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Secretin-Modulated Potassium Channel Trafficking as a Novel Mechanism for Regulating Cerebellar Synapses

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SECRETIN-MODULATED POTASSIUM CHANNEL TRAFFICKING AS A NOVEL MECHANISM FOR REGULATING CEREBELLAR SYNAPSES

A Dissertation Presented

by

Michael R. Williams

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Specializing in Neuroscience

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Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, specializing in Neuroscience.

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ABSTRACT

The voltage-gated potassium channel Kv1.2 is a critical modulator of neuronal physiology, including dendritic excitability, action potential propagation, and neurotransmitter release. However, mechanisms by which Kv1.2 may be regulated in the brain are poorly understood. In heterologous expression systems Kv1.2 is regulated by endocytosis of the channel from the plasma membrane, and this trafficking can be modulated by adenylate cyclase (AC). The goal of this dissertation was to determine whether AC modulated endocytic trafficking of endogenous Kv1.2 occurred in the mammalian nervous system.

Within the brain, Kv1.2 is expressed at its highest levels in the cerebellar cortex. Specifically, Kv1.2 is expressed in dendrites of Purkinje cells (PC), the sole efferent neurons of the cerebellar cortex; Kv1.2 is also expressed in axon terminals of Basket cells (BC), which make inhibitory synapses to Purkinje cells. The loss of functional Kv1.2 in PC dendrites or BC axon terminals causes profound changes in the neurophysiology of Purkinje cells, and aberrant loss of Kv1.2 produces cerebellar ataxia. Therefore, the cerebellum offers a brain structure where Kv1.2 is abundant and has known and important roles in synaptic physiology. A candidate regulator of Kv1.2 trafficking in cerebellar synapses is the secretin peptide receptor: the receptor is also located in both PC dendrites and BC axon terminals, and ligand binding to the secretin receptor stimulates AC. Although secretin affects cerebellar neurophysiology and cerebellar dependent behavior, the mechanisms are not well resolved.

By cell-surface protein biotinylation and subsequent immunoblot quantitation of secretin treated rat cerebellar slice lysates, secretin was found to decrease cell-surface Kv1.2. This effect could be mimicked by stimulating AC with forskolin, and could be occluded by inhibition of the secretin receptor, AC, or protein kinase A. The secretin receptor stimulated loss of surface Kv1.2 was not accompanied by decreased total Kv1.2 protein levels, but did involve enhanced channel endocytosis. Microscopy studies using two novel independent techniques provided evidence that both BC axon terminals and PC dendrites are sites of AC-stimulated Kv1.2 endocytosis. The physiological significance of secretin mediated suppression of Kv1.2 was supported by collaborative studies which found infusions into the cerebellar cortex of either a toxin that inhibits Kv1.2, or of secretin, enhanced eyeblink conditioning, a form of cerebellar dependent learning, in rats.

These studies provided the first evidence that Kv1.2 is regulated by endocytic trafficking in the brain. However, to address the role of that trafficking in synaptic physiology requires knowledge about the determinants of Kv1.2’s endocytic potential, and non-destructive assays to measure Kv1.2 endocytosis in neural circuits. This dissertation therefore concludes with preliminary studies that explore an ancient motif regulating Kv1.2 trafficking, and that discuss a novel dual fluorescent fusion protein reporter of Kv1.2’s subcellular localization.
CITATION

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

Introduction

Electrically excitable cells, such as neurons, display changes in voltage across their plasma membrane due to regulated ion flux between intra- and extra-cellular environments. Active electrogenic pumps and passive leak channels permeant to potassium establish an extracellular environment with higher in sodium and an intracellular environment that is conversely high in potassium and has negative voltage with respect to the extracellular space (Hodgkin and Huxley 1952; Skou 1989; Lesage, Guillemare et al. 1996; Enyedi and Czirjak 2010). Therefore, while sodium influx is a major depolarizing force, the passive efflux of potassium limits depolarization, giving the function of potassium channels a critical role in neurophysiology.

Structural Classes Of Potassium Channels

There are multiple types of potassium (K) channels, and one level of diversity is manifest in their physical structure (reviewed in (Perney and Kaczmarek 1991; Choe 2002). The structurally simplest have two transmembrane domains and one pore (2TM/1P), such as inward rectifier potassium (KIR) channels, the G-protein coupled inward rectifier potassium (GIRK) channels, and the (in complex with sulphonylurea receptors), KATP channels. Slightly more complex are the background or “leak” K2P channels such as TREK or TWICK, having the structure 4TM/2P, consisting of two fused 2TM/1P domains. A third class, the 6TM/1P channels, retain the 2TM/1P moiety but also have an additional 4TM domain that harbors a voltage sensor, and include voltage-
gated potassium (Kv) channels, as well as the small and intermediate conductance calcium-gated potassium channels. (While there is also a large conductance calcium gated potassium (BK) channel, this has a unique structure with a 7th TM domain that places the N-terminus of the protein extracellularly.) A more recently discovered class appears to be a hybrid form, which has an 8TM/2P structure, consisting topologically of a 6TM/1P and a 2TM/1P domain in sequence, found only so far in fungi and yeast, but not mammalian species (Ketchum, Joiner et al. 1995).

**Kv Channels**

Of the structural classes of potassium channels, 6TM/1P Kv channels are the most abundant and diverse class (Roberds, Knoth et al. 1993; Benatar 2000; O'Connell and Tamkun 2005). These Kv channels exist in nature as protein complexes of both the 6TM/1P alpha subunits and auxiliary subunits. While the alpha subunits tetramerize to form the pore, auxiliary subunits modulate the biosynthesis, subcellular targeting, and biophysical properties of the composite holochannel (Pourrier, Schram et al. 2003; Pongs and Schwarz 2010). There are three classes of such non-pore forming subunits: single-pass transmembrane proteins derived from KCNE-type genes, dubbed MinK-related peptides (MiRPs) (Abbott and Goldstein 2001; Kanda and Abbott 2012); calcium sensing/binding proteins derived from the KCNIP genes, called KChIPs (An, Bowlby et al. 2000); and the Kvβ class, encoded by the KCNAB genes, (Scott, Rettig et al. 1994; McCormack, McCormack et al. 1995).
The pore-forming alpha subunits of Kv channels are an even more diverse class. The first potassium channel to be cloned was found in Drosophila, dubbed *Shaker* (Papazian, Schwarz et al. 1987). Under the current naming convention, the many homologous channels that have since been discovered are named KvX.Y, channels, K for the potassium ion the channels are specifically permeant to, v, to indicate that they are gated by voltage, X refers to the family, and Y is the specific member of that family (Chandy 1991). Reflecting their historical discovery in Drosophila, following *Shaker* class channels, those of the *Shab* lineage are Kv2, those of *Shaw* are Kv3, and those of *Shal* are Kv4, however there have now been identified functionally significant channels up through Kv12 (Zhang, Bertaso et al. 2010). Because of this diversity, it is more useful to discuss the different types of channels the various genes of the many families form than to review the products of each Kv gene.

Classically, a functional Kv channel is a protein complex of four alpha subunits of the same family, with the subunits being either the same (homomultimers) or different (heteromultimers) members of that family. For example, Kv1.1 alpha subunits may heteromultimerize with Kv1.2, or form a homomultimeric channel composed only of Kv1.1 alpha subunits (Shen and Pfaffinger 1995). For some exceptional alpha subunits however, the association with alpha subunits of members of another family is not only possible, but necessary for channel function. This includes Kv8.2, which is not a functional channel as a homotetramer, but which can form a functional heterotetramer with Kv2 members (Ottschytsch, Raes et al. 2002). In most instances however, alpha subunits of the *same* family come together to form a channel.
The functionally defined families of 6TM/1P voltage gated potassium channels include the aforementioned modulators/silent channels; the inward rectifiers such as the human ether-a-go-go related (hERG) channel (Trudeau, Warmke et al. 1995), the cyclic-nucleotide gated (CNG) and hyperpolarization and cyclic-nucleotide gated (HCN) channels that have varying permeability to potassium and sodium, important for cardiac rhythmicity and vision (Craven and Zagotta 2006); the rapidly-inactivating channels important for action potential back-propagation in neurons and the cardiac action potential (Birnbaum, Varga et al. 2004); and the delayed rectifiers, typically non- or slowly inactivating channels that limit and define temporal aspects of changes in membrane voltage by providing a repolarizing current that is elicited, with minor lag, by depolarization (Cole and Moore 1960; Gonzalez, Rosenman et al. 2000). One delayed rectifier potassium channel abundantly expressed throughout the body is Kv1.2.

**The Diverse Roles Of Kv1.2**

With the great diversity of potassium channels, it may be surprising that any one alpha subunit serves an indispensable function. Yet, this appears to be the case for Kv1.2. Mice which are genetically manipulated to lack Kv1.2 have reduced sleep (Douglas, Vyazovskiy et al. 2007), are susceptible to seizure, and die prematurely (Brew, Gittelman et al. 2007). Mice with an induced mutation in Kv1.2 that reduces the proteins stability and abundance likewise develop neurological disease and have reduced lifespan (Xie, Harrison et al. 2010). For humans, there are no known tolerated mutations of the KCNA2 gene according to the Online Mendelian Inheritance in Man Database, and people who develop auto-antibodies against Kv1.2 develop neuromyotonia and limbic encephalitis.
The criticality of Kv1.2 may be related to its broad distribution throughout tissues of the body and the multiple processes it apparently supports: it is expressed in the smooth muscle of the esophagus and gut, where it influences contractility and motility (Hart, Overturf et al. 1993; Koh, Ward et al. 1999; Wade, Laurier et al. 1999); it is expressed in uterine smooth muscle, where it influences contractility (Smith, McClure et al. 2007); it is expressed in the vasculature, where it may contribute to regulation of blood pressure and arterial diameter (Belevych, Beck et al. 2002; Albarwani, Nemetz et al. 2003; Fergus, Martens et al. 2003; Plane, Johnson et al. 2005); it is expressed in microglia where it may contribute to their chemokine and reactive oxygen species generation (Li, Lu et al. 2008); and Kv1.2 is abundantly expressed in the nervous system.

**Function Of Kv1.2 In Neurons**

Kv1.2 may be found, in various cell populations, to be expressed in every critical functional compartment of neurons: dendrites, soma, the initial axon segment, and: axons, their transition zones, and their terminals. Neurons are functionally polarized, and information is conveyed to them primarily via their receptive dendrites. Dendritic depolarization may be opposed by postsynaptic potassium channels, such as Kv1.2 expressed in the dendrites of cerebellar Purkinje cells (Khavandgar, Walter et al. 2005) or hippocampal pyramidal neurons (Sheng, Tsaur et al. 1994). Through passive spatial and/or temporal summation, dendritic input may cause the membrane to reach a depolarized threshold at which an action potential (AP) is triggered. However, this process can be opposed by somatic potassium channels, such as Kv1.2 expressed in the
soma of sensory and medium spiny neurons (Andrews and Kunze 2001; Shen, Hernandez-Lopez et al. 2004). In the cases where this threshold potential is reached, the ability to initiate an AP depends upon specialized regions of the neuron, the axon initial segment and the axon hillock. In these domains, there is a larger abundance of voltage gated sodium channels than is found somatically, and the specific types and amounts of potassium channels there dictate the nature of depolarization. In multiple types of neurons these regions express Kv1.2 (Lorincz and Nusser 2008), where the channel influences the degree of depolarization necessary to initiate an AP and its waveform (Dodson, Barker et al. 2002; Glazebrook, Ramirez et al. 2002; Kole, Letzkus et al. 2007; Goldberg, Clark et al. 2008).

The AP occurs over small domains of the plasma membrane, but for information to be conveyed the depolarization must be passed down the efferent aspect of the neuron, the axon. Some axons are simply ensheathed by plasma membrane processes of glial cells. In these instances, the depolarization would quickly decay over distance (Cole and Curtis 1939; Cole and Hodgkin 1939; Hodgkin and Rushton 1946). Therefore, to propagate the depolarization, there must be a complement of voltage gated sodium and potassium channels along the length of the axon with relatively high spatial density to constantly regenerate the action potential down the axons length. However, Kv1.2 is not known to have such a continuous axonal localization nor is there electrophysiological evidence supporting a role for Kv1.2 in this form of AP propagation.
In contrast, some axons are not merely ensheathed, but are instead myelinated. Again, the axon is enveloped by glial plasma membrane, but in myelination, the process wraps around many times, forming a very high resistance and low capacitance segment through which the AP may pass with little decrement. At regularly spaced intervals, there are nodes where myelin is absent, voltage gated sodium and potassium channels are abundant, and in which the action potential is generated anew. Kv1.2 is expressed in flanking domains known as the juxtaparanode, where these channels are believed to modulate axonal excitability, not through repolarization, but through preventing recurrent nodal excitation (Rasband, Trimmer et al. 1998; Vabnick, Trimmer et al. 1999).

In either ensheathed or myelinated axons, the axon may branch to multiple efferent targets. Here too, at axon branch points, Kv1.2 channels may serve an important role, such as in the branching points of cerebellar basket cell axons (Tan and Llano 1999; Zhang, Messing et al. 1999). As Kv1.2 channels are opened by depolarization, these clusters of channel provide points where the invading depolarization may open the channels, increasing membrane permeability and providing a shunting pathway for the depolarization, decreasing the likelihood a depolarization will be faithfully transmitted to the final aspect of the neuron, the axon terminals (Dodson, Billups et al. 2003; Fulton, Thibault et al. 2011).

Axons terminate in the formation of electrical or chemical synapses. In the case of electrical synapses, specialized transmembrane proteins, connexins (or the related pannexins), form a hemichannel that, together with a counterpart on the postsynaptic
target, make a “gap junction” that makes the cytoplasm of the two cells continuous. While this direct coupling allows for very fast transmission and is abundant in cardiac myocytes, neuron to neuron gap junctions are relatively scarce (Reviewed in (Connors and Long 2004; Dere and Zlomuzica 2012)). Instead, most synapses are chemical. In this class, the depolarizing electrical signal is converted into a diffusible chemical signal, primarily through depolarization induced calcium flux and subsequent vesicular transmitter release. Here too, near or at the axon terminal, Kv1.2 channels may resist terminal hyper-excitability during a depolarizing after stimulus that would otherwise permit additional transmitter release (Wang, Kunkel et al. 1994; Dodson, Billups et al. 2003). To summarize, Kv1.2 may be found in different neuronal subcellular domains, ultimately influencing neurotransmission. However, Kv1.2 is most typically localized at or near axon terminals where it serves that function most directly.

**Kv1.2 Controls Transmitter Release In The Brain**

In the Calyx of Held, presynaptic Kv1.2 channels regulate glutamate release by preventing hyper-excitability in the axon terminal after an action potential invades (Dodson, Billups et al. 2003). Neurons of the striate terminating in the substantia nigra pars reticulata also express high levels of presynaptic Kv1.2, where the channel controls gamma-aminobutyric acid (GABA) release (Chung, Shin et al. 2000; Shimada, Uta et al. 2007). Likewise, Kv1.2 can regulate acetylcholine release in the striatum (Fischer and Saria 1999) and GABA release from the entorhinal cortex (Cunningham and Jones 2001). Also, presynaptic Kv1.2 regulates GABA release at the cerebellar basket cell: Purkinje cell synapse (Southan and Robertson 1998; Southan and Robertson 2000). While Kv1.2
is enriched at presynaptic sites throughout the brain and influences transmitter release, it may not simply have a static role: Kv1.2 regulation might be mechanistically involved in tuning synaptic function.

**Change in Kv1.2 Function Is Important For Changes In Neurophysiology**

A number of studies have shown that changes in synaptic function require a change in Kv1.2 function; however all of these studies have relied on toxicological blockade of Kv1.2 to infer this regulatory role. For example, serotonin can increase glutamate release from thalamocortical neurons, but this action requires suppressible Kv1.2 (Lambe and Aghajanian, 2001). Within the paraventricular nucleus, nitric oxide facilitates GABA release; however this facilitation requires presynaptic Kv1.2 available for blockade (Yang et al., 2007). At the basolateral amygdala, mu opioid receptor mediated changes in GABAergic transmission require presynaptic Kv1.2 function (Faber and Sah, 2004; Finnegan et al., 2006). Similarly, the auto-receptor mediated modulation of dopamine release in the dorsal striatum is occluded by Kv1.2 inhibition (Fulton, Thibault et al. 2011). So, while Kv1.2 regulation has been proposed to be an important intermediate step in the modulation of transmitter release, mechanisms by which endogenous synaptic Kv1.2 might be regulated have not been demonstrated. Manipulations involving gross insult to neuronal tissue have provided evidence however that neuronal Kv1.2 can be regulated, allowing for speculation that physiological processes might regulate the channels functional abundance as well.
Kv1.2 Can Be Regulated In The Brain

Early evidence for regulation of Kv1.2 in the central nervous system came from an observation of reduced Kv1.2 mRNA levels in the hippocampus following chemically (Tsaur, Sheng et al. 1992) or electrically (Pei, Burnet et al. 1997) induced convulsion. Other large insults also provided evidence that the brain contains mechanisms for regulating Kv1.2 abundance. After ischemia, Kv1.2 protein levels are elevated throughout many regions of the brain (Chung, Kim et al. 2001), and the induction of Kv1.2 in ischemia is paralleled by post-translational modification of the channel (Qiu, Zhang et al. 2003). Kv1.2 is also regulated in injury, axotomy decreases the abundance of Kv1.2 in dorsal root ganglia (Yang, Takimoto et al. 2004). Likewise, Kv1.2 levels and distribution are altered in diabetes (Zenker, Poirot et al. 2012) and in amyotrophic lateral sclerosis (Shibuya, Misawa et al. 2011). Therefore, there are mechanisms within the central nervous system for bidirectional regulation of Kv1.2, at least with pathological perturbations. Since Kv1.2 is a regulator of neurotransmission, the major goal of this dissertation was to determine if there are non-pathological cellular pathways for regulating endogenous synaptic Kv1.2 in the mammalian nervous system. One mechanism of ion channel regulation in the brain is endocytosis from, and trafficking back to, the plasma membrane.

Trafficking Of Ion Channels As A Mechanism Of Plasticity

As ion channels are pores for charged species to cross the plasma membrane, endocytosis of channels is a physical mechanism for regulating excitability. Indeed, many studies have demonstrated that trafficking of ion channels is an important mechanism in
the modulation of synaptic function. Compared to sparse knowledge on voltage-gated ion channels, a large body of literature concerns studies that have examined how the ionotopic α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) type, and the N-methyl-D-aspartic acid (NMDA) type, glutamate receptors are regulated by endocytosis and recycling at the plasma membrane to induce changes in synaptic function. Perhaps the best studied example is endocytosis of the AMPA receptor, whose regulated trafficking is important in both hippocampal and cerebellar plasticity. Cerebellar long term depression (LTD) can be induced when both parallel fiber and climbing fibers are simultaneous stimulated. Subsequently, the efficacy of parallel fiber input is reduced, and there is an accompanying reduction in postsynaptic function of AMPA receptors (Ito, Sakurai et al. 1982; Crepel and Krupa 1988; Linden and Connor 1991; Linden, Dickinson et al. 1991). The decrease in AMPA receptor responsiveness was found to require endocytosis (Wang and Linden 2000), and it was proposed that activated protein kinase C physically interacts with AMPA receptors that contain GluR2/3 subunits to stimulate their endocytosis from the postsynaptic membrane (Xia, Chung et al. 2000). It was then found that the PKC mediated endocytosis of AMPA receptors required dissociation of the GluR2 subunit of AMPA receptors from its adaptor protein, glutamate receptor interacting protein, which led to decreased stability of AMPA receptors at the cell surface, and to the channels internalization during the induction of LTD (Hirai 2001). Just as long term depression was found to involve increased AMPA channel endocytosis from the plasma membrane, long term potentiation was found to involve an increased insertion of AMPA receptors into the plasma membrane (Kakegawa
and Yuzaki 2005). Although this is a small sample of the literature, it provides an example of how regulated ion channel trafficking at the plasma membrane can be an important physiological mechanism for tuning synaptic function. Though Kv1.2 also modulates synaptic function, it is unknown whether endocytic trafficking is a form of Kv1.2 regulation in the brain, and therefore what cell and molecular pathways may be involved. However, in heterologous expression systems, Kv1.2 is known to be regulated by G-protein coupled receptors (GPCR) and the mechanism involves channel endocytosis.

**Regulation Of Kv1.2 By Channel Trafficking At The Plasma Membrane**

Receptor mediated regulation of Kv1.2 has been shown in numerous experimental paradigms. Initially, studies in Xenopus oocytes and in mammalian cell lines demonstrated that activation of the G_q protein coupled M1 muscarinic receptor reduces Kv1.2 ionic current (Huang, Morielli et al. 1993; Tsai, Morielli et al. 1997). Similarly, in Xenopus oocytes, it was found that stimulation of the endothelin GPCR inhibits Kv1.2 function (Murakoshi, Ishii et al. 1994), and the beta-2 adrenergic receptor, which couples to G_s, was also found to regulate Kv1.2 function (Huang, Morielli et al. 1994). Further support for a central role for GCPRs in the regulation of Kv1.2 was the demonstration that over-expression of RhoA, a GPCR effector, could suppress Kv1.2 current, and that RhoA inhibitors blocked M1 receptor mediated suppression of Kv1.2 current (Cachero, Morielli et al. 1998). These studies provided several examples that Kv1.2 function can be modulated by GPCR signaling. Mechanistically, GPCR mediated Kv1.2 regulation was found to involve trafficking of the ion channel to and from the plasma membrane.
Changes In cAMP Can Regulate Kv1.2 Channel Trafficking

Several studies in an immortalized human embryonic kidney (HEK293) cell line have demonstrated how GPCR activation can change Kv1.2 function by regulating the channels trafficking. Stimulation of the $G_q$ coupled M1 receptor was known to cause a reduction in Kv1.2 current, and it was discovered that this occurred by stimulating endocytosis of the channel from the plasma membrane (Nesti, Everill et al. 2004; Williams, Markey et al. 2007). Similarly, activation of the $G_{12/13}$ effector RhoA, known to reduce Kv1.2 channel function (Cachero, Morielli et al. 1998), was found to stimulate endocytosis of Kv1.2 from the plasma membrane (Stirling, Williams et al. 2009).

Activation of GPCRs which modulate adenylate cyclase were also found to regulate Kv1.2 trafficking at the plasma membrane, and it was proposed that increases in cyclic adenosine monophosphate (cAMP) levels could induce increases or decreases in Kv1.2 surface expression, depending on Protein Kinase A (PKA) and basal cAMP levels (Connors, Ballif et al. 2008). There is further evidence that the Gs/cAMP/PKA pathway can regulate Kv1.2 surface expression: Kv1.2 can be serine-phosphorylated by PKA (Johnson, El-Yazbi et al. 2009), and serine phosphorylation of Kv1.2 alters its surface expression (Yang, Vacher et al. 2007; Connors, Ballif et al. 2008). In summary, Kv1.2 function can be regulated through GPCR signaling and the mechanisms involve Kv1.2 trafficking to and from the plasma membrane. Most salient to this dissertation is the model that GPCR mediated stimulation of adenylate cyclase can regulate Kv1.2 function by increasing channel trafficking to, or trafficking from, the plasma membrane.
Evidence That Presynaptic Kv1.2 May Be Regulated Via cAMP

There is data to suggest that there may be AC/cAMP mediated regulation of Kv1.2 in the brain. In the striatum, the G_i coupled D2 receptor modulates dopamine release through a mechanism apparently involving an increase in functional Kv1.2 (Fulton, Thibault et al. 2011). Likewise, for neurons of the basolateral amygdala, the G_i coupled Mu opioid receptor seems to oppose neurotransmission through a mechanism involving increases in functional Kv1.2 (Zhu and Pan 2005; Finnegan, Chen et al. 2006). Conversely, in cerebellar basket cells, which abundantly express Kv1.2 in their synaptic terminals, the G_s coupled secretin receptor potentiates neurotransmitter release and the suppression of potassium channels has been raised as potential mechanism (Yung, Leung et al. 2001). Collectively, these studies hint that in the brain, AC/cAMP signaling may mechanistically regulate Kv1.2 functional abundance. The main goal of the research herein was to test this hypothesis by experimentally addressing whether AC stimulation could stimulate endocytosis of synaptic Kv1.2 in the mammalian nervous system.

Kv1.2 Is Enriched In Cerebellar Basket Cell Axon Terminals

This dissertation focused on the regulation of Kv1.2 in a region of the brain where its expression is high and some of its functional roles have been established: the cerebellum. The first demonstration of a presynaptic localization for a voltage gated potassium channel was the finding that Kv1.2 is enriched in a specialized structure formed by cerebellar inhibitory interneuron basket cell axon terminals, termed the pinceau by Ramón y Cajal (McNamara, Muniz et al. 1993). Basket cells makes a GABAergic synapse upon the soma and initial axon segment of Purkinje cells, the only
efferent neuron of the cerebellar cortex. The related voltage gated potassium channel, Kv1.1, is also enriched in the pinceau, though Kv1.2 expression is considerably higher (Chung, Joo et al. 2005). In fact, although Kv1.2 is found throughout the brain, its expression is highest at the pinceau (Chung, Shin et al. 2001). Within this structure, Kv1.2 channels are enriched at “septate-like junctions”, where axon collaterals branch and coalesce with axon collaterals from other basket cells (Laube, Roper et al. 1996). This enrichment of Kv1.2 to the pinceau is not exclusive, as Kv1.2 is also found in Purkinje cells dendrites (Khavandgar, Walter et al. 2005) but not in the granule cell layer, and only sparsely and variably in the neuropil of certain deep cerebellar nuclei (McNamara, Averill et al. 1996; Chung, Shin et al. 2001). Cerebellar Kv1.2 containing channels consist mainly of Kv1.1: Kv1.2 heteromultimers (80%), and of Kv1.2 homomultimers (20%) (Koch, Wanner et al. 1997). Therefore, the cerebellum offers an abundance of Kv1.2 channels of relatively well described distribution and composition whose potential regulation by AC stimulated endocytic trafficking can be investigated.

**Presynaptic Kv1.1 And Kv1.2 Control GABA Release From Basket Cells**

Not only are Kv1.1 and Kv1.2 channels enriched at basket cell axon terminals, they play a critical role in regulating transmitter release there. Curiously though, compared to basket cell axon terminals about PC soma, the distal aspects of basket cell axons that form the pinceau and abundantly express Kv1.1 and Kv1.2 are relatively depleted in neurotransmitter vesicles and immunohistochemical markers of functional presynaptic sites. Together with electrophysiological experiments and comparisons to Mauthner cell physiology, it has been proposed that the basket cell pinceau formation
exert inhibition upon the initial axon segment of Purkinje cells not through neurotransmitters, but through passive hyperpolarizing potential field effects ((Korn and Axelrad 1980; Iwakura, Uchigashima et al. 2012). However, direct evidence for these field effects, their physiological importance, or the ion channels perhaps responsible, is not yet available. Thus, while Kv1.2 may be most abundant in an aspect of the basket cell axon theoretically suited for electrical inhibition, presynaptic Kv1.2 has an experimentally confirmed role in classical chemical inhibition of the postsynaptic Purkinje cell.

The role of Kv1.2 channels in the basket cell axon terminal has been demonstrated primarily using channel blocking toxins. Micromolar concentrations of alpha-dendrotoxin (α-DTX), which inhibits Kv1.2 and Kv1.1 containing channels, increases the frequency of inhibitory post-synaptic currents (IPSC’s) recorded in Purkinje cells (Southan and Robertson 1998; Tan and Llano 1999). The α-DTX sensitive channels are only found in the terminals, and not the soma, of basket cells, which is consistent with the staining pattern for Kv1.2 in the pinceau. Further, there are no α-DTX sensitive channels within the Purkinje cell soma (Southan and Robertson 2000). Therefore application of α-DTX, and the subsequent loss of Kv1.2 function, enhances the frequency of IPSC’s in Purkinje cells by a presynaptic mechanism, again consistent with the known localization of Kv1.2 at the pinceau. Two presynaptic mechanisms by which blocking Kv1.2 could increase the frequency of GABA release from basket cells are controlling calcium influx by setting terminal excitability, or decreasing the number of synaptic failures (Southan and Robertson 1998; Southan and Robertson 1998). The latter
role is supported by the finding that Kv1.2 channels do not contribute to regulating action potential induced calcium influx at the basket cell axon terminals (Tan and Llano 1999). However the temporal resolution of those imaging studies may be below the rate of calcium fluxes which control neurotransmitter release from basket cells, as the calcium flux at the basket cell terminal following an action potential is estimated to have a half width as low as 1 ms (Sakaba 2008). Therefore, whether Kv1.2 controls transmitter release by influencing calcium dynamics is unclear. However, the hypothesis that presynaptic Kv1.2 containing channels control failure rate has strong support.

Kv1.1 And Kv1.2 May Modulate Basket Cell Axon Branch Point Failures

Evidence that Kv1.2 may control failure probability in the basket cell terminal comes largely from studies of Kv1.1 knockout and Kv1.1 mutant mice. In mice harboring a reduction of function mutation (V408A) within Kv1.1, there is increased IPSC frequency within Purkinje cells, without a change in basket cell firing frequency or a change in mini IPSCs (Herson, Virk et al. 2003). In Kv1.1 knockout mice, Purkinje cell IPSC frequency is also increased, without any change in basket cell firing rate, in IPSC amplitude, or in mini IPSCs (Zhang, Messing et al. 1999). In both of these publications, the model proposed is that loss of Kv1.1 function decreases the likelihood of conduction failure at the axon branch points where the Kv1.1/Kv1.2 channels are enriched in the basket cell presynapse. A similar role for Kv1.2 is supported by experiments in which application of the Kv1.1 and Kv1.2 channel blocking toxin, a-DTX, similarly increased the frequency of Purkinje cell IPSC frequency just as the Kv1.1 knockout did, without changing basket cell firing rate (Zhang, Messing et al. 1999). Since Kv1.2 inhibition does
not alter basket cell firing, but since the presence of tetrodotoxin prevents the \( \alpha \)-DTX mediated enhancement of IPSCS recorded in Purkinje cells, the likely function of Kv1.2 in the pinceau is to increase the failure rate at the unique axon branch points of the pinceau where these channels are enriched (Southan and Robertson 1998). Knowing the consequence of cerebellar BC Kv1.2 suppression led to an informed search for \( G_s \) coupled receptors expressed in BC axon terminals and PC dendrites whose activation mimicked the consequences of Kv1.2 suppression. Identifying a candidate AC/cAMP system to test whether endocytic suppression of Kv1.2 occurred natively in the cerebellum was advanced by research into Purkinje cell released factors which act retrogradely to regulate GABAergic transmission at the pinceau.

**Basket Cell GABA Release Can Be Potentiated By Retrograde Secretin Release**

A form of synaptic regulation at the BC:PC synapse is depolarization induced potentiation of inhibition (DPI) (Diana and Marty 2003). In this phenomenon, depolarization of the PC is thought to lead to the release of factors which diffuse retrogradely to enhance presynaptic GABA release (the same consequence as presynaptic Kv1.2 suppression). The identity of the retrograde factor which enhances GABA release in DPI has been a matter of debate, and there is a proposed role for glutamate. Glutamate released directly from depolarized PC somas, or spillover from its excitatory afferents, may reach presynaptic AMPA and NMDA receptors at the BC, increasing the release of calcium from internal stores to enhance the probability of transmitter release (Duguid and Smart 2004). This model is consistent with the observation that internal calcium stores can regulate transmitter release from basket cells (Galante and Marty 2003). However,
evidence of somatic glutamate release from Purkinje cells in mature animals and in physiologically relevant settings is wanting (Tanimura, Kawata et al. 2009). Therefore, any glutamate mediated effects that may occur are considered to be an indirect pathway in DPI (Lee, Chen et al. 2005). A second retrograde factor thought to be released from Purkinje cells is the peptide secretin.

The Peptide Secretin Is Expressed In The Brain

Secretin was the first hormone described, and it has long established functions in stimulating pancreatic secretions in the gastrointestinal system (Bayliss and Starling 1902; Bayliss and Starling 1905). The role of secretin in the central nervous system, however, is a more recent line of research. Early studies showed that brain extracts could act like secretin when tested for their ability to stimulate pancreatic secretions (Mutt, Carlquist et al. 1979). Reciprocally, it was shown that secretin from the gastrointestinal tract could modulate neurotransmitter release in the brain (Fuxe, Andersson et al. 1979). These studies suggested that the brain contained functional secretin and secretin receptors. Subsequent immunohistochemical studies demonstrated secretin immunoreactivity in the central nervous system of diverse organisms, including the hover fly (El-Salhy, Abou-el-Ela et al. 1980), sea squirt (Fritsch, Van Noorden et al. 1982), tobacco hornworm moth (El-Salhy, Falkmer et al. 1983), rats and pigs, (Charlton, O'Donohue et al. 1981; O'Donohue, Charlton et al. 1981), and humans (Koves, Kausz et al. 2004). Within an organism, the secretin peptide and its receptor are thought to be identical whether they are found in the gastrointestinal tract or in the brain (Chang, Berger-Ornstein et al. 1985; Itoh, Furuya et al. 1991; Ohta, Funakoshi et al. 1992).
Secretin Is Expressed In Purkinje Cells, The Secretin Receptor In Basket Cells

Within the brain, secretin expression is highest in the cerebellum (O'Donohue, Charlton et al. 1981), and in particular, is abundantly expressed in Purkinje cells (Koves, Kausz et al. 2002; Koves, Kausz et al. 2004; Lee, Yung et al. 2005). The receptor for secretin is also enriched in the cerebellum. In fact, the highest degree of secretin binding in the brain is in the cerebellum, and these binding sites are a thousand fold selective for secretin over other related peptides such as vasoactive intestinal peptide and peptide histidine isoleucine (Fremeau, Jensen et al. 1983; Nozaki, Nakata et al. 2002). Consistent with a high degree of secretin binding, secretin receptor mRNA is abundantly found in the cerebellum (Tay, Goulet et al. 2004), and transcripts for the secretin receptor are localized to Purkinje cells and to basket cells. In the cerebellum then, secretin is expressed in Purkinje cells, while the secretin receptor is in the presynaptic basket cell and in the Purkinje cell. This organization suggested that secretin maybe a retrograde factor from the Purkinje cell for modulating basket cell function (Yung, Leung et al. 2001) and could therefore potentially regulate the Kv1.2 expressed there.

Secretin: Released From Purkinje Cells, Enhances Basket Cell GABA Release

Evidence for secretin as the retrograde factor in DPI initially came from studies that showed exogenous secretin increased IPSC’s in Purkinje cells, just as other studies had shown Purkinje cell depolarization did. The secretin induced increase in GABA release from basket cells was specific, as it was not induced by peptides in the same family, such as by vasoactive intestinal peptide or by pituitary adenylate cyclase activating peptide. Consistent with the fact that the secretin receptor is Gs coupled, the
enhancement of inhibition by secretin required adenylate cyclase (Yung, Leung et al. 2001). Although exogenous secretin could enhance GABA release from basket cells, just as depolarization of Purkinje did, there was no evidence that secretin was indeed released from depolarized Purkinje cells. However, studies on blocks of freshly dissected cerebella demonstrated that not only was secretin released basally, but that this release could be enhanced by depolarizing the tissue. Furthermore, it was demonstrated that electrophysiological activation of Purkinje cells could enhance basket cell GABA release when other mediators of the BC:PC synapse were blocked but secretin signaling was left intact (Lee, Chen et al. 2005). Therefore, there is evidence that exogenous or endogenous secretin acts at basket cells through AC to increase GABA release from basket cells.

**Unknown Mechanisms For Secretin Modulated GABA Release From Basket Cells**

The mechanisms by which secretin enhances basket cell GABA release are largely unknown. While the secretin receptor is found both presynaptically at the basket cell terminal and at Purkinje cell dendrites, evidence points to a presynaptic site of action for secretin in the enhancement of basket cell GABA release. Specifically, secretin reduces the paired pulse ratio, and increases the frequency of sIPSC's recorded in Purkinje cells, without influencing basket cell firing rate; secretin is thought then to act by decreasing the failure rate of the basket cell, in a process that depends on the stimulation of adenylate cyclase (Yung, Leung et al. 2001). Indeed, the secretin receptor is thought to couple preferentially, if not exclusively, to $G_s$ (Reviewed in (Siu, Lam et al. 2006)). Since the secretin receptor and Kv1.2 are localized to the pinceau, and as the activation of the $G_s$ coupled secretin receptor mimics Kv1.2 suppression (enhancing
GABA release), the cerebellum offered a strong platform to ask whether AC mediated suppression of Kv1.2 via the enhancement of channel endocytosis occurred in the mammalian brain.
CHAPTER 2: CELLULAR MECHANISMS AND BEHAVIORAL CONSEQUENCES OF KV1.2 REGULATION IN THE RAT CEREBELLMUM.

Abstract

The potassium channel Kv1.2 alpha-subunit is expressed in cerebellar Purkinje cell (PC) dendrites where its pharmacological inhibition increases excitability (Khavan, Walter et al. 2005). Kv1.2 is also expressed in cerebellar basket cell (BC) axon terminals (Sheng, Tsaur et al. 1994), where its blockade increases BC inhibition of PCs (Southan and Robertson 1998). Secretin receptors are also expressed both in PC dendrites and BC axon terminals (reviewed in (Yuan, Lee et al). The effect of secretin on PC excitability is not yet known, but, like Kv1.2 inhibitors, secretin potently increases inhibitory input to PCs (Yung, Leung et al. 2001). This suggests secretin may act in part by suppressing Kv1.2. Receptor-mediated endocytosis is a mechanism of Kv1.2 suppression (Nesti, Everill et al. 2004). This process can be regulated by protein kinase A (PKA) (Connors, Ballif et al. 2008). Since secretin receptors activate PKA (Wessels-Reiker, Basiboina et al. 1993), we tested the hypothesis that secretin regulates Kv1.2 trafficking in the cerebellum. Using cell surface protein biotinylation of rat cerebellar slices, we found secretin decreased cell-surface Kv1.2 levels by modulating Kv1.2 endocytic trafficking. This effect was mimicked by activating adenylate cyclase (AC) with forskolin, and was blocked by pharmacological inhibitors of AC or PKA. Imaging studies identified the BC axon terminal and Purkinje cell dendrites as loci of AC-dependent Kv1.2 trafficking. The physiological significance of secretin regulated Kv1.2 endocytosis is supported by our finding that infusion into the cerebellar cortex of either
the Kv1.2 inhibitor Tityustoxin-Ka, or of the Kv1.2 regulator secretin, significantly enhances acquisition of eyeblink conditioning in rats.

Introduction

Kv1.2 is an alpha subunit of voltage gated potassium channels that provide outward rectifying currents in diverse cell types. In the brain, Kv1.2 is abundantly expressed in the cerebellar cortex (Wang, Kunkel et al. 1994; Chung, Shin et al. 2001) where it strongly affects Purkinje cell (PC) activity. Kv1.2 influences excitatory synaptic input to PCs by opposing their dendritic excitability, most likely through its effect on membrane potential (Khavandgar, Walter et al. 2005; McKay, Molineux et al. 2005). Kv1.2 also affects inhibitory synaptic input to Purkinje cells; pharmacological blockade of Kv1.2 in axon terminals of basket cells (BC) increases inhibitory post synaptic currents (IPSCs) recorded in PCs. This occurs in part by removing a shunting pathway normally provided by Kv1.2 in BC axon terminals (Laube, Roper et al. 1996; Southan and Robertson 1998).

Since Kv1.2 affects excitation and inhibition of PCs, and since PCs are the sole output of the cerebellar cortex, changes in Kv1.2 functional abundance would be expected to affect cerebellar dependent behaviors. This idea is supported by the identification of a mutation within Kv1.2 that reduces the channel’s surface expression and causes cerebellar ataxia in mice (Xie, Harrison et al. 2010). In HEK293 cells, surface expression and electrophysiological function of Kv1.2 is regulated by its endocytic trafficking (Nesti, Everill et al. 2004). In this heterologous expression system, Kv1.2 trafficking is regulated by adenylate cyclase (AC) and protein kinase A (PKA).
(Connors, Ballif et al. 2008), raising the possibility that AC-dependent trafficking of Kv1.2 occurs in the cerebellum. The Gs-coupled secretin receptor is a candidate for AC mediated regulation of cerebellar Kv1.2 because, like Kv1.2, it is expressed in PCs and BC axon terminals (reviewed in (Yuan, Lee et al.). There is functional support for this idea, as application of secretin to rat cerebellar slices increases inhibitory postsynaptic current activity recorded from PCs (Yung, Leung et al. 2001), a phenomenon that can also be evoked by pharmacological inhibition of Kv1.2 in BC axon terminals (Southan and Robertson 1998; Southan and Robertson 1998). Lastly, while the downstream targets of secretin stimulated AC are not well characterized, the regulation of presynaptic potassium conductance has been raised as a potential mechanism (Yung, Leung et al. 2001). We therefore tested the hypothesis that cerebellar Kv1.2 is regulated by secretin.

Here we report that secretin decreases cell surface expression of cerebellar Kv1.2 through a process involving AC/PKA-dependent channel endocytosis. We provide evidence that Kv1.2 trafficking occurs in the pinceaus of BC axon terminals and in Purkinje cell dendrites. A functional role for this regulation is supported by our finding that infusion of Tityustoxin-Kα (TsTx) into the cerebellar cortex enhances eye-blink conditioning (EBC) and that infusion of secretin has the same effect, likely through the net inhibition of PC output. Taken together our data support the hypothesis that secretin regulates Kv1.2 in vivo and that this regulation is behaviorally significant.
Materials And Methods

Generation Of Cerebellar Slices

Parasagittal 250-400 micron thick cerebellar sections were generated from 3-6 week old male Sprague-Dawley rats using a Leica VT1000S vibratome. Dissection and slice generation was in ice-cold modified artificial cerebrospinal fluid (ACSF) in which NaCl was replaced by sucrose (in mM: 250 sucrose, 26 NaHCO₃, 2.5 KCl, 3 MgCl₂, 1 CaCl₂, 10 glucose, 1.25 NaH₂PO₄, 0.4 sodium ascorbate, gassed with 95% O₂, 5% CO₂) (Moyer and Brown 1998; Southan and Robertson 1998). After generation, slices were briefly maintained in room temperature ACSF (in mM: 125 NaCl, 26 NaHCO₃, 2.5 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose, 1.25 NaH₂PO₄, 0.4 sodium ascorbate, gassed with 95% O₂, 5% CO₂) until transferred to interface culture (De Simoni and Yu 2006). Pharmacological agents were added directly to culture media after slices had recovered in culture for at least an hour. Slices were cultured and exposed to pharmacological manipulations individually. For biotinylation assays (except the Gabazine series), and for the imaging studies, slices were pretreated with 1 μM Tetrodotoxin (EMD Chemicals) to prevent effects from altered sodium action-potential activity by the other drug treatments.

Biotinylation Of Surface Proteins

After drug treatments, cerebellar slices were individually biotinylated with 1 mL of sulfo-nhs-ss-biotin, “biotin”, (Pierce) at 2 mg/mL in ice cold Hank’s Balanced Salt Solution (HBSS), a concentration we determined sufficient to recover all Kv1.2 from a permeabilized slice. Unreacted biotin was quenched with 50mM TRIS. For
internalization assays, slices were biotinylated, quenched, then returned to interface culture for pharmacological treatment, after which remaining surface biotinylated proteins were cleaved by two 20 minute incubations in ice cold 50mM glutathione, 75 mM NaCl, 10 mM EDTA, 0.1 % bovine serum albumin, 0.075 N NaOH (Yang, Huang et al. 2005). In the indicated experiments of figure 4, slices were extracted in acetone after biotinylation and quenching, before lysate generation.

**Preparation Of Biochemical Samples**

Biotinylated cerebellar slices were individually sonicated in lysis buffer (50 mM TRIS, 150 mM NaCl, 1 mM EDTA, 0.25% deoxycholate, 1% NP40, 10% glycerol, mammalian protease inhibitor cocktail (Sigma), pH 8.0), and the lysate was then clarified by centrifugation. A portion of clarified “Total” lysate was reserved and the remainder was incubated with high-capacity neutravidin bead slurry (Pierce). Neutravidin beads and bound biotinylated proteins were isolated by centrifugation and washed repeatedly with lysis buffer. In some experiments, a portion of the post-neutravidin supernatant lysate was analyzed as a “Post” sample. Biotinylated proteins were eluted from neutravidin beads by incubation at 100° C in Laemmlli sample buffer containing 0.1M dithiothreitol (Sigma) to produce the “Eluant”. Total and Post lysate samples were similarly prepared for immunoblot by incubation at 100°C in sample buffer and dithiothreitol.

**Biotinylation Sample Analysis**

For each slice, Total and Eluant samples were resolved by poly-acrylamide gel electrophoresis and protein immunoblot as previously published (Williams, Markey et al.)
2007). Primary antibodies were mouse monoclonal α-Kv1.2 (K14/16, Neuromab) and rabbit polyclonal α-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), (ab9485, Abcam).

According to the supplier, the α-Kv1.2 antibody immunogen is rat Kv1.2, giving no reported reactivity towards Kv1.1, Kv1.3, Kv1.4, Kv1.5, or Kv1.6, or towards brain derived samples from the Kv1.2 knockout mouse.

Infrared secondary antibody signals were detected and quantified using an Odyssey Infrared Imager (LiCor). Surface Kv1.2 was calculated by dividing the density of Kv1.2 signal in the Eluant by the density of Kv1.2 signal in the Total for that slice. Total Kv1.2 was determined by dividing the density of Kv1.2 signal in the Total by the density of GAPDH signal in the same Total sample. The proportion of Kv1.2 bound to neutravidin was calculated by dividing the density of Kv1.2 signal in the Post fraction by that in the Total fraction, normalized to the ratio from a non-biotinylated sample in the same experiment to control for non-specific loss.

Biotinylation Sample Statistics

Within an experiment, Surface and Total Kv1.2 values were normalized to the average of vehicle-treated slices. Each experiment was repeated using slices derived from multiple rats, and normalized values from individual experiments were pooled for analysis. Because each slice was cultured, drug treated, biotinylated, lysed, and evaluated by western blot individually, “n” is the total number of slices from all animals per pharmacological condition. The n for each condition is identified in the text as “n (vehicle), n (treatment); from x rats”. Effects of pharmacological manipulations are
expressed graphically and in text as the mean percent change relative to vehicle. In
graphs, error bars indicate standard error of the mean. Statistical significance is indicated
by an asterisk (*), defined as a p value less than or equal to 0.05 by unpaired t-test.
Welch’s correction was used if the variance was unequal between control and stimulus
groups (Ruxton 2006).

**ATTO 594-Tityustoxin-Kα**

A linear 37mer peptide for Tityustoxin-Kα (ATTO-TsTx) with an Alanine to
Cysteine substitution at site 20 was synthesized by CelTek Bioscience, Nashville, TN.
Conjugation to the fluorescent dye ATTO 594, refolding, purification, and validation of
toxin activity by electrophysiology on rat Kv1.2 expressed in Xenopus Oocytes was
performed by Alomone Labs, Jerusalem, Israel.

**ATTO-TsTx Microscopy**

Rat cerebellar slices that were vehicle or forskolin treated as above were gently
rinsed in ice cold HBSS before fixation on ice in 4% formaldehyde in HBSS. Slices were
incubated overnight in 3 nM ATTO-TsTx, rinsed, and post-fixed. Slices were mounted
with Prolong Gold anti-fade (Invitrogen) and imaged using the DeltaVision Restoration
Microscopy System (Applied Precision). Images were processed with ImageJ software.
Parallel experiments compared α-Kvβ2 (Neuromab, 75-021) labeling in slices fixed under
the same conditions, followed with or without permeabilization by ice cold acetone.
Positive Kvβ2 staining was compared to IgG control, detected by an Alexa 647-
conjugated secondary antibody (Invitrogen).

**Kv1.2 Immunofluorescence**

Rat cerebellar slices that were vehicle or forskolin treated as above were gently rinsed in ice cold HBSS, then extracted with ice cold acetone before by fixation in 4% formaldehyde in HBSS. Slices were then incubated in blocking buffer (10% normal goat serum in phosphate buffered saline (PBS)), then in primary and secondary antibodies. Primary antibodies were mouse monoclonal α-Kv1.2 (Neuromab) or isotype matched mouse IgG. The secondary antibody was goat anti-mouse Alexa 568 (Invitrogen). Slices were mounted with Prolong Gold anti-fade (Invitrogen) and imaged using the DeltaVision Restoration Microscopy System (Applied Precision). Images were processed with ImageJ software.

**Microscopy Analysis**

Within distinct areas of the cerebellar cortex, multiple Fields of View (FOV) were acquired. The sum projections of serial z-sections for each Field of View (FOV) were generated to collapse dimensionality. Within each FOV projection, a Region of Interest (ROI) was defined for the molecular layer, each pinceau, and within the granule cell layer. The mean intensity of ATTO-TsTx in granule cell ROI’s was subtracted from the intensity measured in molecular layer and in pinceau ROI’s to control for local variations in slice thickness and for experimental variability. For experiments using α-Kv1.2, the background was defined by isotype matched IgG incubated slices. These background corrected Kv1.2 intensity measurements from an individual animal in the vehicle treatment for the same area of interest (Molecular Layer or Pinceau) were
averaged together for normalization of the percent change observed in forskolin treated slices. Effects of pharmacological manipulations are expressed graphically and in text as the mean percent change relative to vehicle, “n” is the total number of ROI’s from all animals per pharmacological condition per area of study. The n for each condition is identified in the text as “n (vehicle), n (treatment); from x rats”. Statistical significance is indicated by an asterisk (*), defined as a p value less than or equal to 0.05 by unpaired t-test.

**Electrophysiology**

Sagittal cerebellar slices generated as described above were maintained for at least 1 hour in room temperature ACSF bubbled with carbogen in a home-made maintenance chamber prior to being transferred to the recording chamber. Slices were visualized using a 60X water immersion objective and infra-red differential interference contrast optics on an upright Olympus microscope. Temperature was maintained at 35 ºC and the recording chamber was perfused with carbogenated ACSF at 1.5 – 2 ml/min. Single-cell extracellular recordings of Purkinje cell action-potential activity was with a patch electrode filled with ACSF and an Axopatch 200D amplifier interfaced via a Digi-Data 1322A D/A converter to a Windows-based computer running the pCLAMP data acquisition program Clampex (Molecular Devices, Sunnyvale, CA). Data analysis was performed with the pCLAMP data analysis program Clampfit (Molecular Devices) and with Minianalysis (Synaptosoft). Action potential activity was measured in a one minute window before secretin (30 nM) and compared to in a one minute window starting at 15
minutes after secretin addition. Each reported recording was from a PC in individual slices derived from 6 animals.

_Eyeblink Conditioning Subjects_

Subjects were 30 male Wistar rats from Harlan (Indianapolis, IN). Rats were between 59 and 63 days old when they arrived in the colony. After arrival, the rats were housed individually for approximately one week prior to surgery with ad libitum chow and water. The colony was maintained on a 12 hour light-dark cycle (lights on at 7 am). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

_Eyeblink Conditioning Surgery_

Rats were anesthetized using 3% isoflurane in oxygen and, using aseptic surgical procedures, each rat was surgically prepared with a 22 gauge guide cannula that was implanted in left cerebellar cortex (lobulus simplex), ipsilateral to the conditioned eye, at the following coordinates: -11.3 Anterior-Posterior (from bregma), +2.5 Medio-Lateral, and -3.1 Dorso-Ventral. Subsequently, rats were fitted with differential electromyographic (EMG) recording wires for recording eyeblinks and a bipolar periocular stimulation electrode for delivering the eye stimulation unconditioned stimulus (US). The EMG wires for recording activity of the external muscles of the eyelid, the orbicularis oculi, were constructed of two strands of ultra-thin (75 μm) Teflon-coated stainless steel wire soldered at one end to a mini-strip connector. 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 the insulation was removed. The bipolar stimulation electrode (Plastics One) was positioned subdermally immediately dorsocaudal to the left eye. The mini-strip connector and the bipolar stimulation electrode were adhered to the skull with dental cement. In addition, a ground wire was connected to three stainless steel skull screws. The wound was salved with antibiotic ointment (Povidone), and an analgesic (buprenorphine) was administered subcutaenously immediately after surgery and twice the following day. Rats were given a minimum of 5 days to recover prior to eyeblink conditioning.

*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), each with a grid floor. The top of each chamber was modified so that a 25-channel tether/commutator could be mounted to it. Each testing chamber was housed within an electrically-shielded, sound-attenuating chamber (45.7 x 91.4 x 50.8 cm; BRS-LVE). 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 house light (off during testing) was mounted in the center of the rear wall of each sound-attenuating chamber. The sound-attenuating chambers were housed within a walk-in sound-proof chamber.

Stimulus delivery and recording of eyelid EMG activity were controlled by a computer interfaced with a Power 1401 high-speed data acquisition unit and running Spike2 software (CED). A 2.8 kHz, 80 dB tone (765-ms for the TsTx experiment; 865-ms for the secretin experiment) served as the conditioned stimulus (CS). A 15 ms, 4.0
mA unipolar periocular stimulation, delivered from a constant current stimulator (model A365D; World Precision Instruments), served as the US. The eyelid EMG signals were amplified (10k) and band-pass filtered (100-1000 Hz) prior to being passed to the Power 1401 and from there to a computer running Spike2 (CED). Spike2 was used to full-wave rectify, smooth (10 ms time constant), and time shift (10 ms, to compensate for smoothing) the amplified EMG signal.

Eyeblink Conditioning Procedure

At the beginning of each session, each rat’s cemented connector was attached 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. On Day 1 (adaptation), rats were plugged in but no stimuli were delivered. They remained in the chamber for 60 min (the approximate length of a training session). Spontaneous eyelid EMG activity was sampled for the same duration and at the same time points as during the subsequent conditioning sessions. On Days 2-8 (conditioning), rats received 100 trials per day, with an average inter-trial interval of 30 sec (range = 20-40 sec). Each block of 10 trials consisted of the following trial sequence: 4 CS-US trials (CS preceding and co-terminating with the US), 1 CS-alone trial, 4 CS-US trials, and 1 US-alone trial.

Two separate behavioral experiments were conducted. In the first experiment, immediately prior to the first 6 days of conditioning, rats underwent an infusion of 1 µl of either 0.25 µg TsTx or phosphate-buffered saline vehicle. In the second experiment, immediately prior to the first 3 days of conditioning, rats underwent an infusion of 1 µl of either 1.0 µg secretin or phosphate-buffered saline vehicle. For infusions, the dummy
cannula was removed and a 28 gauge internal cannula was inserted into the guide cannula. The internal cannula protruded 1 mm below the guide cannula tip; thus, infusions were delivered at 4.1 mm below bregma. Infusions were made with a 10 µl Hamilton syringe loaded onto an infusion pump (KD Scientific) set to deliver 1 µl over a period of 2 minutes. At the end of the infusion period, the internal cannula remained in place for an additional 1 min to allow diffusion of the solution away from the cannula tip. Subsequently, the internal cannula was removed, the dummy cannula was replaced, and the conditioning procedure began. Rats were infused and tested in groups of 4 (2 from each group).

**Histology For Cannula Placement**

Following the last day of training, rats were overdosed with sodium pentobarbital (150 mg/kg) and transcardially perfused with 0.9% saline followed by 10% formalin. A small amount of direct current (100 µA for 10 sec) was passed through an insulated 00 (0.3 mm diameter) stainless steel insect pin inserted into the cannula so that the tip of the insect pin, which was uninsulated, protruded approximately 1 mm below the guide cannula tip. Subsequently, the brain was removed and stored in 10% formalin.

Approximately 3 days prior to sectioning, brains were transferred to a 30% sucrose/10% formalin solution. On the day of sectioning, cerebella were embedded in albumin-gelatin, frozen, and 60-µm coronal sections were taken with a cryostat through areas with cannula placements. The tissue was mounted on gelatin-subbed slides, and stained with cresyl violet (for cell bodies) and Prussian blue (for iron deposits from the
marking lesions). Slides were coverslipped with Permount and examined under a microscope by an observer blind to group membership to confirm cannula placement.

**EBC Analysis**

CS-US trials were subdivided into three time periods: (1) a “baseline” period, 280 ms prior to CS onset; (2) a “startle” period, 0-80 ms after CS onset and a (3) a CR period, 81-750 ms (TsTx experiment) or 81-850 ms (secretin experiment) after CS onset.

Eyeblinks that exceeded mean baseline activity by 0.5 arbitrary units (range = 0.0 to 5.0) during the CR period were scored as CRs. This time point was also defined as CR onset. The difference in time (in milliseconds) between CS onset and CR onset represents CR onset latency. The maximum eyelid closure during the CR was CR amplitude. Eyeblinks that met the response threshold during the startle period were scored as startle responses (SRs). UR amplitude was assessed on US-alone trials 65-165 after US onset (the first 65 ms was obscured by the shock artifact). Data were analyzed using SPSS 20.0.0. An alpha level of 0.05 was used as the rejection criterion for all statistical tests.

**Results**

**Characterization Of Kv1.2 Trafficking In The Cerebellum**

**Quantification of surface and internal Kv1.2 pools using a biotinylation assay**

Pharmacological and genetic studies demonstrate the importance of Kv1.2 to cerebellar function. Kv1.2 electrophysiological activity is controlled by receptor-mediated regulation of its trafficking at the cell surface (Nesti, Everill et al. 2004;
As a first step towards examining Kv1.2 trafficking in the cerebellum, we used a biotinylation assay to quantify the proportion of total Kv1.2 at the cell surface in rat cerebellar slices. Application of the biotinylation reagent sulfo-nhs-ss-biotin (Pierce) to cerebellar slices labeled surface but not intracellular protein. By immunoblot we found that Kv1.2, but not GAPDH, was present in the Eluant from neutravidin beads incubated with lysates of biotinylated cerebellar slices, although both proteins were present in the Total lysate (Figure 1A). Kv1.2 was not present in the neutravidin eluant without biotinylation, confirming that that biotinylation allowed selective recovery of cell surface Kv1.2 (Figure 1A). Incubation with neutravidin removes biotinylated surface Kv1.2 from the Total lysate. Therefore, the abundance of Kv1.2 found in the post-neutravidin Post fraction, relative to the abundance in the Total fraction, defines the measurable surface pool of Kv1.2. Determining this ratio for non-biotinylated samples allowed normalization for non-specific protein loss. Using this method, we found the proportion of Kv1.2 in the Post versus Total fractions was significantly different in biotinylated samples (-36.46 ± 9.40%, p=0.0006, n=15, 15; 3 rats). By comparison, in these same samples, there was no difference in the proportion of GAPDH between these non-biotinylated and biotinylated samples (8.04 ± 6.58%, p=0.23, n=15, 15; 3 rats) (Figure 1B), validating the selective labeling of cell surface proteins. Our finding that the majority of Kv1.2 is not biotinylatable by these methods suggests the existence of regulated trafficking of Kv1.2 between the plasma membrane and intracellular compartments.
Secretin enhances inhibitory output from basket cells to PCs. Pharmacological inhibition of Kv1.2 (Southan and Robertson 1998; Tan and Llano 1999) mimics this effect, suggesting that secretin suppresses Kv1.2. Because suppression of Kv1.2 ionic current can be caused by channel endocytosis (Nesti, Everill et al. 2004), we measured the effect of exogenous secretin on surface Kv1.2 levels using cell surface protein biotinylation of rat cerebellar slices (Figure 1C). Secretin (30 nM, 15 min) (Anaspec) decreased surface Kv1.2 relative to vehicle (-13.71 ± 3.54%, p =0.0002, n=52, 51; 12 rats). In the presence of a secretin receptor antagonist, 5-27 secretin (Anaspec) (30 μM), secretin had no effect (-0.96 ± 10.68%, p=0.93, n=12, 9; 3 rats). To determine whether the secretin-induced decrease in surface Kv1.2 involved endocytosis, we measured the secretin modulated internalization of biotinylated Kv1.2 (Figure 1D). Secretin increased the amount of internalized Kv1.2 relative to vehicle (+8.24 ± 4.65%, p=0.047, n=22, 22; 3 rats). In the same slices, total levels of Kv1.2 (Figure 1E) protein did not differ (-0.86 ± 3.65%, p=0.82, n=22, 22; 3 rats). Endocytic trafficking of Kv1.2 involves both endocytosis from, and recycling back to, the plasma membrane (Stirling, Williams et al. 2009). Therefore, the increased internalized pool of Kv1.2 observed in secretin treated slices could be the result of either a net increase in Kv1.2 endocytosis from, or a net decrease in its recycling back to, the plasma membrane. While the internalization assay used here does not distinguish between these mechanisms, the results presented here demonstrate that secretin receptor signaling modulates the endocytic trafficking of Kv1.2 in the cerebellum.
Secretin Regulates Kv1.2 Trafficking Through The Gs/CAMP/PKA Pathway

Having found that secretin regulates cerebellar Kv1.2, we sought to determine the cellular mechanisms involved. Secretin receptors preferentially couple to Gs (Reviewed in (Siu, Lam et al. 2006)) and secretin enhancement of BC neurotransmission involves AC (Yung, Leung et al. 2001). Therefore, we hypothesized secretin acts through AC to regulate Kv1.2. In support of this, the AC activator, forskolin (100 μM, 15 minutes (Calbiochem) reduced surface Kv1.2 (-21.36 ± 9.71%, p=0.036, n=13, 17; 3 rats) (Figure 2). As with secretin, there was no corresponding change in total Kv1.2 (+8.17 ± 11.65%, p=0.48, n=16, 17; 3 rats). Confirming the role of AC, pretreatment with the AC inhibitor SQ-22536 (Calbiochem) (100 μM, 20 minutes), blocked secretin’s (+1.07 ± 6.08 %, p=0.86, n=17, 17; 3 rats), and forskolin’s (-2.26 ± 8.27 %, p=0.78, n=13, 13; 4 rats), effect on surface Kv1.2.

A previous study in HEK-293 cells suggests PKA enhances Kv1.2 endocytosis (Connors, Ballif et al. 2008). We therefore asked whether PKA was the effector of AC in the regulation of cerebellar Kv1.2. After pretreatment with the PKA inhibitor KT-5720 (5 μM, 30 minutes) (Calbiochem), neither secretin (-7.49 ± 5.17 %, p=0.16, n=15, 14, 3 rats), nor forskolin (-11.49 ± 10.30%, p=0.26, n=20, 21; 5 rats) altered surface expression of Kv1.2. In HEK cells, Kv1.2 endocytosis is dynamin dependent (Nesti, Everill et al. 2004) (Stirling, Williams et al. 2009). Consistent with this, we found that in cerebellar slices, pretreatment with the dynamin inhibitor, dynasore, (Calbiochem), (80 μM, 30 minutes), prevented the forskolin stimulated reduction of surface Kv1.2 (+7.57 ± 5.50 %, p=0.181, n=18, 17; 3 rats).
In contrast to PKA dependent endocytosis, cAMP generated by AC activity in HEK293 cells can also cause a PKA independent increase of surface Kv1.2 (Connors, Ballif et al. 2008). This bidirectional modulation increases the dynamic range of surface Kv1.2 that can result from AC stimulation. Therefore we tested whether forskolin stimulation could stimulate an increase in surface Kv1.2 independent of PKA dependent endocytosis in cerebellar slices. We found that, in cerebellar slices pre-treated with both KT-5720 (5 μM) and dynasore (80 μM) for 30 minutes, application of forskolin elicited a significant increase in surface Kv1.2 relative to vehicle control (+23.88 ± 7.32%, p=0.0032, n=18, 18; 3 rats) (Figure 2). Thus, although the cellular mechanisms for the bidirectional regulation of surface Kv1.2 by AC exist in the cerebellum, under our experimental conditions, stimulation of the secretin receptor results predominantly in an AC mediated PKA dependent decrease in surface Kv1.2.

**Regulation Of Kv1.2 By Endogenous Secretin**

The previous experiments demonstrate that activation of secretin receptors with exogenous secretin can regulate cerebellar Kv1.2 trafficking. We therefore asked whether *endogenously* released secretin regulates Kv1.2. The proposed mechanism for the endogenous activation of secretin receptors is depolarization induced release of secretin from PCs (Koves, Kausz et al. 2002; Koves, Kausz et al. 2004; Lee, Chen et al. 2005; Lee, Yung et al. 2005). Purkinje cell activity is normally constrained by GABA<sub>A</sub> mediated inhibition; blocking GABA<sub>A</sub> receptors can increase PC firing in culture (Gahwiler 1975). Therefore, the GABA<sub>A</sub> receptor antagonist, Gabazine (SR-95531) (Sigma-Aldrich), could increase endogenous secretin release from depolarized PCs. We
hypothesized that secretin so released would activate secretin receptors and thereby reduce surface Kv1.2. Supporting this model, Gabazine (20 μM, 45 minutes) decreased surface Kv1.2 (-20.41 ± 6.19%, p=0.003, n=13, 13; 3 rats) (Figure 3), without changing total Kv1.2 (-6.22 ± 7.71%, p=0.43, n=13, 13; 3 rats). The mechanism by which depolarization of PCs enhances secretin release involves activation of voltage-gated calcium channels (VGCC’s) (Lee, Chen et al. 2005). Consistent with this, we found that Gabazine had no effect on surface Kv1.2 in the presence of nimodopine (20 μM) (Calbiochem), an L-type VGCC blocker (-1.39 ± 6.43%, p=0.83, n=12, 12; 3 rats), or ω-agatoxin-IVA (100 nM) (Tocris), a P-type VGCC blocker (+9.45 ± 8.58%, p=0.28, n=12, 12; 3 rats). These results are consistent with a model wherein secretin released from PCs regulates Kv1.2. However, the blockade of VGCC’s could reduce GABA release, precluding an effect of Gabazine on Kv1.2. To address this, we left calcium channel function intact, and directly inhibited the secretin receptor. We found Gabazine had no effect on surface Kv1.2 in the presence of the secretin-receptor antagonist, 5-27 secretin (30 μM), (-2.36 ± 6.66%, p=0.73, n=15, 15; 3 rats). A previous study has shown cerebellar secretin release occurs constitutively (Lee, Chen et al. 2005). Given this, our model predicts that antagonizing the secretin receptor would elevate surface Kv1.2. Indeed, the secretin antagonist 5-27 secretin (30 μM, 15 minutes), relative to vehicle, significantly elevated surface Kv1.2 (+18.35 ± 9.82%, p=0.038, n=24, 16; 3 rats). Collectively, these findings support the hypothesis that endogenous secretin, like exogenous secretin, reduces surface Kv1.2.
Identification Of The Locus Of Kv1.2 Trafficking In The Cerebellar Cortex

Detection of surface Kv1.2 by labeling with a cell surface specific marker

Kv1.2 is abundantly expressed in the molecular layer of the cerebellar cortex, particularly in PC dendrites (Sheng, Tsaur et al. 1994) where it affects excitatory input integration (Khavandgar, Walter et al. 2005) and may contribute to PC output (McKay, Molineux et al. 2005). Kv1.2 is also strongly expressed in the pinceau formation of BC axon terminals where it affects inhibitory synaptic transmission to PCs (Southan and Robertson 1998; Southan and Robertson 1998). Since endogenous secretin regulates Kv1.2, and since secretin acts through AC, we investigated whether AC regulates surface Kv1.2 in Purkinje cell dendrites, pinceaus of BC axon terminals, or both. To accomplish this, we developed a fluorescent dye-conjugated derivative of the relatively specific Kv1.2 specific toxin Tityustoxin-Kα (TsTx) (Werkman, Gustafson et al. 1993; Rodrigues, Arantes et al. 2003). We used a strategy similar to that developed to generate fluorescent conjugates of a less specific potassium channel inhibitory peptide, Hongotoxin (Pragl, Koschak et al. 2002). The linear peptide for TsTx was generated by CelTek Bioscience with a cysteine instead of the native alanine at site 20. Subsequently, the fluorescent dye ATTO 594 was conjugated to the engineered cysteine, and the toxin was refolded, purified, and validated by Alomone Labs.

After vehicle or forskolin treatment, cerebellar slices were fixed without permeabilization, and surface Kv1.2 was labeled with ATTO 594 Tityustoxin- Kα (3nM, overnight), rinsed, post-fixed, and imaged by fluorescence microscopy. A positive signal was detected in the molecular layer in the territory of Purkinje cell dendrites and in
pinceau formations (Figure 4 A). We confirmed that slices fixed without permeabilization were grossly intact by the near absent labeling for the cytoplasmic Kv1 beta-subunit Kvβ2, compared to slices permeabilized with acetone after fixation (Figure 4 I,J).

AC stimulation with forskolin decreased the mean intensity of ATTO 594 signal in pinceau ROI’s (-27.56 ± 4.33%, p≤0.0001, n=63, 48; 4 rats) (Figure 4B). A similar analysis of ROI’s within the molecular layer also revealed a significant decrease in ATTO 594 signal (-22.9 ± 7.77, p=0.0023, n=32, 37; 4 rats) (Figure 4C). Therefore, AC stimulation reduces surface Kv1.2 in both the BC axon terminals and in PC dendrites.

_Detection of surface Kv1.2 by differential extraction with acetone._

We next sought to support our finding that forskolin reduces surface Kv1.2 in the cerebellar cortex with a second assay. To do so we developed a method to preferentially extract non-surface Kv1.2 relative to surface Kv1.2, thereby permitting detection of changes in surface channel levels using immunofluorescence. Biotinylation of surface proteins and quantification of the proportion of surface to total channel was performed as in the experiments for Figure 1, with the difference here that some slices were extracted with acetone after biotinylation of surface proteins. Biotinylated, surface, Kv1.2 is removed from the “Total” lysate by incubation with neutravidin beads, while non-surface Kv1.2 remains in the post-neutravidin supernatant “Post”. Compared to slices incubated in saline, slices incubated in acetone after biotinylation showed proportionally less Kv1.2 in this non-surface “Post” fraction (Figure 4G). Quantifying this, we found that for slices not extracted with acetone, the surface proportion of total Kv1.2 was 35.39% (similar to
in dependent experiments shown in Figure 1). In contrast, for slices extracted with acetone, the surface proportion of total Kv1.2 was 66.76% (+31.37 ± 7.13%, p=0.012, n=3, 3; 3 rats) (Figure 4H). If acetone had extracted both non-surface and surface Kv1.2 equally, this value would have been the same between acetone extracted and non-extracted slices. However, by preferentially extracting non-surface Kv1.2, acetone enhances the representation of surface channel, facilitating detection of changes in surface Kv1.2 by immunofluorescence.

Using this method, we examined the effect of forskolin on surface Kv1.2 levels in the molecular layer and in pinceaus by immunofluorescence. As expected, a positive α-Kv1.2 signal was observed in both regions (Figure 4D). Furthermore, forskolin reduced the mean intensity of surface-enriched Kv1.2 immunofluorescence in cerebellar pinceaus (-17.3 ± 3.58%, p≤0.0001, n=99,102; 7 rats) (Figure 4E), and in the molecular layer of the cerebellar cortex (-16.02 ± 8.69%, p=0.037, n=19, 19; 7 rats) (Figure 4 F).

Thus, by two separate assays, we found that forskolin reduced surface Kv1.2 in the molecular layer and in BC axon terminal pinceaus. The functional significance of the decreased expression of Kv1.2 in pinceaus is indicated by published studies showing that, in rat cerebellar slices, secretin or forskolin (Yung, Leung et al. 2001) mimic the effect of pharmacological inhibition of Kv1.2: enhancing IPSC frequency recorded in Purkinje cells (Southan and Robertson 1998; Southan and Robertson 1998). In contrast, the effect of secretin on PC excitability was not known. Khavandgar et al. demonstrated that pharmacological inhibition of Kv1.2 causes an increase in PC dendritic excitability (Khavandgar, Walter et al. 2005). Because secretin stimulates AC, and AC stimulation
with forskolin decreased surface Kv1.2 in PC dendrites, it would be expected that secretin would mimic pharmacological inhibition of Kv1.2 to some degree, increasing dendritic excitability. We therefore tested the effect of secretin on two indices of enhanced dendritic excitability in PCs identified by Khavandgar et al: 1) the appearance of brief transient increases in action potential frequency of PCs exhibiting the tonic firing pattern, and 2) decreased cycle duration of PCs exhibiting the trimodal pattern of firing (Khavandgar, Walter et al. 2005). PC action potential activity was recorded in the presence of CNQX (100 μM) and Gabazine (10 μM) to block excitatory and inhibitory synaptic input respectively, using single-cell extracellular recording from PC soma to prevent disruption of PC firing caused by whole-cell recording (Womack and Khodakhah 2003). Secretin (30 nM) elicited an effect in four of seven cells tested, two of which started in a tri-modal pattern of cyclic firing, and two of which started in a tonic firing pattern. A tri-modal pattern of cyclic firing is defined as including tonic, bursting and quiescent phases. The number of such cycles recorded in a five minute period before secretin addition, and in the five minute period starting 10 minutes after secretin application, were 2:8 and 3:8 in each respectively. In the two cells that exhibited tonic firing, secretin elicited the appearance of high frequency bursts with the following characteristics for cell 1 and cell 2 respectively: frequency = 215 and 228 hZ, burst duration = 48 and 32 ms, and 14 and 9 events per burst. The baseline firing rate for was unchanged by addition of secretin (cell 1 = 48.2 vs. 48.4 hZ; cell 2 = 102 vs. 100 hZ). Thus, in four out of seven cells, secretin affected PC firing patterns in a manner consistent with it having enhanced dendritic excitability. Interestingly, two of the three
cells that did not respond to secretin exhibited transient high frequency action potential bursts during the pre-secretin baseline recording period, suggesting that in these cells Kv1.2 may have already been at least partially suppressed.

*Kv1.2 Function In Cerebellar Cortex Affects Cerebellum-Dependent Learning In Rats.*

*Intra-cerebellar cortex infusion of either a Kv1.2 inhibitor (TsTx) or secretin enhances eyeblink conditioning*

The AC-induced reduction of surface Kv1.2 in pinceaus likely causes an increase in inhibition to the PCs (Xie, Harrison et al. 2010). PC inhibition has been demonstrated to be important for adaptation of the vestibulo-ocular reflex, a form of cerebellar dependent learning (Wulff, Schonewille et al. 2009). This suggests that suppression of Kv1.2 function might be an endogenous mechanism for regulating learned behaviors controlled by the cerebellum. Eyeblink conditioning (EBC) is a well-studied form of cerebellum-dependent learning for which the entire brainstem-cerebellar circuit has been identified (Thompson 1986; Medina, Nores et al. 2000; De Zeeuw and Yeo 2005; Thompson and Steinmetz 2009; Freeman and Steinmetz 2011). Briefly, the tone CS is relayed through the pontine nuclei to the cerebellar cortex via mossy fiber (MF) projections to cerebellar granule cells (GCs) and to one of the deep cerebellar nuclei, the interpositus nucleus (IP), via MF collaterals. Tone CS information reaches PCs via parallel fiber (PF) axons from granule cells. The periorbital stimulation US is relayed through the inferior olive to the cerebellar cortex via climbing fiber (CF) projections to PCs and to the interpositus nucleus via CF collaterals. Purkinje cells tonically inhibit the deep cerebellar nuclei. A standard model has been that EBC leads to plastic changes in
cerebellar cortex that disinhibit the IP and allow the eyeblink conditioned response (CR) to be generated in response to the tone CS. Given the fact that BCs are in a position to regulate PCs, BCs are positioned to play a critical role in EBC. In this model, suppression of Kv1.2 function in BCs, by enhancing GABA release, would inhibit PCs, thereby disinhibiting the IP and facilitating EBC by permitting MF-IP plasticity to occur more quickly.

We therefore tested whether suppression of Kv1.2 would enhance cerebellar dependent learning. In the first experiment, we infused, via a pre-positioned cannula, the Kv1.2 inhibitor TsTx into lobulus simplex of cerebellar cortex prior to EBC sessions. All cannula tips were located in lobulus simplex ipsilateral to the eye that received the US. Rats that received TsTx infusions into lobulus simplex immediately prior to Sessions 1-6 of eyeblink conditioning learned faster than vehicle rats (Figure 5A). These observations were confirmed by a 2 (Group) x 7 (Session) repeated-measures ANOVA on the percentage of CRs on CS-US trials which revealed a significant main effect of Group, F(1,12) = 5.41, p < 0.04. We further analyzed performance in Session 1 on a block-by-block basis, where each block consisted of 8 CS-US trials. A 2 (Group) x 10 (Block) repeated-measures ANOVA on percentage of CRs on CS-US trials revealed a significant main effect of Block, F(9,108) = 4.71, p < 0.01 but no difference between groups. A series of 2 (Group) X 7 (Session) repeated-measures ANOVAs on CR onset latency, CR amplitude, and UR amplitude did not reveal any differences between groups (p values > 0.13). A 2 (Group) X 7 (Session) repeated-measures ANOVA on percentage of startle responses revealed a significant main effect of Session, F(6,72) = 2.80, p < 0.02 and a
marginally significant main effect of Group, F(1,12) = 4.56, p = 0.054. However, percentage of trials with a startle response was very low (<3%) in both groups across sessions.

The finding that pharmacological inhibition of Kv1.2 enhances EBC suggested that inhibition of Kv1.2 via a more physiological mechanism would have a similar effect. To test this idea, we infused secretin into the lobulus simplex and assessed its effects on EBC. Rats that received secretin infusions into lobulus simplex immediately prior to Sessions 1-3 of eyeblink conditioning learned faster than vehicle rats (Figure 5B). These observations were confirmed by a 2 (Group) X 7 (Session) repeated-measures ANOVA on the percentage of CRs on CS-US trials which revealed a significant main effect of Session, F(6,84) = 12.61, p < 0.01 and a significant Group x Session interaction effect, F(6,84) = 3.03, p < 0.01. Post-hoc tests of the significant interaction effect with a series of one-way ANOVAs revealed that secretin infused rats displayed a significantly greater percentage of CRs in Sessions 2 (p = 0.016) and 3 (p = 0.006) of conditioning. We further analyzed performance in Session 1 on a block-by-block basis, where each block consisted of 8 CS-US trials. A 2 (Group) X 10 (Block) repeated-measures ANOVA on the percentage of CRs on CS-US trials revealed a significant main effect of Block, F(9,126) = 4.45, p < 0.01 but no difference between groups. A series of 2 (Group) X 7 (Session) repeated-measures ANOVAs on CR onset latency, UR amplitude, and the percentage of startle responses failed to reveal any differences between groups (p values > 0.10). A 2 (Group) X 7 (Session) repeated-measures ANOVA on CR amplitude revealed a significant main effect of Session, F(6,84) = 3.88, p < 0.01 and a significant
Group X Session interaction effect, F(6,84) = 3.12, p < 0.01. Post-hoc tests of the significant interaction effect with a series of one-way ANOVAs failed to reveal any significant differences between Groups in any Session (p’ values > 0.09).

Discussion

The voltage-gated potassium channel, Kv1.2, modulates excitatory and inhibitory synaptic input to PCs (Southan and Robertson 1998; Southan and Robertson 1998; Tan and Llano 1999; Southan and Robertson 2000). In this study we provide the first evidence that cerebellar Kv1.2 is regulated by endocytic trafficking, that this process is modulated by AC, and that this channel regulation may have significance as a component of cerebellum-dependent learning.

In Figures 1-3 we used biotinylation of cell surface proteins to demonstrate that cerebellar Kv1.2 is regulated by AC-dependent endocytic trafficking. While this approach is powerful because it detects changes in Kv1.2 trafficking throughout an entire slice, it does not provide information about which population of Kv1.2, BC axon terminal or PC dendritic, is sensitive to AC-mediated trafficking. It was important to investigate the location of the AC-modulated pool of surface Kv1.2 because suppression of Kv1.2 at each site would likely have distinct effects on PC activity and cerebellar function.

Using two independent microscopy techniques, we found that stimulating AC decreased surface Kv1.2 within pinceaus (Figure 4). The physiological role of this decrease is suggested by previous studies that examined the effect of secretin or pharmacological block of Kv1.2 on inhibitory input to PCs. Forskolin and secretin cause an AC-dependent increase in IPSC frequency and amplitude recorded in PCs (Yung,
Pharmacological suppression of Kv1.2 also increases inhibitory input to PCs (Southan and Robertson 1998; Southan and Robertson 1998), suggesting a link between secretin and Kv1.2 effects on PC inhibition. Our data, in conjunction with a previous study showing that Kv1.2 endocytosis suppresses Kv1.2 ionic current (Nesti, Everill et al. 2004), suggests a mechanism for this link whereby secretin stimulation of AC increases channel endocytosis. It is important to note, however, that the increase in inhibitory input to PCs caused by secretin involves an AC-independent component (Yung, Leung et al. 2001), which would not be expected to involve Kv1.2 since the AC-inhibitor SQ22536 blocks the ability of secretin to modulate surface Kv1.2 levels.

The stimulation of AC by forskolin also potentiates excitatory transmission at the parallel fiber to PC synapse and multiple lines of evidence point to a pre-synaptic mechanism (Salin, Malenka et al. 1996; Chen and Regehr 1997). However, the PF:PC synapse is also governed by a post-synaptic mechanism involving Kv1.2. Inhibition of Kv1.2 within the molecular layer increases dendritic excitability and enhances PF:PC evoked postsynaptic currents (Khavandgar, Walter et al. 2005). A pre-synaptic role for Kv1.2 in this phenomenon seems unlikely since no effect was observed on presynaptic calcium. Rather, Kv1.2 blockade appears to enhance PF:PC evoked currents by increasing dendritic excitability directly (Khavandgar, Walter et al. 2005). Consistent with this, we show AC stimulation with secretin also increased dendritic excitability (Figure 5), and decreased surface Kv1.2 levels in the molecular layer (Figure 4). Thus, AC-dependent potentiation of the PF:PC synapse could include a post-synaptic mechanism.
The data in Figures 1-4 identifying cellular mechanisms for secretin mediated suppression of Kv1.2 in BCs and PCs raises the question of whether this regulation impacts cerebellum-dependent behavior. The data in Figure 5A showing that infusion of TsTx into the cerebellar cortex enhanced EBC indicates inhibition of Kv1.2 does indeed affect cerebellum-dependent behavior. Our finding that secretin infusion mimicked this behavioral effect (Figure 5B) suggests that suppression of Kv1.2 by endogenous secretin, as shown in Figure 3, could be a mechanism for regulating cerebellar EBC. Our infusions likely influenced both BCs and PCs, given the presence of secretin receptors (Yung et al., 2001) and Kv1.2 (Chung et al., 2001) in both cell types. However, it is important to note that, as detailed below, the finding that EBC was enhanced rather than impaired suggests that inhibition of PCs may have dominated at the systems level. Indeed, our behavioral experiments were designed with this prediction in mind, using a long-delay procedure to enhance resolution of potential facilitation of EBC.

The synaptic mechanisms of plasticity in cerebellar cortex that underlie EBC have remained somewhat elusive. PF:PC long-term depression (LTD) has been the most studied candidate. On one hand, mutant mice with deficient PF:PC LTD induction due to lack of mGluR1 (Aiba, Kano et al. 1994; Kishimoto, Kawahara et al. 2001) or GluRδ2 (Kishimoto, Kawahara et al. 2001; Kakegawa, Miyazaki et al. 2008) in PCs show poor EBC. This is consistent with the necessity for removal of PC inhibition of the IP for EBC to proceed normally. On the other hand, there are data that call into question the strength of the relationship between PF:PC LTD and EBC. For example, mutant mice with enhanced, rather than impaired, PF:PC LTD also shows deficient EBC (Koekkoek,
Yamaguchi et al. 2005), pharmacological disruption of LTD in rats does not affect EBC (Welsh, Yamaguchi et al. 2005), and a study of three different strains of mutant mice with deficient cerebellar LTD showed these mice conditioned normally (Schonewille, Gao et al. 2011). Thus, PF:PC LTD appears to be only part of the mechanisms underlying EBC.

Our results lead us to propose that a new mechanism of cerebellar cortical synaptic plasticity important in EBC may involve secretin dependent suppression of Kv1.2. During EBC, converging CS (mossy/parallel fiber) and US (climbing fiber) inputs to a subset of PCs would be expected to depolarize these cells and thereby enhance their release of secretin (Lee, Chen et al. 2005). Once released, secretin might act both as a retrograde transmitter to regulate Kv1.2 in the BC axon terminal and as an autocrine/paracrine signal to regulate Kv1.2 in PC dendrites (Yung, Leung et al. 2001; Ng, Yung et al. 2002; Lee, Chen et al. 2005).

Secretin, acting as a retrograde messenger, could activate receptors on BC axon terminals to stimulate AC-dependent endocytic trafficking of Kv1.2. This loss of surface Kv1.2 is hypothesized to reduce Kv1.2 ionic current, enhancing GABA release and thus inhibition of these same PCs (Southan and Robertson 1998; Southan and Robertson 1998). This mechanism may partially counter the LTD of inhibitory inputs to PCs that is also induced by CF input (Mittmann and Hausser 2007), which would presumably occur during EBC. LTD of inhibitory inputs opposes the increase in inhibition produced by CF-driven LTD at PF:PC synapses (Mittmann and Hausser 2007). Thus, it is possible that regulation of Kv1.2 at BC terminals contributes, along with other mechanisms (Bao,
Reim et al. 2010), to ensure that inhibition of the PCs activated by CF input eventually dominates. This inhibition of PCs would relieve inhibition of select deep cerebellar nuclei neurons in the IP, allowing MF-IP synapses to strengthen more quickly, enhancing EBC.

Secretin, acting as an autocrine/paracrine signal, could also enhance EBC. A previous report suggested that Kv1.2 opposes dendritic calcium influx and therefore the induction of LTD (Khavandgar, Walter et al. 2005). The regional reduction of dendritic Kv1.2 function by secretin stimulated channel endocytosis could enhance PF:PC LTD by increasing sensitivity of specific PF synaptic inputs during coincident CF inputs. Under this model, dendritic Kv1.2 suppression would have the same net effect as BC axon terminal Kv1.2 suppression: reducing PC output and disinhibiting the IP.

Our data do not inform whether secretin facilitates EBC through facilitation of PF:PC LTD, by elevation of PC inhibition, both means, or by alternate mechanisms. However, the proposed models give plausible mechanisms by which the regulation of Kv1.2 may be an important contributing factor. Determining the mechanisms behind how secretin enhances cerebellar dependent learning is of interest, especially since, paralleling our animal studies, in studies of patients with schizophrenia, secretin also enhances EBC (Bolbecker, Hetrick et al. 2009).

In this study we focused on secretin receptor mediated regulation of Kv1.2. However, the cerebellum contains a variety of other receptors likely to regulate Kv1.2. One logical candidate is the Gi-coupled CB1 receptor. CB1 receptors are expressed in BC axon terminals, where their activation reduces inhibition of PCs (Szabo, Than et al. 2004). One mechanism may be CB1 receptor mediated inhibition of AC and a resulting
increased surface expression of presynaptic Kv1.2. Further, like secretin, endocannabinoids are thought to be released from depolarized PCs (Yoshida, Hashimoto et al. 2002). Because secretin and cannabinoids would have opposing effects on surface Kv1.2 in BC axon terminals, PCs might regulate inhibition they receive by altering the ratio of secretin and cannabinoids they release. It is interesting to speculate this ratio could be determined by changes in PC firing patterns.

Kv1.2 may also be regulated by other receptors expressed in BC axon terminals or PC dendrites, including c-Kit receptor tyrosine kinase (Manova, Bachvarova et al. 1992; Kim, Im et al. 2003), metabotropic glutamate (Neale, Garthwaite et al. 2001; Neale, Garthwaite et al. 2001), NMDA (Glitsch and Marty 1999), metabotropic GABA (Batchelor and Garthwaite 1992), purinergic (Kocsis, Eng et al. 1984; Wall, Atterbury et al. 2007), or adrenergic receptors (Saitow, Satake et al. 2000; Hirono and Obata 2006). Further regulatory diversity could result from differences in alpha and beta subunit composition of Kv1.2 containing holochannels, which might differ between BCs and PCs (Koch, Wanner et al. 1997). For example, Kv1.2 in BC axon terminals consists mostly of Kv1.2/Kv1.1 heteromultimers (Koch, Wanner et al. 1997). Less is known about the composition of PC Kv1.2 containing channels; however, PCs express Kv1.5, suggesting the existence of Kv1.2/Kv1.5 heteromultimers in these cells (Chung, Shin et al. 2001).
References


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Additional Unpublished Data Related To The Published Manuscript

The following experiments were conducted as part of the dissertation research project that led to the preceding published manuscript. However, the following data in this section has not been subject to peer-review, and the material mostly represents preliminary studies. They are included here to provide evidence of the scope of the research, and to, more importantly, provide an expanded experimental perspective for the critical analysis of the published data. Except where otherwise indicated, the studies were performed and analyzed with the same methods as in the manuscript.

The Source Of Tonic Inhibition Opposing Secretin Mediated Kv1.2 Suppression

Antagonizing GABA$_A$ receptors decreased surface Kv1.2 in the rat cerebellar slice preparations through a secretin-receptor dependent pathway, suggesting that tonic GABAergic inhibition opposes secretin release from Purkinje cells, and therefore suggesting further a potential homeostatic role for Kv1.2 regulation. In this model, tonic inhibition of PC’s decreases their secretin release, but when this tonic inhibition is diminished or overcome, PC secretin release is enhanced, which suppresses presynaptic BC Kv1.2, restoring GABAergic inhibition to the PC’s. This raises the question of the origin of the tonic GABAergic inhibition to Purkinje cells.

Basket cells make inhibitory synapses on to the Purkinje cell. The identity of these synapses as GABAergic has been well established by a variety studies. The distribution of GABA receptors in the rat brain was first explored using light microscopy and autoradiography with a radio-labeled GABA agonist, muscimol. The highest amount
of binding in the brain was within the cerebellum, at the soma and initial axon segment of
the Purkinje cell, the territory of basket cell axon terminals (Chan-Palay, Plaitakis et al.
1977). The enrichment of labeled muscimol binding sites at the postsynaptic element of
the pinceau, on the Purkinje cell, was confirmed by electron microscopy (Chan-Palay and
Palay 1978). Subsequent studies used antibodies raised against GABA, in combination
with light and electron microscopy, to directly visualize presynaptic GABA in the basket
cell axon terminals (Ottersen, Storm-Mathisen et al. 1988). The high affinity GABA
transporter, GAT-1, was also found to be highly enriched to the basket cell axon
terminals (Morara, Brecha et al. 1996). In fact, it was calculated that a single pinceau
structure may contain nearly eight million GAT1 molecules (Chiu, Jensen et al. 2002).
The GABA synthase, glutamic acid decarboxylase isoform 67 (GAD 67), which is
sufficient and necessary for basket cell GABA production, is enriched in the pinceau
(Obata, Fukuda et al. 1999). Therefore, the transmitter GABA, its synthase, and its
transporter, are all localized to the BC: PC presynapse, at the pinceau.

Postsynaptically, Purkinje cells express mRNA for ionotropic GABA\textsubscript{A} receptor
subunits (Gambarana, Beattie et al. 1991), in particular, the GABA\textsubscript{A} alpha 1 subunit is
enriched here (Takayama and Inoue 2003). The inhibition of Purkinje cells is thought to
be solely mediated by ionotropic GABA\textsubscript{A} receptors containing the alpha 1 subunit:
Purkinje cells from knockout mice lacking the subunit have no recordable inhibitory post
synaptic currents (IPSCs) (Fritschy, Panzanelli et al. 2006). In summary, basket cells
mediate Purkinje cell output, and therefore, cerebellar function, through their release of
GABA. However, there appears to be two classes of GABAergic inhibition in the
cerebellar cortex: classical vesicular release and channel-mediated release mediated by a protein known as bestrophin.

Initially identified as a gene mutated in Best’s macular dystrophy, bestrophin was categorized as a multi-transmembrane protein of unknown function (Bakall, Marknell et al. 1999; Caldwell, Kakuk et al. 1999). A bioinformatics approach suggested that Bestrophin may be a chloride channel, and subsequently it was found that the human genome encodes four forms, at least one member of which was validated to serve as an ion channel, Best1 (Bakall, Marknell et al. 1999; Gomez, Cedano et al. 2001; Tsunenari, Sun et al. 2003). The regulation of Bestrophin chloride conductance was found to be complex, being sensitive to voltage, volume, and calcium in various experiments (Qu, Wei et al. 2003; Fischmeister and Hartzell 2005; Chien and Hartzell 2007). The sites of Best1 expression grew as well, being found not just in the eye, but in airway and olfactory epithelia (Duta, Szkotak et al. 2004; Pifferi, Pascarella et al. 2006), and in excitable cells, including myocytes of the heart (O'Driscoll, Hatton et al. 2008) and neurons (Al-Jumaily, Kozlenkov et al. 2007; Boudes, Sar et al. 2009). The notions of Best1 function were expanded with the finding that these channels were very permeable to bicarbonate anions, therefore the channel was permissive to both inorganic and organic anions (Qu and Hartzell 2008; Yu, Lujan et al. 2010). In a critical advance, it was determined that Best1 was also permeable to GABA, and indeed mediated, through this conduction, tonic GABAergic inhibition of granule cells in the cerebellum (Lee, Yoon et al. 2010). While the tonic Best1 mediated inhibition of granule cells was suggested to be mediated largely through astrocytic GABA conductance, this result is contentious (Diaz,
Wadleigh et al. 2011). However, Best1 was also found to be expressed in cerebellar basket cells, (Lee, Yoon et al. 2010), suggesting that, in addition to classic vesicular GABA release, Best1 mediated inhibition of PC’s may also be a mechanism of BC GABAergic inhibition to PC’s. While the classic vesicular release of GABA is largely dependent upon action potentials of spontaneously firing basket cells, and can be therefore be blocked by tetrodotoxin, the GABA conductance through Best1 channels may be inhibited by 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), (Glitsch and Marty 1999; Zhang, Messing et al. 1999; Lee, Yoon et al. 2010). In the rat cerebellar slices, inhibiting GABA_A receptors with Gabazine reduced surface Kv1.2 (through secretin), so towards determining the source of that tonic GABA release, vehicle or Gabazine was added in the presence of TTX or of NPPB, and it was tested whether Kv1.2 was still suppressed.

Preformed and analyzed using the methods of the preceding manuscript, (i.e. by pharmacological treatment, cell surface protein biotinylation, and evaluation by protein immunoblot and densitometry of rat cerebellar slices), Gabazine, relative to vehicle, did not reduce Surface Kv1.2 in the presence of NPPB (-0.43±9.90%, n=12(Vehicle),12(Gabazine); p=0.48). In contrast, in the presence of TTX, Gabazine, versus Vehicle, did significantly reduce surface Kv1.2 (-18.36±10.48%, n=12(Vehicle), 13(Gabazine); p=0.047), Figure 6. Therefore, the source of GABA opposing secretin mediated suppression of Kv1.2 may not be vesicular in origin, but channel mediated.

*The Complex Roles Of AC In The Regulation Of Surface Kv1.2 Expression*
While the source of PC inhibition opposing secretin mediated Kv1.2 endocytosis appears to be channel mediated, rather than vesicular, GABA release, a second open question from the preceding manuscript is how secretin/AC may modulate the bidirectional regulation of cerebellar Kv1.2 regulation. While secretin appears to act through AC/PKA to reduce surface Kv1.2 channel expression, the inhibition of PKA and dynamin revealed that AC stimulation could increase surface Kv1.2 (Williams, Fuchs et al. 2012). Towards supporting the model that AC has a role in forward trafficking of Kv1.2 to the plasma membrane as well, inhibiting AC with SQ22536 (20 minutes, 100μM), was found to decrease surface Kv1.2 in the biotinylation assay (16.14±3.80%, n=32(Vehicle), 12 (SQ); two-tailed t-test p=0.0001), Figure 7A. The G\textsubscript{i} coupled CB1 cannabinoid receptor is expressed in basket cell axon terminals (though not PC dendrites), suggesting an endogenous mechanism for inhibiting AC, and therefore another means and mechanism for testing whether inhibiting AC reduces cerebellar surface Kv1.2 (Herkenham, Groen et al. 1991; Carey, Myoga et al. 2011) To test this, cerebellar slices were treated with vehicle or with the mixed CB1 agonist WIN 55212-2 mesylate (Cayman Chemical) 10μM, 15 minutes (Felder, Joyce et al. 1995). Again by the biotinylation assay, the WIN compound, compared to vehicle, reduced surface Kv1.2 (30.99±12.19%, n=12(Vehicle), 11(WIN); two-tailed t-test p=0.02). If inhibiting AC reduces surface Kv1.2 in one population of synapses or subcellular environment, and in a separate population/environment stimulating AC reduces surface Kv1.2 as well, it would be expected that the reductions in surface Kv1.2 caused by WIN and secretin might be additive. Therefore, in a pilot experiment, slices were treated with vehicle, secretin, WIN-
55212, or secretin and WIN-55212 as above. However, in this small sample, by one way analysis of variance, there was no difference between the means of the treatment groups (F (3, 41) = 1.10, p=0.36).

These results might suggest a relatively simple model whereby AC stimulation, independent of PKA, increases surface Kv1.2, while activation of AC that results in PKA stimulation, decreases surface Kv1.2. Non-PKA effectors of AC include the guanine-nucleotide-exchange factor EPAC, which can be relatively selectively activated with the cyclic AMP analogue 8-(4-chlorophenylthio)-2′-O-methyl-cAMP (8-pCPT-2′-O-Me-cAMP), while PKA may be relatively selectively activated by N6-benzyladenosine-3′,5′-cyclic monophosphate (6-Bnz-cAMP) (de Rooij, Zwartkruis et al. 1998; Grandoch, Roscioni et al. 2010). As inhibiting AC decreased surface Kv1.2, and as stimulating AC (under inhibition of PKA and Dynamin) increased surface Kv1.2, it is perhaps consistent with a role for EPAC as the AC effector for forward Kv1.2 trafficking that 8-pCPT-2′-O-Me-cAMP treatment (15 minutes, 100μM), relative to vehicle, by the biotinylation assay, increased surface Kv1.2 (+26.25±14.24%, n=4(Vehicle),5(CPT-cAMP); though this did not reach significance, one-way t-test p=0.054), Figure 7B. Therefore, perhaps EPAC activity is responsible for AC mediated increases in surface Kv1.2.

In the manuscript, because PKA inhibition (by KT5720) prevented forskolin or secretin stimulated reductions in surface Kv1.2, PKA was proposed as the AC effector for Kv1.2 endocytic trafficking. However, it is likely not simply the case that, downstream of AC, PKA stimulation generically reduces surface Kv1.2: in a pilot
biotinylation study, treatment with the PKA stimulator 6-Bnz-cAMP (15 minutes, 100μM), actually increased surface Kv1.2 (+22.45±0.11%, n=4(vehicle), 5(Bnz-cAMP); one-tailed t-test p=0.036) Figure 7D. In further support of a role for PKA in forward trafficking of Kv1.2 to the plasma membrane, again by biotinylation, inhibition of PKA with KT-5720 (30 minutes, 5μM) decreased surface Kv1.2 (-9.47±3.67%, n=32(Vehicle), 14(KT); two-tailed t-test p=0.013) Figure 7C.

Therefore AC, perhaps through both EPAC and PKA, can bi-directionally modulate surface Kv1.2 in the cerebellum. Intriguingly, PKA activity, beyond the role proposed in the manuscript for stimulating net Kv1.2 endocytosis, seems important for forward trafficking of Kv1.2 as well. Determining the differences in the populations of Kv1.2 that show AC mediated increases versus decreases in surface expression will be informative, perhaps they are of different holochannel composition, or in different membrane domains, or associated with different adaptor protein complexes.

**Basal Kv1.2 Endocytosis, The Involvement Of Dynamin, But Perhaps Not RhoA**

In the manuscript, inhibiting the endocytic GTPase, dynamin, with dynasore was sufficient to prevent forskolin stimulated decreases in cerebellar surface Kv1.2. In HEK cells also, dynamin is important for AC stimulated changes in surface Kv1.2 (Connors, Ballif et al. 2008; Stirling, Williams et al. 2009). However, in HEK cells, the endocytosis of Kv1.2 from the plasma membrane happens not just under stimulated conditions, but constitutively as well: there is basal internalization of surface Kv1.2, and correspondingly, inhibiting dynamin with dynasore increases surface Kv1.2 (Stirling,
Williams et al. 2009). Therefore, towards testing whether there may also be dynamin-dependent constitutive endocytosis Kv1.2 in the cerebellum, slices in a pilot experiment were incubated with vehicle or with dynasore (80μM, 30 minutes), and surface Kv1.2 expression was examined by biotinylation and protein immunoblot. Compared to vehicle treatment, dynasore treatment significantly enhanced surface Kv1.2 (+24.50±8.13, n=6(Vehicle), 6(Dynasore); two-tailed t-test p=0.03). Therefore, there appears to be cerebellar Kv1.2 that is regulated by basal dynamin-dependent endocytosis, as in HEK293 cells.

The dynamin dependent basal internalization of Kv1.2 in HEK293 cells occurs through a pathway that is dependent upon RhoA/Rho Kinase: inhibition of RhoA with cell permeable C3 exoenzyme, or inhibition of Rho Kinase with Y-27632 results in an increase in surface Kv1.2 that involves decreased internalization of Kv1.2 (Stirling, Williams et al. 2009). Since there appears to be dynamin dependent basal internalization of Kv1.2 in the cerebellum, it was tested whether, like in HEK cells, inhibiting RhoA or Rho Kinase with C3 or Y27632 respectively, would increase surface Kv1.2. Unlike all other cerebellar biotinylation experiments, this early pilot experiment occurred before protocols utilized TTX pretreatment. Cerebellar slices were treated with vehicle, with 2μg/mL cell permeable C3 exoenzyme (Cytoskeleton Inc.), or with 10μM Y-27632 (EMD Biosciences), for six hours. In a biotinylation assay, by one-way analysis of variance, there was a significant difference amongst the means, (F (2, 10) = 10.08, p=0.004). Bonferroni’s Multiple Comparison post-hoc test revealed significant differences between vehicle and C3 treatment (-19.95%, 95% CI [-3.03 to -36.78].
p<0.05) between vehicle and Y-27632 treatment (-24.45%, 95% CI [-7.53 to -41.37], p<0.01), but not between C3 and Y-27632 (treatment (+13.34%, 95% CI [-22.34 to 13.34], NS). Therefore, to the extent the preliminary data indicate that RhoA or Rho Kinase inhibition in the cerebellum decreases surface Kv1.2, the results suggest that, unlike in HEK293 cells, these proteins do not apparently stimulate/permit constitutive Kv1.2 endocytosis.

**ATTO-TsTx Labeling After Cardiac Perfusion Fixation**

The aforementioned pilot biotinylation experiments, and in those of the manuscript, provided the first evidence that cerebellar Kv1.2, or any native Kv1.2, can undergo regulated trafficking. However, these biochemical studies involved homogenization and examination of global average changes across slice preparations, and therefore a loss of information about the localization of the changes (i.e. whether they were in BC pinceaus, PC dendrites, or both). It was a significant advance then, that the collaborative generation of fluorescently labeled Typustoxin-Ka (TsTx), and the optimization of fixation conditions, allowed the preferential visualization of surface Kv1.2 in cerebellar slices by microscopy. This led to the published observation that AC stimulation reduced surface expression of both the BC pinceau and PC dendritic populations of Kv1.2.

We found that artificial suppression of Kv1.2 by unlabeled TsTx or secretin infusion into rat cerebellar cortices enhanced the eyeblink conditioned response, that is, that suppressing Kv1.2 enhanced a measure of learning. This raises the possibility that
reduced surface expression (suppression) of Kv1.2 may natively accompany learning, perhaps mechanistically. To test this hypothesis, a change in methodology from the manuscript may be in order. This is because the learning subjects were older and healthy slices from mature animals are difficult to generate (Moyer and Brown 1998) and because of the additional time and disruption that accompany attempting to following learning trials with slice generation, fixation, and eventual surface Kv1.2 labeling with ATTO-TsTx. It was therefore tested whether animals could be transcardially perfused in such a way that did not result in gross permeabilization of the plasma membranes, but that would still allow for ATTO-TsTx binding, collectively permitting a snapshot of surface Kv1.2 expression as it had existed in the animal.

As performed by Jason Fuchs of Dr. John Green’s lab, male rats were heavily sedated by overdose of sodium pentobarbital before transcardial perfusion with Hank’s buffered salt solution with calcium and magnesium but no phenol red (HBSS), after this flushing, the perfusate was switched to HBSS with 2% methanol-free formaldehyde. After rinsing off excess fixative, 200 micron thick sagittal slices were then generated by vibratome at minimum speed and maximum amplitude in cool HBSS and collected to HBSS on ice. After again rinsing in HBSS, some slices were intentionally permeabilized in ice cold acetone for 5 minutes. All slices were again rinsed in HBSS and probed with labeled TsTx, no label, control IgG, or mouse monoclonal α-Kvβ2 and examined by microscopy as described in the manuscript. Under these conditions, thresholded above IgG-defined background, α-Kvβ2 signal was strongly enhanced in slices that were intentionally permeabilized with acetone, suggesting that transcardially perfusing alone
had not resulted in comparable gross, maximal, permeabilization, displayed in Figure 8, A vs. B. Without acetone, compared to no label, ATTO-TsTx did give a positive signal in the pinceau pattern (FIGURE 8, C versus D). Therefore, it should be technically feasible to have animals undergo similar eyeblink conditioning as in the manuscript, followed by cardiac perfusion fixation and ATTO-TsTx labeling to study regional Kv1.2 surface expression changes in a learning paradigm.
Figure Legends

Figure 1. Secretin Regulates Kv1.2 Surface Expression In The Cerebellum. A, In cerebellar slices treated with 2 mg/ml biotin (+Biotin) or not (-Biotin), both GAPDH and Kv1.2 are present in the Total lysate. Kv1.2 is present in neutravidin bead Eluant of +Biotin slices, while GAPDH is not, indicating selective biotinylation of cell-surface proteins. Kv1.2 is diminished in the Post fraction after the Total is incubated with neutravidin. B, The percentage decrease in GAPDH or Kv1.2 in the Post versus Total fraction from slices biotinylated or not following incubation with neutravidin. Compared with -Biotin slices, ~36% of all Kv1.2 is neutravidin bound in +Biotin slices (*p ≤ 0.0006). In contrast, there is no significant difference in the amount of neutravidin-bound GAPDH between -Biotin and +Biotin slices (p ≥ 0.23). C, Change in mean surface Kv1.2 as determined by biotinylation and immunoblot of drug-treated cerebellar slices. A 15 min incubation in 30 nM secretin, relative to vehicle, reduces Kv1.2 at the cell surface in cerebellar slices (*p ≤ 0.0002). In the presence of 30 μM secretin receptor antagonist, 5–27 secretin, secretin does not change the surface expression of Kv1.2 (p ≥ 0.93). D, The change in mean amount of Kv1.2 internalized from the plasma membrane as determined by biotinylation and immunoblot. Compared with vehicle, secretin increases the mean internalized population of Kv1.2 (*p ≤ 0.05). E, As determined by immunoblot of the samples in which internalization was measured, secretin does not alter mean total Kv1.2 levels relative to vehicle (p ≥ 0.82).
**Figure 2. Secretin Regulates Kv1.2 Via AC/PKA.** Change in mean surface Kv1.2 as determined by biotinylation and immunoblot of drug-treated cerebellar slices. A 15 min incubation in the AC activator forskolin (100 μM) reduces mean surface Kv1.2 (*p ≤ 0.05). In the presence of the AC inhibitor SQ-22536 (100 μM) neither secretin (*p ≥ 0.86) nor forskolin (*p ≥ 0.78), relative to their vehicle, changes surface Kv1.2. In the presence of the PKA inhibitor KT-5720 (5μM) neither secretin (*p ≥ 0.16) nor forskolin (*p ≥ 0.26), relative to their vehicle, changes surface Kv1.2. The dynamin inhibitor dynasore (80μM) prevents forskolin-induced reduction in surface Kv1.2 (*p ≥ 0.18). Under inhibition of both PKA (KT-5720, 5μM) and dynamin (dynasore, 80μM), forskolin, compared with vehicle, increases surface Kv1.2 (*p ≤ 0.005).

**Figure 3. Endogenous Secretin Regulates Cerebellar Kv1.2 Surface Expression.**

Change in mean surface Kv1.2 as determined by biotinylation and immunoblot of drug-treated cerebellar slices: a 45 min incubation with GABA_A receptor antagonist, Gabazine (20μM) reduces surface Kv1.2 (*p ≤ 0.003). The L-type calcium channel blocker nimodipine (20μM; *p ≥ 0.83) or the P-type calcium channel blocker ω-agatoxin-IVA (100 nM; *p ≥ 0.28) blocks gabazine’s effect on surface Kv1.2. Gabazine-induced reduction of surface Kv1.2 is also prevented by the secretin receptor antagonist, 5–27 secretin, (30 μM; *p ≥ 0.73). Antagonism of endogenous secretin with 5–27 secretin (30μM for 15 min) increases surface Kv1.2 relative to vehicle (*p s≤ 0.05).
Figure 4. Stimulation Of AC Reduces Surface Kv1.2 At Cerebellar Pinceaus. A, Overnight incubation of fixed but not permeabilized rat cerebellar slices in ATTO-TsTx (3 nM) gives positive signal in the molecular layer and in the pinceaus of BC axon terminals. Scale bar, 10μm. B, Mean intensity of ATTO-TsTx in pinceau ROIs is lower in forskolin than in vehicle-treated slices (*p ≤ 0.001). C, In the same slices, the mean intensity of ATTO-TsTx in molecular layer ROIs is also reduced in forskolin versus vehicle-treated slices (*p ≤ 0.005). D, α-Kv1.2 staining in acetone extracted cerebellar slice to enrich for surface Kv1.2. Scale bar, 20μm. E, Forskolin significantly reduces the mean intensity of surface-enriched Kv1.2 in pinceau ROIs (*p ≤ 0.0001). F, Forskolin also reduces the intensity of surface-enriched Kv1.2 in the molecular layer (*p_0.05).G, Cerebellar slices are first biotinylated then incubated in saline or acetone. Kv1.2 is preferentially lost from the non-biotinylatable internal population in the Post fraction after incubation of the Total lysate with neutravidin beads pulls out biotinylated surface Kv1.2, as seen in the Eluant. H, Quantification of Western blots as in G. Neutravidin removes more Kv1.2 from the lysates of slices that were incubated with acetone as opposed to saline after biotinylation (*p ≤ 0.02), indicating preferential extraction of non-surface channel. I, J, Cytoplasmic α-Kv1.2 staining thresholded to IgG control yields weak staining in the cortex of cerebellar slices without intentional permeabilization, demonstrating that cell membranes are grossly intact with the fixation protocols used for ATTO labeling as in A–C. Scale bars: 20μm. K, Single-cell extracellular action-potential recordings taken during ACSF perfusion (top) and after (bottom) addition of secretin
show representative secretin-induced transient high-frequency bursts of action potentials in a tonically firing PC.

**Figure 5. Secretin And Kv1.2 Inhibition With TsTx Similarly Affect Cerebellum-Dependent Learning.**

A, B, The percentage of CRs on CS–US trials. Error bars indicate SEM. A, TsTx, versus vehicle infusion, into lobulus simplex of rat cerebellar cortex enhances EBC. A 2 (Group) × 7 (Session) repeated-measures ANOVA on the percentage of CRs on CS–US trials revealed a significant main effect of Group, F(1,12) = 5.41, p < 0.04. In Session 1, on a block-by-block basis where each block consisted of 8 CS–US trials, a 2 (Group) × 10 (Block) repeated-measures ANOVA on percentage of CRs on CS–US trials revealed a significant main effect of Block, F(9,108) = 4.71, p < 0.01 but no difference between groups. B, Secretin, versus vehicle, infusion into lobulus simplex of rat cerebellar cortex enhances EBC. A 2 (Group) × 7 (Session) repeated measures ANOVA on the percentage of CRs on CS–US trials revealed a significant main effect of Session, F(6,84) = 12.61, p < 0.01 and a significant Group × Session interaction effect, F(6,84) = 3.03, p < 0.01. In Session 1, on a block-by-block basis where each block consisted of eight CS–US trials, a 2 (Group) × 10 (Block) repeated-measures ANOVA on the percentage of CRs on CS–US trials revealed a significant main effect of Block, F(9,126) = 4.45, p < 0.01, but no difference between groups.
**Figure 6. The Source Of Tonic Inhibition Opposing Secretin Mediated Kv1.2 Endocytosis.** A,B: Bar graphs of change in mean surface Kv1.2 as determined by biotinylation and immunoblot of drug-treated cerebellar slices. A) In the presence of 1 μM TTX, Gabazine (45 minutes, 20μM), versus vehicle, significantly reduced surface Kv1.2 (-18.35%, *p*≤0.05). B) In contrast, in the presence of NPPB (50 μM), Gabazine, versus vehicle, did not reduce surface Kv1.2 (-0.43%, *p*=0.48).

**Figure 7. The Complex Regulation Of Surface Kv1.2 By AC And PKA.** A-D: Bar graphs of change in mean surface Kv1.2 as determined by biotinylation and immunoblot of drug-treated cerebellar slices. A) Treatment with the adenylate cyclase inhibitor, SQ 22536 (20 minutes, 100 μM), relative to vehicle, decreases surface Kv1.2 (-16.14%, *p*≤0.0001). B) Stimulation of the cAMP effector, EPAC, by 8-pCPT-2-Me-cAMP (15 minutes, 100μM), non-significantly increases surface Kv1.2 (+26.25%, *p*=0.054). C) Inhibition of PKA with KT 5720 (30 minutes, 5 μM), relative to vehicle, decreases surface Kv1.2 (-9.47%, *p*≤0.05), while D) stimulation of PKA with 6-Bnz-cAMP (15 minutes, 100μM), increases surface Kv1.2 (+22.45%, *p*≤0.05).

**Figure 8. Labeling Surface Kv1.2 In The Rat Cat Cerebellum Following Fixation By Cardiac Perfusion.** A,B: Photomicrographs of rat cerebellar cortex showing background (IgG) thresholded immunofluorescence for α-Kvβ2 following perfusion by cardiac perfusion A) without intentional permeabilization or B) with intentional permeabilization.
by incubation in ice cold acetone. While dim labeling can be seen in A), there is predominant staining in B), consistent with the known expression of Kvβ2 in basket, granule, and Purkinje cells. C, D: Cerebellar slices from the same experiment which were not intentionally permeabilized were either incubated without C), or with D), ATTO 594-Tityustoxin-Kα to label cell surface Kv1.2 potassium channel pores. C) Without the label, there is only vague puncta, but D) in the presence of ATTO-TsTx, there is evident staining in the classic pinceau pattern of basket cell axon terminals, where Kv1.2 is known to be expressed.
Figure 1. Secretin regulates Kv1.2 surface expression in the cerebellum.
Figure 2. Secretin regulates Kv1.2 via AC/PKA.
Figure 3. Endogenous secretin regulates cerebellar Kv1.2 surface expression.
Figure 4. Stimulation of AC reduces surface Kv1.2 at cerebellar pinceaus.
Figure 5. Secretin and Kv1.2 inhibition with TsTx similarly affect cerebellum-dependent learning.
Figure 6. The source of tonic inhibition opposing secretin mediated Kv1.2 endocytosis.
Figure 7. The complex regulation of surface Kv1.2 by AC and PKA.
Figure 8. Labeling surface Kv1.2 in the rat cat cerebellum following fixation by cardiac perfusion.
CHAPTER 3: COMPREHENSIVE DISCUSSION

Overview

In the preceding paper, the hypothesis that Kv1.2 was regulatable by adenylate cyclase mediated endocytic trafficking in the mammalian brain was tested. By pharmacological manipulations and subsequently quantifying immunoblots of biotinylated cell-surface proteins from rat cerebellar slices, it was found that the peptide secretin enhanced the net endocytosis of Kv1.2 through a SCTR/AC/PKA/dynamin dependent pathway. By microscopy techniques, pharmacological activation of AC was found to decrease the apparent abundance of plasma membrane localized Kv1.2 not only in pinceau structures of basket cell axon terminals, but also within the molecular layer, presumptively reflecting Kv1.2 in Purkinje cell dendrites. Through collaborations with the other authors, the electrophysiological (Morielli) and behavioral (Green and Fuchs) consequences of such regulation were explored.

This work collectively demonstrates the first example of endocytic regulation of Kv1.2, and indeed of any Shaker family channel, in the mammalian nervous system. However, the discovery of the regulation of cerebellar Kv1.2 by secretin raises multiple questions.

Determinants Of AC-Mediated Kv1.2 Endocytic Potential

Alpha Subunit Composition

A main question arising out of this research concerns the generality of the central phenomenon. That is, cerebellar Kv1.2 appears regulatable by adenylate cyclase through
the modulation of its endocytic trafficking. As was reviewed prefacing the article, Kv1.2 is abundantly expressed throughout the body, including other neuronal populations such as the hippocampus, and in other excitable cells, such as smooth muscle. Is the trafficking phenomenon restricted to cerebellar Kv1.2, or is it an intrinsic property of the channel to be regulated by AC modulated endocytosis? This may depend upon the identity of the holochannel of which Kv1.2 is a part. In the cerebellum, Kv1.1 is expressed presynaptically, among other places, in pinceaus, and Kv1.5 is expressed postsynaptically in Purkinje cells (Chung, Shin et al. 2001; Khavandgar, Walter et al. 2005). Since both populations seemed to have reduced surface Kv1.2 expression following AC stimulation, it may be that only homomeric populations both pre- and postsynaptically are so regulatable, or it may be that heteromultimeric channels of Kv1.2 with either Kv1.1 or Kv1.5 are also modulated by AC dependent endocytosis. Unpublished work in the Morielli lab on Kv1.2 surface expression in HEK cells, under conditions where it is co-expressed with Kv1.1 or with Kv1.5, has found that its surface expression can still be modulated by AC. Therefore, Kv1.2 may confer the ability of the heteromultimers to be regulated by AC dependent trafficking.

**Beta Subunit Composition**

However, endocytic potential may not simply be dictated by the molecular composition of the alpha subunits. Both the Kvβ1 and Kvβ2 subunits are expressed in the cerebellum, and although Kvβ1 seems to be a minor constituent of the protein complexes (Rhodes, Strassle et al. 1997), it is unknown what role the accessory subunit composition might play in determining the polarity of cerebellar Kv1.2 channels by AC. However, at
least in HEK cells, Kvβ2 is not required for cAMP mediated increases in Kv1.2 expression (Connors, Ballif et al. 2008). Likewise, Kv1.2 containing channels may interact with a variety of other molecules that dictate whether and how they are endocytically regulated.

Accessory Proteins

Cerebellar pinceaus are rich in actin (Capani, Ellisman et al. 2001), with which Kv1.2 may physically associate through the adaptor molecular cortactin, a protein known to modulate the endocytic potential of Kv1.2 downstream of GPCR and AC signaling (Williams, Markey et al. 2007; Connors, Ballif et al. 2008). Kv1.2 may also interact, through a C-terminal PDZ binding domain (reviewed in Tiffany, Manganas et al. 2000), with members of the membrane-associated guanylate kinase (MAGUK) family that are expressed in both pinceaus and PC dendrites, such as PSD-95. Since these proteins may alter the subcellular localization of Kv1 family channels, it is conceivable that they regulate whether Kv1.2 is susceptible to endocytosis (Tiffany, Manganas et al. 2000; Ogawa, Horresh et al. 2008). This is the case for AMPA receptors, which also associate with PSD-95, and through the latter’s recruitment of enzyme such as A-kinase-anchoring protein 150 (AKAP-150), dictate the endocytic potential of glutamate receptors (Bhattacharyya, Biou et al. 2009). Determining the macromolecular identity of endocytosed versus not-endocytosed Kv1.2 channels shed light on the molecular determinants of Kv1.2 regualtability by AC.

Adenylate Cyclase Isoforms
The experiments within the manuscript provided evidence that the stimulation of AC is capable and necessary for enhancing net endocytic trafficking of cerebellar Kv1.2. While PKA, through pharmacological inhibition, was inferred to be necessary for observing an AC mediated decrease in surface Kv1.2, PKA inhibition (along with dynamin inhibition) actually unmasked an AC stimulated increase in surface Kv1.2. Since AC stimulation therefor appears capable of producing either increases or decreases in surface Kv1.2 expression, this raises the question of the determinants of the polarity of Kv1.2 trafficking following AC stimulation. Adenylate cyclase has been referred to thus far in this dissertation as a unitary entity, when in fact there are ten vertebrate forms (Defer, Best-Belpomme et al. 2000; Sadana and Dessauer 2009). It would be interesting to determine what AC isoforms are behind the regulation of Kv1.2 surface expression, as at least types I, II, V, VII, VIII are expressed in the cerebellum, of which isoform 1 is responsible for the calcium sensitive pool (Xia, Choi et al. 1993; Mons, Yoshimura et al. 1998; Defer, Best-Belpomme et al. 2000; Sadana and Dessauer 2009). This determination would be informative, i.e. perhaps Kv1.2 endocytosis is natively stimulated by calcium dependent AC, serving as a response to coincidence detection (Choi, Xia et al. 1993), or perhaps Kv1.2 endocytosis is stimulated by the ethanol stimulated AC isoform VII, partially causative of discoordination with alcohol consumption, just as partial loss of Kv1.2 in the pgu mouse causes gait disturbances (Xie, Harrison et al. 2010). It may be even the case that different isoforms of AC are involved in the different polarities of AC mediated Kv1.2 trafficking.

*Protein Kinase A Forms And Effectors*
In this dissertation thus far, PKA, like AC, has been simply referred to as a single entity. In reality, PKA is a multimeric protein complex consisting of diverse regulatory and catalytic subunits that constitute the holoenzyme. Each holoenzyme consists of two catalytic subunits and of two regulatory subunits, the latter of which bind cAMP and liberate the former. There is diversity in both regulatory and catalytic subunits: there are alpha, beta, and gamma isoforms of the catalytic subunit, and there are RIα and RIβ, as well as RIIα and RIIβ isoforms of the regulatory subunits. The RI vs. RII identity of the regulatory subunit defines the identity of the holochannel as PKA Type 1, which is largely cytoplasmically located or Type 2, which is chiefly localized to organelles and cytoskeletal components. The RII proteins are also important for associating the holochannel with AKAPs, which are known to coordinate PKA and related phosphatases to HERG potassium channels, and AKAPs may even dictate whether cAMP elevations enhance or suppress potassium channel function (Kurokawa, Motoike et al. 2004; Chen, Kurokawa et al. 2005; Chen and Kass 2006; Li, Sroubek et al. 2008). Perhaps as been done in cardiac monocytes, AKAP inhibitors could be delivered via an electrophysiological recording pipette to test if they have a role in the secretin dependent suppression of Kv1.2 and the subsequent increase in IPSC frequency in BC terminals if delivered presynaptically, or in the secretin mediated increase in PC dendritic excitability, if delivered postsynaptically (Potet, Scott et al. 2001). In general, determining the protein complexes of both the internalized and surface pool of Kv1.2 after AC stimulation may add to understanding the mechanisms that determine what populations of Kv1.2 channels may be so modulated. Indeed, it is known that for Kv4.2
association with AKAPs and the AKAPs’ phosphorylation state dictate the trafficking and subcellular targeting of the potassium channel complex downstream of PKA activation (Lin, Sun et al. 2011). Conversely, it should be noted that there are likewise a multiplicity of enzymes that oppose the AC/PKA pathway, such as phosphodiesterases and phosphatases (Fimia and Sassone-Corsi 2001; Baillie, Scott et al. 2005; Pidoux and Tasken 2010). So, in addition to identifying the targets that drive the AC stimulated, PKA dependent endocytosis of Kv1.2, experiments which complement these by elucidating pathways that reverse or oppose AC stimulated Kv1.2 endocytosis should be considered.

**Potential Mechanisms Of PKA Modulated Kv1.2 Endocytosis**

**Direct Phosphorylation**

Beyond identifying the relevant isoforms of AC and PKA, there is also the question of the effector downstream of PKA that is responsible for stimulating net Kv1.2 endocytosis. At least in vitro, Kv1.2 can be serine phosphorylated by PKA at site 449 (Johnson, El-Yazbi et al. 2009). It seems unlikely however that direct phosphorylation of the channel by PKA is responsible for endocytosis. This is because mutagenesis of this site to preclude phosphorylation does not result in enhanced surface expression, but rather diminished surface expression: phosphorylation at this site appears to be involved in biosynthesis of the channel, serving as a hallmark of mature channel competent for surface expression (Yang, Vacher et al. 2007), and is unrelated to acute changes in Kv1.2 surface expression after AC stimulation (Connors, Ballif et al. 2008). An alternate residue, T46, may (Huang, Morielli et al. 1994) or may not (Minor, Lin et al. 2000) be a
site for PKA phosphorylation, but its mutation does prevent cAMP mediated increases in surface Kv1.2 in HEK cells (Connors, Ballif et al. 2008).

**Modulation of the cytoskeleton**

Besides direct phosphorylation, one mechanism for how PKA activity may enhance net endocytosis of Kv1.2 is through modulation of the actin cytoskeleton. Indeed, PKA modulation of the closely related channel Kv1.5 occurs through a cytoskeletal dependent mechanism (Mason, Latten et al. 2002). PKA may modulate cytoskeletal dependent Kv1.2 endocytosis in the cerebellum through the adaptor protein cortactin. Cortactin physically interacts with Kv1.2 (Hattan, Nesti et al. 2002; Williams, Markey et al. 2007), and cortactin knockdown in HEK cells prevents AC mediated changes in Kv1.2 surface expression (Connors, Ballif et al. 2008). Mechanistically, AC may stimulate PKA to induce a loss of surface Kv1.2 through the regulation of calpain mediated cortactin proteolysis (Mingorance-Le Meur and O'Connor 2009).

**Protein tyrosine kinases**

An alternative mechanism by which PKA may enhance Kv1.2 endocytosis is activation of protein tyrosine kinases, as tyrosine phosphorylation is known to be a signal for Kv1.2 endocytosis in HEK cells (Nesti, Everill et al. 2004), and as PKA has been found to activate the tyrosine kinase Fyn in neurons (Yang, Yang et al. 2011). Other kinases may be directly or indirectly involved as well: PKA modulates Kv4.2 through the intermediate regulation of MEK/ERK (Anderson, Adams et al. 2000; Morozov, Muzzio et al. 2003).
Another target of PKA is the small GTPase RhoA, which physically associates with Kv1.2 (Cachero, Morielli et al. 1998) and which, through Rho Kinase, regulates both Kv1.2 endocytosis and recycling (Stirling, Williams et al. 2009). Although PKA classically inhibits RhoA, RhoA or Rho Kinase inhibition increases surface Kv1.2 in HEK cells (Stirling, Williams et al. 2009). In contrast, pilot studies as described earlier have shown that inhibition of RhoA or Rho Kinase reduces surface Kv1.2. Therefore, it is possible that that stimulation of PKA enhances cerebellar Kv1.2 endocytosis through inhibition of RhoA; testing whether secretin reduces surface Kv1.2 in the presence of C3 exoenzyme could test this model.

**Inhibition of Forward Trafficking**

Alternatively, PKA may not elicit endocytosis per se, but may retard the exocytic delivery of constitutively recycling Kv1.2 by influencing its localization at the plasma membrane to ongoing endocytic sites, or by modulating its targeting once internalized to fast versus slow recycling paths, as has been described for PKA’s ability to modulate EGFR trafficking (Salazar and Gonzalez 2002). Furthermore, PKA may enhance net endocytosis of Kv1.2 by interfering with recycling of Kv1.2 back to the plasma membrane from multi-vesicular bodies, where PKA has recently been found to be targeted in an activity- and isoform-dependent fashion (Day, Gaietta et al. 2011).

**Overview of multiple roles for PKA in ion channel trafficking**
While the work provides support for a role of PKA in the regulated trafficking of cerebellar Kv1.2, the actual mechanisms are likely to be complex, as PKA is not simply a universal driver of endocytosis. On the one hand, PKA is established to regulate membrane protein endocytosis, i.e.: PKA is required for endocytosis of the sodium/proton exchanger NHE3 (Hu, Fan et al. 2001), for glycine receptor internalization in retinal cells (Velazquez-Flores and Salceda 2011), and for endocytosis of low density lipoprotein receptors (Li, van Kerkhof et al. 2001). On the other hand, PKA activity protects certain proteins from endocytosis, such as metabotropic and ionotropic glutamate receptors (Ehlers 2000; Mundell, Pula et al. 2004) and aquaporins (Katsura, Gustafson et al. 1997). Therefore, while the work provides the first evidence that Kv1.2 can be regulated by endocytic trafficking in the nervous system, and describes that the ubiquitous AC/PKA pathway is involved, there are several lines of research that could follow up by further examining the molecular mechanisms. There are, similarly, experiments that could flow from this work to determine what forms of endocytosis are involved in the trafficking of neuronal Kv1.2.

**Potential Dynamin Sensitive Forms Of Cerebellar Kv1.2 Endocytosis**

*Dynamin Overview*

In the manuscript, it was demonstrated that the AC mediated loss of surface Kv1.2 is accompanied by an increase in internalized channel, and that this decrease in surface channel could be prevented by pretreating cerebellar slices with dynasore, a small molecule inhibitor of the GTPase dynamin (Macia, Ehrlich et al. 2006). Dynamin was initially identified in bovine brain extracts as a protein which bound microtubules and
which exhibited GTPase activity (Shpetner and Vallee 1992), but it is now recognized that there are at least three vertebrate genes encoding nearly thirty dynamin proteins via alternative splicing (Faire, Trent et al. 1992; Cook, Urrutia et al. 1994; Gray, Fourgeaud et al. 2003). Since the initial proposal that a homologous protein in Drosophila was associated with endocytic processes (van der Bliek and Meyerowitz 1991), it has been demonstrated over the past decades that dynamin is involved in many forms of endocytosis. Endocytosis involves the invagination of the plasma membrane and internalization of extracellular matter and plasma membrane embedded molecules, and there are several forms, well reviewed in (Doherty and McMahon 2009), by which cerebellar Kv1.2 may have been regulated in a dynamin dependent fashion.

**Macropinocytosis**

Macropinocytosis is a process by which invaginations can close off to form vesicles up to several microns wide, that cells utilize for regulating bulk fluid exchange and is important in immune cell function (Kerr and Teasdale 2009; Lim and Gleeson 2011). Neurons are capable of macropinocytosis (Kabayama, Takeuchi et al. 2011) and the involvement of this process in secretin mediated cerebellar Kv1.2 endocytosis could be tested by inhibition with amiloride derivatives (West, Bretscher et al. 1989; Koivusalo, Welch et al. 2010). Inhibition of dynamin GTPase activity as in the manuscript may have inhibited macropinocytosis, as dynamin 2 has been found to regulate macropinocytosis (Schlunck, Damke et al. 2004) and the related micropinocytosis (Cao, Chen et al. 2007); but dynamin 1 has been found to not have a role in some forms of macropinocytosis (Noguchi, Matsumoto et al. 2005).
**Phagocytosis**

Phagocytosis is a process largely limited to specialized cell types such as macrophages, whereby large particles can be engulfed, though this process has been observed in neurons as well (Esselens, Oorschot et al. 2004; Bowen, Ateh et al. 2007). At least in macrophages, dynamin is required for this form of endocytosis (Gold, Underhill et al. 1999), and accordingly, dynasore can block phagocytic engulfment of pathogens by macrophages (Barrias, Reignault et al. 2010). However, the role of dynamin in phagocytosis may require dynamin’s actin modulatory roles, not simply its ability to facilitate vesicle scission (Huynh and Grinstein 2008), and the extent to which phagocytosis is involved in normal neuronal physiology is largely unknown. To the degree which phagocytosis may exist in neurons and be dynamin dependent; dynasore treatment would have been expected to inhibit this form of cerebellar Kv1.2 endocytosis.

**Caveolin/Flotillin Dependent Endocytosis**

Another class of endocytosis involves proteins that are typically found in cholesterol enriched microenvironments of the plasma membrane: caveolin/flotillin dependent endocytosis. Though caveolin proteins can form the structural invaginations known as caveolae, they have roles in trafficking and endocytosis that are unrelated to these formations (Lajoie, Kojic et al. 2009). Caveolin appears to play a role in endocytic trafficking of metabotropic glutamate receptors in the cerebellum (Kimura, Watanabe et al. 1990), and interaction with caveolin can regulate potassium channels (Lin, Lin et al. 2008; Brainard, Korovkina et al. 2009; Davies, Purves et al. 2010) including their surface expression (Alioua, Lu et al. 2008) and endocytosis (Jiao, Garg et al. 2008; Massaeli, Sun et al. 2010).
et al. 2010). Indeed, while such a role has not been described in the central nervous system, caveolin can influence the subcellular targeting of the Shaker family potassium channel Kv1.5 (Folco, Liu et al. 2004; Abi-Char, Maguy et al. 2007; McEwen, Li et al. 2008), as well as the targeting of PKA to potassium channels (Sampson, Hayabuchi et al. 2004), and dynamin is required for some (Huang, Wang et al. 2011; Moskovich, Herzog et al. 2012) but not all forms of endocytosis that are caveolin dependent (Balkrishna, Broer et al. 2010; Khan, Pickl-Herk et al. 2011). Determining the involvement of caveolin type endocytosis proteins in cerebellar Kv1.2 trafficking will be interesting, especially since in HEK cells, dynamin dependent Kv1.2 endocytosis appears to involve both cholesterol dependent and independent endocytosis pathways (Stirling, Williams et al. 2009), and as caveolin is involved in the former (Le Lay, Hajduch et al. 2006; Gerondopulos, Jackson et al. 2010). While the dynamin dependency of AC mediated Kv1.2 endocytosis does not inform on the involvement of caveolin/flotillin dependent processes, the roles for caveolin dependent processes in cerebellar Kv1.2 endocytosis could be investigated experimentally. This could be accomplished with commercially available cell permeant peptides that compete for caveolin scaffolding domains (Bucci, Gratton et al. 2000; Zhu, Schwegler-Berry et al. 2004), while the dependence on cholesterol might be investigated with cholesterol depletion via methyl-beta-cyclodextrin, or by cholesterol elevation via incubation of free cholesterol loaded with the same, as has been done in neuronal cultures (Nicholson and Ferreira 2009).
Clathrin Mediated Endocytosis

The most well characterized form of endocytosis, especially with respect to its dependence on dynamin, is clathrin-mediated endocytosis. Clathrin is a protein which exists in both heavy and light chain forms, and by formation of a triskelion lattice, creates coats about certain forms of endocytic vesicles (Pearse 1976). Sometimes used synonymously with receptor mediated endocytosis, a classic understanding of this process involves cargo, for example a potassium channel, interacting with adaptor proteins that then complex to clathrin, driving the formation of a budding endosome along with the activity of membrane deforming proteins (reviewed in (McMahon and Boucrot 2011)). Dynamin has an important function in terminating this process, which both by its interactions with actin and by its pinchase activity, provides energy to break the endocytosing vesicle off from the plasma membrane (McNiven 1998; Sweitzer and Hinshaw 1998; Takei, Slepnev et al. 1999; Hill, van Der Kaay et al. 2001; Marks, Stowell et al. 2001; Merrifield, Feldman et al. 2002). Intriguingly, cortactin, which regulates Kv1.2 trafficking, is required for some forms of dynamin and clathrin dependent endocytosis (Cao, Orth et al. 2003). Though cortactin and dynamin are also involved in clathrin-independent endocytosis (Sauvonnet, Dujeancourt et al. 2005) and actin dynamics are critical to many forms of endocytosis, both forms clathrin dependent ((Yarar, Waterman-Storer et al. 2005; Mooren, Galletta et al. 2012) and independent (Romer, Pontani et al. 2010)). Testing the requirement of clathrin in the endocytosis of cerebellar Kv1.2 might be approached pharmacologically: hypertonic sucrose inhibits coated pit formation (Heuser and Anderson 1989), while hydrophobic amines such as
chlorpromazine or monodansylcadaverine aberrantly stabilize clathrin coats (Phonphok and Rosenthal 1991). However, at this stage, the dynamin dependency again does not clarify whether the type of endocytosis involved in cerebellar Kv1.2 trafficking is clathrin dependent.

Because of the diversity of endocytic pathways and their frequent but not utter reliance on dynamin, there is a great deal of work that could be done to understand the molecular mechanisms guiding the endocytosis of Kv1.2 in the brain. At the least, it will be interesting to distinguish whether the prevention of Kv1.2 endocytosis under inhibition of dynamin occurs through the loss of the dynamin’s pinchase or dynamin’s cytoskeletal regulation properties, or both (Itoh, Erdmann et al. 2005; Tanabe and Takei 2009; Gu, Yaddanapudi et al. 2010), as Kv1.2 subcellular distribution and endocytosis is regulated by actin dynamics (Hattan, Nesti et al. 2002; Denisenko-Nehrbass, Oguievetskaia et al. 2003; Nesti, Everill et al. 2004; Williams, Markey et al. 2007).

Potassium Channels In Learning And Memory

Beyond the cellular mechanisms responsible for the endocytic suppression of cerebellar Kv1.2, the very finding that suppression of this channel improves learning in least in one paradigm is an important addition to a generally small field. The finding, in the preceding manuscript, that direct inhibition of Kv1.2 function with Tityustoxin-Kα (TsTx) enhanced the acquisition of the eyeblink conditioned response is the first indication that acute modulation of this channel’s function can alter learning. The research on the role of Shaker-family potassium channels in learning and memory in
general is sparse, but previous studies do support the contention that potassium channels are either regulated in learning, or that modulation of their function alters learning.

Extending back nearly thirty years, in Drosophila, *Shaker* mutants did not display classical conditioning in courtship behavior (Cowan and Siegel 1984), and subsequent studies by the same group demonstrated defective degree and speed of performance in a conditioned odorant avoidance task (Cowan and Siegel 1986). Interestingly, in Drosophila, learning deficits from altered adenylate cyclase signaling or *Shaker* function have been proposed to be mechanistically related (Zhong, Budnik et al. 1992). In a sea slug, classical conditioning has been found to lead to down regulation of potassium channel function (Etcheberrigaray, Matzel et al. 1992). In rats, the mRNA of Kv1.1 was found to be down regulated differentially over time and space in the hippocampus by an associative learning task (Kourrich, Manrique et al. 2005). Also in rats, inhibition of Kv1.1 and Kv1.3 with kaliotoxin improved performance on an olfactory associative learning task (Kourrich, Mourre et al. 2001). In mice, loss of Kv1.1 impaired a passive avoidance and spatial learning task (Meiri, Ghelardini et al. 1997; Gratacos, Ghelardini et al. 1998). Perhaps most germane to this dissertation, it was found that in the rabbit cerebellum, an eyeblink conditioning task decreased the functional abundance of 4-aminopyridine (4-AP) sensitive potassium channel function in Purkinje cell dendrites (Schreurs, Gusev et al. 1998). Voltage-gated potassium channels likely subserve a variety of cerebellar physiological responses, since 4-AP has been found in the rabbit cerebellum to not only enhance conditioning per se, but also to elevate the degree of non-conditioned responsivity in general (Wang, Darwish et al. 2006). The results presented here and in the
referenced literature collectively suggest that localized suppression of specific Shaker family potassium channels can influence behavior, and further suggest that suppression of these channels may be a largely unexplored mechanism of mammalian learning. Research which parcel(s) out specific pathways that modulate potassium channels in discrete brain regions could then elucidate novel pathways in learning and memory.

**Potential Roles For The Regulation Of Cerebellar Kv1.2**

With respect to the neurobiology of learning, the classes of cerebellar neurons, their afferents and efferents, and the mechanisms regulating those synapses are relatively well documented. While plasticity has been observed at many different cerebellar synapses, as detailed in the preceding manuscript, Kv1.2 expression is essentially limited to Purkinje cell (PC) dendrites, and inhibitory basket cell (BC) axon terminals about the Purkinje cell’s initial axon segment and soma. How might the description of AC mediated suppression of Kv1.2 in both PC dendrites and BC axon terminals inform potential novel mechanisms for established synaptic phenomena?

Beginning at the Purkinje cell itself, depolarization of the Purkinje cell elicits two related phenomena: a transient suppression of inhibitory input (DSI), followed by a potentiation of inhibitory input (DPI) (As reviewed in (Tzingounis and Nicoll 2004). The DSI phenomenon is mediated through the release of cannabinoids from the PC, which act retrogradely at CB1 receptors in the BC axon terminal, activating Gi and reducing GABA release. As Gi activation, as described in the manuscript, suppresses the BC axon terminal Kv1.2, perhaps CB1 mediated Gi activation leads to enhanced surface
expression of Kv1.2 as a mechanism to transiently oppose transmitter release. However, as indicated in the pilot data following the manuscript, inhibiting AC or stimulating CB1 seems also to reduce surface Kv1.2 in the cerebellum, so rather than a causative mechanism, perhaps Gi mediated decreases in surface Kv1.2 temporally limit DSI.

Conversely, DPI occurs via retrograde release of secretin from Purkinje cells, which potentiates GABA release through the activation of Gs coupled secretin receptors in the BC axon terminal. Since it was demonstrated here that secretin reduces surface Kv1.2, and since we, in unpublished pilot work, and others (Southan and Robertson 1998; Southan and Robertson 1998), have shown that suppression of Kv1.2 also enhances GABA release, one mechanism for DPI may be SCTR/Gs stimulated endocytosis of presynaptic Kv1.2. Since other Gs coupled receptors also potentiate GABA release from BC’s, such as beta-adrenergic receptors, it will be interesting to explore whether these pathways stimulate Kv1.2 endocytosis too (Saitow, Satake et al. 2000).

Similarly, AC mediated endocytic suppression of Kv1.2 in PC dendrites may provide additional mechanisms for established synaptic phenomena. Forskolin application enhances the strength of the granule cell parallel fiber (PF) to Purkinje cell synapses, and this mechanism has been proposed to be largely presynaptic due to unaltered amplitude of neurotransmitter induced excitatory postsynaptic currents, coupled with evidence for an increase in presynaptic release probability but not site number (Saitow, Satake et al. 2000). However, direct postsynaptic recordings were not performed, so forskolin could increase strength at the PC-PC synapses in part by
enhancing intrinsic excitability of PC dendrites by suppressing Kv1.2 there. Indeed, in the paper that first described the role of Kv1.2 in opposing PC dendritic hyperexcitability, (Khavandgar, Walter et al. 2005) suggested that suppression of Kv1.2 and the accompanying increase in PC dendritic excitability could be a novel postsynaptic mechanism of spatially limiting PC dendritic excitatory responses. It is conceivable then that forskolin may enhance PF-PC strength through AC mediated suppression of postsynaptic Kv1.2, enhancing the spread of response to PF input over space or time. In the manuscript it was described, for first time, that there is a physiological mechanism for suppressing PC dendritic Kv1.2. Therefore, Gi coupled receptors in PC dendrites, such as SCTR, that affect cerebellar neurophysiology may do so in part through stimulating Kv1.2 endocytosis there.

Although not explored in the manuscript, Kv1.2 is variably expressed in some deep cerebellar or vestibular nuclei, so it is possible that AC mediated Kv1.2 endocytosis regulates cerebellar physiology at this level as well (Chung, Shin et al. 2001; Popratiloff, Giaume et al. 2003; Chung, Joo et al. 2005; Popratiloff, Shao et al. 2011; Iwasaki, Nakajima et al. 2012). This would be interesting to explore in future research, as some models propose that the long term changes for cerebellar dependent learning, while reliant on the cortex initially, are ultimately relayed to and dependent upon the deep nuclei (Shutoh, Ohki et al. 2006; Nagao and Kitazawa 2008; Kellett, Fukunaga et al. 2010). The cerebellar cortex also plays an important role in consolidation (Attwell, Cooke et al. 2002; Cooke, Attwell et al. 2004). Particularly, regulation of pinceau localized Kv1.2 would be expected to powerfully influence cerebellar dependent
learning, as presynaptic Kv1.2 function opposes basket cell GABA release, and as GABAergic inhibition of PC’s has been demonstrated to be critical for the consolidation of at least one form of cerebellar dependent learning (Wulff, Schonewille et al. 2009). Therefore, regulation of Kv1.2, whether in the cortex or deep nuclei, would be expected to modulate consolidation of cerebellar dependent learning.

**Discussion Summary**

By providing evidence that Kv1.2 is subject to AC modulated endocytic trafficking in the cerebellum, the manuscript provides support for the contention that there are endogenous mechanisms for acute suppression of Shaker family voltage gated potassium channels in the central nervous system. To move beyond describing the phenomenon of endocytic suppression of Kv1.2, towards understanding how this process is regulated over space and time, the neurophysiological and behavioral consequences of Kv1.2 endocytosis, and understanding why it is that this channel is so regulated will require experiments that supplement the pharmacological approaches utilized here. Globally manipulating the secretin pathway provided intelligible results and testable models of Kv1.2 regulation in the cerebellar slice preparation because of the relatively restricted expression and co-localization of the secretin receptor and Kv1.2. However, experimentally addressing some of the points raised in this discussion, such as globally manipulating clathrin, cholesterol levels, AKAP’s, etc., have the major limitation that they may not affect Kv1.2 trafficking directly. Instead, manipulation of such global pathways is likely to alter other aspects of neuronal function and cell biology, which might obscure or indirectly change Kv1.2 regulation. One approach that may be taken
then is not to manipulate pathways that might affect Kv1.2, but to instead manipulate
Kv1.2’s ability to be regulated by those pathways through targeted mutagenesis of the
channel. Preliminary unpublished research on an attempt to engineer AC responsiveness
by Kv1.2 mutagenesis is covered in APPENDIX A.

Another limiting aspect to the approach taken in the manuscript is that AC/PKA
and endocytic signaling cascades are dynamic with respect to space and time. In contrast,
the assays used herein are destructive, limiting their utility to inform how Kv1.2 is
likewise regulated spatiotemporally. For example, biotinylation offers only a global
average of Kv1.2 surface expression that can be obtained for a single time point.
Similarly while the Kv1.2 surface microscopy, whether by acetone enrichment for
surface channel or by ATTO-TsTx labeling, provided the large advantage of looking at
surface Kv1.2 in specific areas of the cerebellum (i.e. PC dendrites or pinceaus), it
required fixation, and so it too lacks the ability to resolve Kv1.2 trafficking over time.
While theoretically ATTO-TsTx could be applied to a live cerebellar slice or even in a
cerebellar window preparation to look at Kv1.2 localization, this would not be practically
informative since Kv1.2 inhibition would dramatically change the neurophysiology under
study. Towards developing a tool to allow the non-destructive study of Kv1.2 trafficking
over time, APPENDIX B details the unpublished preliminary work on the creation of a
genetically encoded bi-fluorescent reporter of Kv1.2’s subcellular environment.
APPENDIX A: A CAVEOLIN-BINDING DOMAIN MOTIF REGULATING Kv1.2 BIOSYNTHESIS

Background

Voltage gated potassium channels containing the alpha subunit Kv1.2 regulate excitability in diverse tissues. Several articles have described how, in heterologous expression systems, Kv1.2 function can be modulated by G-protein coupled receptor signaling, which mechanistically involves changes in Kv1.2 surface expression (Nesti, Everill et al. 2004; Williams, Markey et al. 2007; Connors, Ballif et al. 2008; Stirling, Williams et al. 2009). Recently, evidence for the GPCR mediated regulation of endogenous Kv1.2 trafficking in the rat cerebellum has been published (Williams, Fuchs et al. 2012). The regulated surface expression of cerebellar Kv1.2 occurred downstream of adenylate cyclase stimulation and involved channel endocytosis.

To the extent it has been studied, stimulation of AC at cerebellar synapses where Kv1.2 is expressed has been found to mimic inhibition of Kv1.2: stimulation of AC in cerebellar basket cell axon terminals as by secretin addition or adrenergic stimulation (Saitow, Satake et al. 2000; Yung, Leung et al. 2001) enhances GABA release. The facilitation of GABA release can also be accomplished by inhibiting Kv1.2 containing potassium channels in the basket cell axon terminal by exogenous pore-blocking toxin’s (Southan and Robertson 1998; Southan and Robertson 1998; Tan and Llano 1999; Southan and Robertson 2000), or by genetic reduction of Kv1.2 (Xie, Harrison et al. 2010). Within cerebellar Purkinje cells (PC), Kv1.2 is expressed in their dendrites. Pore-
blocking toxins that are specific for Kv1.2 increase the excitability of PC dendrites (Khavandgar, Walter et al. 2005), as does stimulation of AC by secretin addition (Williams, Fuchs et al. 2012). Therefore, our finding that AC stimulation stimulates endocytosis of cerebellar Kv1.2 (Williams, Fuchs et al. 2012), suggests that channel endocytosis could be a mechanism for AC mediated changes in cerebellar synaptic function.

**Point Mutations Which Alter Kv1.2 Trafficking**

To test this model requires asking whether cerebellar synapses expressing forms of Kv1.2 resistant to AC mediated endocytosis show reduced changes in synaptic physiology upon AC stimulation. A number of point mutations have been described that influence Kv1.2, but they are not specific to AC mediated regulation of the channel. While the T46V mutation does not show forskolin induced increases of Kv1.2 surface expression, this site does not appear to be a site of PKA phosphorylation in vitro or in vivo, and it shows very low surface expression, so it may have a dominant negative effect not just on AC/PKA regulatability of the channel, but on basal function as well (Yang, Vacher et al. 2007; Connors, Ballif et al. 2008; Johnson, El-Yazbi et al. 2009). What other mutations may affect Kv1.2 trafficking potential? Clathrin-mediated endocytosis is involved at least in the G12/13 coupled receptor modulated surface expression of Kv1.2 (Stirling, Williams et al. 2009). Tyrosine-based motifs, through their interaction with adaptor proteins, can confer clathrin-mediated endocytic potential to membrane proteins, classically as mediated by these motifs binding to the μ2 subunit of the AP2 protein complex (Chen, Goldstein et al. 1990; Canfield, Johnson et al. 1991; Collawn, Lai et al.
Kv1.2 contains multiple tyrosine based potential endocytosis motifs in its C-terminus, and indeed, mutation of Y458 alters the endocytic potential of Kv1.2 downstream of muscarinic receptor stimulation; however these tyrosine residues are also involved in the axonal targeting, cortactin binding, and basal surface expression of Kv1.2 (Hattan, Nesti et al. 2002; Gu, Jan et al. 2003). Furthermore, these mutations may not be the best target, as AP2 independent endocytosis occurs at neuronal synapses (Kim and Ryan 2009). The T1 domain of Kv1.2 also influences channel targeting and trafficking, but mutation of this domain would be inappropriate to attempt to uncouple it from AC mediated modulation, as it is involved in the formation and biophysical function of functional Kv1 tetramers (Yi, Minor et al. 2001; Gu, Jan et al. 2003; Long, Campbell et al. 2005; Rivera, Chu et al. 2005). Other trafficking motifs have been identified in Kv1.2, such as aforementioned C-terminal serines, but mutation of these sites change channel biosynthesis and basal surface expression (Yang, Vacher et al. 2007). Within the pore, there are Shaker family specific signals that regulate Kv1.2 trafficking, however they act through binding endoplasmic reticulum (ER) localized proteins and regulate channel progression through the biosynthetic machinery (Zhu, Gomez et al. 2005; Vacher, Mohapatra et al. 2007; Utsunomiya, Tanabe et al. 2010). Overall, then, there are no known mutations that can be made to Kv1.2 which would specifically modulate endocytic potential, precluding directly testing the role of AC regulated Kv1.2 endocytosis in neuronal physiology. The initial goal of the research in this appendix therefore was to test the effect of a novel mutation on Kv1.2 trafficking. Identification of
critical residues specifically involved in AC/PKA modulated trafficking of Kv1.2 would be a powerful tool for testing the role of this regulation in synaptic physiology or learning, as it has catalyzed work for Kv4.2, where a single point mutation prevents PKA dependent trafficking of the channel (Hammond, Lin et al. 2008).

Caveolin Regulates AC/PKA Modulation Of Ion Channels

What unexplored domains of Kv1.2 may regulate its AC/PKA mediated endocytic potential? Again drawing inspiration from studies on Kv4.2, PKA mediated regulation of is dependent upon channel interaction with caveolin, which is also known to scaffold and regulate the activity of components of the AC signaling cascade such as peptide receptors, $G\alpha$, AC, PKA, AKAPs , and phosphatases, (Razani, Rubin et al. 1999; Heijnen, Waaijenborg et al. 2004; El-Yazbi, Cho et al. 2006; Levin, Coroneus et al. 2006; Syme, Zhang et al. 2006; Ohkubo and Nakahata 2007; Allen, Yu et al. 2009; Alday, Urrutia et al. 2010; Macdougall, Agarwal et al. 2012). The role of caveolin in PKA activity is likely complex; in some cases loss of caveolin is associated with elevated PKA activity (Cohen, Razani et al. 2004; El-Yazbi, Cho et al. 2006), while in other cases, loss of caveolin appears to reduce PKA activity (Engelman, Zhang et al. 1999; Razani and Lisanti 2001; Levin, Murase et al. 2007). However, since caveolin is thought to spatially organize PKA to potassium channels, such as inward rectifiers, it was hypothesized that delocalizing Kv1.2 from caveolin may prevent regulation of the channels trafficking by the AC/PKA pathway (Sampson, Hayabuchi et al. 2004).
Caveolin Background

Initially identified as a coat component of caveolae with an antibody against an unknown v-src kinase substrate, caveolins are encoded by three genes, and the resultant transmembrane proteins have relatively well deduced functional domains (Rothberg, Heuser et al. 1992; Scherer, Okamoto et al. 1996; Tang, Scherer et al. 1996). Caveolins structurally consist of cytoplasmic N- and C- termini that contain membrane associating domains and that mediate protein-protein interactions, and consist of an intervening hairpin that may mediate insertion within the intracellular aspect of cholesterol rich regions of the plasma membrane (Murata, Peranen et al. 1995; Sargiacomo, Scherer et al. 1995; Das, Lewis et al. 1999; Krajewska and Maslowska 2004; Epand, Sayer et al. 2005; Spisni, Tomasi et al. 2005). The N-terminus of caveolin (amino acids 61-101) contains the domain that is required for caveolin-caveolin oligomerization. A section of that domain, proximal to the first transmembrane section, contains the caveolin scaffolding domain (amino acids 82-101), through which caveolin interacts with proteins that have a caveolin binding domain motif: ΦXΦXXXΦ, ΦXXXXΦXXΦ, or the composite ΦXΦXXXXΦXXΦ, where Φ is an aromatic hydrophobic amino acid: tyrosine, phenylalanine, or tryptophan (Li, Couet et al. 1996; Couet, Li et al. 1997).

Engineering Caveolin Binding Through Mutagenesis

Mutation of the Φ residues to alanines prevents the proteins which host these motifs from interacting with caveolin (Couet, Li et al. 1997), and this approach has been taken to probe the involvement of caveolin in the regulation of calcium activated potassium channels (Alioua, Lu et al. 2008), the metabotropic glutamate receptor subunit
mGluR1 (Francesconi, Kumari et al. 2009), the glucagon-like peptide 1 receptor (Syme, Zhang et al. 2006), the sonic hedgehog receptor Patched (Karpen, Bukowski et al. 2001), the insulin receptor (Nystrom, Chen et al. 1999), and the D1 dopamine receptor (Kong, Hasbi et al. 2007), among others.

Similar mutations have not been made in Shaker family channels, perhaps because it has been (erroneously) reported that at least Kv1.5 lacks a caveolin binding motif (Martens, Sakamoto et al. 2001). To address this scientific gap, and towards the goal of producing a mutagenized form of Kv1.2 normal but for being uncoupled from caveolin, and therefore perhaps AC/PKA dependent regulation, mutagenesis of a putative caveolin binding domain motif in Kv1.2 was conducted, and the consequences on channel trafficking were explored.

Methods

Mutagenesis

A mammalian expression vector (EGFP-C) (Clontech) encoding rat Kv1.2 was used as a template (Doczi 2010), along with a site-specific mutagenic primer

\(5'\text{GCATCATCTGGTCTCCGCTGAGGCTCTGGTTAGATTCGCTGCCTGTCCCA}\ GC\)

and its reverse complement, to create the F145A/W150A/F153A mutation in GFP-Kv1.2 using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent) according to the manufactures instructions. The product was transformed, again according to manufacturer’s instructions, into chemically competent XL10 Gold cells (Agilent) under kanamycin (40 μg/mL) selection. Kanamycin resistant clones were used
to inoculate cultures in Luria Broth with kanamycin, and the resultant cultures were used for cryopreservation and for DNA production for subsequent transfection (as indicated below) after the final sequence was confirmed by the UVM DNA Analysis Core Facility.

**Cell Culture**

Human Embryonic Kidney (HEK293) (ATCC) cell lines stably expressing the M1 muscarinic receptor under G418 selection (HEK293-M1) were maintained at 37° Celsius in an environment of humidified atmosphere plus 5% carbon dioxide. The culture media consisted of DMEM/F-12 supplemented with penicillin, streptomycin, glutamine, 10% fetal bovine serum, sodium bicarbonate, and G418. In indicated experiments, for which HEK-293 M1 cells also stably expressing the Kvβ2 subunit (HEK293-β) were used, Zeocin was also present in the cell culture media to maintain selection of Kvβ2 expression under the Zeo+ plasmid. Plastic, cell culture treated, dishes from Corning were used for all experiments, and as, indicated, were coated with Poly-D-Lysine (PDL).

**Transfection**

Nearly confluent plates of either HEK293-M1 or HEK293-β cells were gently rinsed with phosphate buffered saline without calcium or magnesium (PBS) twice, then incubated with Trypsin-EDTA for 5-10 minutes. After resuspension by gentle trituration in cell culture media lacking Zeocin and/or G418 (No Select Media), cells were plated to ~70% confluence and allowed to recover in culture for at least thirty minutes. During this time, for each dish to be transfected, in a low retention tube (Fisher Scientific), cell
culture media lacking Zeocin and/or G418 and also lacking serum (Zero Serum) was incubated with the manufacture recommended amount of dsDNA, gently mixed, then incubated with the manufacture recommended amount of LTX lipofection reagent (Invitrogen), after gentle mixing, for at least 30 minutes at room temperature. In all experiments, Kv1.2 was one quarter of the total DNA used, and in experiments when an additional protein was overexpressed, an amount equal to Kv1.2 was used, and the balance was empty plasmid vector. The following day, transfected cells at confluence were trypsinized as above and plated to low confluence on PDL coated tissue culture dishes, or onto PDL coated glass cover slips in tissue culture dishes and cultured in No Select Media for at least 30 minutes. Subsequently, dishes were gently rinsed twice with PBS and incubated overnight in Zero Serum before being processed for immunoprecipitation, cell surface protein biotinylation and/or protein immunoblot, flow cytometry, fluorescence microscopy, or electrophysiology.

Cell Surface Protein Biotinylation And Western Blot Sample Preparation

Culture dishes were removed from the incubator directly to ice/water slurry. Media was aspirated and cultures were rinsed three times with ice-cold Hank’s Buffered Salt Solution with calcium and magnesium but not phenol red (HBSS, Invitrogen). Primary amines of cell surface proteins were then biotinylated by incubating the cultures with 1 mg/ml sulfo-nhs-ss-biotin (“Biotin”, Pierce) dissolved in HBSS, for 30 minutes on ice with gentle agitation. The biotin solution was then aspirated, and unreacted biotin was quenched by rinsing the cultures three times with 50 mM TRIS in HBSS. Biotinylated cultures were lifted by scraping with the wide end of a disposable 100-1000 μl pipettor
tip, in a lysis buffer (50 mM TRIS, 150 mM NaCl, 1 mM EDTA, 0.25% deoxycholate, 1% NP40, 10% glycerol, mammalian protease inhibitor cocktail (Sigma), pH 8.0), and the lysate was transferred to a low retention tube. After brief sonication, samples were incubated on ice before being clarified by refrigerated centrifugation for 30 minutes at 16 kRCF. A portion of clarified “Total” lysate was reserved and a fixed majority of the remainder, without disturbing the pellet, was incubated with high-capacity neutravidin bead slurry (Pierce). Neutravidin beads and bound biotinylated proteins were isolated by centrifugation and washed repeatedly with lysis buffer. Biotinylated proteins were eluted from neutravidin beads by incubation at 100ºC in Laemmli sample buffer containing 0.1M dithiothreitol (Sigma) to produce the “Eluant”. Total lysate samples were similarly prepared for immunoblot by incubation at 100ºC in sample buffer and dithiothreitol. For some experiments, biotinylation was omitted, but HBSS rinsed samples were similarly lysed, clarified, and incubated with Sample buffer and heated to analyze the Total lysate.

Samples were resolved by poly-acrylamide gel electrophoresis and protein immunoblot as previously published (Williams, Markey et al. 2007). To summarize, samples were resolved via Precise TRIS-HEPES Pre-Cast gels of either 10% or 4-20% gradient polyacrylamide content (Pierce), ran at 100V, and then transferred in a TRIS/Glycine/Methanol buffer onto nitrocellulose. After blocking in 5% non-fat dry milk reconstituted in TRIS-buffered Saline with 0.1% Tween-20 (TBST), blots were incubated with primary antibodies diluted in 3% bovine serum albumin (BSA) in TBST. After rinsing in TBST, blots were incubated in infrared dye-coupled secondary antibodies diluted in TBST with 5% non-fat milk. Goat Anti-Rabbit 800 and Goat Anti-Mouse 800
antibodies were acquired from Rockland, while Goat Anti-Rabbit 700 and Goat Anti-Mouse 700 antibodies were acquired from Invitrogen. Following incubation in secondary antibodies, and after rinsing in TBST, infrared signals were detected and quantified using an Odyssey Infrared Imager (LiCor).

Primary antibodies were mouse monoclonal α-Kv1.2 (K14/16, Neuromab) and rabbit polyclonal α-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ab9485, Abcam). Also used was mouse monoclonal α-GFP (N86/8 or N86/38 Neuromab), mouse monoclonal α-Kv1.2 phospho-serines 440/441 (A kind gift from Dr. James Trimmer, (Yang, Vacher et al. 2007)), and rabbit polyclonal α-PAN Kv1.2 (44703G, Chemicon/Invitrogen Discontinued). In pilot experiments, both GFP-Kv1.2 and GFP-Kv1.2-Φ were confirmed to generate qualitatively consistent banding patterns whether probed with α-GFP, α-Kv1.2, or α-PAN Kv1.2, so throughout the text, “α-Kv1.2” refers non-specifically to utilization of any of these similarly preforming antibodies in immunoblot studies unless specifically indicated otherwise.

Immunoprecipitation

Total lysates were generated as above from HEK293-M1 and from HEK293-β cells transiently transfected with vector only, with GFP-Kv1.2, or with GFP-Kv1.2-Φ. A portion of Total lysate was reserved; the remainder incubated with mouse monoclonal α-Kv1.2 (K14/16, Neuromab). The immunoprecipitate was collected by incubation with Protein G beads (Invitrogen), which were then washed with lysis buffer before elution and evaluation by protein immunoblot as above. Blots were probed with rabbit polyclonal
anti-PAN Kv1.2 (44703G, Chemicon/Invitrogen Discontinued) and with mouse monoclonal anti-Kvβ2 (K17/70, Neuromab).

*Biotinylation Sample Statistics*

Surface Kv1.2 (Absolute) was calculated by dividing Kv1.2 signal in the Eluent by the GAPDH in the Total. Surface Kv1.2 (Proportion) was calculated by dividing the density of Kv1.2 signal in the Eluent by the density of Kv1.2 signal in the Total. Total Kv1.2 was determined by dividing the density of Kv1.2 in the Total by the density of GAPDH in the Total. The molecular identity of cell-surface Kv1.2 was characterized by dividing the intensity of the high molecular weight form in the Eluent by the sum of the intensities of both the high- and low- weight forms in the Eluent. Background intensity, measured using the same Region of Interest, within a lane at a molecular weight with no banding, was subtracted for each channel.

Within an experiment, Surface and Total Kv1.2 values were normalized to the wild type or vehicle control, or average of multiple control samples, and “n” is the total number of plates per transfection condition. Effects of pharmacological manipulations or mutation are expressed graphically and in text as the mean percent change relative to control. In graphs, error bars indicate standard error of the mean. Statistical significance is indicated by an asterisk (*), defined as a p value less than or equal to 0.05 by the statistical test identified in the text.

*Flow Cytometry*

Transfected cells at low confluence in Zero Serum on PDL coated plates were analyzed by flow cytometry as previously described (Stirling, Williams et al. 2009).
Briefly, after incubation in 154 mM sodium azide for 30 minutes, cells were lifted by scraping into low retention tubes, gently pelleted by centrifugation at 4 kRCF for 4 minutes, rinsed and resuspended in PBS with 0.1% bovine serum albumin and 0.1% sodium azide, then pelleted again. All but 100 μl of supernatant was removed and the cells incubated with 0.33 μg/ml rabbit polyclonal α-Kv1.2 directed against the first extracellular loop (Developed in-house in collaboration with Biosource) or with non-specific control Rabbit IgG. After rinsing, cells were incubated with Goat Anti-Rabbit Spectral Red (R-phycoerythrin: Cy5 tandem dye, Southern Biotech). After rinsing, labeled cells were dispensed to a 96-well plate treated with SigmaCote (Sigma), and GFP and Spectral Red fluorescent positive cells were counted using the EasyCyte Flow Cytometer with 488nm laser stimulation (Guava). For GFP, positive signal was thresholded against cells transfected with only empty vector. In GFP positive cells, Spectral Red positive signal for cells treated with α-Kv1.2 antibody was thresholded against cells treated with control IgG. Alternatively, positive Kv1.2 Spectral Red signal was determined by incubating cells not expressing Kv1.2 with the α-Kv1.2 antibody. The percentage of GFP positive cells that were also Spectral Red positive after background subtraction was compared between wild type and mutant forms of Kv1.2, and/or between drug treated and vehicle treated samples. This control percentage was normalized to one for pooling replicate experiments, and the mean with SEM is reported in the text, with comparison between conditions with t-test and significance indicated by *, set at p≤0.05.

Electrophysiology
These experiments were conducted and analyzed by Dr. Anthony Morielli using previously described methods (Connors, Ballif et al. 2008). Biophysical properties of wild-type or mutant forms of GPP-Kv1.2 were compared using transiently transfected HEK293-β cells that had been incubated overnight in Zero Serum on glass PDL-treated coverslips as described earlier, in bath media of (in mM) NaCl (100), KCl (2.5), CaCl₂ (1.8), MgCl₂ (0.8), glucose (23), and NaHEPES (5), pH 7.4, maintained at 35-37°C. The pipette solution for whole-cell voltage clamp contained (in mM): KCl (120), CaCl₂ (3.69), MgCl₂ (0.094), BAPTA (5), EDTA (5), NaHEPES (5), glucose (5), pH 7.1. Data was collected via an Axopatch 200D amplifier through a Digi-Data 1322A D/A converter on a Windows-based computer running Clampex, with analysis performed by Clampfit (Connors et al., 2008). To determine the voltage-current relationship, the voltage was stepped from -70 to 50 mV in increments of 10 mV for a 75 ms time period, followed by a return to -40 mV to elicit tail currents. To determine the voltage dependence of activation, currents elicited by the final voltage step were normalized to their maxima and plotted as a function of the preceding command voltage, and the resultant curves fit (Connors et al., 2008).

Microscopy

Transiently transfected HEK293-β cells were incubated overnight in Zero Serum on glass PDL-treated coverslips as described earlier. After a gentle rinse in warm HBSS, cells were incubated in 1μM DPX-ER Tracker (Invitrogen) in HBSS for 30 minutes at 37°C, then rinsed in HBSS and fixed at 37°C with 4% formaldehyde in HBSS. Following multiples washes in HBSS, slips were mounted onto slides using ProLong Gold AntiFade
(Invitrogen). Images were acquired using the DeltaVision deconvolution-restoration microscopy system (Applied Precision), and processed using ImageJ (NIH).

**Results**

*Mutation Of The N-Terminal Caveolin-Binding Domain Motif In Kv1.2 (Kv1.2-Φ) Reduces Surface Expression*

Kv1.2 containing three point mutations in the cytoplasmic N-terminus: F145A/W150A/F153A (Kv1.2-Φ) replaces the large aromatic hydrophobic side chains in the Φ XXXX Φ XX Φ canonical, putative, caveolin binding domain with alanines, which has been shown, for ligand gated ion channels, to dissociate them from caveolin dependent trafficking (Francesconi et al., 2009). In biotinylation experiments of HEK293-β expressing GFP-Kv1.2 or Kv1.2-Φ, the Absolute Surface expression of Kv1.2-Φ was reduced (20.45±4.65% of wild type, one-sample t-test p<0.0001), as was the Proportional Surface Kv1.2-Φ expression (21.29±4.26% of wild type, one-sample t-test p<0.0001). Relative to wild type Kv1.2 however, Total Kv1.2-Φ was not different (105.9±2.81%, one-sample t-test p=0.840. These results suggest the mutation of the caveolin-binding domain motif substantially reduced basal surface expression, not by a gross loss of total channel, but due to an enhanced internalization or reduced forward trafficking to the plasma membrane.

*ER Tracker*

To assess whether the intracellular localization of Kv1.2-Φ reflected accumulation within the endoplasmic reticulum, GFP Kv1.2-Φ or GFP Kv1.2 were transfected into
HEK293β cells for microscopy as above. Wild type Kv1.2 showed a normal subcellular
distribution with some intracellular puncta, apparent plasma membrane labeling,
localization to leading-edge type extensions, and sparse apparent co-labeling with the
ER-Tracker (FIGURE 10A). In contrast, Kv1.2-Φ localization was diffuse, juxta-
nuclear, lacked leading-edge type targeting, and was apparently more highly co-localized
with the ER-Tracker in a mesh-like pattern (RED) (FIGURE 10B).

*The Kv1.2-Φ Mutant Does Not Undergo Normal Maturation*

The apparent co-localization with an endoplasmic reticulum (ER) marker
suggested that Kv1.2-Φ was abnormally retained in the ER. Towards distinguishing this
from mature channel which was retrogradely targeted to the ER, the post-translational
modifications of the wild-type and mutant forms were compared. Normally, Kv1.2
undergoes glycosylation at a single extracellular asparagine residue (N-type
glycosylation) within the ER, acquiring a “high-mannose” form; the channel is then
processed within the Golgi apparatus wherein the antennary structure of the glycan is
further modified, including sialylation which causes an increase in the apparent molecular
weight for the fully mature channel (Manganas and Trimmer 2000; Zhu, Watanabe et al.
2003) . In addition to complex glycosylation processing, Kv1.2 biosynthesis involves
serine-phosphorylation: phosphorylation at a pair of adjacent residues (pS440/pS441) is
associated with channel that is competent for cell surface expression, and is thought to be
limited to channel that is in the post-ER pool (Yang, Vacher et al. 2007). Therefore, to
biochemically interrogate the localization and maturation of Kv1.2-Φ, the abundance of
the high molecular weight mature form and presence of serine phosphorylation was investigated by biotinylation and protein immunoblot.

Neither α-Kv1.2 nor α-pS440-pS441-Kv1.2 antibodies produced a positive signal at the relevant molecular weight ranges for Vector transfected cells (Figure 10C, Vector, Input). In cells expressing GFP wild type Kv1.2, scant amount of low molecular weight were detected as they were for the mutant condition, likely reflecting newly synthesized channel (Figure 10C, Input, Band 1). At a slightly higher molecular weight, a strong band was observed that appears to correspond to the “high-mannose” form for wild-type Kv1.2, and also appeared, perhaps at higher amounts, in the mutant form of the channel (Manganas and Trimmer 2000) (Figure 10C, Input, Band 2). Most obviously, however, was the near total loss of the highest molecular weight form of the mutant, which was clearly present in the wild type condition corresponding to the Golgi processed form of Kv1.2 (Shi and Trimmer 1999; Watanabe, Zhu et al. 2007) (Figure 10C, Input, Band 3).

Examining the Eluent samples, again no specific banding was present in the Vector transfected conditions. For the wild type Kv1.2 condition, the predominant form of cell surface Kv1.2 was the fully mature, upper molecular weight band (Band 3), though the “high-mannose” band was present (Band 2), while the lowest, presumptively non-glycosylated, form was not (Band 1) (Figure 10C, Eluent). For the mutant form of the channel, there was detectable surface channel, as reported in previous experiments, but surprisingly, this form was predominantly the immature “high-mannose” form (Band 2). However, enrichment of the surface channel by biotinylation in this Eluent sample did reveal that there is apparently a minor amount of mutant channel at the fully-mature
molecular weight, suggesting that while this channel is capable of biosynthetic maturation, it mainly fails to do so (Figure 10C, Eluent, Band 3).

The relative abundance of immature Kv1.2 in the mutant condition suggests that the channel is not progressing past the endoplasmic reticulum. Evaluating these samples with an antibody against phosphorylated-S440/S441 revealed no specific band at the expected molecular weight for Kv1.2 in Vector transfected samples, but a single predominant band at the fully-mature molecular weight for wild type, but not mutant Kv1.2 (Figure 10C, Input). This phosphorylation is thought to occur only in the post-ER pool, reflecting mature channel competent for cell-surface expression, and it was found to be enriched in the Eluent for wild type, but not present for the mutant forms of Kv1.2 or the vector only sample (Yang, Vacher et al. 2007) (Figure 10C, Eluent). Collectively, these data suggest that Kv1.2-Φ is not efficiently processed in the biosynthetic machinery, and is largely retained within the endoplasmic reticulum. However, the minor mutant channel that is found at the cell surface is predominantly the immature form.

Quantifying the proportion of the fully mature form in the eluent, the average was 81.23±3.59% for the wild type versus 37±6.58% for the mutant form (n=10,10; two-tailed t-test p<0.0001). Therefore, the predominant form of surface channel for wild-type Kv1.2 is the fully mature form, while for the mutant it is the immature high-mannose form. Since a caveolin-binding-motif was mutated, it was hypothesized that caveolin was a chaperone for the biosynthesis of Kv1.2. If this was the case, it was predicted that overexpressing caveolin-2 (the predominant form in HEK cells (Cha, Jung et al. 2004))
would increase the surface expression of, and proportion of fully mature channel at the
cell surface, for wild type, but not mutant, Kv1.2.

*Caveolin-2 Overexpression Inhibits Maturation Of Kv1.2 And Kv1.2-Φ*

The Absolute and Proportional surface expression of wild type and Kv1.2-Φ
mutant forms of Kv1.2 were examined under conditions of Caveolin-2 (isoform 1)
overexpression. Percent changes are in terms of how they differ from the transfection
condition which expressed empty vector rather than caveolin-2. In contrast to the initial
hypothesis, caveolin-2 overexpression *reduced* the Absolute Surface expression of wild
type Kv1.2 compared to vector co-expression (21.95±3.17%, n=4, one sample t-test
p<0.0001). However, caveolin-2 overexpression did not reduce the Proportional surface
expression (84±12.4%, n=4, p=0.29), or the Total Kv1.2 expression (69.25±11.28%, n=4,
one sample t-test p=0.072).

Also contrasting the initial hypothesis, caveolin-2 overexpression did have an
effect on Kv1.2-Φ, which harbored the point mutations in the putative N-terminal
caveolin binding domain. In Kv1.2-Φ with caveolin-2 overexpression, Absolute surface
Kv1.2 was reduced even further (1.13±3.5%, compared to the 12.4% Kv1.2-Φ with
vector, n=4, one sample t-test p<0.0001). However, the Proportion of Kv1.2 at the cell
surface was higher in the mutant condition with caveolin-2 overexpression (63.5±8.6%,
compared to the 32% of Kv1.2-Φ with vector, n=4, one sample t-test p<0.035). As was
the case with wild type Kv1.2, caveolin-2 overexpression did not change Total Kv1.2
(77.1±27.68%, compared to the 133.5% of Kv1.2-Φ with vector, n=4, p=0.134).
Therefore, while caveolin-2 overexpression reduces surface Kv1.2, it has this effect on the mutant form as well, and although it did not reach significance for wild type, caveolin-2 overexpression reduced Total Kv1.2 for both wild type and mutant forms. The only apparent difference in the quality of the consequence of caveolin-2 overexpression between wild-type and mutant forms of Kv1.2 was that caveolin-2 increased the Proportion of surface Kv1.2. To examine whether, like the impact of the mutation itself, this reflected a change in the balance of mature vs. immature forms of channel at the cell surface, the proportion of mature channel at the cell surface was quantified across multiple experiments as before.

In the Eluent of biotinylated samples expressing GFP wild-type or mutant forms of Kv1.2, protein immunoblot experiments again revealed that, for the wild-type form, the fully mature, upper molecular weight, species predominated, while for the mutant form, the immature form is most abundant. Rather than caveolin-2 facilitating the representation of mature channel, it had the opposite result: decreasing the mature form and increasing the abundance of the immature form. For the mutant form of the channel, caveolin-2 overexpression had a similar result: the scant fully mature form in the Kv1.2-Φ transfection is no longer apparent under caveolin-2 overexpression, while the amount of immature Kv1.2-Φ at the cell surface increases further (Figure 11A). This could provide a mechanism by which caveolin-2 reduces the proportional surface expression of the wild type form, but increases the proportional surface expression of the mutant form: in the wild-type case, there is a loss of mature form that is greater in degree than the gain in immature form at the cell surface, while for the mutant form, there is very little mature
channel to be reduced, but there is more immature channel to be facilitated to the plasma membrane. Quantifying this across experiments revealed that the proportion of the fully mature form of wild type Kv1.2 at the cell surface was 49.42 ± 4.87% lower with caveolin-2 overexpression, compared to vector, (n=4,4; p<0.0001) (Figure 11B). For Kv1.2-Φ, caveolin-2 overexpression, relative to vehicle, also reduced the proportion of surface channel that was in the fully mature form, (-27.99 ± 6.57% , n=4,4; p=0.024) (Figure 11C). Thus, contrary to the initial hypothesis, caveolin-2 overexpression facilitated the surface expression of immature Kv1.2 at the expense of mature channel, and similarly altered Kv1.2-Φ, which harbored the putative n-terminal caveolin binding domain motif mutation.

To explore the specificity of this effect, caveolin-1 or dopamine receptor interacting protein 78 (DRiP78) were overexpressed, and the effects on the maturity of surface Kv1.2 were observed. Caveolin-1 overexpression was to examine the specificity with respect to a different caveolin, while DRiP78 was selected because it is capable of recognizing caveolin-binding motifs and is localized to the ER (Bermak, Li et al. 2001; Leclerc, Auger-Messier et al. 2002). Similar to caveolin-2 overexpression, caveolin-1 (versus vector control), qualitatively decreased the abundance of mature, while increasing the amount of immature, Kv1.2 in the Eluent. This pattern was also observed with the Kv1.2-Φ mutant. For both wild type and mutant Kv1.2, however, DRiP78 overexpression did not yield such a result, giving specificity to the consequence of caveolin overexpression (FIGURE 11B).

*Kv1.2 And Kv1.2-Φ Are Capable Of Binding To Kvβ2*
Since both caveolin-1 and caveolin-2 overexpression similarly affected wild type and mutant forms of Kv1.2, the effects of the mutation may be unrelated to a lost ability to bind to caveolin, if such a property exists at all. As mutating the putative caveolin binding motif within Kv1.2 decreased surface channel primarily through causing net retention within the endoplasmic reticulum, what was the cause? One protein which has been proposed to have a role as biosynthetic chaperone for Kv1.2 is Kvβ2 (Manganas and Trimmer 2004). Perhaps the mutant form could no longer interact with Kvβ2, and was therefore ER retained. To investigate this possibility, wild type or mutant Kv1.2 was immunoprecipitated from transiently transfected HEK293-β cells, and these samples were probed by immunoblot for Kvβ2. In parallel, this experiment was also done using HEK293-M1 cells, which lacked Kvβ2, and so served as a negative control. As expected, Kvβ2 was present in all HEK293-β Input samples, while in none of the HEK293-M1 samples (α-Kvβ2, Input, Figure 12). In HEK293-β and in HEK-293-M1 Input samples, Kv1.2 was detected in both wild-type and mutant transfections, but not vector alone (α-Kv1.2, Input, Figure 12). When the α-Kv1.2 immunoprecipitate was evaluated, a similar pattern was observed: Kv1.2 was pulled down from all samples, with no immunoreactivity in the vector transfection, confirming the specificity of the antibody for Kv1.2 (α-Kv1.2, Immunoprecipitate, Figure 12). In the α-Kv1.2 immunoprecipitate, Kvβ2 was detected in none of the HEK293-M1 samples, but in all of the HEK293-β samples, except for the vector transfection, which indicates that Kvβ2 was not simply pulled down non-specifically by the α-Kv1.2 antibody (α-Kvβ2, Immunoprecipitate, Figure 12). Therefore, both wild type and mutant forms of the channel are capable, in this
assay, of binding to Kvβ2. This suggests that a lack of Kvβ2 binding is not the reason for poorer surface expression and channel maturation for the mutant channel. To test this, wild type or mutant channel were transfected into HEK293-M1 cells (which lack Kvβ2) and were subject to surface protein biotinylation as before.

The general effects of the mutation were similar in the absence of the beta subunit: In biotinylation experiments of HEK293-M1 cells expressing GFP-Kv1.2 or GFP-Kv1.2-Φ, the Absolute Surface expression of Kv1.2-Φ was lower (14.16±6.58% of WT, n=6, one-sample t-test p<0.0001) (Figure 13A), as was the Proportional Surface Kv1.2-Φ expression lower than wild-type (17.65±5.98%, n=6, one-sample t-test p<0.0001)(Figure 13B). Relative to wild type Kv1.2, Total Kv1.2-Φ was also reduced (71.3±8.21%, one-sample t-test p=0.017) (Figure 13C). Therefore, while the mutant form can bind to the beta subunit, it still expresses more poorly at the cell surface relative to wild-type even in the absence of the beta subunit, suggesting that Kvβ2 might not confer a chaperone quality to the mutant channel complex.

*Kv1.2-Φ Has Altered Trafficking Response To Chaperone Overexpression*

To test this, HEK293-M1 cells were transfected with the wild-type or mutant form of the channel, with or without transient stoichiometric transfection of Kvβ2 or calnexin, which is thought to be able to substitute as a chaperone for Kvβ2 in the maturation of Kv1.2 (Manganas and Trimmer 2004). In these experiments chaperone co-transfection on each form of the channel was compared to that same form of the channel co-transfected with empty vector. Five experiments were conducted, but analysis was
limited to three where Kvβ2 was found to enhance surface expression of wild type Kv1.2 (Shi, Nakahira et al. 1996; Accili, Kiehn et al. 1997; Connors, Ballif et al. 2008).

For the wild type channel, neither Kvβ2 nor calnexin increased Absolute Surface Kv1.2, although the average was in this direction (Kvβ2: 178.1±36.03%, n=3, one sample t-test, p=0.16; calnexin: 137.7±39.78%, n=3, one sample t-test p=0.44). In the case of the mutant form of the channel, chaperone expression did have a significant effect on Absolute Surface Kv1.2: transfection of either chaperone decreased surface expression (Kvβ2: 63±6.38%, n=3, one sample t-test, p=0.029; calnexin: 52.98±4.46%, n=3, one sample t-test p=0.0089).

For both forms of the channel, either chaperone expression increased Total Kv1.2, though this only reached significance for the effect of Kvβ2 on wild type Kv1.2 (213±17.03%, n=3, one sample t-test p=0.022). In comparison, calnexin over-expression on Total wild type Kv1.2 was not significant: (133±83.69%, n=3, one sample t-test p=0.25), nor was the effect of Kvβ2 on Total mutant Kv1.2 (125.2±25.72%, n=3, one sample t-test p=0.43), nor the effect of calnexin on Total mutant Kv1.2 (116.3±22.04%, n=3, one sample t-test p=0.54).

In examining the effect of chaperones on the Proportional Surface Kv1.2 expression, neither chaperone altered the wild type channel (Kvβ2: 82.6±13.67%, n=3, one sample t-test p=0.33; calnexin: 61.51±12.14%, n=3, one sample t-test p=0.09). In contrast, chaperone over-expression did significantly alter the mutant form of the
channel: the chaperones reduced the Proportion of Surface Kv1.2 (Kvβ2: 52.55±5.5%, n=3, one sample t-test p=0.013; calnexin: 49.07±9.69%, n=3, one sample t-test p=0.034).

Therefore, while either wild type or mutant forms can bind at least Kvβ2, in contrast to the trend of increased surface expression with chaperone expression for wild type channel, the mutant channel showed the opposite effect, being more highly retained intracellularly in the presence of chaperone overexpression. Therefore the mutant channel appears largely defective in normal biosynthetic export, but is this channel simply “broken” with respect to forward trafficking in general? Wild type forms of Kv1.2 are regulatable by both adenylate cyclase and Rho-kinase, whose stimulation or inhibition, respectively, increases the cell surface expression of Kv1.2 (Connors, Ballif et al. 2008; Stirling, Williams et al. 2009). If the Kv1.2-Φ mutant form had a global inability to undergo net forward trafficking of both biosynthetic and endocytic types, it would be predicted that surface expression would not increase upon AC stimulation or Rho Kinase inhibition.

Kv1.2-Φ Is Capable Of Stimulated Trafficking To The Plasma Membrane

To test this hypothesis, the result of AC stimulation on Absolute surface expression was tested by biotinylation, while the result of Rho Kinase inhibition on surface Kv1.2 levels was tested via flow cytometry. For the biotinylation assay, HEK293-β cells transfected with either GFP-Kv1.2 or GFP-Kv1.2-Φ were treated with 10 μM forskolin or with dimethyl sulfoxide (DMSO) vehicle for ten minutes. These experiments were down pairwise to compare the effect of forskolin for each of the channel forms. For
wild-type Kv1.2, forskolin significantly increased Absolute Surface Kv1.2
(208.8±76.74% versus vehicle 100±27.76%, n=6, 6; paired one-tailed t-test p=0.043)
(Figure 14A). For the mutant form, forskolin also caused a significant increase in
Absolute Surface Kv1.2 (129.6±27.55% vs. vehicle 100±24.26%, n=6, 6 (vehicle,
forskolin), paired one-tailed t-test p=0.0014) (Figure 14B).

For flow cytometry, the cells were treated with Y-27632 to inhibit Rho kinase for
30 minutes at 10μM before sample preparation as described earlier (Stirling, Williams et al. 2009). Values are expressed as percentages normalized to the average of vehicle
treated wild type GFP-Kv1.2 surface expression. Treatment with Y-27632 significantly
increased the surface expression of wild type Kv1.2 (122.5±8.39% vs.100±9.51% for
vehicle, n=21,20 (vehicle, Y-27632), one-tailed t-test p=0.044) (Figure 14C) and also
increased the surface expression of Kv1.2-Φ (83.74±24.58% vs. 33.41±9.39%, n=21,21
(vehicle,Y-27632), one-tailed t-test p=0.032) (Figure 14D). Therefore, pharmacological
manipulations of two pathways known to enhance surface expression of wild-type Kv1.2
also enhance surface expression of Kv1.2-Φ, suggesting that forward trafficking defects
are specific to biosynthesis. The Shaker family channels are known to have related amino
acid determinants of their biosynthetic trafficking (Li, Takimoto et al. 2000; Ma,
Zerangue et al. 2001) and as the Kv1.2-Φ mutation seems to result in biosynthetic
defects, the conservation of the mutated motif was investigated.

*The Caveolin-Binding-Domain Of Kv1.2 Is Similar In Other Kv1 Channels*
Using a short stretch corresponding to a dozen amino acids from the NCI reference published protein sequences for rat Kv1.1-Kv1.7, WebLogo was used to visualize the aligned sequences (Crooks, Hon et al. 2004). This was used solely as a convenient way to look qualitatively at this short domain, as the number and length of the sequences are unconventionally low for motif discovery and quantitative analysis (Schneider and Stephens 1990). As seen in Figure 15, flanking the ΦXXXXΦXXΦ motif on either side was typically a glutamate (E), while the most common hydrophobic bulky amino acid of the first and last position of the ΦXXXXΦXXΦ motif was phenylalanine, and in all cases, the central Φ was tryptophan. While the intervening XXXX motif was less fixed, the XX motif was in all but one case a di-leucine, except Kv1.5, which harbors an isoleucine. It is interesting to note that not just the motif, but is localization is conserved within the Kv1 family as well: based on known or predicted structural annotations in the NCBI Reference Sequence database, the motif for each of the rat Kv1.1-1.7 sequences begins at a cytoplasmic position 19 amino acids prior to the first transmembrane residue. For the mannose-6-phosphate receptor, a 19 amino acid juxtamembrane domain encodes determinants of sorting and targeting (Bresciani, Denzer et al. 1997).

Since this motif appears to generally be similar across the Shaker within a species, other species were searched to see if they harbor it as well. Using the NCBI database, sequences for Shaker or Kv1.2 potassium channels from twenty species listed in Figure 15C were used to seed a similar WebLogo query (Figure 15B). The most obvious result is the preservation of the tryptophan as the core of the motif, the
downstream dileucine-FEY, and constant valine upstream of the tryptophan. While, in these selected sequences, the generic motif is conserved, is it is lost in some of these samples. The first bulky hydrophobic Φ is instead a postively charged amino acid in two insect cases, arginine for the Ostrinia corn borer, and lysine for Drosophila. Although there was not conclusive evidence for a role of this domain in the caveolin mediated regualtion of Kv1.2, it is interesting to note that this motif is lost for Drosophila, which also lacks caveolin (Galbiati, Volonte et al. 1998). This domain appears important for Shaker channels, as it is present in many members across many species. Beyond influencing biosynthetic trafficking what role might in play in regulating the biophysical properties of the channel?

For inward rectifying (Kir) potassium channels, interactions with cholesterol have been proposed to stablize plasma-membrane localized channels in a functionally “silent” state. Furthermore, some Kir channels interact with caveolin, which associates with these channels in those cholesterol rich regions, and which may regulate Kv1 channel targetting to cholesterol rich domains as well (Folco, Liu et al. 2004; McEwen, Li et al. 2008). If the Caveolin binding motif was resonisible for targetting Kv1.2 to lipidic environments that, like has as referenced for some Kir channels, stabilize Kv1.2 in a “silent” state, there should be an increased population of functional Kv1.2. To test this, the biophysical properties of GFP-Kv1.2 or GFP-Kv1.2-Φ were tested in transently transfected HEK293-β cells.

*Altered But Functional Electrophysiology Of Kv1.2-Φ*
As seen in Figure 16 A vs. B, there appears to be a greater lag to peak current upon depolarization for the mutant form of Kv1.2, though the mutant form does appear to retain the normal property of being non-inactivating under these conditions. Surprisingly, although flow cytometry and biotinylation revealed a dramatically reduced surface expression of Kv1.2-Φ (~80-90% reduction), there was not a correspondingly gross decrease in the peak current. While there was a decreased current amplitude of approximately half, this did not reach significance (mean steady state GFP-Kv1.2 amplitude at +50 mV: 7.18±1.61 nA versus 3.91±1.11 nA for GFP-Kv1.2-Φ, one-tailed t-test; p=0.1) (Figure 16C). Although single-channel recordings were not acquired, and therefore a large increase in conductance cannot be ruled out, the disconnect between grossly decreased surface expression and moderate current reduction could be due, as speculated, to a decreased relative abundance of “silent” channels.

Discussion

The goal of the mutagenesis of Kv1.2 was to make a form of the channel normal but for inhibited regulation by adenylate cyclase mediated trafficking. Instead, mutation of the N-terminal putative Caveolin-binding domain resulted in a channel which still shows AC sensitive trafficking, but had altered biosynthetic and biophysical properties. Furthermore, the Caveolin binding domain motif is not specific to Kv1.2, and is instead an apparently ancient and conserved ΦXXXXΦXXΦ motif in other Shaker family channels. Exploring the mechanisms behind why mutation of this hydrophobic domain
disrupts biosynthesis and alters the biophysical properties will require experiments that tease apart the two phenomom.

*The Role Of Glycosylation In Kv1.2 Function*

There is a single N-linked glycosylation site for Kv1.2 at residue N207, a NXT/S motif (Kornfeld and Kornfeld 1985). For both Kv1.2 and Kv1.1, glycosylation at this extracellular residue seems to have a role in modulating biophysical properties of the channel. Mutation of the arginine, culture in cell lines deficient in glycosylation, or enzymatic digestion of the glycan results in channels whose kinetics of activation are slowed and for which the voltage of half-activation is shifted towards more depolarized potentials; the primary apparent mechanism appears to be the loss of sialic acid addition to the sugar tree in the Golgi apparatus (Thornhill, Wu et al. 1996; Watanabe, Wang et al. 2003; Zhu, Watanabe et al. 2003; Watanabe, Zhu et al. 2007). Thus, altered biophysical properties in the Kv1.2-Φ mutant could be due to the absence of Golgi mediated post-translational modifications, perhaps due to poor export to that organelle. If this was the case, siladase would be expected to make wild-type channel behave more like the Kv1.2-Φ mutant, and have little or no effect on the mutant channel; also, there should be an apparent decrease in the molecular weight upon such digestion for the wild type but not the mutant form (Shi and Trimmer 1999; Manganas and Trimmer 2000; Zhu, Watanabe et al. 2001). The possibility that the altered biophysical properties are due to changes of the N-linked glycan species could also be tested by examining Kv1.2 harboring just the N207Q mutation versus Kv1.2 harboring the N207Q mutation and the Kv1.2-Φ motif
mutation. In this case, there would be no N-linked species, and presumably any observed differences would be due to the mutation itself.

**Possible Roles For The Dileucine Within The Caveolin Binding Domain**

Whether or not the biophysical differences are due to altered biosynthetic modifications, why does the mutant form of the channel apparently fail to progress from the endoplasmic reticulum to the Golgi apparatus? A loss of caveolin binding does not seem likely, as caveolin overexpression decreased maturation of wild type and mutant forms of Kv1.2. Alternatively, it is possible that the mutation unmasked an ER retention motif or disturbed an ER export motif. Within the mutated putative caveolin binding domain, there are two consecutive leucines. Initially identified as a regulator of CD3 endocytosis and lysosomal targetting (Letourneur and Klausner 1992), dileucine based motifs have been found to be involved in both endocytic and biosynthetic trafficking. Early work identified functionally important dileucine motifs in the related CD4 and also in the insulin receptor, where these motifs, like in Kv1.2, are found in a juxtamembrane domain (Aiken, Konner et al. 1994; Haft, Klausner et al. 1994). The most established function of dileucine containing motifs is mediating endocytosis: a dileucine motif is needed for endocytosis of the beta 2 adrenergic receptor (Gabilondo, Hegler et al. 1997), the cytokine receptor subunit GP130 (Thiel, Behrmann et al. 1998), the epidermal growth factor receptor (Kil, Hobert et al. 1999), and the GABA<sub>A</sub> receptor (Herring, Huang et al. 2003). Critically though, it is not simply the case that dileucine motifs are signals for endocytosis; for some proteins, such as the lutropin/choriogonadotropin receptor, these motifs inhibit endocytosis (Nakamura and Ascoli 1999). The involvement of dileucine
motifs has also been established for forward trafficking: a dileucine motif is important for the ER exit, complex glycosylation, and surface expression of the V2 vasopressin receptor (Schulein, Hermosilla et al. 1998), for the D1 dopamine receptor (Guo and Jose 2011), and for the oranic anion transporter-1 (Zhang, Wu et al. 2011). Likewise, for synaptic adhesion-like molecule 1 (Seabold, Wang et al. 2012), and for calcium activated chloride channels (Huan, Greene et al. 2008), a dileucine motif influences ER retention. Therefore, perhaps the Kv1.2-Φ mutant does not mature normally because the steric or chemical environment of the nested dileucine is disturbed.

Mechanistically, Kv1.2 with mutations about the dileucine motif may fail to progress to the Golgi because of a reduced ability to bind the AP1 complex, as dileucine motifs can mediate cargo protein interaction with this adaptor protein that is important for sorting within the Golgi network (Bresnahan, Yonemoto et al. 1998; Rapoport, Chen et al. 1998; Mattera, Boehm et al. 2011). Related, a second class of candidate dileucine interactors may be Golgi-localized, γ -ear–containing, ARF-binding proteins (GGA’s), which regulate procession of proteins through the Golgi apparatus (Puertollano, Aguilar et al. 2001; Takatsu, Katoh et al. 2001). Yet another potential trafficking explanation may be an altered ability to bind a particular RAS family GTPase Rab, as a dileucine motif of the beta 2 adrenergic receptor mediates Rab8 binding, and as Rab1 is important in regulating ER export of membrane proteins (Stenmark and Olkkonen 2001; Zhang, Wang et al. 2009; Dong, Yang et al. 2010). Lastly, rather than having reduced permisivesness for export due to occluded dileucine mediated interactions, it is also possible that the
Kv1.2-Φ mutant can not mask the dileucine motif, as dileucine masking is important for surface expression of CD3 containing T-cell receptors (Lauritsen, Bonefeld et al. 2004).

It is interesting to speculate that the Kv1.2-Φ mutation may perturb maturation due to an altered ability to bind a kinase or phosphatase. For Kv1.2, between the embedded dileucine motif and the first transmembrane residue, there are conserved serines and tyrosines, and juxtamembrane residue phosphorylation is required for the dileucine mediated trafficking of some proteins (Geisler, Dietrich et al. 1998; Gibson, Schiemann et al. 2000). Although detecting transient protein interactions or posttranslational modifications is technically challenging, it may be mechanistically informative to immunoprecipitate wild type or Kv1.2-Φ mutants for submission to mass spectoscopy to facilliate indenfication of interacting proteins or altered phosphorylation.

The Kv1.2-Φ channel may fail to mature not due to a specific loss of a trafficking signal or protein interaction, but more simply because the mutation induces a folding defect that fails ER quality control. Indeed, mutating dileucine sequences has been found to reduce maturation and surface expression through this abberant folding mechanism for the aforementioned V2 receptor (Krause, Hermosilla et al. 2000), for a cholestrol transporting protein (Alpy, Stoeckel et al. 2001), and for P-glycoprotein (Loo, Bartlett et al. 2005). Perhaps consistent with this model, in a pilot experiment, mutation of the dileucines within Kv1.2 to dual alanines resulted in primarily the high-mannose immature form of the channel, reminiscent of the Kv1.2-Φ mutant (data not shown). Testing a folding defective model further could involve incubation of wild type or mutant Kv1.2
transfected cells at lowered temperature or with chemical chaperones to attempt to rescue channel maturation (Brown, Hong-Brown et al. 1996; Brown, Hong-Brown et al. 1997).

**Alternative Mechanisms For Altered Kv1.2-Φ Biosynthesis**

Mutating the bulky hydrophobic amino acids within the putative caveolin binding domain motif reduced surface expression and Kv1.2 maturation, and as discussed, alteration of the environment of the nested dileucines which made up the XX of the ΦXXXXΦXXФ motif may be responsible. However, the altered chemical environment or masking/unmasking of other residues within the XXXX domain may be responsible for the biosynthetic defect of Kv1.2-Φ. For Kv1.2/Shaker, of many species examined, including mouse, rat, and human, the XXXX of the ΦXXXXΦXX is QRQV, however nothing in this domain resembles classic lysine based KDEL-type ER retention signals (Teasdale and Jackson 1996), the FCYENE potassium channel ER export motif of Kir channels (Ma, Zerangue et al. 2001), or the C-terminal VXXSL type motif which regulates Kv1 glycosylation and surface expression (Li, Takimoto et al. 2000), nor does it encode a consensus arginine based ER trafficking motif (Michelsen, Yuan et al. 2005). Intriguingly, however, the motif (FQRQVWLLFEY) sequence within Kv1.2 is very similar to a well conserved membrane proximal motif that regulates the ER exit and surface expression of multiple GPCR’s: F(X)₆LL, though in the case of the Shaker channels, the sequence is F(X)₅LL (Duvernay, Zhou et al. 2004). It would be interesting to test whether appending Kv1.2-Φ with a known GPCR F(X)₆LL motif would resuce surface expression and or maturation of the channel. Lastly, both RhoA and cortactin are known to be capable of binding Kv1.2’s N-terminus through unknown sites, perhaps one
or both of these interaction are disturbed in Kv1.2-Φ (Cachero, Morielli et al. 1998; Hattan, Nesti et al. 2002), resulting in reduced ER export.

Perhaps it is not modulation of a specific motif that induces the apparent biosynthetic arrest of Kv1.2-Φ. Of the full domain investigated (FQRQVWLLFEY) that harbors the ΦXXXXΦXX motif, it is notable that 7 of the 12 residues have hydrophobic side chains. The ER associated heat shock protein family member DRiP78, recognizes hydrophobic motifs within the D1 dopamine receptor to influence the receptors glycosylation and surface expression (Bermak, Li et al. 2001), acts through a caveolin binding domain motif to regulate trafficking of the angiotensin II AT1 receptor (Leclerc, Auger-Messier et al. 2002), and functions a chaperone of Gγ (Dupre, Robitaille et al. 2007). Although DRiP78 did not apparently influence maturation of wild type or mutant Kv1.2, an alternate ER localized analogous heat shock type protein may likewise act through the mutated hydrophobic residues of Kv1.2-Φ to regulate maturation and membrane protein surface expression of wild type Kv1.2. As one candidate, unpublished work from this lab (Morielli) has provided initial evidence that Hsp70 and Kv1.2 co-immunoprecipitate (Connors 2009). Therefore, perhaps the Kv1.2-Φ mutant has aberrant biosynthesis due a diminished interaction with specific chaperones, or the mutant form may even have enhanced ER resident chaperone interaction, thus causing its deficient forward trafficking. This may partially explain why Kvβ2 or calnexin overexpression actually reduced surface expression of Kv1.2-Φ.
The mutation of the hydrophobic domain may disrupt Kv1.2 biosynthesis due not to perturbed chaperone interaction, but due to altered localization to a particular plasma membrane domain. Kv1 family channels are differentially targeted to lipid rafts (see (Martens, O'Connell et al. 2004) for a review). Although the hydrophobic domain of Kv1.2 under investigation may not be a caveolin binding domain, since caveolin affected both wild type and Kv1.2-Φ similarly, the domain may mediate interaction with other molecules that can be found enriched in lipid raft-like regions such as those that harbor stomatin/prohibitin/flotillin/HflK-C (SPFH) domains (Tavernarakis, Driscoll et al. 1999). Of these family of proteins, Erlins are ER localized (Browman, Resek et al. 2006), but nothing is known about what motifs may regulate Erlin interactions. Regardless of the potential adaptor, some enzymes and chaperones of the ER are also differentially localized to particular lipid domains, so it is possible that due to altered hydrophobicity, Kv1.2-Φ has aberrant partitioning to ER microdomains where the channel may otherwise normally exit the ER or be able to be targeted to enzymes that confer posttranslational modifications that would permit ER exit (Fu and Sztul 2003; Brignac-Huber, Reed et al. 2011; Waugh, Minogue et al. 2011). Ultimately, experiments which interrogate ultrastructure, such as immuno-electron microscopy, may allow an unbiased assessment of the exact localization of Kv1.2-Φ versus wild-type channel, and may therefore lead to new testable hypothesis about the mechanisms of its defective biogenesis. Similarly, an exact understanding of the glycan species may inform what biosynthetic step is aberrant. While the mutation is not naturally occurring, the presence of this motif in Shaker potassium channels from invertebrate trematodes and arthropods to humans suggests this
unexplored region of the channel may serve an indespensible, if for now elusive, function.
Figure Legends

Figure 9. Mutation Of A Caveolin Binding Domain Motif Reduces Surface Kv1.2 Expression. A) Diagram representing the N-terminal GFP-Kv1.2 (Rat) fusion protein used as a template for generating the GFP-Kv1.2-Φ mutant, which harbors three point mutations: F145A/W150A/F153A, substituting alanines for the aromatic hydrophobic residues comprising a putative ΦXXXXΦXXΦ caveolin binding domain. B) As determined by quantification of protein immunoblots of biotinylated cell surface proteins from HEK293 cells expressing Kvβ2 and either GFP-Kv1.2 or GFP-Kv1.2-Φ, the absolute surface expression of GFP-Kv1.2-Φ is decreased (20.45% of WT, *p≤0.0001), as was the proportion of all channel at the cell surface also decreased (21.29% of WT, *p≤0.0001), though the total level of mutant form did not differ significantly (105.9% of WT, p≥0.05).

Figure 10. Evidence That GFP-Kv1.2-Φ Is Retained In The Endoplasmic Reticulum. A, B: Photomicrographs of optical sections through HEK293 cells expressing Kvβ2 and either GFP-Kv1.2 or GFP-Kv1.2-Φ (Green) labeled with DPX-ER-Tracker (Red). A) The wild type GFP-Kv1.2 (Green) pattern includes weak peri-nuclear, intracellular puncta, and apparent cell surface localization. B) In contrast, the GFP-Kv1.2-Φ pattern includes more prominent peri-nuclear localization, reduced puncta, and a diffuse trabecular intracellular pattern without obvious apparent plasma membrane localization, but higher apparent co-localization with ER-Tracker. C) Protein
immunoblots of “Input” lysates from cell surface protein biotinylated HEK293 cells expressing Kvβ2 and either empty vector (Vector), GFP-Kv1.2 (Kv1.2) or GFP-Kv1.2-Φ (Kv1.2-Φ) were probed with α-Kv1.2 or with α-Kv1.2: phosphorylated serines 440/441 (α-pS440-pS441-Kv1.2). For α-Kv1.2, no band is observed in the Vector only transfection condition, while for the Kv1.2 condition, multiple bands are observed: “1”, corresponding to un-glycosylated newly synthesized channel; “2”, a high-mannose form associated with endoplasmic reticulum localized channel; and “3”, a fully mature form which is associated with surface expression after complete processing in the Golgi apparatus. For Kv1.2-Φ, only the newly synthesized and ER localized “1” and “2” bands are evident. Parallel Input lysates were purified by incubation with neutravidin beads to enrich for biotinylated surface protein, whose “Eluents” were also evaluated. No band is present for α-Kv1.2 in the Vector only transfection, while the fully mature form of Kv1.2 predominates at the cell surface for wild type channel. In contrast, the high mannose immature form is the predominant form of surface channel for Kv1.2-Φ, though scant fully mature channel is also detectable. For both the Input and the Eluent, an α-pS440-pS441-Kv1.2 signal, associated with post-ER processing, is only apparent for wild type Kv1.2, not observed in the Vector or Kv1.2-Φ transfections.

Figure 11. Caveolin Over-Expression Reduces Maturation Of Kv1.2 And Kv1.2-Φ. A, B): Protein immunoblots of the neutravidin Eluents from lysates of cell surface protein biotinylated HEK293 cells expressing Kvβ2 and either Vector only, GFP-Kv1.2 (Kv1.2), or GFP-Kv1.2-Φ (Kv1.2-Φ), with or without stoichiometric overexpression of caveolin-2 isoform 1 (Cav2), caveolin-1 isoform 1 (Cav1), or dopamine receptor-interacting protein.
78 (DRiP78) probed with α-Kv1.2. A) While no labeling is evident in the Vector transfection, the fully-mature form of Kv1.2 predominates at the cell surface with scant amounts of the high-mannose form, and this pattern is reversed with Cav2 overexpression. The results with Kv1.2-Φ are similar: Cav2 overexpression further enhances the surface representation of high-mannose form, and diminishes the abundance of the already minor presence of fully mature mutant channel. B) Overexpression of Cav1, but not DRiP78 (which can also bind caveolin-binding-domain motifs), also reduces the levels of fully mature wild type and mutant forms of Kv1.2, while increasing the levels of the immature form. C, D) Bar graphs of the mean proportion in the eluent of the fully mature versus immature form of Kv1.2, and the effect of Cav2 overexpression on this “Maturity of Surface Channel” metric. C) Overexpression of Cav2, versus vector, reduces the relative abundance of fully mature Kv1.2 at the cell surface (-49.42%, *p≤0.0001), D) Overexpression of Cav2 also reduces the abundance of the fully mature form of Kv1.2-Φ (-27.99%, *p≤0.05).

Figure 12. Both Kv1.2 And Kv1.2-Φ Can Interact With Kvβ2. A) Protein immunoblots of Input lysates of HEK293 cells stably expressing Kvβ2 or not, transfected with Vector (V), GFP-Kv1.2 (Kv1.2), or GFP-Kv1.2-Φ (Kv1.2-Φ), probed with α-Kv1.2 or α-Kvβ2. A) There is the expected α-Kv1.2 signal in cells expressing wild type or mutant forms of Kv1.2, but not in the Vector transfection condition, regardless of Kvβ2 expression, which is apparent by the α-Kvβ2 signal in the + Kvβ2 cell line. B) Using a separate α-Kv1.2 antibody, the immunoprecipitates show the expected positive α-Kv1.2
signal in all non-vector transfection conditions. When the α-Kv1.2 immunoprecipitate is probed with α-Kvβ2, the vector transfection condition for the +Kvβ2 cell line does not have a signal, though both mutant and wild type forms of Kv1.2 do. No Kvβ2 co-immunoprecipitate was observed in either wild type or mutant forms of Kv1.2 immunoprecipitated from the -Kvβ2 cell line.

**Figure 13. Reduced Surface Expression Of Kv1.2-Φ Does Not Require Kvβ2.** A-C: bar graphs of mean changes in Absolute or Proportional Surface, or Total, expression of GFP-Kv1.2 compared to GFP-Kv1.2-Φ as determined from quantification of protein immunoblots of biotinylated HEK293 cells not expressing Kvβ2, transfected with either GFP-Kv1.2 (Kv1.2) or GFP-Kv1.2-Φ (Kv1.2-Φ). A) Absolute surface Kv1.2 was significantly lower for Kv1.2-Φ (14.16% of Kv1.2, p≤0.0001), as was B) the Proportion of all Kv1.2 that was at the cell surface lower for Kv1.2-Φ (17.65% of Kv1.2, *p≤0.0001). Unlike what was observed in cells expressing Kvβ2, Kv1.2-Φ Total protein levels were also decreased (71.83% of Kv1.2, *p≤0.05).

**Figure 14. Kv1.2-Φ Is Capable Of Stimulated Trafficking To The Plasma Membrane.** A,B: Bar graphs of mean percent change in Absolute Surface Kv1.2 following a 10 minute, 10 μM forskolin treatment, determined by quantification of protein immunoblots of biotinylated cell surface proteins from HEK293 cells expressing Kvβ2 and transfected with GFP-Kv1.2 (Kv1.2) or GFP-Kv1.2-Φ (Kv1.2-Φ). A)
Stimulation of adenylate cyclase with forskolin increases absolute surface expression of wild type Kv1.2 (208.8±76.74% versus vehicle 100±27.76, *p≤0.05). B) Forskolin treatment also increased absolute surface expression of Kv1.2-Φ (129.6±27.55% vs. vehicle 100±24.26%, *p≤0.01) C, D: Bar graphs of mean percent change in Surface Kv1.2 expression as detected by flow cytometry utilizing fluorescently labeled antibody against an extracellular epitope of Kv1.2, following a 30 minute 10 μM Y-27632 treatment of HEK293 cells expressing Kvβ2 GFP-Kv1.2 or GFP-Kv1.2-Φ. A) Inhibition of Rho kinase with Y-27632 increased surface expression of wild type Kv1.2 (+22.33±12.73%, *p≤0.05), and B) treatment with Y-27632 also increased surface expression of Kv1.2-Φ (+50.32±26.32%, *p≤0.05).

Figure 15. Amino Acid Logo Of The Putative N-Terminal Kv1.2 Caveolin Binding Domain. A) A Logo of the dozen amino acids housing the ΦXXXXΦXXΦ motif within Kv1.1-Kv1.7 (Rattus Norvegicus), providing a qualitative view of relative similarity of this domain across multiple channels. B) A logo of the dozen amino acids encompassing the ΦXXXXΦXXΦ motif of Kv1.2 or Shaker from twenty species, showing qualitative similarity of the region across the varied species listed in C), a list of the species names and the National Center for Biotechnology Information (NCBI) Protein reference sequence identification codes for the analyzed Kv1.2 and Shaker sequences.

Figure 16. Electrophysiological properties of Kv1.2-Φ are altered. A,B) Plots of
mean current amplitude versus time relationships of HEK293 cells expressing Kvβ2 transfected with either GFP-Kv1.2 or with GFP-Kv1.2-Φ under whole patch clamp. C) Bar graph of the mean currents of GFP-Kv1.2 or GFP-Kv1.2-Φ evoked at +50 mV revealed that the mutant form had a reduced peak current, but this was not significant (7.18±1.61 nA versus 3.91±1.11 nA for GFP-Kv1.2-Φ, one-tailed t-test; p=0.1).
Figure 9. Mutation of a caveolin binding domain motif reduces surface Kv1.2 expression.
Figure 10. Evidence that Kv1.2-Φ is retained in the endoplasmic reticulum.
Figure 11. Caveolin over-Expression reduces maturation of Kv1.2 and Kv1.2-Φ.
Figure 12. Both Kv1.2 and Kv1.2-Φ can interact with Kvβ2.
Figure 13. Reduced surface expression of Kv1.2-Φ does not require Kvβ2.
Figure 14. Kv1.2-Φ is capable of stimulated trafficking to the plasma membrane.
Figure 15. Amino acid Logo of the putative N-terminal Kv1.2 Caveolin binding domain.
Figure 16. Electrophysiological properties of Kv1.2-Φ are altered.
APPENDIX B: A BI-FLUORESCENT FUSION PROTEIN IS AN OPTICAL REPORTER OF KV1.2 LOCALIZATION

Background

Mutated forms of enhanced green fluorescent protein (GFP) that are sensitive to physiological changes in pH are called pHluorins. Super-ecliptic pHluorin (SEpH) is strongly fluorescent at the neutral pH of the typical extracellular environment but does not fluoresce appreciably at the acidic pH of intracellular vesicles (Sankaranarayanan, De Angelis et al. 2000; Sankaranarayanan and Ryan 2000). Endocytosis of membrane proteins can expose extracellular protein domains to the acidic lumen of the vesicle. As a result, fusions of SEpH to the extracellular aspect of AMPA receptors (Ashby, De La Rue et al. 2004), metabotropic glutamate receptors (Pelkey, Yuan et al. 2007), GABA_A receptors (Jacob, Bogdanov et al. 2005; Smith, Muir et al. 2012), D2 dopamine receptors (Li, Roy et al. 2012), vesicular monoamine transporter 2 (Pan and Ryan 2012), L-type calcium channels (Di Biase, Tuluc et al. 2011), nicotinic acetylcholine receptors (Richards, Srinivasan et al. 2011), and A1 adenosine receptors (Baines, Correa et al. 2011) has allowed the visualization of endocytosis of these proteins as a reduction in SEpH intensity. Although it was recently published that Kv1.2 is also regulated by endocytic trafficking in the mammalian brain (Williams, Fuchs et al. 2012), the understanding of the spatiotemporal nature of that trafficking, and how it relates to changes in neurophysiology are limited, as to date, only destructive assays have been used to assess surface Kv1.2 localization in the brain. The goal of research presented in
this appendix then, was to work towards generating a fusion protein of Kv1.2 and SEpH that may ultimately be used as an optical non-destructive reporter of Kv1.2 surface expression in neurons.

**Rationale Of SEpH Fusion Site**

It is the goal to have the fusion protein report changes in subcellular localization with as little disruption to Kv1.2 function as possible. To be exposed to the neutral extracellular space and thus fluoresce when the ion channel is at the cell surface, the SEpH moiety must be incorporated into one of the three extracellular loops of Kv1.2. As determined from glycosylation site scanning mutagenesis, disrupting the first extracellular loop has the least potential to change Kv1.2 properties, including trafficking, activation, and conductance, as compared to disruptions at the second or third extracellular loops (Zhu, Recio-Pinto et al. 2009). Within this first loop extracellular domain, amino acids 195 to 210 are known to be in the aqueous environment of the extracellular environment, and it has been determined that a glycosylation site at 207 must be maintained (Zhu, Watanabe et al. 2003). Therefore, to maintain the relative position of the native glycosylation site near the C-terminal aspect of the first extracellular loop, it would be ideal to insert SEpH as far upstream as possible while remaining in the domain known to be in an aqueous environment. Conveniently, the sequence of Kv1.2 amino acids 196-197 encode a digest site for the restriction enzyme Nsi1, and this is the only such consensus digestion sequence in Kv1.2 (Roberts 1985). Flanking the SEpH construct with the flexible amino acid glycine is a typical and successful strategy (Miesenbock, De Angelis et al. 1998). The amino acids 197, 198, and
199 of Kv1.2, which would follow the C-terminus of the pHluorin insert, are each natively glycine, therefore, inserting SEpH at the NsiI site between amino acids 196 and 197 should produce a flexible fusion in the aqueous environment.

**Rationale Of An N-Terminal mCherry Fusion**

The nature of SEpH to have a strongly reduced fluorescent intensity in acidic environments is central to its use in tracking changes in the surface expression of fusion proteins, but this property also poses a challenge, as a change in SEpH intensity may also be due to changes in total protein. Towards overcoming this problem, the red fluorescent protein mCherry has also added to the cytoplasmic aspect of pHluorin-fusion proteins, such as the vesicular glutamate transporter 1 (Kim and Ryan 2010). The characteristics of mCherry are useful in this strategy because its fluorescent properties are not altered by the physiological changes in pH that dictate SEpH fluorescence alteration, it is monomeric and of good photostability, and its excitation/emission are spectra are readily resolved from that of SEpH by standard microscopy filter sets (Shaner, Campbell et al. 2004). The N-terminus of Kv1.2 tolerates large additions of proteins, such as members of the green fluorescent protein family, without disrupting channel function (Li, Takimoto et al. 2000; Gu, Zhou et al. 2006; Williams, Markey et al. 2007). Furthermore, other voltage-gated potassium channels of the *Shaker* family, such as Kv1.5, have already been successfully tagged with mCherry to the channel’s N-terminus without disrupting channel function (Zadeh, Xu et al. 2008; Dou, Balse et al. 2010).
This body of preliminary work describes the initial development and characterization of mCherry-super ecliptic pHluorin-Kv1.2 (CP-Kv1.2), with mCherry fused to the channel N-terminus, and SEpH encoded between amino acids 196 and 197 (Figure 17).

**Methods:**

*mCherry-Super Ecliptic Phlourin-Kv1.2 (CP-Kv1.2) Construct Generation*

Generation of the CP-Kv1.2 construct was through the technical expertise of Dr. Sheryl White at the University of Vermont Cellular and Molecular Biology Core. A starting plasmid of Rat Kv1.2 with an N-terminal mCherry fusion provided a template from which the ion channel and fluorescent protein were excised using EcoR1 and Xba1 respectively. The Kv1.2 and mCherry fragments from this digest were isolated by gel electrophoresis. The Kv1.2 fragment was then ligated into pBluescript. Super ecliptic pHluorin (SEpH) in a pCI vector was a kind gift from Dr. Gero Miesenboeck, and the SEpH plasmid was used as a template for PCR with primers that corresponded to the N- and C-termini of SEpH, and that were flanked by NsiI sites. The resulting PCR fragment was sub-cloned into pSC_ampaKan, and the subsequent plasmid was digested with NsiI to yield SEpH with NsiI ends. This product was gel purified and ligated into the pBluescript-Kv1.2 construct which had been cut with NsiI. Transformant colonies were screened using a Kv1.2 forward and a pHluorin reverse primer to identify colonies with the insert in the correct orientation. Verified plasmid was digested with EcoR1 and Xba1, isolated and gel purified, and ligated it back into the mCherry vector that had been
digested with EcoRI and Xba1. This produced the desired mCherry-SEpH-Kv1.2 (CP-Kv1.2) construct, the sequence confirmed by the UVM DNA Analysis Core Facility.

**Cell Culture**

As described earlier and published elsewhere, HEK293 cells stably expressing Kvβ2 (HEK293-β) were cultured in Full Select media comprised of DMEM/F-12, Sodium Bicarbonate, and 10% Fetal Bovine Serum, supplemented with Zeocin, G418, and Penicillin/Streptomycin/Glutamine at 37°C in a humidified atmosphere plus 5% CO₂. When cells were plated for transient transfection, this was in No Select, identical in composition to Full Select but lacking Zeocin and G418. Lipofectamine transfection reactions and serum-starving periods were conducted in Zero Serum, which further lacked Fetal Bovine Serum, as described in Appendix A. Cells were passaged at or before reaching full confluency, transfected at approximately 70% confluence, and all experiments were conducted on transfected serum starved cells maintained overnight at low confluence (Williams, Markey et al. 2007; Connors, Ballif et al. 2008; Stirling, Williams et al. 2009).

**Transient Transfection**

The CP-Kv1.2 plasmid was transiently transfected into HEK293-β cells using the LTX lipofectamine reagent (Invitrogen), and subsequently serum stared on poly-d-lysine coated tissue culture dishes or glass coverslips in the same as described in Appendix A.
**Stable Transfection**

Into ten 100mm plates of HEK293-M1 cells that did not express Kvβ2 (ATCC), CP-Kv1.2, and Kvβ2 in the Zeo+ plasmid were transfected in a 1:1 ratio using LTX. The day after transfection, each of the ten transfected plates was split to a 150 mm plate with No Select. On the third day, No Select media was replaced with Full Select media to select for Zeocin resistant cells. Once discrete colonies emerged, they were manually screened for mCherry fluorescence. Of the ten plates of colonies, 2 clones were identified as mCherry positive. These two clones were each isolated by trypsin digestion into a plate of Full Select media. Both cultures were overtaken by apparent fungal infection, one of which survived Nystatin treatment. After maintenance in Full Select plus Nystatin for at least a week, subsequent culture in Full Select alone did not reveal any further infection/contamination. By gross microscopic evaluation, all cells were apparently mCherry positive, and stocks of this HEK293-CP-Kv1.2 clonal line were cryopreserved and used for further experiments.

**Western Blot**

HEK293 cells stably expressing wild type Kv1.2 or CP-Kv1.2 were lysed by sonication in an ice cold lysis buffer of (50 mM TRIS, 150 mM NaCl, 1 mM EDTA, 0.25% deoxycholate, 1% NP40, 10% glycerol, and mammalian protease inhibitor cocktail (Sigma), pH 8.0). The lysate was clarified by centrifugation at 16kRCF at 4°C before dilution in 5x Laemlli Buffer with 0.1 M DTT. Samples were incubated at 100°C for ten
minutes, and evaluated by protein immunoblot using TRIS-HEPES-SDS gels (Pierce). Resolved proteins were transferred to nitrocellulose in a buffer of TRIS, Glycine, and Methanol. Nitrocellulose blots were blocked in a solution of 5% milk in TRIS-buffered saline + 0.1% TWEEN-20 (TBST), then incubated in primary antibodies diluted in TBST with 3% bovine serum albumin. The antibodies used were mouse monoclonal anti-Kv1.2 (K14/16), anti-GFP (N86/8) or anti-Kvβ2 (K17/70) from Neuromab with rabbit polyclonal anti-GAPDH (ab9485, Abcam); or rabbit polyclonal anti-DsRed (632496, Clontech) and mouse monoclonal anti-GAPDH (Millipore). Monoclonal antibody binding was visualized by goat anti-mouse 800 infrared secondary antibody (Rockland), while polyclonal rabbit antibody binding was visualized by goat anti-rabbit 700 infrared secondary antibody (Invitrogen), secondary antibodies were diluted in TBST with 5% milk. Scans were acquired using the Odyssey scanner and associated software (LiCor).

Imaging Solutions

HEK293 cells stably expressing Kvβ2 and stably or transiently expressing CP-Kv1.2 were imaged after serum starving on PDL coated glass coverslips. A pH 7.4 media consisted of, (in mM), NaCl (137), KCl (2.4), CaCl$_2$ (2), MgCl$_2$ (1), Glucose (25), HEPES (20). A pH 8.5 media consisted of the same, but having NaHCO$_3$ (20) in place of HEPES. A pH 5.5 media was prepared that was the same as the pH 7.4 media except for having NaH$_2$PO$_4$ (4.5) in lieu of HEPES. For each of the 7.4, 8.5, and 5.5 medias, a second version (+NH$_3$) was made that contained NH$_3$Cl (50) and reduced NaCl (87). The purpose of the acidic media is to quench surface SEpH fluorescence, and the purpose of
the basic media was to enhance surface SEpH fluorescence. The inclusion of ammonium chloride is to collapse the pH gradient across the plasma membrane, and in conjunction with different pH medias, to manipulate the pH environment and thus the fluorescence of the total SEpH population (Miesenbock, De Angelis et al. 1998; Sankaranarayanan and Ryan 2000; Ashby, De La Rue et al. 2004). Solutions were pH adjusted as necessary with NaOH or HCl, sterile filtered, and heated to 37°C before use. In some experiments, 0.5% sodium azide was used to prevent Kv1.2 endocytic trafficking during the course of solution changes (Williams, Markey et al. 2007; Connors, Ballif et al. 2008; Stirling, Williams et al. 2009). While azide inhibits mitochondrial F-ATPase activity, a similar inhibition is not likewise clearly established for V-ATPases important for the acidification of endosomes (Forgac, Cantley et al. 1983; Vasilyeva and Forgac 1998; Bowler, Montgomery et al. 2006). Drug additions were made directly to the pH 7.4 control media at the indicated concentrations and times. Fluid exchanges were accomplished manually by means of flexible tubing attached to a 100-1000μl pipette tip upon a P1000 pipettor.

Image Acquisition

Transmitted light, SEpH intensity, and mCherry fluorescence were detected with the Delta Vision Restoration Microscopy System (Applied Precision) and standard techniques for epifluorescence utilizing mercury arc lamp illumination. For the excitation set of filters, green fluorescent signals (SEpH) were stimulated with a 490nm filter with a 20nm bandpass, while red fluorescence (mCherry) was stimulated with a 555nm filter.
with a 28nm bandpass. The emission wheel filter for green fluorescence was a 528nm filter with a 38 nm bandpass, while for red fluorescence a 605 nm filter with a 52 nm bandpass was used. Oil-immersion lenses were used. The sample, stage, and objectives were housed in an environmental control chamber to maintain a physiological temperature (37°C).

*Quantification Of Ph Responsiveness*

HEK293 cells stably expressing Kvβ2 and stably or transiently expressing CP-Kv1.2 were prepared for imaging as above. Fields of view encompassing at least one entire cell were used, and within these fields of view, a Z-series was captured for both SEpH and mCherry fluorescence in each of the pH 7.4, 5.5, 8.5 and then 7.4+NH₃, 5.5+NH₃, 8.5+NH₃ solutions. Neutral density filters were exchanged and exposure times were adjusted as required to provide dynamic range and prevent saturation. For each cell under each solution, a single, similar, in-focus, unsaturated, z-section was chosen, and section maximums for each channel (SEpH and mCherry) were recorded. These raw values were corrected to the neutral density filter and/or exposure time under which they were acquired, and for each cell, the values were normalized to 1 for the pH 7.4 solution without NH3. In this way, normalized fold changes for SEpH and mCherry fluorescence under different pH manipulations could be compared across multiple cells.

*Electrophysiology*

These experiments were conducted and analyzed by Dr. Anthony Morielli.

Determination of peak amplitude and the voltage dependence of activation was
determined as described in Appendix 1, and as in (Connors, Ballif et al. 2008). Briefly, HEK293-β cells stably expressing CP-Kv1.2 were serum starved at low density on PDL coated glass coverslips. Coverslips were then transitioned to a recording media composed of (in mM) NaCl (100), KCl (2.5), CaCl$_2$ (1.8), MgCl$_2$ (0.8), glucose (23), and NaHEPES (5), pH 7.4, and maintained at 35-37°C. Seals were achieved using glass electrodes and an internal solution of (in mM): KCl (120), CaCl$_2$ (3.69), MgCl$_2$ (0.094), BAPTA (5), EDTA (5), NaHEPES (5), glucose (5), pH 7.1. Using whole-cell voltage clamp, currents were stimulated by 75-ms voltage changes in 10-mV steps from -70 to +50 mV, as collected by an Axopatch 200D amplifier interfaced with a computer utilizing Clampex (Molecular Devices). Analysis was conducted using both Clampfit (Molecular Devices) and Origin (Microcal).

**Results**

**Western Blot**

Using an α-Kv1.2 antibody, compared to wild type Kv1.2 which ran as a triplet, the principle of which had an apparent molecular weight of ~ 65kD, CP-Kv1.2 was recognized as a triplet with the most intense band having an apparent molecular weight of ~119 kD (Figure 18A). As mCherry has a molecular weight of ~29kD, and SEpH of ~27, the CP-Kv1.2 appears to run at the approximate predicted molecular weight of the sum of the three proteins (119 kD). These blots resolved the molecular weights down to ~20kD, and there were no apparent α-mCherry or α-GFP signals at the predicted molecular weight of the free fluorescent proteins (Figure 18 A, B, C). Likewise, there were no α-mCherry or α-GFP signals evident for the wild type Kv1.2 cell line, but there were bands
at the predicted fusion protein weight, suggesting these signals were specific to an intact CP-Kv1.2 fusion (Figure 18 B,C). The gross levels of GAPDH were visually comparable for both the Kv1.2 wild type and CP-Kv1.2 cell line across all blots, and both cell lines gave positive signal for α-Kvβ2, suggesting that levels of protein were similar for both samples, and that the two cell lines were comparable with respect to their abundance of the beta subunit (Figures 18 D). Therefore, CP-Kv1.2 appears to be successfully translated as an intact protein.

**The pH Responsiveness Of CP-Kv1.2**

Quantifying the normalized pH responsiveness as described earlier in Methods, the mean change in fluorescence for either SEpH or mCherry of nine samples was compared to the hypothetical value of 1, the normalized fluorescence of the pH 7.4 sample without NH3 in each experiment. The changes are reported in terms of % change relative to pH 7.4 without NH3.

As illustrated in Figure 19 A, acidifying the extracellular space with pH 5.5 saline significantly decreased SEpH fluorescence (48.94±5.89%, n=9, p<0.0001). Conversely, alkalization of the extracellular space with pH 8.5 media saline significantly enhanced SEpH fluorescence (142.2±9.87%, n=9, p=0.002). Neutralizing the intracellular environment with a pH 7.4 media containing NH3 significantly enhanced SEpH fluorescence (317.8±86.69%, n=9, p=0.036). By paired sample two-tailed t-test, the average change in SEpH fluorescence was also compared between pH 5.5 and pH5.5+NH3, and there was no significant difference (-2.17%, n=9,9 ; p=0.85), suggesting
that in these samples either any SEpH fluorescence from intracellular compartments was minor, or that extracellular acidification resulted in intracellular pH acidification even in the absence of NH$_3$ (Khiroug, Pryazhnikov et al. 2009). Further confirming the acid sensitivity of SEpH intensity, by paired sample two-tailed t-test, the average change in SEpH fluorescence was also compared between pH 7.4 with NH$_3$ and pH 5.5 with NH$_3$; there was a significant decrease (-271%, n=9, p=0.017). Therefore entire cell population of CP-Kv1.2 shows acid sensitive SEpH diminution.

As illustrated in Figure 19B, the analysis of mCherry fluorescence changes was carried out and is reported in the same manner, but as expected, there were no significant changes in mCherry intensity with pH perturbations. The changes are reported in terms of normalized mCherry intensity percent change relative to pH 7.4 without NH$_3$. Acidifying the extracellular space with pH 5.5 saline did not change mCherry fluorescence (88.13±7.06%, n=9, p=0.13). Likewise, alkalization of the extracellular space with pH 8.5 media saline also did not significantly change mCherry fluorescence (98.49±8.06%, n=9, p=0.86). Unlike for SEpH, neutralizing the intracellular environment with a pH 7.4 media containing NH$_3$ did not change mCherry fluorescence (96.6±7.38%, n=9, p=0.66), nor did acidifying the intracellular pH environment with pH 5.5 media +NH$_3$ (108.9±12.08%, n=9, p=0.48), or alkalinizing the intracellular pH environment with pH 8.5 media +NH$_3$ (117.6±9.18%, n=9, p=0.09). Even comparing the total acidification to total alkalization conditions by paired sample two-tailed t-test failed to reveal a pH sensitive change in mCherry intensity (-8.74%, n=9, p=0.43). Therefore, under these conditions, mCherry intensity is pH insensitive.
**Electrophysiology**

Since fusing mCherry or SEpH to Kv1.2 did not apparently interfere with the fluorescence proteins optical properties, it was tested whether the fluorescent fusion proteins in turn did not disrupt Kv1.2 biophysical properties. The biophysical properties of wild type Kv1.2 in HEK cells expressing Kvβ2 have been previously described (Connors, Ballif et al. 2008), including YFP-Kv1.2 (Williams, Markey et al. 2007). It was therefore tested whether CP-Kv1.2 had similar properties. The CP-Kv1.2 construct was found to have a mean amplitude at +50 mV of 6.3 +/- 0.8 mV, a voltage of half-activation of -3.8 +/- 0.9 mV, and no obvious inactivation (See current-time trace in Figure 20.) These correspond approximately to the reported values of half activation of approximately -15 mV and amplitude of near 7 nA for YFP-Kv1.2 (Williams, Markey et al. 2007). Therefore, CP-Kv1.2 is still grossly functional and displays a typical activation voltage and a lack of inactivation.

**CP-Kv1.2 Fluorescent Response To Endocytic Stimuli**

Endocytosis of Kv1.2 from the plasma membrane into endosomes can be stimulated by phorbol 12-myristate 13-acetate (PMA) (Nesti, Everill et al. 2004). Since such endosomes have a progressively acidic pH due to the action of V-ATPase (Geisow and Evans 1984; Cain, Sipe et al. 1989; Lafourcade, Sobo et al. 2008), PMA stimulated entry of Kv1.2 into acidic endosomes should result in a decrease in the CP-Kv1.2 SEpH fluorescence. To test this, transmitted light, as well as SEpH and mCherry fluorescence were recorded from HEK293 cells expressing Kvβ2 and CP-Kv1.2 before and ten
minutes after application of 100 nM PMA (Figure 21). As can be seen by whole cell projection, in transmitted light (Figure 21 Top Row: Left vs. Right) the cells are still present and in focus before (Left) and after PMA treatment (Right). As revealed by mCherry (Figure 21, Middle Row), Kv1.2 is present in both an apparent cell surface and punctate appearance before PMA exposure (Left), while after PMA treatment (Right), the mCherry signal becomes more punctate in appearance, particularly in the bottom cell. Changes in SEpH intensity are most apparent (Figure 21 Bottom Row), while before PMA treatment (Left), SEpH intensity is seen in the top and bottom cells about the edges (Figure 21 Bottom Row), after PMA treatment (Right) SEpH intensity is greatly diminished about the periphery, particularly in the top cell. Thus qualitatively, the evidence is consistent with the interpretation that a loss of surface Kv1.2 by stimulated entry into endosomes may be optically determined by reduction in SEpH intensity, while changes in total Kv1.2 distribution sub-cellularly could be interrogated via mCherry.

Discussion

The work on validating CP-Kv1.2 remains in preliminary stages, but upon initial examination, there are several reasons for optimism that the construct may allow non-destructive optical tracking of changes in surface and total Kv1.2 in living cells in real time. The dual fluorescent fusion protein appears to be translated intact, and retains the desired pH insensitivity of mCherry but the desired pH sensitivity of SEpH. Biophysically, the channel appears normal, insofar as the SEpH or mCherry moieties do not grossly diminish the amplitude of the channel’s current, nor alter is voltage dependent activation, nor abnormally confers inactivation. Furthermore, the pilot PMA experiment
suggests that CP-Kv1.2, like wild type Kv1.2, may undergo endocytosis from the plasma membrane into acidic endosomes. However, a number of future experiments must be directed at scrutinizing the nature of CP-Kv1.2 before it can reliably be employed.

**Considering Biological Modulators Of CP-Kv1.2 Function**

In terms of channel biophysics, CP-Kv1.2 must be subject to conductance measurements to distinguish whether the apparently wild type current amplitude results from a normal abundance of channels with typical conductance, from a reduced amount of channels with enhanced function, or a greater abundance with reduced function. Not only the ability to activate the channel, but inhibit it as well, should be examined. The CP-Kv1.2 construct could therefore also be tested for its ability to undergo normal pharmacological inhibition, such as by the intracellularly acting open-channel blocker 4-aminopyridine ((Kirsch and Narahashi 1983; Russell, Publicover et al. 1994; Kerr, Clement-Chomienne et al. 2001)), or extracellularly acting snake or scorpion toxins such as α-Dendrotoxin or Tityustoxin-Kα (Stansfeld, Marsh et al. 1987; Newitt, Houamed et al. 1991; Werkman, Kawamura et al. 1992; Werkman, Gustafson et al. 1993; Harvey 1997).

The ability of CP-Kv1.2 to undergo normal biological inactivation should also be investigated. Although Kv1.2 is generally non-inactivating, it is capable of undergoing both N-type fast inactivation through association with Kvβ1.2, and a slower C-type inactivation through alterations in confirmation of the pore face, a process which Kvβ species can also modulate (Hoshi, Zagotta et al. 1990; Hoshi, Zagotta et al. 1991; Rettig,
Heinemann et al. 1994; Liu, Jurman et al. 1996; Morales, Wee et al. 1996). In contrast, association with the Lgi1 accessory subunit prevents KvB1 mediated inactivation to the related Kv1.1 channel (Schulte, Thumfart et al. 2006). It will therefore be important to test whether CP-Kv1.2 can associate normally with various auxiliary subunits, as their differential association with alpha subunits in heteromultimers finely tunes holochannel function (Accili, Kiehn et al. 1997).

Because CP-Kv1.2 harbors the SEpH moiety in the extracellular domain where glycosylation occurs, it would also be prudent to test whether the glycan species of CP-Kv1.2 is the same as for wild type Kv1.2, because this too can influence the trafficking and gating of the channel (Watanabe, Zhu et al. 2007). Likewise, the interaction between the Kv1.2 pore architecture and biosynthetic anchors may be physically occluded by the SEpH moiety (Vacher, Mohapatra et al. 2007).

Testing the biophysical properties of physiologically relevant heteromultimers including CP-Kv1.2 will further be important if manipulation of extracellular pH will continue be used, because while Kv1.2 function is resistant to acid and alkaline pH shifts, Kv1.5 shows enhanced inactivation after exposure to acidic medias (Steidl and Yool 1999), therefore experimental manipulations to alter CP-Kv1.2 fluorescence may alter biophysical properties of other Kv1 alpha subunits with which CP-Kv1.2 may associate. Overall, the experimentally addressable concern is that CP-Kv1.2 containing holochannels, including accessory and beta subunits, and association with other alpha-subunits, may function normally in HEK293 cells as tested here, but not in the context
where the reporter would be perhaps most intriguing: within neurons of the mammalian nervous system.

** Strategies For Introduction Of CP-Kv1.2 To Neural Tissues **

Attempting to generate a transgenic rat or mouse line that expresses CP-Kv1.2 is a long-term goal which would likely be a time and resource intensive process. An alternative strategy could be transient expression using chemical, biolistic, or viral transduction of a CP-Kv1.2 construct. These strategies have their drawbacks though, since they preclude the use of acutely generated tissue slices, requiring first the generation of slices and then a waiting period until proteins are expressed, or instead require survival surgery to introduce the construct. Further, chemical and viral transductions can be associated with neuronal toxicity, while the efficiency of biolistic methods is typically low in brain slices (Washbourne and McAllister 2002; McAllister 2004).

** Potential Consequence Of Overexpressing CP-Kv1.2 **

Beyond the technical hurdles of introducing this reporter, regardless of the method used, the result will still be introduction of CP-Kv1.2 into a neuronal population that already expresses the channel. Conceivably this could exert undesired changes through the sequestration of regulatory elements, or by simple enhanced abundance of the potassium channel, inducing a hypo-excitabile phenotype, as has been observed in myocyte, skeletal muscle, and neuronal cells with Kv1.1, Kv1.2, Kv1.4, or Kv1.5 potassium overexpression (Falk, Kilani et al. 2001; Dou, Balse et al. 2010; Cazzin,
Piccoli et al. 2011; Wykes, Heeroma et al. 2011). Alternatively, a non-conducting version of CP-Kv1.2 could be used to strictly report trafficking of the channel, but since functional Kv1.2 channels form from the assembly of separate alpha subunits, this could cause a dominant negative effect on any channel into which one such CP-Kv1.2 alpha subunit is incorporated (Perozo, MacKinnon et al. 1993; Wollnik, Schroeder et al. 1997; Grunnet, Rasmussen et al. 2003). Alternatively, constructs which encode the reporter could also be used to encode short hairpin RNA sequences to selectively knockdown the endogenous but not the introduced, channel (Zhang, Adams et al. 2007).

**Issues For Resolving CP-Kv1.2 Changes In Neural Tissue**

Despite the hurdles that remain to be overcome, as has been reported for pHluorin fusions of the AMPA, NMDA, and GABA_A receptors, it is anticipated that CP-Kv1.2 will accurately report its pH environment, and that its utilization may expand our understanding of the mechanisms and consequences of potassium channel trafficking in neurophysiology. However, even if the reporter functions perfectly, and even if it can be introduced successfully into desired neuronal populations without grossly changing their physiology, the changes in CP-Kv1.2 fluorescence intensity may not accurately report changes in surface Kv1.2 if the vesicles to which the construct may be endocytosed do not undergo acidification (Cocucci, Racchetti et al. 2004). Further, if the degree of variability in the measured CP-Kv1.2 SEpH fluorescent intensity is greater than the amount of change in surface expression, these changes may not be detected accurately. That is, the signal to noise of this assay may be insufficient to report physiologically relevant amounts of channel trafficking to or from the plasma membrane. However,
studies of pHluorin fusions to the NMDA and AMPA type glutamate receptors suggest a relatively high fidelity of this approach, detecting changes in the surface proportion within an error of two percent (Gong and De Camilli 2008).

Summary

While biotinylation assays and fluorescent microscopy techniques have provided the first evidence that Kv1.2 may be regulated by endocytic trafficking in the mammalian brain, these assays are destructive, and so only one manipulation may be made per slice, per time point (Williams, Fuchs et al. 2012). This is a low throughput approach which has the further downside of requiring additional tissue, and therefore subjects, to resolve changes over time. In contrast, an optical method of measuring surface Kv1.2 would allow changes to be measured at individual synapses over time and various manipulations, and if combined with electrophysiology or functional assays, would inform the understanding of the contribution of Kv1.2 trafficking to neurophysiology. Though work remains to be done in the validation of CP-Kv1.2, the potential is compelling: the successful utilization of other pHluorin fusions in cranial window preparations suggests that the regulation of Kv1.2 trafficking may ultimately be observable in freely behaving animals (Bozza, McGann et al. 2004).
**Figure Legends**

**Figure 17. The CP-Kv1.2 Construct.** A) To the N-terminus of Kv1.2, mCherry is fused; within the first extracellular loop of Kv1.2, super-ecliptic pHluorin (SEpH) is inserted. When facing the pH neutral extracellular environment, SEpH fluoresces. B) Upon endocytosis, the SEpH moiety is in an acidic environment, and SEpH fluorescence is quenched. Across physiological ranges, mCherry intensity is pH insensitive and is a constant signal for total Kv1.2 regardless of the channels pH environment.

**Figure 18. Protein Immunoblots Of CP-Kv1.2.** A-D) Protein immunoblots of HEK293 cells stably expressing Kvβ2 and either wild type rat Kv1.2 (WT) or CP-Kv1.2 (CP). The WT and CP forms of Kv1.2 are both recognized by an α-Kv1.2 antibody, with the CP form running at an apparent higher molecular weight of ~119kD compared to the ~65kD of the WT form, consistent with an increased molecular weight from the mCherry and SEpH moieties. No α-Kv1.2 signal is apparent for CP at the WT molecular weight or lower, suggesting efficient production and no aberrant degradation of the CP construct. B) For CP Kv1.2, but not for WT, α-GFP immunoreactivity is found at the expected molecular weight (~119) of the fusion protein. C) As for α-GFP immuno-reactivity, α-DsRed (which recognizes mCherry), gives a prominent band for the CP, but none for the WT, form of Kv1.2 at the expected fusion protein molecular weight. D) Samples from both the WT and CP Kv1.2 cell lines demonstrate grossly equivalent α-Kvβ2, and all
blots have similar total protein loading as seen by α-GAPDH intensity.

**Figure 19. The pH Sensitivity Of CP-Kv1.2.** A,B: Plots of mean change in maximal SEpH (A) or mCherry (B) intensity within a single optical slice through HEK293 cells expressing Kvβ2 and CP-Kv1.2 when the extracellular media is of pH 5.5, 7.4, or 8.5, with or without NH3, normalized to the intensity in pH 7.4 media without NH3. As illustrated in Figure 19A, extracellular acidification decreased (48.94%, *p≤0.0001) while alkalization increased (142.2%, *p≤0.005) SEpH fluorescence, as they did in the presence of NH3 (46.77%, *p≤0.005), (546.4%, *p≤0.05), respectively. Neutralizing the intracellular pH (pH 7.4 with NH3 also increased SEpH fluorescence (317.8%, *p≤0.05). C) In contrast, as seen in Figure 19B, neither acidification nor alkalization changed mCherry signal in the absence (88.13%, p=0.13; 98.49%, p=0.86) or presence of NH3 (108.9%, p=0.66; 117.6%, p=0.09). Neutralizing the intracellular pH (pH 7.4 with NH3) also did not change mCherry fluorescence (96.6%, p=0.66).

**Figure 20. Electrophysiology Of CP-Kv1.2.** A) Plot of mean current amplitude versus time relationship of HEK293 cells stably expressing Kvβ2 and CP-Kv1.2 under whole patch clamp. As reported in text, the CP-Kv1.2 construct was found to have a mean amplitude at +50 mV of 6.3 +/- 0.8 mV, a voltage of half-activation of -3.8 +/- 0.9 mV, and no obvious inactivation.
**Figure 21. PMA Stimulated Changes In CP-Kv1.2 Fluorescence. A)**

Photomicrographs of a maximum projection of a Z-series through an HEK293 cell expressing Kvβ2 and CP-Kv1.2, showing transmitted light (top), and SEpH fluorescence (middle, Green), and mCherry fluorescence (bottom, Red), before (left column) and ten minutes after (right column) 100 nM phorbol-12-myristate-13-acetate (PMA) treatment. As seen by Transmitted light, both before and after PMA treatment, the cells are present and in focus. The SEpH signal is strongly decreased, losing intensity from a ring like pattern in the top cell, and a diffuse signal in the bottom cell after PMA treatment. As by mCherry signal, gross Kv1.2 levels appear similar before and after PMA treatment, though a more punctate pattern becomes apparent after the stimulus in the bottom cell.
Figure 17. The CP-Kv1.2 construct.
Figure 18. Protein immunoblots of CP-Kv1.2.
Figure 19. The pH sensitivity of CP-Kv1.2.
A.

Figure 20. Patch clamp recording of CP-Kv1.2
Figure 21. PMA stimulated changes in CP-Kv1.2 fluorescence.
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