells cultured in media containing arginine and lysine amino acids with heavy isotopes (K\( ^{13} \)C\( _6 \) & \( ^{15} \)N\( _2 \) and R\( ^{13} \)C\( _6 \) & \( ^{145} \)N\( _4 \) (Figure 6B). Cells were lysed in buffer conditions equivalent to tissue MS trial 3 of Figure 1 and equal protein concentration from both light and heavy populations were pooled together prior to electrophoresis. Lysates were immunoprecipitated for Kv1.2 and processed for LC-MS/MS exactly as the cerebellar homogenates with the addition of reduction (.1M DTT, 30min, 55 degrees C).
and alkylation (55mM, 45 minutes RT) iodoacetamide) prior to digestion for proper quantification of cysteine containing peptides.

In agreement with the flow cytometry experiments, no ubiquitylated peptides of Kv1.2 were detected in ether the absence or presence of Trim32 in the highly confluent cells. Because Kv1.2 is highly phosphorylated at several serine residues adjacent to the cerebellar ubiquylation site K437, we examined whether Trim32 overexpression alters these states of phosphorylation. Interestingly, the presence of Trim32 caused a dramatic decrease of phosphorylated S434 with a corresponding increase of the non-phosphorylated S434 peptide (at both +2 (Figure 6C, top) and +3 (not shown) charge states), while S440 was unchanged (Figure 6C, bottom). It should be noted that the nonphoshorylated peptides of 1048.98 (light) and (1052.99 heavy) m/z were confirmed by MS2 fragmentation, however the detected peptides reported here of 1089.00 and 1093.01 m/z as the phosphorylated versions were not given their overall low abundance in the MS1 process and the non-targeted LC-MS/MS approach performed. The peptide m/z was in agreement with previous reports of the same peptide (Yang et al., 2007) as well as previous LC elution retention times previously detected by our lab (data not shown). In all of our experiments in tissue and cultured cells, this phosphopeptide was always localized to S434 and not nearby T433.

Like K437 ubiquitylation, S434 and S440 phosphorylation are consistently detected in cerebellar tissue. We investigated if these modification sites, like K437, have a role in surface trafficking of the channel in HEK cells. To avoid compensation by nearby T433 and S441 (and account for potential ambiguity of LC-MS/MS spectra matching), we compared Kv1.2WT to Kv1.2T433V/S434A and Kv1.2S440A/S441A
double mutants. In cells plated to low confluence, Kv1.2T433V/S434A mutation caused a dramatic reduction in surface Kv1.2 (-80%, q(df) = 17.92(47), \( p < 0.0001 \), \( n = 6 \) each). Kv1.2S440A/S441A had a more subtle but significant decrease of surface Kv1.2 compared to the wild-type channel alone (-14%, q(df) = 3.25(47), \( p < 0.001 \), \( n = 6 \) each) that was not significant by individual t-test. These results suggest that availability of S434 (and possibly T433) for phosphorylation may be critical for getting Kv1.2 to the surface (the functional role of S434 is unknown). Additionally, adding Trim32 overexpression, we see the expected increase of surface Kv1.2, however Kv1.2T433V/S434A amplified the effect by 10 fold (mean difference between Kv1.2WT + Trim32 and Kv1.2T433/VS434A + Trim32 by 1-way ANOVA multiple comparisons = 151.12%, \( p < 0.0001 \)) (Figure 6D, left and middle insert). Unexpectedly, the Kv1.2S440A/S441A mutant, which caused a modest decrease of surface Kv1.2 alone, reversed the effect of Trim32 to a significant decrease, suggesting that the increase of Kv1.2 surface expression by Trim32 may first require phosphorylation of S440/S441.

Discussion

Although historically viewed as a regulator of motor function, a growing body of research indicates the cerebellum is essential for a wide range of non-motor functions. These include modulation of autonomic function to processing of emotional information and other cognitive functions. The link to emotional and cognitive function may be especially important for understanding behavioral and cognitive symptoms of autism since defects in cerebellar structure are highly correlated with autism (Allen, Muller, & Courchesne, 2004; Fatemi et al., 2012; Whitney, Kemper, Bauman, Rosene, & Blatt,
Interestingly, ASD is not typically associated with motor dysfunction, suggesting a particularly important role for non-motor cerebellar effects. Genetics studies suggest a key role for disruption in cerebellar inhibitory neurotransmission, but the neurophysiological mechanisms linking cerebellar function with ASD remain unclear. Understanding the neurophysiological roles of autism-related genes, such as Trim32, is therefore essential.

Trim proteins are involved in diverse biological processes throughout the body, and Trim32 is no exception. The links of Trim32 mutation to degenerative muscular disease led to the magnitude of discovered Trim32 functions within skeletal muscle tissue, however more Trim32 resides within the brain than all other organs combined, suggesting that Trim32 likely has important neurological roles that are at this time, overlooked (Kudryashova et al., 2009). The work in this study proposes a unique and exciting role for Trim32 in the adult brain: From purified cerebellar tissue, we first took an unbiased proteomic approach to identify Trim32 as an interacting protein with Kv1.2, an ion channel key for regulating neuronal excitability. Next, we used mutagenesis and overexpression across both biochemistry and flow cytometry experiments to provide evidence that Trim32 regulates Kv1.2’s surface trafficking through multiple mechanisms.

Trim32’s most understood role is its ubiquitin ligase activity, particularly in catalyzing polyubiquitin chains on proteins to signal their degradation by the proteasome. Here, we have shown that Trim32 can regulate Kv1.2 in a ubiquitin-dependent but degradation-independent mechanisms. In heterologous systems, our data suggest that Trim32 increases channel surface expression by a monoubiquitlyation like mechanism.
Comparing Trim32 overexpression experiments (both biochemistry and flow cytometry) with those of wtUb and mUb, the increased surface Kv1.2 appears to work through mono or multiubiquitylation of the channel. Non-degradative trafficking of ion channels by mono or multiubiquitylation has been previously reported, however, usually the ubiquitin tagged channels are endocytosed into primary vesicles and trafficked to early endosomes where ubiquitin is removed by deubiquitinating enzymes (W. H. Wang, 2006). Monoubiquitylation of ROMK1 (Kir1.1) decreases its surface but not total expression (Lin et al., 2005), while overexpression of an E3 ligase (Nedd4-2) also caused decreased surface Kv1.3 and Kv1.5 in Xenopus Oocytes (Boehmer et al., 2008; Henke, Maier, Wallisch, Boehmer, & Lang, 2004). Monoubiquitylation is recognized as a sorting signal throughout the endocytic pathway, however a mechanism to promote expression at the plasma membrane through endocytic trafficking, perhaps by increased recycling, has not been reported. Our finding that Trim32 increases surface Kv1.2 is consistent with such a mechanism although other mechanisms, including decreased constitutive endocytosis or increased biosynthetic trafficking to the plasma membrane are also possible.

How Trim32 affects Kv1.2 in vivo and what the effects of that are on brain function remains unknown. As mentioned previously, large-scale genetic reports have determined Trim32 is both a risk factor for ASD/ADHD as well as a genetic link for ASD/ADHD comorbid with epilepsy, a disease state with known Kv1 channel involvement. A point mutation in Trim32s B-Box domain (P130S) leads to the less commonly developed Bardet-Biedl Syndrome (Chiang et al., 2006) that, interestingly, some patients with Bardet-Biedl syndrome report cerebellar dysfunction (Keppler-
Noreuil et al., 2011), including a case of cerebellar ataxia (Kowal & Sikora, 1989), a channelopathy that can be caused by KCNA2 (Kv1.2) mutation (G. Xie et al., 2010). The findings reported here linking Trim32 with Kv1.2 function suggests novel avenues for the development of therapeutics and pharmaceuticals for these diseases.

**Materials and Methods**

**Tissue Harvest and Membrane Fractionation**

Cerebellar tissue was harvested from adult (5-9 week) Sprague Dawly rats and homogenized in 10 mM HEPES (pH 7.4), 0.3M sucrose, 2mM EDTA, 1mM DTT, 1mM NaF, 1mM NaOrthovanadate, 20mM BAPTA, 5mM NEM, 10 µM MG-132 and protease inhibitors (HALT cocktail of 100mM AEBSF*HCl, 80µM Aprotinin, 5mM Bestatin, E-64, 1.5mM EDTA, 2mM Leupeptin, and 1mM Pepstatin A) using a 15mL dounce homogenizer (10-15 strokes). Nuclear proteins were removed by low speed spin (1000g for 10 min at 4°C) and samples were separated into membrane fractions by ultracentrifugation (200,000 for 90 min at 4°C). The supernatant containing cytosolic proteins was discarded and remaining non-nuclear, membrane fraction was then re-suspended and lysed in 50mM Tris (pH 7.41), 150 mM NaCl, 1mM EDTA, 0.25% deoxycholate, 1% NP-40, 1mM DTT, 1mM NaF, 1mM NaOrthovanadate, 20mM BAPTA, 5mM NEM, 10 µM MG-132 and protease inhibitors (HALT).

**Co-immunoprecipitation**

Immunoprecipitation (IP) with soluble lysates was performed with magnetic Dynabeads (Gibco, 30µl bead/ 1µg antibody) for 1 hr at 4°C. IPs were washed three times with lysis
buffer. Lysates and IPs were analyzed by SDS-PAGE and Western blotting using anti-Kv1.2 (K14/16 mouse monoclonal, UC Davis, 1.5µg/cerebellum) and anti-Trim32 (Rabbit polyclonal, GeneTex, 1.5µg/cerebellum) antibodies. For IPs processed for LC-MS/MS analysis, proteins were separated by 1D SDS-PAGE electrophoresis and visualized with Coomassie staining.

**In Gel Digestion and Mass Spectrometry Analysis**

Coomassie stained gel pieces were excised, diced, washed, destained, dehydrated, and digested with 1.25ng/µl trypsin as previously described (Connors et al., 2008) Extracted peptides were analyzed by LC-MS/MS using an LTQ linear ion trap mass spectrometer alone or as a LTQ-Orbitrap hybrid mass spectrometer (SILAC). Identification of post-translational modifications included dynamic modification of 79.96 to S/T/Y and 114.0429 to K amino acids. A detailed description of mass spectrometric conditions are presented in the Extended Experimental Procedures.

**Immunofluorescence**

Cerebellar/hippocampal slice images: Adult rat cerebellar slices (50 µM) were fixed (2% formaldehyde cardiac perfusion), permeabilized (0.2% Triton X-100), blocked, and stained for Kv1.2 (1:200) and Trim32 (1:100), and imaged using a DeltaVision Restoration Microscopy System (Applied Precision). Images were acquired 20X, 60X, or 100X oil immersion lenses using the SoftWorks software and analyzed with NIH Image J. Colocalization was determine using the Colocalization Point plugin using automated threshold detection that was manually verified.
In Vitro Ubiquitylation Assay

Purified recombinant FLAG-ubiquitin, ubiquitin-activating enzyme (E1), and E2 ubiquitin-conjugating enzymes (UbcH5a) were purchased from Boston Biochemical. GST-Trim32 was expressed in E. coliBL21(DE3)pLysS strain (Stratagene), and isolated from cells lysed in 10 mMTris/HCl, pH 8.0, 10% glycerol, 1% Triton X-100, 100 mM NaCl, 10 mM MgCl2, 10 μM ZnCl2, and protease inhibitors using glutathione-Sepharose 4B beads in bulk suspension. Prior to use, Trim32 was released from the GST-bead complex with thrombin in buffer containing 50 mM Tris, 5 mM MgCl2, 100 mM NaCl, 1 mM DTT and 0.5. In vitro ubiquitination reactions were done by combining FLAG-ubiquitin, E1 and E2 (UbcH5a) ligase, ATP and Trim32 in 50 mM Tris/HCl, pH 8.0, 5 mM MgCl2, and 0.5 mM dithiothreitol. Reactions were incubated at 37 °C for 120 min and stopped by heating to 100 °C in protein sample buffer.

Cell Culture and Flow Cytometry

Kv1.2 was overexpressed in HEK293 cells stably expressing the beta subunit HEKβ2. For detection of cellular surface expression, an antibody that binds an external portion of Kv1.2 was used for fluorescent detection by flow cytometry as previously described (source). For high confluence detection, cells were allowed to grow for an additional 1-2 days following transfection.

SILAC Mass Spectrometry

Culture media in the Light population contained lysine and arginine amino acids with K = $^{12}\text{C}_6 & ^{14}\text{N}_2$ and R = $^{12}\text{C}_6 & ^{14}\text{N}_4$) while Heavy populations contained them with K =
$^{13}\text{C}_6$ & $^{15}\text{N}_2$ and R $^{13}\text{C}_6$ & $^{15}\text{N}_4$. Cells were grown to full confluence and split 5 times in respective media before plating for transfection. In repeat experiments, the Light population was overexpressed with Kv1.2 + empty vector and the Heavy population with Kv1.2 + Trim32.

**Supplementary Materials**

Supplementary material can be found in Appendix A.

**Figure Legends**

**Figure 1. The ubiquitin ligase Trim32 interacts with Kv1.2 in the cerebellum. A.)** Coomassie stained gel showing successful immunoprecipitation of proteins in the predicted range of neuronal Kv1 channels (~90kDa) and Trim32 (65-100kDa). B.) Trim32 can be detected by LC-MS/MS after immunoprecipitation of Kv1.2 from rat cerebellum tissue. Throughout multiple experiments, no Trim32 peptides were detected in parallel IgG antibody control IPs. C.) The Trim32 interaction with Kv1.2 in cerebellar tissue can also be detected by Western blot co-IP analysis. Left: WBs showing IP of Kv1.2 (top) with corresponding Trim32 Co-IP (middle) and lysate (bottom) confirming both proteins.

**Figure 2 Trim32 is expressed in cerebellar localizations with Kv1.2. A.)** Cartoon depiction of cells within the cerebellum and localizations of Kv1.2. B.) Trim32 is diffuse throughout the cerebellum as examined immunofluorescence at 10X magnification (data
not shown). Closer examination (60X) with co-staining of Parvalbumin (yellow), a marker for molecular layer neurons and PCs, show Trim32 is found within BCs. C.) Kv1.2 is of highest expression in BC pincesoae structures, and high magnification (100X) show Trim32 also localizes to these structures.

Figure 3. Trim32 can directly ubiquitylate Kv1.2 in vitro. A.) Schematic image of the in vitro ubiquitylation reaction. Recombinant purified E1, E2 and Trim32 (E3) proteins were combined with a recombinant purified GST-fusion protein of the C or N-terminus of Kv1.2 (GST-Kv1.2N/C). The ubiquitylation reaction was initiated by addition of ATP. B.) Immunoblot analysis shows incremental upward shifts in electrophoretic mobility of GST-Kv1.2N/C indicative of the increased molecular weight conferred by covalent attachment of ubiquitin. These shifts are absent in reactions lacking ATP. C.) Repeat experiment with Kv1.2C after GST cleavage showing increased electrophoretic band shifts that overlap in both Flag (ubiquitin) and Kv1.2 channels.

Figure 4. Kv1.2 is ubiquitylated in the rat cerebellum. LC-MS/MS output of Kv1.2 K437 ubiquitylation showing liquid chromatography elution peaks (top insert), representative MS1 detection of tryptic peptide at \( m/z \) 1064.54 (top), and second MS fragmentation spectrum (bottom). Table insert lists the Sequest XCorr values for the peptides detected, all at charge state +3.
Figure 5. **Trim32 increases Kv1.2 surface expression through ubiquitylation.** Flow cytometry was used to monitor Kv1.2 surface expression by whole cell fluorescent detection after incubation with an external loop tagged Kv1.2 antibody.  

**A.** Co-transfection with wild type ubiquitin (WT-Ub) causes a significant decrease in surface Kv1.2, while co-transfection with a form of ubiquitin that cannot form chains (mUb), causes an increase in surface Kv1.2.  

**B.** Like mUb, overexpression of Trim32 also caused increased surface Kv1.2, an effect that was attenuated by single point mutation of K437 (the prominent site of cerebellar ubiquitylation).  

**C.** In cells plated to low confluence, Trim32 repeatedly caused a significant increase of surface Kv1.2, an effect that is dependent on Kv1.2 lysines shown by replacement of Kv1.2WT with Kv1.2KR.  

**D.** Mutation of Trim32’s ubiquitylation catalytic RING domain, like Kv1.2KR, diminished the increase of surface Kv1.2 after Trim32 coexpression.  

**E.** Using WB analysis, co-expression of Trim32 (middle lane compared to left lane) appears to cause monoubiquitylation bandshifts (~8.5kDa) of Kv1.2 similarly to monoUb (right lane compared to left lane). Taken together, Trim32 appears to cause an increase in surface Kv1.2 through a mechanism involving monoubiquitylation of the channel. * = p <0.05  ** = p <0.01  *** = p <0.001 by students’ t-test or two-way ANOVA. Sample size (n) for each experiment or combined experiments are listed inside the bar graphs.

Figure 6. **Trim32 lowers surface Kv1.2 independent of ubiquitylation.**  

**A.** The transient transfections of Figure 5C and D were repeated using HEK293 cells that were plated and grown to high confluence and flow cytometry assessment demonstrated that
under these conditions, Trim32 causes a significant decrease of surface Kv1.2 that is independent of both Kv1.2 lysines and Trim32 ubiquitin ligase activity. **B.** Stable Isotope Labeling of Cells in Culture (SILAC) was used to quantitatively examine whether co-expression of Kv1.2 with Trim32 can alter Kv1.2’s phosphorylation state in highly confluent cells. In the light population, Kv1.2 was co-transfected with empty vector, while in the heavy population Kv1.2 was co-transfected with Trim32, and the relative change in the MS1 intensity spectra in the light vs. heavy fractions provided a quantitative measure of Trim32’s effect on Kv1.2 phosphopeptide abundance. **C.** We find, in repeated experiments, that Trim32 strongly decreases phosphorylation of serine 434 within Kv1.2 (C, top), but has no effect on another prevalent phosphorylation site serine 440 within the channel (C, bottom). The functional role of S434 phosphorylation remains to be elucidated. Using flow cytometry with T433V/S434A and S440A/S441A double mutants, we found the S434 but not S440 double mutant caused a dramatic decrease in surface Kv1.2, which facilitates Trim32’s surface Kv1.2 increasing mechanism (D, left and insert). Co-expression of Trim32 with S440A/S441A now caused surface Kv1.2 to significantly decrease. Double mutants were used to avoid MS2 ambiguity potential phosphorylation compensation.* = p <0.05 ** = p <0.01*** = p <0.001 by students’ t-test or two-way ANOVA. Sample size (n) for each experiment or combined experiments are listed inside the bar graphs. Heavy and Light SILAC peaks were extracted under equal LC elution peaks, and MS peaks were averaged across multiple peaks where statistical analysis was used to determine significance between relative intensities.
Figure 7. Proposed model for Trim32 induced regulation of Kv1.2. The following model of surface Kv1.2 regulation by Trim32 is proposed: Under certain cellular conditions (low confluence in HEK293 cells), Trim32, through its E3 ligase RING domain, catalyzes ubiquitylation of Kv1.2 at lysine 437 in coordination with S440/S441 phosphorylation to promote Kv1.2 at the plasma membrane. Under different cellular conditions (high confluence in HEK293 cells), Trim32, independent of its RING domain, facilitates the removal of S434 phosphorylation to promote internalization of Kv1.2 from the plasma membrane.
Figure 2:1 Ubiquitin ligase Trim32 interacts with Kv1.2 in cerebellar extracts.
Figure 2:2 Trim32 is expressed in cerebellar localizations with Kv1.2.
Figure 2:3 Trim32 can directly ubiquitylate Kv1.2 in vitro.
Figure 2: Kv1.2 is ubiquitylated at K437 in the cerebellum.
Figure 2:5 Trim32 increases Kv1.2 surface expression through ubiquitylation.
Figure 2:6 Trim32 lowers surface Kv1.2 independent of ubiquitylation.
Figure 2:7 Model of Trim32 induced trafficking of Kv1.2.
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CHAPTER 3: CLORIDE-SENSING WNK1/SPAK AS MODULATORS OF A VOLTAGE-GATED POTASSIUM CHANNEL IN THE BRAIN.

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Running Title: WNK signaling links Kv channel trafficking and chloride regulation in the cerebellum.

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Abstract

WNKs are a family of serine/threonine kinases widely expressed in the brain where they are associated with several neurological disorders, including autism and schizophrenia. They have been most extensively studied in kidney where they regulate cation-chloride cotransporters. In the brain, WNK1 and its downstream target SPAK also regulate cation-chloride co-transporters, and are hypothesized to influence chloride homeostasis during inhibitory neurotransmission. Here we report that in the cerebellum WNK1 and SPAK complex with and regulate the voltage-gated potassium channel Kv1.2, indicating a new mechanism for WNK-SPAK signaling and identifying a novel cellular pathway for regulating neuronal excitability. Using mass spectrometry and immunoblot, we identified WNK1 and SPAK as components of the cerebellar Kv1.2 protein interactome. Immunofluorescence studies revealed that WNK1 is expressed, along with Kv1.2, in cerebellar Purkinje cells. Kv1.2 function is regulated by its endocytic trafficking to and from the cell surface, and flow cytometry analysis of surface Kv1.2 in HEK293 cells showed that both WNK1 and SPAK influence Kv1.2 expression at the cell surface. In the presence of functional SPAK, WNK1 over-expression reduced surface Kv1.2 as did over-expression of SPAK. In contrast, WNK1 increases surface Kv1.2 levels when uncoupled from SPAK using either the pharmacological inhibitor STOCK-1S50669 or expression of a version of SPAK lacking the phosphorylation site required for its activation by WNK1. Evidence that the interaction between WNK1 and Kv1.2 is regulated in vivo comes from our finding that significantly less WNK1 co-
immunoprecipitated with Kv1.2 of rats that had been subject to eye-blink conditioning, a type of cerebellar learning that we have previously shown to be influenced by Kv1.2 regulation. We conclude that WNK1/SPAK regulation of Kv1.2 is a novel cellular mechanism governing cerebellar function.

**Introduction**

Mammalian with-no-lysine [K] (WNK) are a class of serine-threonine protein kinases that detect changes in intracellular chloride concentration \([\text{Cl}^-]\). In the kidney, where WNK signaling is the most studied, WNKs have been shown to both govern the kinase-regulation of ion transport and contribute to the development of inherited and acquired forms of salt-sensitive hypertension (for review, see (Hoorn & Ellison, 2012)). All four WNK kinase isotypes are found in the brain (Holden, Cox, & Raymond, 2004; Kahle et al., 2004; O'Reilly, Marshall, Speirs, & Brown, 2003; Rinehart et al., 2011; Verissimo & Jordan, 2001), although WNK1 and WNK2 are the most abundantly expressed. Despite their widespread expression in the brain, comparatively little is known about their function there (Delaloy et al., 2006; Rinehart et al., 2011).

Most studies indicate that WNKs are the chloride sensing unit of a cascade of kinases that ultimately control intracellular ion homeostasis. Immediately downstream of WNK kinases are two structurally similar kinases: oxidative stress-responsive kinase 1 (OSR1) and STE20/SPS1-related proline/alanine-rich kinase (SPAK), both of which are phosphorylated and consequently activated by WNKs (Welling, Chang, Delpire, & Wade, 2010). SPAK and OSR1 in turn phosphorylate cation-chloride co-transporters, regulating their activity (Dowd & Forbush, 2003; Gagnon, England, & Delpire, 2006;
Piechotta, Lu, & Delpire, 2002). In the adult brain, these transporters consist mainly of the Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter NKCC1, which drive chloride influx, and the K\(^+\)-Cl\(^-\) co-transporter KCC2 which drive efflux of intracellular chloride (de Los Heros et al., 2014). The main inhibitory neurotransmitters in the brain are gamma-amino butyric acid (GABA) and glycine, both of which activate ligand gated chloride channels. Thus, WNK/SPAK regulation of cation-chloride co-transporters has a major influence on excitability in the brain. Mutation or alteration of SPAK/OSR1 are correlated with increased neuronal activity leading to epilepsy (reviewed in (Alessi et al., 2014)), while defects in KCC2 are also implicated in disease state involving hyper-excitability and deficits in GABA\(_A\) receptor mediated neuronal inhibition (Silayeva et al., 2015). Additionally, models of acquired epilepsy show elevated SPAK expression in hippocampal neurons (L. Yang et al., 2013).

WNK and SPAK induced trafficking of cation-chloride co-transporters is well defined, particularly in the kidneys. Recent studies, however, have shown that SPAK is also able to affect trafficking other types of membrane proteins (Elvira et al., 2014; Elvira, Warsi, Munoz, & Lang, 2015; Fezai et al., 2014; Warsi et al., 2014). One target, chloride channel protein 2 (ClC-2), is a voltage -and chloride-sensitive channel that contributes to the regulation of neuronal excitability (Foldy, Lee, Morgan, & Soltesz, 2010; Ratte & Prescott, 2011; Rinke, Artmann, & Stein, 2010; Warsi et al., 2014). In inhibitory basket cells of the hippocampus, ClC-2 was demonstrated to extrude Cl\(^-\) under intense neural firing to maintain efficacy of GABAergic inhibition (Foldy et al., 2010). Other studies have proposed that under physiological conditions, ClC-2 channels permit
Cl- entry into the neurons to reduce neuronal excitability (Ratte & Prescott, 2011). Recently, overexpression of SPAK kinase was shown to modulate Kv1.5 surface levels in a *Xenopus* oocyte expression system (Elvira et al., 2015). This finding is particularly interesting because it suggests roles for WNK/ SPAK that extends beyond their direct effects on chloride homeostasis.

Voltage-gated potassium channels are expressed throughout the brain and have diverse electrophysiological effects. In the cerebellum, Kv1 family of voltage-gated potassium channels, in particular those harboring the Kv1.2 alpha subunit, contribute to excitability of Purkinje cells (PC), the main computational unit and sole output of the cerebellar cortex (Southan & Robertson, 1998, 2000). In a previous study we showed that suppression of Kv1.2 by the neuromodulator secretin increases dendritic excitability of PCs. We also reported that such suppression of Kv1.2 enhanced cerebellum-dependent eye blink conditioning (EBC). Thus, the regulation of Kv1.2 impacts core neurophysiological and computational functionalities of the cerebellum, including those governing behavior. (Williams et al., 2012). *In this study, we identified WNK1 and SPAK kinases as components of the cerebellar Kv1.2 interactome, suggesting a new mechanism for regulating excitability in the brain.* We report that WNK1 is present in cerebellar Purkinje cells along with Kv1.2. Using a cell culture system we show that both kinases can regulate Kv1.2 expression at the cell surface. Finally, we demonstrate that the physical association between WNK1 and Kv1.2 decreased in cerebellum from rats trained in an associative conditioning task, suggesting that WNK1 regulation of Kv1.2 contributes to higher order functions in the brain.
Materials and Methods

Materials

Antibodies: The following primary antibodies were used in this study: Flow Cytometry: A rabbit polyclonal antibody directed against the first extracellular loop of Kv1.2 (α-Kv1.2e) developed in conjunction with BioSource International (Camarillo, CA)” (Stirling et al., 2009), Immunoprecipitation, immunofluorescence, and immunoblotting: mouse monoclonal Kv1.2 (K14/16; Neuromab) rabbit polyclonal C-terminal WNK1 (GTX30695; GeneTex), rabbit polyclonal SPAK (2281S; Cell Signaling), rabbit polyclonal SPAK (Pierce, PA5-17338), and rabbit polyclonal GAPDH (ab9485; Abcam). The following secondary antibodies were used in this study: Immunofluorescence: Goat anti-mouse Alexa Fluor 488 (Invitrogen), Goat anti-rabbit Alexa Fluor 568 (Invitrogen), Goat anti-rabbit Alexa Fluor 647 (Invitrogen); Immunoblotting: Goat anti-mouse 700 (Invitrogen), Goat anti-mouse 800 (Rockland); Flow Cytometry: Goat anti-rabbit antibody conjugated to phycopychrythin/Cy5, with an excitation peak of 488 nm and an emission peak of 667 nm (Southern Biotech, Birmingham, AL).

Constructs: The following DNA constructs were used in this study: Human pCMV5-myc-WNK1 was a generous gift by Dr. Chou-Long Huang. SPAK constructs GFP SPAK T233/A (DU6190) and GFP SPAKWT (DU6188) were purchased at MRC-PPU Reagents, University of Dundee, UK. Kv1.2 and KvB2 were developed in our lab previously.
Treatments: STOCK1S-50699 was purchased from MolPor (MolPort-002-144-727, Riga, Latvia) and NaCl salt (58.44 g/mol, ≥99.0%) was purchased from Fisher Scientific (BP358-10).

Cell Culture and Transfections

Human embryonic kidney 293T cells (HEKT-cells), Human embryonic kidney 293T cells with WNK1 knocked out (WNK1KO cells) by CRISPR genomic editing (generated as described in (Roy et al., 2015)), or human embryonic kidney cells stably expressing Kvβ2 were used and cultured as previously reported (Nesti et al., 2004). Cells were plated onto 60 mm petri dishes pre-coated in polyethylenimine and allowed to grow to ~60-80% confluence. Experimental plasmids were transfected into cells using a PEI transfection method: Diluted DNA (5µg) was added to PEI (1µg/µL) at a 1:1 ratio and immediately mixed by pipetting. Mixture was allowed to sit at room temperature for 15 minutes before being added drop wise to cells. Transfections were checked manually with an arc-lamp microscope prior to proceeding (via 0.1µg EGFP DNA added to DNA mixture). For immunoblotting, transfected cells were lysed 48 hours later. For flow cytometry, 24 hours following transfection cells were re-plated to a density of 3.3 × 10^4 cells/cm^2 onto 35 mm petri dishes. Once cells adhered, media was aspirated, plates were rinsed with sterile PBS (GIBCO life sciences), and media and new serum-free media was given for overnight serum starvation (10-16 hours). In all experiments comparing multiple cells lines, each were maintained at similar passage and treated equally from plating to treatment. Results comparing the cell lines were analyzed blindly.

Surface Detection of Kv1.2 and Flow Cytometry
Surface Kv1.2 detection: Cells were given a drug, vehicle, or no treatment before application of 154mM solium azide as described previously (Nesti et al., 2004). Cells were then lifted, put in a microcentrifuge tube, labeled for surface Kv1.2 by application of 0.33µg/ml α-Kv1.2e, and antibody binding detected with fluorescently conjugated anti-rabbit immunoglobulin G (IgG) (0.1µg/ml). Whole cell fluorescence was detected by a single laser flow cytometer and distribution selection and background subtraction was performed as previously described (Stirling et al., 2009).

Biochemical Sample Preparation

Immunoblot: For HEK293 cells, after lifting cells and removing a fraction for flow cytometry plating, the remainder of transfected cells were allowed to settle and re-grow to full confluence. 24-48 hours later, cells were rinsed (2 x ice-cold PBS), lifted in a lysis buffer (described below for tissue homogenization), incubated for 10 minutes at 55°C in Laemmli sample buffer containing 0.1 M dithiothreitol (DTT) (Sigma S3401-10VL), and resolved by SDS-PAGE and protein immunoblot as previously described (Williams et al., 2007).

Immunoprecipitation: Immunoprecipitation with soluble lysates from male, rat cerebellar tissue (5-10 week old, Sprague Dawley, Chargles River, Quebec, Canada) was performed with magnetic Dynabeads (Gibco, 30µl bead/ 1µg antibody) for 1 hr at 4°C. IPs were washed three times with lysis buffer. Lysates and IPs were analyzed by SDS-PAGE and Western blotting using anti-Kv1.2 (K14/16 mouse monoclonal, UC Davis), anti-WNK1 (rabbit polyclonal C-terminal WNK1 (GTX30695; GeneTex), anti-SPAK (rabbit polyclonal SPAK (2281S; Cell Signaling), and anti-GAPDH (rabbit polyclonal GAPDH
Infrared secondary antibody signals were detected and quantified using an Odyssey Infrared Imager (LI-COR). For co-IP analysis by immunoblot in Figure 1D, female rats (5 week old, Charles River, Quebec, Canada) were used. For IPs processed for LC-MS/MS analysis, following SDS-PAGE electrophoresis gels were stained with Coomassie Brilliant Blue G-250 (cat. #20279, Lifetechnologies) to produce protein bands for excision.

**Immunofluorescence**

**Multiphoton:** Adult rat cerebellar slices (400 µM) were fixed (4% formaldehyde cardiac perfusion followed by drop-in of room temperature 4% formaldehyde for 15 minutes), permeabilized (10% ice-cold acetone, 5 minute), blocked (10% normal goat serum in PBS, 1 hour), stained for Kv1.2 (1:200) and WNK1 (1:100) or SPAK (1:50) overnight, rinsed (5 x 5 minute rocking in NGS, given secondary antibodies with conjugated fluorescent probes (Alexa 488 and Alexa 586/647, 1 hour) and rinsed again (5 x 5 minute rocking in NGS). Images were obtained using a Zeiss LSM 7 multiphoton microscope. The Zeiss LSM-7 Dedicated Multiphoton has four non-descanned PMT detectors, two of which make up the highly sensitive Zeiss gallium arsenide phosphide BiG detector. Multiphoton excitation is generated by a Coherent Chameleon Vision II Titanium Sapphire pulsed IR laser, with dispersion compensation. The stage is a motorized Prior Z-deck, which is fully integrated into the Zeiss Zen software. A 20X Plan Apo 1.0 NA DIC VIS-IR water immersion lens is the main objective lens. The system frame is a Zeiss Axio Examiner, specifically optimized for multiphoton microscopy. Images were analyzed in Image J (NIH) and Zen Light 2012 software (Zeiss, Germany).
Co-localization was determined using the Image J Colocalization Point plugin with automated threshold detection that was manually verified.

**Widefield with Deconvolution:** Adult rat cerebellar slices (50 µM) were fixed (4% formaldehyde cardiac perfusion followed by drop-in of room temperature 4% formaldehyde for 15 minutes), permeabilized (0.2% Triton X-100), blocked 10% normal goat serum in PBS, and stained for Kv1.2 (1:200) and SPAK (1:50), and imaged using a DeltaVision Restoration Microscopy System (Applied Precision). Images were acquired with a 100X oil immersion lens using the SoftWorks software and analyzed with NIH Image J. Colocalization was determined using the Colocalization Point plugin using automated threshold detection that was manually verified.

**Tissue Harvest and Membrane Fractionation**

Cerebellar tissue was harvested from male (5-9 wk) Sprague Dawley rats and homogenized in 10 mM HEPES (pH 7.4), 0.3M sucrose, 2mM EDTA, 1mM DTT, 1mM NaF, 1mM NaOrthovanadate, 20mM BAPTA, 5mM NEM, 10 µM MG-132 and protease inhibitors (HALT) using a 15mL dounce homogenizer (10-15 strokes). Nuclear proteins were removed by low speed spin centrifugation (1000g for 10 min at 4°C) and samples were separated into membrane fractions by ultracentrifugation (200,000 for 90 min at 4°C). The supernatant containing cytosolic proteins was discarded and remaining non-nuclear, membrane fraction was then re-suspended and lysed in 50mM Tris (pH 7.41), 150 mM NaCl, 1mM EDTA, 0.25% deoxycholate, 1% NP-40, 1mM DTT, 1mM NaF, 1mM NaOrthovanadate, 20mM BAPTA, 5mM NEM, 10 µM MG-132 and protease inhibitors.
inhibitors (HALT cocktail of 100mM AEBSF·HCl, 80µM Aprotinin, 5mM Bestatin, E-64, 1.5mM EDTA, 2mM Leupeptin, and 1mM Pepstatin A).

**In Gel Digestion and Mass Spectrometry**

Coomassie stained gel pieces were excised, diced, and subjected to in-gel digesting with 1.25ng/µl sequence grade modified trypsin. Peptides were extracted, re-suspended, loaded, and separated by HPLC also as previously described (Connors et al., 2008). MS/MS spectra obtained by the LTQ-XL linear ion trap mass spectrometer (Thermo Electron) were also determined as previously described (Connors et al., 2008). Instrument control was performed using the Xcalibur software package (version 2.0, SR2, Thermo Electron). Raw data were searched against a rat sequence data base using Sequest software (version 27, revision 12, Thermo Electron) with trypsin enzyme specificity and a 2-Da mass tolerance. Cysteine residues had a static requirement of 71 Da for acrylamide adduction. Differential modifications of 79.96 Da for serine, threonine, and tyrosine residues, as well as 114.0429 for lysines and 16 Da for methionine residues, were permitted, and phospho-specific neutral loss increases to XCorr scoring were turned off.

**Statistical Analysis**

Bar graphs within figures contain descriptive statistics representing the sample mean with error bars representing standard error of the mean (SEM). Determination of statistical difference between two independent measurements was detected by one-way Student’s t test. Comparison of percent changes between pairs of independent measurements was by
a two-way analysis of variance. Sample populations were considered to be significant at $p < 0.05$: NS = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

**Eyeblink Conditioning**

**Subjects**

Male Wistar rats were obtained from Charles River (Quebec, Canada) and housed in pairs upon arrival with access to food and water ad libitum. Rats were single housed after surgery. The colony room was maintained on a 12 hour light-dark cycle (lights on at 7:00 AM and off at 7:00 PM). Rats weighed 200-300g prior to surgery. All behavioral testing took place during the light phase of the cycle and all procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Vermont.

**Surgery**

Surgeries took place 4-6 days after arrival. Surgeries were performed under aseptic conditions. Rats were anesthetized with 3% isoflurane in oxygen. Once the wound had been cleaned and the skull was dried, four skull screw holes were drilled and skull screws were placed as anchors for the head stage. The eyeblink portion of the surgeries consisted of implantation of a bipolar stimulation electrode (Plastics One, Roanoke, VA) that was positioned subdermally immediately dorsocaudal to the left eye. Two electromyogram (EMG) wires for recording activity of the orbicularis oculi muscle were constructed from two strands of 75-µm Teflon coated stainless steel wire soldered at one end to gold pins fitted into a plastic threaded pedestal connector (Plastics One). The other end of each wire was passed subdermally to penetrate the skin of the upper eyelid of the
left eye and a small amount of insulation was removed so that the bare electrodes made contact with the orbicularis oculi muscle. A ground wire was wrapped around two skull screws at one end and the other end was also soldered to a gold pin fitted into the pedestal connector. Once the ground wire and electrodes were placed, all were cemented to the skull with dental cement. The wound was numbed with a local injection of 0.15 ml bupivacaine, spread out over three points around the wound. The wound was salved with antibiotic ointment (Povidone). At the end of the surgery, rats received subcutaneous injections of Lactated Ringers (1ml) for hydration and ~0.3ml buprenorphine for post-operative analgesia. A second injection of the same concentration was given ~24-hours after surgery. Rats were post-operatively checked daily for 4-5 days after surgery. Rats were given 5-6 days to recover prior to eyeblink conditioning.

Apparatus

Eyeblink conditioning took place in one of four identical testing chambers (30.5 x 24.1 x 29.2 cm; Med-Associates, St. Albans, VT), each with a grid floor. The top of the chamber was altered so that a 25-channel tether/commutator could be mounted to it. Each testing chamber was kept within a separate electrically-shielded, sound attenuating chamber (45.7 x 91.4 x 50.8 cm; BRS-LVE, Laurel, MD). A fan in each sound-attenuating chamber provided background noise of approximately 60 dB sound pressure level. A speaker was mounted in each corner of the rear wall and a light (off during testing) was mounted in the center of the rear wall of each chamber. The sound-attenuating chambers were housed within a walk-in sound-proof chamber.
Stimulus delivery was controlled by a computer running Spike2 software (CED, Cambridge, UK). A 2.8 kHz, 80 dB tone, delivered through the left speaker of the sound-attenuating chamber, served as the conditioned stimulus (CS). The CS was 295-ms in duration. A 15-ms, 4.0 mA uniphasic periorbital stimulation, delivered from a constant current stimulator (model A365D; World Precision Instruments, Sarasota, FL), served as the unconditioned stimulus (US) during conditioning. Recording of the eyelid EMG activity was controlled by a computer interfaced with a Power 1401 high-speed data acquisition unit and running Spike2 software (CED, Cambridge, UK). Eyelid EMG signals were amplified (10k) and bandpass filtered (100-1000 Hz) prior to being passed to the Power 1401 and from there to the computer running Spike2. Sampling rate was 2 kHz for EMG activity. The Spike2 software was used to full-wave rectify, smooth (10ms time constant), and time shift (10ms, to compensate for smoothing) the amplified EMG signal to facilitate behavioral data analysis.

_Eyeblink Conditioning Procedure_

At the beginning of each session, each rat was plugged in, via the connectors cemented to its head, to the 25-channel tether commutator, which carried leads to and from peripheral equipment and allowed the rat to move freely within the testing box. Group Conditioned (n = 11) received 100 CS-US trials per day (intertrial interval = 20-40 sec) for 3 days. Group Untrained (n = 9) spent the equivalent amount of time in the conditioning chamber, but without any stimuli and eyelid EMG activity was sampled during 100 “no stimulus” trials per day for 3 days.

_Behavior Analysis_
For Group Conditioned, CS-US trials were subdivided into four time periods: (1) a “baseline” period, 280-ms prior to CS onset; (2) a non-associative “startle” period, 0-80ms after CS onset; (3) a “CR” period, 81-280ms after CS onset; and (4) a “UR period,” 65-165ms after US onset. For Group Untrained, “no stimulus” trials were subdivided using the same time periods. In order for a response to be scored as a CR, eyeblinks had to exceed the mean baseline activity for that trial by 0.5 arbitrary units during the CR period. Eyeblinks that met this threshold during the startle period were scored as startle responses and were analyzed separately. The primary behavioral dependent measure for all experiments was the percentage of trials with an eyeblink response during the “CR period” across all 100 CS-US Paired trials (Conditioned group) and comparable “trial periods” for the Untrained group for each session.

For the percentage of CRs, the data were analyzed using repeated measures ANOVAs comparing between groups across sessions. As a confirmation, a one-way ANOVA was run comparing the groups in the final day of acquisition. The criterion for a significant effect was an α-level of 0.05 or below.

**Results**

**WNK1 and SPAK interact with Kv1.2 in the Brain**

Kv1.2 is expressed within the mammalian central nervous system, where, like other ion channels, it interacts with protein complexes that influence its function. Kv1.2 function can be modulated by endocytic trafficking triggered by protein-protein interaction in cultured cells (Hattan et al., 2002; Williams et al., 2007), however the mechanisms governing Kv1.2 trafficking in the brain remain largely unknown. Recently,
we have demonstrated that Kv1.2 trafficking is modulated, at least in part, by adenylate
cyclase/protein kinase A (AC/PKA) mediated trafficking. Regulation of Kv1.2 has
important consequences for cerebellar function, affecting not only PC dendritic
excitability but cerebellum-dependent associative learning as well (Williams et al., 2012).
To expand our understanding of Kv1.2 regulation beyond known or suspected
mechanisms, we used liquid chromatography tandem mass spectrometry (LC-MS/MS)
analysis of Kv1.2 isolated from cerebellar tissue extracts to systematically screen and
identify candidate proteins that may interact and modulate with Kv1.2 in the brain. Kv1.2
was immunoprecipitated from rat cerebellum and resolved using gel electrophoresis
(Figure1A). Protein components of the immunoprecipitate were identified using a high
mass accuracy LTQ-Orbitrap hybrid mass spectrometer (Thermo Electron) (Figure 1B).
The criteria for identification were 3 or more unique peptides with a Sequest cross-
correlation score (X-corr) of 2.4 or greater after first filtering all peptides to a less than
1% false discover rate using a concatenated reverse database during Sequest searches. A
parallel immunoprecipitation was done using mouse IgG and those non-specific
interactions were removed from the analysis. Among the most frequently observed
peptides are those from Kv1.2 itself, as well as proteins that we expect to interact with
Kv1.2, such as Kv1.1 (a closely related alpha subunit that multimerizes with Kv1.2) and
Kvβ2 (a Kv1.2 associated beta subunit), thus confirming the general methodology. We
also identified unexpected proteins. Four of these are not readily explained (Skiv2l2,
ZCCHC8, Acly and Pfkp). However, three others have functions suggesting they may be
potential novel modulators of Kv1.2, including the protein kinases WNK1 and SPAK or
STK39 (Trim32 was investigated in a separate study). Using an LTQ Linear Quadrupole Ion Trap Mass Spectrometer Plus Liquid Chromatography (LCMS), which can more accurately detect lower-abundant protein peptides, we detected approximately 30 additional Kv1.2-specific interacting proteins (data not shown), with WNK1 and SPAK detected in five of five experiments with this instrument. Figure 1C provides peptides identified in one experiment, highlighting those which suggest potential WNK1/SPAK interaction. Immunoblot analysis corroborated the presence of WNK1 and SPAK in Kv1.2 immunoprecipitated from the cerebella of both male and female adult rats (Figure 1D).

**WNK1 Localizes with Kv1.2 in Purkinje Cells**

WNK1 and SPAK are known to physically interact with each other and to regulate ion transporter function in the brain (McCormick & Ellison, 2011; B. E. Xu, Lee, et al., 2005), however their potential regulation of ion channels in the brain has been unexplored. As a first step in determining whether WNK1 and SPAK are involved in cerebellar Kv1.2 function we sought to characterize their relative anatomical localization. Kv1.2 protein is expressed in two anatomical regions of the cerebellar cortex: the axon terminals of basket cells (BC) where they influence inhibitory synaptic input to PCs, and in the dendrites of PCs where they influence excitatory synaptic input (Khavandgar et al., 2005; Koch et al., 1997). Previous *in situ hybridization* studies have identified WNK2 mRNA in cerebellar granule cells and PCs as well as neocortical pyramidal cells and thalamic relay cells (Rinehart et al., 2011). A WNK1 reporting transgenic mouse found WNK1 expression in cerebellar granule and PCs (Delaloy et al., 2006). Using
immunofluorescence (IF) in cerebellar slices we confirmed WNK1 is highly expressed in the cerebellum, with strong signal in PCs as well as some detection in granule cells and molecular layer interneurons (Figure 2A). WNK1 signal was prominent in PCs soma (Figure 2A, circle) as well as in PC dendrites within the molecular layer (Figure 2A, arrows). Little WNK1 signal was detected in BC axon terminal (Figure 2A, box), a site with dense Kv1.2 expression, suggesting that the WNK1 detected by LC-MS/MS was associated with Kv1.2 originating in PCs but not BCs. Multiple attempts were made to image SPAK as well, however the commercially available antibodies used gave poor signal above background. Thus, the relative localization of SPAK and Kv1.2 in the cerebellum was not determined (images depicting potential SPAK localization are provided in Appendix B, Figure B:2).

**WNK1 Influences Expression of Kv1.2 in Cells Stably Expressing Kvβ2**

To determine if WNK signaling influences expression of Kv1.2, we first performed a series of transient transfection experiments in cultured cells stably expressing Kvβ2, an auxiliary Kv channel subunit important for Kv1.2 biosynthetic trafficking (Manganas & Trimmer, 2000; Rhodes et al., 1996). First, we asked whether WNK1 alters the surface expression of Kv1.2 measured using flow cytometry as previously described (Nesti et al., 2004; Stirling et al., 2009). Images depict normalized count which we interpret as an objective measure of surface Kv1.2. Kv1.2 surface expression was significantly lower in cells expressing WNK1 relative to those expressing Kv1.2 alone (−41%, t(df) = 12.45(46), p < 0.0001, n = 24 each) (Figure 3, top). WNK kinases have been shown to decrease surface expression of ion channels through
trafficking, such as the WNK1-induced clathrin-mediated endocytosis of renal outer medullary potassium channel (ROMK) channels (He et al., 2007; C. L. Huang et al., 2008); however, WNK kinases can also decrease the total expression of ion channels, such as the WNK4 driven increase of lysosomal degradation of renal large-conductance Ca(2+)-activated K(+) (BK) channels (Z. Wang et al., 2013; Yue et al., 2013). We therefore next asked if co-expression of WNK1 alters total Kv1.2 protein levels in these cells. Interestingly, while co-expression of WNK1 significantly decreased channel surface expression, total expression of Kv1.2 significantly increased (+110%, t(df) = 12.79(4), p < 0.0005, n = 5 (+ vector), 6 (+ WNK1) (Figure 3, middle and bottom).

**Knockout of WNK1 Alters WNK1-induced Modulation of Kv1.2 Expression**

HEK293 cells contain endogenous WNKs, including WNK1, which could confound interpretation of experiments involving WNK1 over-expression. To address this we used HEK293 cells in which endogenous WNK1 expression had been disrupted (WNK1KO cells) using Clustered Regularly Interspaced Short palindromic Repeats (CRISPRs)/Cas-mediated genome editing (developed and characterized in (Roy et al., 2015)). These cells harbor the large-T antigen and are derived from a parent line of HEK293T cells (Roy et al., 2015). Over-expression of WNK1 in the parent HEKT-cell line produced a small but still significant decrease in surface Kv1.2 relative to cells transfected with empty vector lacking the WNK1 gene (Figure 4, top left) (-9.2%, t(df) = 4.61(22), p < 0.0001, n = 12 each). We next tested the effect of WNK1 on Kv1.2 in WNK1KO cells. Interestingly, baseline surface Kv1.2 was significantly lower in the WNK1KO relative to their parent HEKT cell line (-21.8%, t(df) = 12.60(22), p < 0.0001,
From that reduced Kv1.2 baseline, expression of WNK1 in the WNK1KO cells caused surface Kv1.2 to increase significantly (+13.1%, \( t(\text{df}) = 2.84(21), p < 0.01 \) \( n = 12 \) each). Immunoblotting detected increased total channel protein following co-expression of WNK1, however the increase was only significant in the HEKT-cells (+33%, \( t(\text{df}) = 4.08(8), p < 0.005, n = 5 \) each) but not the WNK1KO cells (+28%, \( t(\text{df}) = 1.75(8), p = 0.119, n = 5 \) each). Together, these results show that co-expression of WNK1 alters Kv1.2 at the cell surface, and that the presence of endogenous WNK1 affects both the baseline amount of Kv1.2 at the cell surface and the manner with which overexpressed WNK1 influences the channel at the surface. WNK1 was not detected to decrease total channel protein under the conditions tested. These results suggest that WNK1 effects on Kv1.2 might be non-linear, with lower WNK1 levels (as in WNK1 expressed in WNK1KO cells) increasing Kv1.2 surface expression and higher levels decreasing it.

**Activation of Downstream SPAK/OSR1 Influences WNK1-induced Modulation of Kv1.2**

We next asked if the downstream kinases SPAK/OSR1 are involved in WNK1 induced alteration of surface Kv1.2. In order for SPAK/OSR1 to become phosphorylated and subsequently activated by WNK kinases, SPAK/OSR1 must first interact with WNK via a conserved carboxy-terminal (CCT) domain which binds to an RF(X)V/I domain within WNK kinase (Villa et al., 2007). A small-molecule pharmacological compound, STOCK1S-50699, disrupts this interaction, preventing WNK from activating SPAK/OSR1, thus inhibiting WNK-dependent activity of SPAK/OSR1 on downstream
targets (de Los Heros et al., 2014; Mori et al., 2013). We therefore used STOCK1S-50699 to investigate whether WNK1 alters surface expression of Kv1.2 independently of OSR1/SPAK (Figure 5A). To our surprise, treatment with STOCK1S-50699 (30 minutes, 20µM) decreased surface Kv1.2 levels in either HEKT-cells (-70%, $t(15) = 15.55(15), n = 12$ (vehicle), 6 (STOCK1S-50699), $p < 0.0001$) or WNK1KO cells (-68%, $t(16) = 13.58(16), n = 12$ (vehicle), 6 (STOCK1S-50699), $p < 0.0001$). From this reduced baseline, co-transfection with WNK1 in HEKT-cells no longer decreases surface Kv1.2 (+13.2%, $t(9) = 1.47(9), p = 0.1762, n = 6$ each), suggesting that the mechanism by which WNK1 decreases surface Kv1.2 may be dependent on activation of SPAK/OSR1 by WNK kinases. Additionally, co-transfection of Kv1.2 with WNK1 in WNK1KO cells treated with STOCK1S-50699 resulted in a significant increase of surface Kv1.2 (+50%, $t(10) = 4.95(10), p < 0.001, n = 6$ each). Thus, expression of WNK1 in a WNK1 knockout background drives an increase in surface Kv1.2, an effect greatly enhanced by blocking SPAK/OSR1 activation. This finding suggests that WNK1 might affect Kv1.2 trafficking in SPAK-dependent and SPAK-independent ways.

**SPAK Influences Surface Kv1.2 Regardless of WNK1**

Given that WNK1 is capable of modulating Kv1.2 via a SPAK-independent mechanism, we asked whether SPAK can affect Kv1.2 independently of WNK1. Both HEKT-cells and WNK1KO cells were transfected with either Kv1.2 alone (+ empty vector) or co-transfected with SPAKWT (Figure 5B). Like WNK1, co-expression of SPAK in HEKT-cells caused a significant decrease in surface Kv1.2 (-26%, $t(16) = 4.53(16), p < 0.0005, n = 6$ (+ vector), 12 (+SPAKWT)). As also observed in Figure 5A,
switching from HEKT-cells to WNK1KO cells significantly decreased baseline surface Kv1.2 levels (-39%, t(df) = 4.89(10), p < 0.001, n = 6 each). However, co-expression with SPAK in WNK1KO cells caused a further significant decrease in surface Kv1.2 (-18%, t(df) = 2.31(16), p < 0.05, n = 6 (+ vector), 12 (+SPAKWT). Interestingly, SPAK over-expression had no significant effect on total Kv1.2 protein levels (+13%, t(df) = 1.42(10) p = 0.189, n = 6 each), but significantly increased total Kv1.2 in WNK1KO cells (+113%, 11.41(10), p < 0.0001, n = 6 each) (Figure 5C). This increase in total Kv1.2 is similar to that caused by over-expression of WNK1 in the Kvβ2 stable HEK cell line (Figure 3), suggesting the effect involved WNK1 activation of endogenous SPAK. To address the possibility that over-expression of SPAK in WNK1KO cells might involve other endogenous WNKS, we substituted SPAKWT with SPAKT233A, a version of SPAK that cannot be phosphorylated and thus activated by WNKS (Vitari, Deak, Morrice, & Alessi, 2005). In HEKT-cells, overexpression of SPAKT233A caused a dramatic decrease in surface Kv1.2 (-38%, p < 0.0001, n = 6 (+ vector), 12 (+SPAKT233A), again implying that SPAK can decrease surface Kv1.2 independently of WNK activation. Interestingly however, in WNK1KO cells, SPAKT233A resulted in a moderate, non-significant decrease of surface Kv1.2 (-10%, t(df) = 1.38(14), p = 0.189, n = 6 (+ vector), 10 (+ SPAKT233A). Therefore, although SPAK is able to regulate surface Kv1.2 without being activated by WNKS, its effects on Kv1.2 trafficking is still influenced by WNK1.
Cerebellum-dependent Learning Decreases WNK1 Interaction with Kv1.2

Neurological WNK1 is highly localized to the cerebellum (Delaloy et al., 2006; Rinehart et al., 2011), but its effect on overall cerebellar function is not known. Since we demonstrated that WNK1 associates with Kv1.2 in the cerebellum (Figure 1), we wondered if we could utilize cerebellar-dependent learning, a process we have shown previously to involve Kv1.2, to demonstrate a functional role for WNK1 in the cerebellum. In a previous study, blocking Kv1.2 via infusion of a specific Kv1.2 blocker, tityustoxin-Kα, into the lobulus simplex of the cerebellum enhanced delay eyeblink conditioning (Williams et al., 2012). Delay eyeblink conditioning (EBC) is a classical conditioning paradigm that engages a discrete brainstem-cerebellar neural circuitry (Thompson & Steinmetz, 2009). Furthermore, the neuropeptide secretin was previously associated with Kv1.2 trafficking (Williams et al., 2012). Intracerebellar infusions of secretin or a secretin receptor antagonist, facilitated and impaired acquisition of EBC, respectively (Fuchs et al., 2014, Williams et al., 2012). Since suppression of Kv1.2 enhances EBC learning, we hypothesized that EBC itself might affect Kv1.2 regulation, potentially by influencing the composition of its protein interactome. To test this, male Wistar rats underwent either no training (Untrained) or a short delay EBC paradigm (Conditioned) and LC-MS/MS analysis was used to identify Kv1.2 interacting proteins. Immediately following the last trial of EBC training, rats were sacrificed and the lobulus simplex region (HVI) of the cerebellum, ipsilateral to the trained eye, was harvested and flash frozen in liquid nitrogen (Figure 6A). Rats in group Conditioned learned to make a conditioned response significantly more than rats that those of group Untrained (Figure 6B). The tissue was pooled within experimental condition and subject to Kv1.2
immunoprecipitation followed by analysis by LC-MS/MS (Figure 6C). Peptides of Kv1.2 detected for each group were roughly equal (Conditioned: 55 peptide, Protein XCorr = 3.76); Untrained 43 peptide, Protein XCorr = 3.66). In both groups, WNK1 and SPAK were detected as interacting proteins, however nearly half as many WNK1 peptides were detected in the Conditioned group (Conditioned: 10 total, 7 unique, Protein XCorr = 3.03 vs. Untrained: 19 total, Protein XCorr = 3.02), with roughly equal SPAK peptides between groups (Paired: 13 total, protein XCorr = 3.25; Unpaired: 9 total, Protein XCorr = 3.10). The level of Kvβ2, which binds Kv1.2 readily, was unchanged between groups (Conditioned: 39 total, Protein XCorr = 3.16; Untrained: 31 total, Protein XCorr = 3.26). Immunoblot analysis and of Kv1.2 and co-immunoprecipitated WNK1 confirmed a learning-induced drop in WNK1 association with Kv1.2 caused by learning, with the Untrained group showing a 3.6 fold increase in Kv1.2 associated WNK1 (Figure 6D).

Discussion

The WNK/SPAK signaling pathway has long been known to regulate cation-chloride cotransporters in the kidney and more recently have been shown to do so in the brain. Cation-chloride co-transporters have a major impact on intracellular chloride concentration, and thus on the driving force for chloride currents through GABA<sub>A</sub> and glycine receptors. The recent finding that WNK1 is a chloride sensor has led to the hypothesis that WNK/SPAK phosphorylation and regulation of cation-chloride co-transporters has a key role in maintaining intracellular chloride levels during neurotransmission involving ligand-gated chloride channels. The findings presented here
expand this paradigm by identifying the voltage-gated potassium channel Kv1.2 as a new target for WNK and SPAK in the brain.

Using mass spectrometry, we identified WNK1 and SPAK as components of the cerebellar Kv1.2 interactome (Figure 1). We then used HEK239 cells as a model system to assess the role of WNK1 and SPAK on Kv1.2 function (Figures 3-5). Since Kv1.2 electrophysiological function is regulated by endocytic trafficking (Nesti et al., 2004), we assessed the effects of WNK1 and SPAK on Kv1.2 levels at the cell surface. Over-expression of either WNK1 or SPAK decreased Kv1.2 surface levels in wild-type HEK293 cells (Figures 3-5). However, in HEK cells in which endogenous WNK1 expression had been ablated using CRISPR genomic editing (Roy et al., 2015), over-expression of WNK1 increased surface Kv1.2 levels (Figures 4-5). We attribute the WNK1 induced drop in Kv1.2 surface levels in wild-type HEK192 cells to its activation of SPAK since the dramatic internalization of Kv1.2 by SPAK over-expression was not eliminated in WNK1 knockout cells (Figure 5). In contrast, disruption of WNK-SPAK interaction by over-expressing a version of SPAK lacking the WNK phosphorylation site (SPAK-T233A) or with the small-molecule inhibitor Stock1S-50699, reversed the polarity of the effect of WNK1 on Kv1.2 surface levels (Figure 5). Collectively, these findings suggest that WNK1 can affect Kv1.2 trafficking not only by activating SPAK, but also by a SPAK-independent mechanism as well.

Given that WNK1 is a chloride sensor, it is possible that WNK/SPAK signaling might link Kv1.2 function to GABA\textsubscript{A} or glycine receptor activity in the brain. Purkinje cell dendrites, which harbor both Kv1.2 and WNK1 (Figure 2), receive abundant input
from GABAergic interneurons. The primary effect of this synaptic input is inhibitory via GABA_A receptor mediated chloride influx. The resulting increase in intracellular chloride levels would be expected to inhibit WNK1 and, as hypothesized for other cell types, relieve WNK1 mediated inhibition of KCC1. This, in consequence, would increase chloride extrusion by KCC1, thus providing a feedback mechanism for maintaining intracellular chloride homeostasis. However, our data suggests a parallel mechanism whereby chloride mediated inhibition of WNK/SPAK activity would also decrease endocytosis of Kv1.2. This would in turn increase Kv1.2 levels at the cell surface, facilitating its ability decrease dendritic excitability. This hypothetical mechanism would suggest a novel role for WNK1 as a mechanism for linking inhibitory transmission with Kv1.2 via a mechanism that does not directly involve voltage sensitivity. Such a mechanism could serve to extend the inhibitory effect of GABAergic transmission on membrane excitability beyond the period of direct GABA_A receptor activation.

Intriguing evidence that WNK1 is involved in Kv1.2 regulation in vivo comes from our finding that the physical interaction between WNK1 and Kv1.2, or at least the stability of the interaction during immunoprecipitation, is significantly altered by experience in live, behaving animals. Pairing of CS and US in an EBC paradigm roughly halved the amount of WNK1 that co-immunoprecipitated with Kv1.2 (Figure 6). Interestingly, the channel’s interaction with SPAK was not decreased. Why this is the case remains to be determined, although it might indicate that WNK1 and SPAK interact with Kv1.2 via different mechanisms, potentially via different sites within the channel. We also note that phosphorylation at two sites within Kv1.2, S434 and S468 was
detected in the CS-US paired but not unpaired condition. Whether this is related to EBC induced changes in WNK1 interaction with Kv1.2 remains to be determined. The functional effect of decreased WNK1 interaction with Kv1.2 can also only be speculated on at this point. In previous studies we have shown that suppressing Kv1.2 activity enhances EBC (Fuchs et al., 2014; Williams et al., 2012). It is possible that, as in HEK cells (Figures 3-5), cerebellar WNK1 might act independently of SPAK to enhance surface Kv1.2 levels. Reducing WNK1 interaction with Kv1.2 would thus reduce surface Kv1.2 levels, possibly enhancing EBC.

Throughout our experiments, we did not deduce whether WNK1 or SPAK directly bind Kv1.2, leaving the possibility that other intermediary proteins may contribute to WNK signaling induced trafficking of Kv1.2. Additionally, the current studies do not directly address the mechanism by which WNK1 or SPAK affect surface Kv1.2 levels. Our findings that the WNK-induced decreases in surface Kv1.2 levels in HEK293 cells was accompanied by an apparent increase in total Kv1.2 protein suggest that WNK modulates endocytic trafficking, either by enhancing endocytosis or decreasing recycling of endocytosed channel back to the plasma membrane. It is likely that WNK1 or SPAK affect Kv1.2 phosphorylation and future in vitro kinase assays or quantitative LC-MS/MS approaches such as Stable Isotope Labeling in Culture (SILAC) could help determine this. Finally, intracranial infusion of Stock-1S-50699 into the lobulus simplex of adult rats prior to EBC training would provide a powerful future experiment to observe whether disruption of WNK:SPAK binding alters the progression of EBC.
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The authors declare no competing financial interests.

Keywords: Kv1.2; WNK1; SPAK; chloride, GABA; potassium, cerebellum

Figure Legends

Figure 1. **Kv1.2 associates with WNK1 and SPAK kinases in the cerebellum.**  A: Representative protein gel scan following immunoprecipitation of Kv1.2 or mouse IgG from cerebellar tissue. Arrows indicate molecular weight range for Kv1 channels (~80-100kDa), WNK kinases (> 250kda), and SPAK (~65Kda).  B: A full interactome of Kv1.2 in the cerebellum as detected by LTQ Linear Quadrupole Ion Trap-Orbitrap Mass Spectrometer Plus Liquid Chromatography (Thermo-Fisher Scientific) following Kv1.2 IP (non-specific proteins detected in IgG IP removed). Peptides detected relative to WNK:SPAK interaction are highlighted for observation.  C: Tryptic peptides from
WNK1 and SPAK detected by LTQ Linear Quadrupole Ion Trap Mass Spectrometer Plus Liquid Chromatography (Thermo-Fisher Scientific) following IP of Kv1.2 in the cerebellum. No peptides of WNK1 or SPAK were detected from IgG controls. WNK peptides containing SPAK binding RF(x)V/I domain and SPAK T-loop phosphorylation are highlighted. Non-Orbitrap interactome of Kv1.2 was performed in 3 total repeats. 

**D:** Immunoblots demonstrating co-IP of both WNK1 (left, 7.5% gel) and SPAK (right, 10% gel) with Kv1.2 from female, cerebellar tissue.

**Figure 2. WNK1 localizes to PC cell bodies and dendrites.** **A:** Multiphoton imaging demonstrating that WNK1 (red) strongly localizes to PC cell bodies and processes of the molecular layer where Kv1.2 is also present (green). Insert and merge show that the overlap within the dendritic processes is not exact, with WNK1 giving a punctate appearance alongside the processes. Insert scale bar represents 10µm. **B:** Secondary only controls showing no non-specific fluorescence within the microscopy. Multiphoton images were obtained on a Zeiss LSM 7 multiphoton microscope with a 20X water immersion lens and analyzed in Image J (NIH) and Zen Light 2012 software. Widefield images were acquired using a 100X oil immersion lenses using the SoftWorks software and analyzed with NIH Image J. Colocalization was determined using the Colocalization Point plugin using automated threshold detection that was manually verified. Neither SPAK nor Kv1.2 was localized to PC pineaus using Alexa 647 by either multiphoton or widefield microscopy.
Figure 3. WNK1 alters both total and surface Kv1.2 expression in HEK293 cells stably expressing Kvβ2. Top: WNK1 co-expressed with Kv1.2 caused a significant decrease in surface Kv1.2 expression in HEK293 cell lines stably expressing beta subunit Kvβ2 (-41%, t(df) = 12.45(46), p < 0.0001, n = 24 each). Middle, bottom: WNK1 co-expressed with Kv1.2 caused a significant increase in total Kv1.2 expression in HEK293 cell lines stably expressing Kvβ2 (+110%, t(df) = 12.79(4), p < 0.0005, n = 5 (+ vector), 6 (+ WNK1).

Figure 4. Knockout of WNK1 alters the response of overexpressed WNK1 in HEK293T cells. Top: In control HEK293T cells (HEKT-cells), WNK1 also but more modestly significantly decreased surface Kv1.2 (-9.2%, t(df) = 4.61(22), p < 0.0001, n = 12 each). In HEK293T cells deficient of endogenous WNK1 (WNK1KO cells), overexpression of WNK1 caused surface Kv1.2 to significantly increase +13.1%, t(df) = 2.84(21), p < 0.01 n = 12 each. Switching from HEKT-cells to WNK1KO cells also caused baseline surface Kv1.2 to drop (-21.8%, t(df) = 12.60(22), p < 0.0001, n = 12 each). Middle, bottom: Co-expression of WNK1 caused a significant increase in HEKT-cells (+33%, t(df) = 4.08(8), p < 0.005, n = 5 each) and a similar although non-significant increase in WNK1KO cells compared to Kv1.2 + vector only ((+28%, t(df) = 1.75(8), p = 0.119, n = 5 each).

Figure 5. SPAK influences Kv1.2 expression in HEK293 cells.
A: After treating either HEKT-cells or WNK1KO cells with the WNK/SPAK binding inhibitor stock-1S50699, Kv1.2 baseline surface expression (Kv1.2 + empty vector) dramatically dropped compared to vehicle treated cells in both HEKT-cells (-70%, t(df) = 15.55(15), n = 12 (vehicle), 6 (STOCK1S-50699), p < 0.0001) or WNK1KO cells (-68%, t(df) = 13.58(16), n = 12 (vehicle), 6 (STOCK1S-50699), p < 0.0001). From this baseline, WNK1 overexpression in HEKT-cells caused a non-significant increase of surface Kv1.2 (+13.2%, t(df) = 1.47(9), p = 0.1762, n = 6 each) and a dramatic, significant increase in WNK1KO cells (+50%, t(df) = 4.95(10), p < 0.001, n = 6 each). B: Top: Co-expression of Kv1.2 with SPAKWT caused a significant decrease in surface Kv1.2 in both HEKT-cells (-26%, t(df) = 4.53(16), p < 0.0005, n = 6 (+ vector), 12 (+SPAKWT) and WNK1KO cells (-18%, t(df) = 2.31(16), p < 0.05, n = 6 (+ vector), 12 (+SPAKWT), and switching from HEKT-cells to WNK1KO cells significantly dropped baseline surface Kv1.2 (-39%, t(df) = 4.89(10), p < 0.001, n = 6 each). In T-cells, replacing SPAKWT with WNK1 insensitive SPAKT233A still caused a significant decrease of surface channel (-38%, p < 0.0001, n = 6 (+ vector), 12 (+SPAKT233A) and in WNK1KO cells, replacing SPAKWT with SPAKT233A caused a non-significant decrease in surface Kv1.2 (-10%, t(df) = 1.38(14), p = 0.189, n = 6 (+ vector), 10 (+ SPAKT233A). Middle, bottom: Co-expression with SPAK did not significantly increase total channel protein in HEKT-cells (+13%, t(df) = 1.42(10) p = 0.189, n = 6 each) but did in WNK1KO cells (+113%, 11.41(10), p < 0.0001, n = 6 each).
Figure 6. WNK1 association with Kv1.2 is decreased in the lobules simplex following EBC learning. A: Wistar were given either a paired conditioning or non-stimulated 3 session training session to develop EBC prior to LC-MS/MS analysis. Approximate region of the cerebellum containing the lobules simplex was dissected and pooled together for each training group while the remainder was discarded (2 paired subjects were removed for equal sample sizes). Protein gel scan of Kv1.2 IPs from trained or untrained lobules simplex dissections demonstrates density of proteins similar to whole cerebellar homogenates (Figure 1). C: Demonstration of successful acquisition training for the conditioned group. B: LC-MS/MS output shows decreased WNK1 peptide detection following acquisition compared to untrained. SPAK (STK39) peptides were lightly increased within a reasonable range of error. Two phosphorylation states of Kv1.2 (S434 an S468) were detected only in the Conditioned group while S440/441 was detected equally. D: Immunoblot of the same IPs detected a 3.6 fold decrease of WNK1 protein that co-IP compared to the Conditioned group.
Figure 3:1 Kv1.2 associates with WNK1 and SPAK kinases in the cerebellum.
Figure 3:2 WNK1 localizes to PC cell bodies and dendrites.
Figure 3: WNK1 alters both total and surface Kv1.2 expression in HEK293 cells stably expressing Kvβ2.
Figure 3: Knockout of WNK1 alters the response of overexpressed WNK1 in HEK293T cells.
Figure 3:5 SPAK influences Kv1.2 expression in HEK293 cells.
Figure 3:6 WNK1 association with Kv1.2 is decreased in the lobules simplex following EBC learning.
References for Chapter 3


CHAPTER 4: COMPREHENSIVE DISCUSSION

Overview

In the preceding chapters, a proteomic assessment of Kv1.2 purified from native tissue extracts enabled the discovery of novel protein associations with Kv1.2 in the cerebellum. A sample of these associations was selected for two studies in which the interactions were further validated and tested in cultured cells which ultimately yielded working models for Kv1.2 modulation in the brain. These models also further utilized the power of mass spectrometry by incorporating post-translational modifications of both Kv1.2 and interacting protein partners that were also identified by mass spectrometry from cerebellar tissue.

In Chapter 2, the hypothesis that the ubiquitin ligase Trim32 modulates surface Kv1.2 through channel ubiquitylation was tested. Using mutagenesis, quantitative proteomics, and biochemistry, a model was developed in which Trim32, through the coordinated interplay of channel phosphorylation and ubiquitylation, modulates expression of Kv1.2 at the plasma membrane.

In Chapter 3, the hypothesis that the chloride-sensitive WNK signaling pathway regulates Kv1.2 was tested. Using a knockout cell line, pharmacology, and mutagenesis, it was determined that both WNK1 and its downstream target SPAK impact Kv1.2 expression at the plasma membrane, and a working model was developed in which WNK1 and SPAK modulate Kv1.2 through complementary mechanisms. In the
conclusion of this study, mass spectrometry and biochemistry demonstrated that the association of WNK1 with Kv1.2 changed following cerebellum-dependent learning. Given that suppression of Kv1.2 can alter cerebellar-dependent learning (Williams et al. 2012), this provided framework for future studies to investigate a potential impact of this association on behavior.

Collectively, this work demonstrates the first evidence to support roles for Trim32 and WNK1 on the expression and trafficking of voltage-gated potassium channels. However, both studies raise several questions.

**Excitability Control as a Role for Trim32 in the Adult Brain**

*Contribution of Trim32 to Neuronal Excitability*

The preceding work in Chapter 2 demonstrated evidence for an interaction between Kv1.2 and Trim32 and proposed a complex model of modulation of Kv1.2 by Trim32 in the brain. We did not, however, investigate the influence of Trim32 on membrane potential. There are currently no reported pharmacological agents that manipulate Trim32 activity or expression, complicating our ability to investigate Trim32 in vivo. Although Trimk32KO mice are not commercially available and we were unable to obtain a donation from other labs possessing Trim32KO colonies, companies such as Applied Biological Materials (ABM) and Santa Cruz have recently made viral constructs that utilize CRISPR technology to genetically remove Trim32 in vivo. Comparing the electrophysiology of neurons of a Trim32KO rodent to that of a normal one would provide a relatively straight forward approach to address this question. Alternatively, and perhaps more simplistically, culture systems such as HEK cells which lack endogenous
Trim32 could be utilized to compare membrane potential from cells transiently transfected with a Trim32 construct to those that were not.

Aside from the work presented in Chapter 2, the most compelling evidence in the literature that links Trim32 to neuronal excitability is the recent connection of Trim32 and Autism co-morbid with epilepsy.

*Epilepsy Co-morbid with Autism*

According to the Center for Disease Control (CDC), one in 68 children in the United States have a form of Autism Spectrum Disorder (ASD), a spectrum of complex neurological diseases characteristic of difficulties in social interaction, verbal and nonverbal communication, and repetitive disorder. Among ASD patients, a seizure occurrence rate of up to 40% of ASD has been reported (Tuchman & Rapin, 2002). Anywhere from 7-46% of ASD patients are also affected with epilepsy (Bolton et al., 2011; Danielsson, Gillberg, Billstedt, Gillberg, & Olsson, 2005; Giovanardi Rossi, Posar, & Parmeggiani, 2000), a seizure disorder which involves disturbances in electrical activity in the brain caused by brain injury, genetics, or as is often the case, for unknown reasons.

Epilepsy is one of the most common neurological diseases occurring in children, with incidence ranging from 41-187/100,000 dependent of demographic factors (reviewed recently in: (Camfield & Camfield, 2015)). Like ASD patients, who may display symptoms of epilepsy, 15-35% of children with epilepsy may show symptoms of ASD (Matsuo, Maeda, Sasaki, Ishii, & Hamasaki, 2010; Saemundsen, Ludvigsson, Hilmarisdottir, & Rafnsson, 2007; Steffenburg, Steffenburg, & Gillberg, 2003). Despite
the frequent co-morbidity of these neurological diseases, the mechanisms behind the co-
ocurrence of epilepsy and ASD phenotypes are unclear and complex. Various
neurobiological mechanism critical to early brain development seem to be altered, and
key neuronal processes such as synaptic plasticity, GABA transmission, and functional
connectivity are impacted (Stafstrom, Hagerman, & Pessah, 2012; Tuchman, Moshe, &
Rapin, 2009).

Epilepsy Co-morbid with Autism May Link Trim32 with Membrane Excitability

Mutations of the Trim32 gene have been identified as a risk factor for ASD, and a
recent study on genetic screening of ASD patients identified Trim32 as one of 12
candidate genes associated with comorbidity of ASD and epilepsy in children. The
majority of genes identified are involved with formation, maintenance, or remodeling of
the synapse, for the first time placing Trim32 in a category of genes that influence
neuronal and synaptic activity (Lionel et al., 2011; Lionel et al., 2014; Lo-Castro &
Curatolo, 2014).

Trim32 is a small gene nested within an intron of ASTN2 located along
chromosome locus 9q33.1. A recent study utilized clinical microarray data to
systematically screen for novel mutations at this loci and found that exonic deletion or
duplication of ASTN2 was significantly enhanced in male patients with
neurodevelopmental disorders (NDD), and 27 of 46 deletions (no duplications) also
affected Trim32. Twelve of the subjects referred for the genetic study were ASD patients.
The authors also utilized comprehensive gene expression data from the BrainSpan
database and found that, in concordance with Trim32KO mice, Trim32 is most highly expressed in the cerebellar cortex (Lionel et al., 2014).

As pointed out by De Smet et al., neuroanatomical, neuroimaging, and clinical studies combined have extended the role of the cerebellum beyond motor control to the modulation of cognitive and affective processing (De Smet, Paquier, Verhoeven, & Marien, 2013). Damage to the cerebellum by injury, birth defect, and even tumor growth can lead to neurological disruptions such as emotional outbursts, mood changes, difficulty understanding social cues, and repetitive behaviors, all of which are symptomatic of ASD. Additionally, reduced PC volume in the cerebellum is a consistent finding of post-mortem ASD patients and rodent models (Kern, 2003; Piochon et al., 2014; Whitney et al., 2008), and mice lacking the Autism-linked genes TSC1/2 displayed both decreased PC expression and Autistic-like behavior (Reith et al., 2013; Tsai et al., 2012).

Altogether, epilepsy within ASD may link a role for Trim32 in neuronal excitability, and the cerebellum provides an appropriate route for investigating this role given the high expression of Trim32 and emerging roles of ASD pathology. Additionally, Kv1 channels, which are also highly expressed in the cerebellum, are strongly involved in seizures, a marked phenotype of epilepsy involving altered neuronal excitability.

*The Role of Kv1.2 in Epilepsy and Seizure*

Mutation or deletion of the genes encoding Kv1.1 and Kv1.2 result in neurological disorders with disrupted excitability in both humans and rodents (Adelman, Bond, Pessia, & Maylie, 1995; Brew et al., 2007; Buffington & Rasband, 2011;
Kullmann & Hanna, 2002; Leao, Berntson, Forsythe, & Walmsley, 2004; Smart et al., 1998; Wimmer, Reid, So, Berkovic, & Petrou, 2010). Both Kv1.1 and Kv1.2 null mice have spontaneous seizures, however the seizure disorder models are different (Robbins & Tempel, 2012). Deletion of Kv1.1 can cause epilepsy in mice with spontaneous seizures throughout life (Smart et al., 1998), and only half of the Kv1.2 null mice survive to adulthood (Wenzel et al., 2007). Kv1.2 null mice present a more severe seizure and are lethal beyond post-natal day 19 (Brew et al., 2007) with seizures characteristic of those in the brainstem (Browning, 1986; Gale, 1992). Seizure activity produces a reduction of Kv1.2, but not Kv1.1, mRNA in dentate granule cells of the hippocampus, and treatment with anticonvulsive drugs both protect animals from seizure and prevent reduction of Kv1.2 (Tsaur, Sheng, Lowenstein, Jan, & Jan, 1992). If epilepsy connects Trim32 to neuronal excitability, then Kv1 channels such as Kv1.2 are clear candidates for involvement in this mechanism.

Conclusion

The work of Chapter 2 provided evidence that the ubiquitin ligase Trim32 associates with Kv1.2 in the brain and modulates the channels’ function. Given the literature linking Trim32 with epilepsy in ASD patients, and the pivotal role Kv1 channels can play in epileptic seizure, further experiments may identify Trim32 as a modulator of neuronal excitability and thus provide a novel physiological role for Trim32 in the brain. Additionally, Appendix A presents data that demonstrates Trim32 also localizes with Kv1.2 in hippocampal neurons, and given the mutual connection of these
proteins with seizure, the hippocampus may also useful venue for future experimental investigations of Trim32 and neuronal excitability.

**GABAergic Inhibition as a Model Role for WNK1/SPAK Modulation of Kv1.2 in the Brain**

The preceding work in Chapter 3 identified components of the chloride-sensitive WNK kinase pathway associating with Kv1.2 in the brain, and proposed a model of WNK1 and its downstream target SPAK providing complementary modulation of the channel at the cell surface. WNK1’s best described role in the literature is to activate SPAK/OSR1 in response to alterations of intracellular chloride, although it can have functionality that may be independent of its kinase domain and SPAK activation. The following section will provide evidence for a testable model of GABAergic inhibition of Purkinje cells through chloride-influenced WNK/SPAK signaling and Kv1.2 trafficking.

**Chloride Inhibits WNK1 Kinase Activation of SPAK**

Chloride inhibits WNK1 kinase activity, which primarily targets downstream kinases SPAK/OSR1. The chloride ion stabilizes the inactive conformation of WNK1 by binding the kinase domain, preventing auto-phosphorylation, and thereby preventing the ability of SPAK to be activated by WNK1 phosphorylation. This mechanism is what defines WNK1 as an intracellular chloride sensor (Piala et al., 2014). However, even with an inactivated kinase domain, WNK1 may still have kinase-independent functionality.

**WNK1 Traffics Membrane Proteins without Kinase Activity**

WNK1 also induces clathrin-dependent endocytosis of ROMK (Zagorska et al., 2007) which requires binding outside its kinase domain, and mutants lacking kinase
activity can still inhibit the channel (H. R. Wang, Liu, & Huang, 2008). Additionally, WNK1 activates the Serum and Glucocorticoid-induced Kinase (SGK) 1 (SGK1) to impact the membrane expression of epithelial sodium channel (ENaC) by requiring only the N-terminal residues and not the kinase domain (B. E. Xu, Stippec, Lazrak, et al., 2005). Given the kinase independence, these functionalities of WNK1 are also likely independent of chloride.

**Chloride May Determine the Route WNK1/SPAK Influence Surface Kv1.2**

As chloride inactivates WNK1 kinase activity, reduced intracellular chloride activates WNK kinases (Piala et al., 2014). Particularly, reduced intracellular chloride has been shown to activate SPAK/OSR1 and eventual major downstream targets of the chloride-response pathway such as NKCC1 in secreting epithelia (Lytle & Forbush, 1996). In the case of NKCC2, the main transporter that extrudes chloride through this pathway in the brain (de Los Heros et al., 2014), the transporter activation by reduced intracellular chloride is blocked by WNK mutants that affect the WNK-SPAK docking interaction (Ponce-Coria et al., 2008).

In relation to the topics of this thesis, decreased extracellular chloride reduces the current of Kv1.5 and Kv2.1, providing the first evidence for Cl-induced modulation of Kv channels. (X. Li, Surguchev, Bian, Navaratnam, & Santos-Sacchi, 2012). Hypotonic low-chloride conditions rapidly activate WNK kinase isoforms, including SPAK/OSR1 (Lenertz et al., 2005). Thus, in conditions where WNK1 kinase activity is expected to be increased, at least some Kv channel currents are also suppressed. This is in line with our conclusions from Chapter 3, where SPAK or WNK1 activated SPAK
activity decreases Kv1.2 at the plasma membrane. If increasing chloride inside the cell increases Kv channel current, and if chloride inactivates WNK1 activation of SPAK, perhaps increased intracellular chloride triggers WNK1 to favor its SPAK-independent increase of Kv1.2 at the membrane surface. Along these lines, decreased intracellular chloride would then activate WNK1 kinase activity and, through SPAK, decrease Kv1.2 from the surface. Experiments that directly manipulate intracellular chloride need to be performed to validate this model, but it seems at least possible that chloride may be the factor with which dictates how this signaling pathway influences Kv1.2. In the brain, fluctuations of intracellular chloride occur often from the neurotransmitter GABA. WNK1 has been hypothesized to be involved with GABAergic inhibition in the brain, but this concept is for the most part speculative (Alessi et al., 2014).

Proposed Model of GABAergic Inhibition of Kv1.2 by WNK/SPAK Signaling

GABA_A receptors (GABA_AR) comprise the main inhibitory neurotransmission system in the brain, eliciting hyperpolarization through chloride ion influx. In a previous publication, treatment of live cerebellar slices with gabazine, a drug that blocks GABA_A receptors, resulted in a reduction of Kv1.2 measured at the cellular surface (Williams et al., 2012). In Chapter 3 and Appendix B, immunofluorescence demonstrated WNK1 and likely also SPAK are expressed in the GABAergic Purkinje cells of the cerebellar cortex which receive GABA_A inhibition from interneurons such as basket cells. Binding of GABA to GABA_A receptors promotes the passage of chloride through the channel and into the cell, and therefore blockade of GABA_A receptors with the drug gabazine is hypothesized to decrease intracellular chloride and therefore increase WNK1 kinase
activity and thus WNK1-activation of SPAK. This suggests the following model illustrated in **Figure 1**: GABA release by cerebellar inhibitory interneurons bind PC GABA_A Rs and cause an increase of [(Cl^-)_i]. The elevation of [(Cl^-)_i] decreases WNK1 kinase activity which in turn results in increases of Kv1.2 expression at the neuronal plasma membrane. Kv1.2 maintained at the surface provides hyperpolarization and thus extends the overall GABAergic inhibition of the PC. Conversely, under conditions where inhibitory neurons release less GABA, less chloride passes into the PC and the decrease of [(Cl^-)_i] allows increased activation of WNK1 kinase activity and thus favors WNK1 to activate SPAK and internalize Kv1.2 from the neuronal membrane. Decreased surface Kv1.2 depolarizes the PC leading to enhanced excitability. Although speculative, this hypothesis suggests a novel and currently unexplored voltage-independent link between GABA_A receptor function and voltage-gated ion channels.
Figure 4:1 Proposed model of Kv1.2 modulation by GABA induced WNK1/SPAK signaling. GABA release by cerebellar inhibitory interneurons bind PC GABA$_A$Rs and cause an increase of [(Cl$_-$)$_i$]. The elevation of [(Cl$_-$)$_i$] decreases WNK1 kinase which in turn results in increases of Kv1.2 expression at the neuronal plasma membrane. Kv1.2 maintained at the surface provides hyperpolarization and thus extends the overall GABAergic inhibition of the PC. Conversely, under conditions where inhibitory neurons release less GABA, less chloride passes into the PC and the decrease of [(Cl$_-$)$_i$] allows increased activation of WNK1 kinase activity and thus favors WNK1 to activate SPAK and internalize Kv1.2 from the neuronal membrane. Decreased surface Kv1.2 depolarizes the PC leading to enhanced excitability.
Are Trim32 and WNK1 Pathways Linked?

Determining whether Trim32 and WNK signaling modulate Kv1.2 through separate or linked pathways is not straightforward. The preceding work demonstrated complex, duel mechanisms for modulating Kv1.2 by both routes. Cellular growth conditions and potential patterns of post-translational modification can sway Trim32’s route, while endogenous WNK1 can influence whether WNK1 activates SPAK and alters it’s route of modulation. Additionally, compensatory effects from other WNK kinases and ubiquitin ligases must be accounted for. It is especially interesting to speculate that WNK1 or SPAK affect phosphorylation proximal to Trim32 ubiquitylation sites, since, as described in Chapter 2, phosphorylation at these sites influence the effect of Trim32 on Kv1.2 trafficking. Figure 2 demonstrates a replicate series of experiments where co-expression of Trim32 and WNK1 together abolished the effects of either enzyme co-expressed individually. Although tempting to infer a connection, the complex mechanisms by which either enzyme modulates the channel makes this result speculative at best. Future experiments are needed to further examine whether Trim32 and WNK1 cooperate to modulate Kv1.2.
Figure 4:2 Co-expression of both Trim32 and WNK1 abolishes individual effects on surface Kv1.2 by each enzyme. As demonstrated in Chapters 2 and 3, co-expression of Trim32 (wild-type) causes Kv1.2 to significantly increase in surface expression (Chapter 2), while, conversely, co-expression of WNK1 causes Kv1.2 to significantly decrease in surface expression (Chapter 3). When equal amounts of Trim32 and WNK1 were both co-expressed in the same cells with Kv1.2 (total amount of each was equal to the individual co-expressions), no significant change was observed to Kv1.2 surface expression. **** indicates $p < 0.0001$. Note: data presented in this graph was also separately presented in graphs of Chapters 2 and 3.

Conclusions

Altogether, this work provides novel mechanisms by which voltage-gated potassium channels may be regulated in the brain. Regulation of Kv1.2, the channel focused on in this work, can influencing neuronal excitability and ultimately behavior in the brain (Williams et al., 2012), and therefore this work provides framework for future studies investigating excitability in regard to brain function of both the normal and disease state. Regulation of Kv1.2 by the WNK/SPAK signaling pathway may provide further understanding of GABAergic inhibition in cerebellar neurons that underlie...
numerous neuronal functionalities. Regulation of Kv1.2 by Trim32 may provide a link for Trim32 and neuronal excitability that could translate into a model addressing the epilepsy connection of Trim32 and ASD.
APPENDIX A: DATA RELATING TO CHAPTER 2

Background

The following data presented was collected during the development of Chapter 2 but was not incorporated into the final seven figures of the publication in preparation. These findings may provide further clarity and breadth to the concepts present in Chapter 2.

Results and Discussion

Mutation of Either C or N Terminal Kv1.2 Lysines Cripple Trim32-induced Increase of Surface Channel

HEK293 cells co-expressing Kv1.2 and Trim32WT plated to low confluence display increased surface Kv1.2 detection by flow cytometry compared to Kv1.2 transient transfection alone (+ empty vector). In Chapter 2, it was demonstrated that mutation of all C and N terminal lysine of Kv1.2 to arginine diminish this effect, and evidence was provided to demonstrate that Trim32 is able to ubiquitylate Kv1.2 in vitro at both N and C termini. To determine if the Trim32 induced increase of surface Kv1.2 is dependent solely on either channel termini, we repeated the co-expression experiments of Chapter 2 at low confluence with Kv1.2WT, Kv1.2KR, and added Kv1.2CKR and Kv1.2NKR (Figure A:1A) and determined that neither mutant appeared to completely eliminate the effect, however the overexpression of Trim32 with Kv1.2CKR was not statistically significant. This suggests both termini of Kv1.2 may be impacted by Trim32 co-expression to produce the final results observed in Chapter 5. Given the sample size (n =
6 each), greater power and repeat experiments are needed to conclude the degree of influence Trim32 has either mutant channel.

*Kvβ₂ is Not Necessary for Trim32-induced Increase of Surface Kv1.2*

Kv1.2 and other Kv1 alpha subunit channels can associate with cytoplasmic accessory proteins called Kvβ subunits typically in a 1:1 stoichiometry. In the cerebellar tissue homogenates of Chapter 2, ~ half as many Kvβ subunits peptides were detected as were alpha subunits. Kvβ subunits, particularly Kvβ₂, can impact modulation of gating, stability, and trafficking of the channels. Co-expression of all Kvβ subunits (1-3) with Kv1.2 alpha subunit promotes surface expression of Kv1.2 (Campomanes et al., 2002). To minimize complexity, overexpression experiments reported in Chapter 2 were performed in HEK cells stably expressing Kvβ₂. To investigate whether Kvβ₂ influences Trim32 modulation of surface Kv1.2, we repeated the co-expression of Kv1.2 and Trim32 in HEK293-ATTC cells lacking Kvβ₂ (Figure A:1B). Kvβ₂ was co-expressed into replicate experiments as a control. Empty vector was also co-expressed in cells not transfected with Kvβ₂. No significant difference was found between the two cell lines under identical conditions, suggesting that Kvβ₂ is not significant contributing to the Trim32-induced increase of surface Kv1.2.

*C24A Mutation of Trim32 Does Not Alter Trim32 Trafficking of Kv1.2*

Mutation of cysteine 24 to alanine within Trim32’s RING domain has been previously reported to cause crippling of Trim32 ubiquitin ligase activity. Within the experiments used to determine Trim32∆RING’s effect on Kv1.2 surface expression in
Chapter 2, Trim32C42A was also co-expressed with Kv1.2 and determined to have no significant change to Trim32 induced modulation of surface Kv1.2 Figure A:1C).

Inhibition of Clathrin-dependent Endocytosis Enhances Trim32-induced Increase of Surface Kv1.2

Chapter 2 did not explicitly investigate whether the effect of Trim32 on surface Kv1.2 influences trafficking of the channel. Clathrin-dependent endocytosis is a major internalization mechanism for decreasing Kv1.2 from the PM in HEK cells (Nesti et al., 2004; Stirling et al., 2009). Given the results of Chapter 2, where Trim32 promoted Kv1.2 at the cellular membrane at lower confluence conditions, we wondered if inhibiting clathrin-dependent endocytosis would enhance the Trim32-induced increase of Kv1.2. To test this, a cell-permeable inhibitor of dynamin, an essential component for clathrin-dependent coated vesicle formation (Macia et al., 2006), was applied to plated cells co-expressing Kv1.2 and either Trim32WT, Trim32ΔRING, Trim32C24A, or mUb and the surface expression of Kv1.2 was observed. As with previous experiments, co-expression of Trim32WT, Trim32C24A, or mUb all significantly increased surface Kv1.2 while Trim32ΔRING did not in vehicle treated cells. In cells treated with the dynamin inhibitor (Dynasore), with the exception of mUb, all of these increases were significantly enhanced after the treatment of Dynasore (Trim32WT **, Trim32ΔRING ***, Trim32C24A***) by two-way ANOVA test. This enhancement may be a result of blocking endogenous trafficking mechanisms in HEK cells, possibly also incorporating Trim32’s mechanism to decrease surface Kv1.2 observed in highly confluent cells, however these details were not investigated and require further experiments.
Co-immunoprecipitate of Trim32 with Kv1.2 was not observed by Immunoblot from Tissue Lysed in Buffer Lacking DUB, Proteasome, and Phosphatase Inhibitors

Chapter 2 detected only three peptides of Trim32 by mass spectrometry following IP of Kv1.2 from tissue lysed in a limited buffer (MS trail 1). To test whether this was a limitation of experimental detection by mass spectrometry, we immunoprecipitated either Kv1.2 or Trim32 from tissue lysed in the same buffer containing protease inhibitors (HALT cocktail of 100mM AEBSF*HCl, 80µM Aprotinin, 5mM Bestatin, E-64, 1.5mM EDTA, 2mM Leupeptin, and 1mM Pepstatin A) but lacking DUB, proteasome, and phosphatase inhibitors and used Immunoblotting to detect a potential co-IP (Figure A:2). Unlike the IB analysis of Chapter 2, which performed the same IPs from tissue lysed also in 1mM NaF, 1mM NaOrthovanadate, 20mM BAPTA, 5mM NEM, 10 µM MG-132, co-IP of either protein under these conditions was not detected. This data suggests that the preservation of the Kv1.2 and Trim32 association is sensitive to removal of post-translational modifications. These inhibitors are not specific to Kv1.2 or Trim32 and further experiments are needed to elaborate the conclusions.

Trim32 Co-expression Promotes High Molecular Weight Bandshifts of Kv1.2 Detected by Immunoblot.

Chapter 2 highlighted the ~8.5kDa bandshift increases of Kv1.2 following co-expression of Trim32 (or mUb). At the top portion of the gel (< 300kDa), faint, high-molecular weight bandshifts of Kv1.2 can also be detected following Trim32 co-expression. These bandshifts were seen in replicate experiments (Figure A:3A left and right) and not seen with co-expression of empty vector.
The RING Domain of Trim32 is not Required for the Trim32 association with Kv1.2

To determine if Trim32s catalytic RING domain is essential for its association with Kv1.2, Kv1.2 was overexpressed alone, with wild-type Trim32, or with Trim32 lacking the RING domain (Figure A:3B). Cells were lysed and prepared for Kv1.2 IP and immunoblot as described in Chapter 2 for tissue IB. Regardless of the RING domain, Trim32 was still detected to co-IP with Kv1.2, suggesting that the catalytic RING domain is not essential for associating with Kv1.2. Red arrows in the Trim32 co-IP channel indicate Trim32 and the decreased MW as a result of the deleted RING domain.

AC Activation by Forskolin Alters the Influence of Trim32 on C-terminal Phosphorylation Sites of Kv1.2

Under identical conditions to those described in Chapter 2, a parallel SILAC experiment was performed to determine the effect of Trim32 and Forskolin treatment, as the AC/PKA activator Forskolin has shown previously shown to influence activity of both Kv1.2 and Trim32 (Connors et al., 2008; Ichimura et al., 2013). The SILAC results of Chapter 2 are also listed as the vehicle treatment (Table A:1). As reported in Chapter 2, co-expression of Trim32 caused no change in S440 phosphorylation but significantly decreased S434 phosphorylation. In cells treated with Forskolin, however, Trim32 now significantly decreased S440 phosphorylation and did not alter S434. These results suggest that AC activation by Forskolin treatment is able to manipulate the influence of Trim32 on Kv1.2 phosphorylation. L = Light amino acid (K/R 12C & 14N) containing culture media and H = Heavy amino acid (K/R 13C & 15N) containing culture media.
Trim32 is Present Throughout the Mammalian Brain

In addition to isolated cerebellar slices of Chapter 2, a whole brain immunofluorescent survey of Trim32 was performed by multiphoton imaging on whole brain, sagittal slices (Figure A:4, top left shows a cropped picture from a whole, sagittal image). Trim32 was found to be highly prevalent in the hippocampus particularly (Figure A:4, top right), where triple staining with Kv1.2 and DAPI showed that Trim32 can also be found within the nuclei of hippocampal cells containing Kv1.2 (Figure A:4, bottom images). Both the cerebellar finding of Chapter 2 and hippocampal findings here are in agreement with recent IHC reports of human (both male and female) tissue provided by proteinatlas.org. Sagittal slices were cut between 10-50 µM thick, permeabilized in 0.3% Triton X. Slices were prepared for immunofluorescence and analyzed by microscopy through methods described in Chapters 2 and 3.

Additional Mass Spectrometry Detection of Trim32

A modified table of detection of Trim32 by LC-MS/MS which includes the addition of the 4 cerebellum homogenate detected by an Orbitrap mass spectrometer listed in Chapter 3 (Table A:2).

Figure Legends

Figure A:1. Additional flow cytometry data pertaining to Trim32 and ubiquitylation surface regulation of Kv1.2. (A) Trim32s influence on Kv1.2 is altered by C or N terminal lysine mutations. Figures 5 and 6 demonstrated that Trim32 modulates surface Kv1.2 differently dependent on cellular conditions. Here, we tested Trim32 overexpression on N and C terminal KR mutants and compared the results to both
Kv1.2WT and KV1.2KR. In this experiment, Trim32 caused a typical significant increase in surface Kv1.2WT also causing a significant decrease in surface Kv1.2KR.

Overexpressing Trim32 with either Kv1.2-CKR or Kv1.2-NKR caused a modest increase in surface channel, with NKR but not CKR being significantly different from Kv1.2WT.

N = 6 for each condition. * denotes significance of p < 0.05 by students t-test.

(B) Trim32 does not require Kvβ2 to increase surface Kv1.2. Chapter 2 focused solely on Trim32 influence toward Kv1.2 alpha subunit. Using HEK293-ATTC cells, we found that removing Kvβ2 decreased baseline surface Kv1.2 but had no significant effect on Trim32 increasing Kv1.2 at the membrane surface. N = 6 for each Kv1.2 + vector condition and n = 12 for Kv1.2 + Trim32 conditions.

(C) GFP-Trim32C24A traffics Kv1.2 similarly to GFP-Trim32WT. It has been suggested that the cysteine within Trim32’s RING domain is responsible for much of its ligase activity, and in some assays, replacement with an alanine can partially or completely cripple ligase activity. Alongside the same experiments run in Figures 5 and 6, we observed that GFP-Trim32C24A altered surface Kv1.2 similarly and not significantly different to GFP-Trim32WT.

(D) Dynamin inhibition enhances Trim32 increase of surface K1.2. To investigate the effect of inhibiting clathrin-dependent endocytosis to plated cells co-expressing Kv1.2 and Trim32WT, Trim32ΔRING, Trim32C24A and mUb, cells were treated the dynamin inhibitor Dynasore and compared to DMSO vehicle controls. Dynasore significantly enhanced the increase of surface Kv1.2 by all Trim32 constructs, but not mUb. N = 6 for
each condition. Data analyzed by 2way analysis of variance. \* = p <0.05 \** = p <0.01 \*** = p <0.001 \**** = p <0.0001.

**Figure A:2. Co-immunoprecipitation of Trim32 with Kv1.2 is not detected in cerebellar extracts without DUB, proteosome, and phosphatase inhibitors present in lysis buffer.**

To determine whether the low Trim32 co-IP detected by LC-MS/MS from tissue homogenized in lysis buffer lacking 1mM NaF, 1mM NaOrthovanadate, 20mM BAPTA, 5mM NEM, 10 µM MG-132, the experiment was repeated and subject to WB analysis. No visible co-IP from either Kv1.2 or Trim32 IP was visibly detected.

**Figure A:3. Kv1.2 interacts with Trim32 in the absence of its RING domain. A:** Wild type Kv1.2 was expressed alone (+empty vector) or co-expressed with either GFP-Trim32WT. Cells were lysed and Kv1.2 protein was detected by immunoblot. Co-expression of Trim32 with Kv1.2 produces high molecular weight bandshifts of Kv1.2 detected in either the cell lysates or following IP of Kv1.2.

**B:** Wild type Kv1.2 was expressed alone (+empty vector) or co-expressed with either GFP-Trim32WT or GFP-Trim32∆RING in HEK293β2cells. Cells were lysed in the presence of MS Trial 3 in Chapter 2, and lysates were IP’d for Kv1.2. Proteins were separated by SDS-PAGE electrophoresis and analyzed by immunoblot. Probing for Trim32, a band of Trim32 (~100kDa) was detected from Kv1.2 + GFP-Trim32WT (Middle lane). A band of Trim32 was still detected from cells co-expressing Kv1.2 + GFP-Trim32∆RING (Right lane) with an appropriate decrease of molecular weight.
corresponding to the deleted RING domain. Lysates show total Kv1.2 and Trim32 proteins in both conditions, and molecular weight for Trim32 was similarly decreased form cultures expressing the RING mutation.

**Figure A:4. Trim32 is present throughout the rat brain.** Beside the cerebellum, Kv1.2 is endogenously expressed in other cortical regions of the brain such as the hippocampus. *Top panel-* multiphoton analysis of fixed cerebellar slices demonstrated that Trim32 is diffuse throughout the brain (left), particularly in the hippocampus where Kv1.2 is highly expressed (right). Full brain sagittal images: Adult rat cerebellar slices (400 µM) were fixed (2% formaldehyde cardiac perfusion), permeabilized (10% acetone), blocked, and stained for Kv1.2 (1:200) and Trim32 (1:100), and imaged using a Zeiss LSM 7 multiphoton microscope and a 20X water lens. Images were analyzed in Zen 2011 Software. *Bottom panel-* Widefield/deconvolution (Deltavision) images demonstrated Trim32 localizes to the nucleus of neurons expressing Kv1.2 within the hippocampus. Images obtained at 60X magnification with the same method and parameters listed in Chapter 2.

**Table A:1. Table of SILAC quantification that includes the result of Chapter 2 and a parallel experiment with Forskolin treatment.** The SILAC results of Chapter 2 are listed as the vehicle treatment and compared to a parallel experiment with both Light and Heavy populations treated with Forskolin. Rather than decreasing S434 phosphorylation, Trim32 co-expressed cells treated with Forskolin now significantly decreased S440
phosphorylation and did not alter S434. Culture media in the Light population contained
lysine and arginine amino acids with $K = {^{12}}C_6 \& {^{14}}N_2$ and $R = {^{12}}C_6 \& {^{14}}N_4$) while Heavy
populations contained them with $K = {^{13}}C_6 \& {^{15}}N_2$ and $R {^{13}}C_6 \& {^{15}}N_4$.

**Table A:2. Additional detection of Trim32 by LC-MS/MS.** A modified table of
detection of Trim32 by LC-MS/MS which includes the addition of the 4 cerebellum
homogenate detected by an Orbitrap mass spectrometer listed in Chapter 3.
Figure A:1 Additional flow cytometry data pertaining to Trim32 and ubiquitylation surface regulation of Kv1.2.
Figure A:2 Co-immunoprecipitation of Trim32 with Kv1.2 is not detected in cerebellar extracts without DUB, proteasome, and phosphatase inhibitors present in lysis buffer.
Figure A:3 Kv1.2 interacts with Trim32 in the absence of its RING domain.
Figure A:4 Trim32 is present throughout the rat brain.
Table A:1 Table of SILAC quantification that includes the result of Chapter 2 and a parallel experiment with Forskolin treatment.

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Table A:2 Additional detection of Trim32 by LC-MS/MS.

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Lionel, A. C., Crosbie, J., Barbosa, N., Goodale, T., Thiruvahindrapuram, B., Rickaby, J., . . . Scherer, S. W. (2011). Rare copy number variation discovery and cross-disorder comparisons identify risk genes for ADHD. Sci Transl Med, 3(95), 95ra75. doi: 10.1126/scitranslmed.3002464


APPENDIX B: DATA RELATING TO CHAPTER 3

Background

The data presented here were collected during the experiments presented in Chapter 3 but were not included in the final six figures. Similarly to Appendix A for Chapter 2, this data is intended to expand the clarity and depth of Chapter 3.
Results and Discussion

**Hypertonic Stress Alters WNK1-Induced Modulation of Surface Kv1.2 in WNK1KO 293T but not Control 293T Cells.**

Since co-expression of WNK1 alters surface Kv1.2 in HEK293 cells, and WNK1 is an intrinsic sensor of cell volume, we wondered if altering extracellular tonicity would impact the influence WNK1 has on Kv1.2 surface expression. High extracellular NaCl produces a hypertonic extracellular environment which has previously been shown to stimulate autophosphorylation and activation of endogenous WNK1 in HEK cells (B. Xu et al., 2000). Hyperosmotic stress also induces a rapid localization of WNK1 to clathrin-coated vesicles (Zagorska et al., 2007). Just as Chapter 3, experiments were performed in both HEK293T-cells and WNK1KO cells co-expressing Kv1.2 and WNK1 (or empty vector). Surface Kv1.2 in cells treated with high NaCl (0.4M, 20 minutes) was compared to isotonic controls treated with vehicle (PBS) (Figure B:1). High extracellular NaCl alone had no effect on Kv1.2 surface expression (+3%, t(df) = 0.44(16), p = 0.6629, n = 12 (isotonic), 6 (hypertonic), and co-transfection of WNK1 caused a significant decrease of surface Kv1.2 similar to isotonic conditions (-12%, t(df) = 2.96(10), p < 0.05, n = 12 (isotonic), 6 (hypertonic). As observed in Chapter 3, switching from HEK293T-cells to WNK1KO cells significantly decreased baseline Kv1.2 (-9%, t(df) = 2.28(10), p < 0.05, n = 12 (isotonic), 6 (hypertonic). However, unlike isotonic conditions, co-transfection of WNK1 in WNK1KO cells caused a significant decrease of surface Kv1.2 (-15%, t(df) = 2.34(10), p < 0.05, 12 (isotonic), 6 (hypertonic) after NaCl exposure, suggesting that the mechanism driving increased surface Kv1.2 does not occur during hypertonic stress. In
addition to activation of WNK kinases, hypertonic stress promotes both the binding and activation of downstream SPAK/OSR1 kinases by WNK kinases (Zagorska et al., 2007), supporting the idea that the decrease of surface Kv1.2 by Trim32 over-expression is through SPAK activation.

*WNK1 Localizes to Ring-like Structures in PC Soma*

In a repeat IF experiment for Chapter 3, again, WNK1 is strongly localized in the molecular layer with Kv1.2, however WNK1 also displays a unique, ring-like structures within the PC bodies (*Figure A:2A Top panel*). Additionally, WNK1 also is found with punctate structures that surround the cell body of the PC which overlap with Kv1.2. A magnification of a PC soma with a surrounding Pinceau is provided for further clarity (*Figure A:2A, insert*). Further experiments are needed to determine whether the ring-like structure is in fact nuclear or peri-nuclear in localization as well as to determine the true function of the puncta.

*SPAK May also Localize to Purkinje Cells*

Given the localization of WNK1 determined in Chapter 3, and the fact that WNK1 and SPAK share a similar signaling pathway, SPAK is likely also localized to PCs and molecular layer neurons. Using multiphoton imaging similar to Chapter 3, SPAK was also detected, although at weaker resolution, within all cerebellar locations, most particularly in PCs and within the molecular layer by multiphoton microscopy (*Figure B:2B, Middle panel*). Additional imaging with high magnification (100X) widefield microscopy with DeltaVision restoration demonstrated SPAK diffuse within
the molecular layer and PCs (Figure B:2B, Bottom panel). Perinuclear ring-like structure similar to that seen with WNK1 by multiphoton (Figure B:2B, Insert).

**SPAK Peptide Fragmentation Spectra with Phosphorylation at Threonine T240**

SPAK is phosphorylated and subsequently activated by WNK1 at an equivalent threonine found across mammals: T233 in human, T243 in mice, and here T240 in rat. Chapter 3 presented several peptides of SPAK T240 that were found phosphorylated in cerebellar tissue enriched for Kv1.2, however the spectra were not shown. Figure 3 provides a representative spectra detected from Chapter 3 experiments. Peptide charge = +3, Xcorr = 2.57, localization determined by manual inspection.

**Figure Legends**

**Figure 1. Extracellular hypertonic treatment of HEK293 cells and response of Kv1.2 surface expression.** HEK293T control or WNK1KO cells co-transfected with Kv1.2 and WNK1 or empty vector were treated with either isotonic or hypertonic (0.4M, NaCl) media for 20 minutes prior to surface expression measure by flow cytometry. Control 293T cells caused a significant decrease of surface Kv1.2 in both conditions, and hypertonic media alone did not alter baseline expression of Kv1.2. Switching from T cells to WNK1KO cells, as before, decreased baseline surface Kv1.2 in both conditions, however co-expression of WNK1 caused a significant decrease of surface channel equivalent to that of the T cells, rather than the significant increase demonstrated under prior experiments and isotonic conditions here.
Figure 2. SPAK localization in the cerebellum and Possible WNK1 trans-golgi and vesicular localization in cerebellar Purkinje cells. A: Multiphoton imaging highlighting the ring-like structure that WNK1 (pseudo colored green) often demonstrates in PC cell bodies. Other cerebellar interneurons (BC, stellate) can be seen as well as what may be Burgmann Glia between (large, green structures between PCs). Note: colors are reversed to those of Chapter 3 imaging. B: Imaging of SPAK by both Multiphoton (top) and Widefield/deconvolution (bottom) Microscopy collectively display what appears as localization to both PCs and the molecular layer, however repeat experiment with improved detection are needed.

Figure 3. SPAK T240 phosphorylation spectra in the cerebellum. SPAK is phosphorylated and subsequently activated by WNK1 at an equivalent threonine found across mammals: T233 in human, T243 in mice, and here T240 in rat. Chapter 3 presented several peptides of SPAK T240 and a representative peptide spectra is shown (Peptide charge = +3, Xcorr = 2.57). All LC-MS/MS preparation and analysis were identical to those described in Chapter 3.
Figure B:1 Extracellular hypertonic treatment of HEK293 cells and response of Kv1.2 surface expression.
Figure B:2 SPAK localization in the cerebellum and Possible WNK1 trans-golgi and vesicular localization in cerebellar Purkinje cells.
Figure B:3 SPAK T240 phosphorylation spectra detected in cerebellar tissue immunoprecipitated for Kv1.2.
### Table B:1 MS detection of SPAK.

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### Table B:2 MS detection of WNK1.

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APPENDIX C: ADDITIONAL MASS SPECTROMETRY DATA

Background

This section provides supplementary mass spectrometry data which was obtained throughout the generation of Chapters 2 and 3.

Results and Discussion

Forskolin Alters S440 Phosphorylation of Kv1.2 in Cerebellar Slices

Recently, application of the neuropeptide secretin to live cerebellar slices was shown to result in altered trafficking of Kv1.2 through AC/PKA activation (Williams et al., 2012). As mentioned in previous chapters, AC stimulation by Forskolin treatment has also been shown to alter S440 phosphorylation in HEK293 cells (Connors et al., 2008). To test whether stimulation of AC/PKA to cerebellar slices leads to altered phosphorylation at S440, we stimulated live slices with Forskolin and used Absolute Quantification of Peptides (AQUA) quantitative MS analysis. The AQUA peptide was identical to the commonly detected doubly charged, singly phosphorylated peptide IPSpSPDLKK with the addition of $^{13}\text{C}_6$ to the leucine (L444) and was detected prior to experimentation at 526.38 m/z. AQUA analysis showed that Forskolin treated slices resulted in ~50% increased S440 phosphorylation compared to DMSO vehicle treated slices (Figure C:1). Because of overall weak detection of Kv1.2 in the samples, MS2 fragmentation spectra was not obtained for all +2 peptides quantified and therefore S441 phosphorylation cannot be ruled out and interpretation may therefore be limited to
combined S440 and S441 phosphorylation. Regardless, AC/PKA activation by Forskolin treatment appears to impact both surface expression (Williams et al., 2012) and phosphorylation of Kv1.2 in cerebellar slices.

Kv1.2 Post-translational Modifications Detected by LC-MS/MS with Unknown Functionality

A recent, large-scale proteomic study reported a peptide of Kv1.2 ubiquitylated at K45 and K63 isolated from mouse tissue (Wagner et al., 2012). In Chapter 2, we reported identification of these two sites on a single peptide detected in MS Trial 2. The spectra is presented here in Figure C:2. Mass addition of lysine 45 was well localized (b5, b6 and y16 ions detected), however, mass addition by di-GG of adjacent K63 and K64 was difficult to localize (although y2, y3, and b19 were suggestive of K63). The peptide charge was +3 with a Sequest Xcorr value of 2.2. Sample preparation and analysis by LC-MS/MS was identical to Chapter 2. Functional roles for these sites remained to be elucidated.

Another site of Kv1.2 modulation detected in mouse tissue by Wagner et. al. was serine 468 phosphorylation. This site has not been reported in rat tissue or localized to the cerebellum, nor does a functional role for this PTM exist. We detected this site abundantly phosphorylated in cerebellar tissue (Xcorr values as high as 5.0). An example spectra is shown in Figure C:3 (Charge = +3, Xcorr = 5.4, A score = 119). Additionally, detection of phosphosites of this peptide by LC-MS/MS analysis following cerebellum-dependent learning in Chapter 3 was noted, however a functional link was not determined.
nor were the results truly quantitative. Further validation is needed to determine whether phosphorylation of S468 is truly related to cerebellum-dependent learning.

*Comparison of Kv1.1 S439 and Kv1.2 Modified Peptides*

Sequence alignment of Kv1.2 with that of Kv1.1 shows that Kv1.1 does not have a homologous lysine or arginine in this region of its C-terminus (*Figure 5*). The resultant tryptic peptide is of similar length to the peptide of Kv1.2 with K437 ubiquitylation. Serine 439, homologous to S440 of Kv1.2, was often detected phosphorylated and produced a tryptic peptide with similar length and general fragmentation pattern for comparison of Kv1.2 K437 ubiquitylation.

**Figure Legends**

**Figure C:1. Forskolin alters S440 phosphorylation of Kv1.2 in vivo.** Treatment of Forskolin (100µM, 15min) to cerebellar slices resulted in approximately twice as many Kv1.2 S440 phosphopeptides compared to DMSO vehicle treated slices as detected by Absolute Quantification LC-MS/MS (run on an LTQ Orbitrap Discovery mass spectrometer with mass accuracy ~0.05Da). Figure shows MS1 spectra and LC elution insert from DMOS condition only. Spectra demonstrates successful detection of both light (endogenous) and heavy (synthetic) peptides, while quantitative ratios of light/heavy peptides listed below demonstrate a change between conditions.
Figure C:2. N-terminal Kv1.2 ubiquitylated peptide detected by MS. Ubiquitylation at K45 and K63 was detected on a single peptide from MS trail 2. Within the peptide, the first –GG motif was well localized to K45 while other was likely at K63 upon manual inspection of fragmentation, although K64 was not able to be ruled out (Peptide charge +3, Xcorr = 2.2). Preparation of sample and analysis of LC-MS/MS was described in Chapter 2 (Also pertains to Figure C:3 and Figure C:4 below).

Figure C:3. Peptide fragmentation spectra of Kv1.2 phosphorylation at S468.
Phosphopeptides containing serine 468 phosphorylation were detected in all tissue experiments and mentioned in the behavioral experiment of Chapter 3. A representative fragmentation spectra of this phosphopeptide is shown (Charge = +3, Xcorr = 5.44, A score = 119).

Figure C:4. Kv1.1 Sequence and fragmentation spectra comparison of Kv1.2 437 ubiquitylation and Kv1.1 S439 phosphorylation. Kv1.1 does not possess a lysine or arginine equivalent to the region of Kv1.2 K437, and tryptic peptides are cut in similar length to the commonly detected +3 K437 ubiquitylated peptide of Kv1.2. This peptide was often detected phosphorylated at a S439, homologous to S440 of Kv1.2. The two peptides are presented together for qualitative assessment.
Table 1. **Total PTMs detected by LC-MS/MS for Kv1.2.** A comprehensive list of all PTMs identified in cerebellar tissue by mass spectrometry. All sites were identified by matching LC-M/MS fragmentation spectra to Sequest database spectra with mass tolerance of +/- 1 Da. Localization of all PTMs were confirmed by manual inspection.

Table 2. **Total PTMs detected by LC-MS/MS for Kv1.1.** A comprehensive list of all PTMs identified in cerebellar tissue by mass spectrometry similarly to Table 1 for Kv1.2.

Table 3. **Complete Linear Quadrapole LC-MS/MS interactome of Kv1.2 from Chapter 2 MS trail 1.** All proteins identified contained at least 3 unique peptides detected in the experimental and none detected in the equivalent controls.

Table 4. **Complete Linear Quadrapole LC-MS/MS interactome of Kv1.2 from Chapter 2 MS trails 2 and 3 combined (3 includes entire gel).** As Table 2, all proteins identified contained at least 3 unique peptides detected in the experimental and none detected in the equivalent controls.
Figure C:1 Forskolin alters S440 phosphorylation of Kv1.2 in vivo.
Figure C:2 N-terminal Kv1.2 ubiquitylated peptide detected by MS.
Figure C:3 Peptide fragmentation spectra of Kv1.2 phosphorylation at S468.
Figure C:4 Kv1.1 Sequence and fragmentation spectra comparison of Kv1.2 437 ubiquitylation and Kv1.1 S439 phosphorylation.
Table C:1 Total PTMs detected by LC-MS/MS for Kv1.2.

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Table C:2 Total PTMs detected by LC-MS/MS for Kv1.1.

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Table C:3 Complete Interactome of Kv1.2 from Chapter 2 MS trial 1.

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Table C:4 Complete Interactome of Kv1.2 from Chapter 2 MS trials 2 and 3.

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Lionel, A. C., Crosbie, J., Barbosa, N., Goodale, T., Thiruvahindrapuram, B., Rickaby, J., . . . Scherer, S. W. (2011). Rare copy number variation discovery and cross-disorder comparisons identify risk genes for ADHD. Sci Transl Med, 3(95), 95ra75. doi: 10.1126/scitranslmed.3002464


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phosphorylate and activate SPAK and OSR1 protein kinases. Biochem J, 391(Pt 1), 17-24. doi: 10.1042/BJ20051180


