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Protein Kinase Mzeta (PKM-ζ) Regulates Kv1.2 Dependent Cerebellar Eyeblink Classical Conditioning

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PROTEIN KINASE MZETA (PKM-ζ) REGULATES KV1.2 DEPENDENT CEREBELLAR EYEBLINK CLASSICAL CONDITIONING

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ABSTRACT

Learning and memory has been a topic that has captured the attention of the scientific and public communities since the dawn of scientific discovery. Without the faculty of memory, mammals cannot experience nor function in the world; among homosapiens specifically, language, relationships, and personal identity cannot be developed (Eysenck, 2012). After all, some philosophers such as John Locke argued we are nothing but a collection of past memories in which we have developed and improved upon (Nimbalkar, 2011).

Understanding the cellular mechanisms behind learning, and the subsequent formation of memory, has been a topic that has garnered scientific interest for many decades. One particular kinase that has been at the center of attention in the last decade is the serine/threonine kinase PKM-ζ, an N-terminal truncated form of PKC-ζ that renders it constitutively active (Hernandez et al., 2003). PKM-ζ has long been implicated in a cellular correlate of learning, long-term potentiation (LTP). Inhibition of PKM-ζ with Zeta-inhibitory peptide (ZIP) has been shown in many brain structures to disrupt maintenance of AMPA receptors, irreversibly disrupting numerous forms of learning and memory that have been maintained for weeks.

The voltage-gated potassium channel Kv1.2 is a critical modulator of neuronal physiology, including dendritic excitability, action potential propagation, and neurotransmitter release. While expressed in various mammalian tissues, Kv1.2 is most prevalent in the cerebellum where it modulates both dendritic excitability of Purkinje cells (PCs) and basket cell (BC) inhibitory input to PCs. Because PCs are the main computational unit of the cerebellar cortex and provide its sole output (Napper et al., 1988; Harvey et al., 1991), regulation of synaptic Kv1.2 is predicted to have a major role in cerebellar function. Pharmacological inhibition of Kv1.2 in cerebellar PC dendrites increases excitability (Khavandgar et al., 2005), while its inhibition in BC axon terminals increases inhibition to PCs (Southan & Robertson, 1998). Interestingly, two prior studies have demonstrated that PKC-ζ, an atypical Protein Kinase C, is able to phosphorylate and bind cerebellar Kvβ2, a Kv1.2 auxiliary subunit. (Gong et al., 1999; Croci et al., 2003).

Delay eyeblink conditioning (EBC) is an established model for the assessment of cerebellar learning. Despite being highly expressed in the cerebellum, no studies have examined how regulation of cerebellar PKM-ζ may affect cerebellar-dependent learning and memory nor have they examined the possible effect PKM-ζ may have on Kv1.2. The goal of this dissertation was to determine whether PKM-ζ could modulate EBC in a Kv1.2 dependent manner. Through the use of microscopy techniques we have shown that PKM-ζ is highly expressed in the cerebellar cortex, primarily in the PC, and by the use of pharmacological manipulations, it was found that PKM-ζ has an important role in regulating the acquisition of EBC. Through the use of biotinylation, flow cytometry, and behavioral manipulations, it was determined that PKM-ζ regulates Kv1.2 during eyeblink conditioning. These studies provided the first evidence that PKM-ζ has a role for learning and memory in the cerebellum, and the first evidence of PKM-ζ regulating a voltage-gated ion channel rather than a ligand-gated ion channel such as AMPA receptors.

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

Introduction

Learning and memory has been a topic that has captured the attention of the scientific and public communities since the dawn of scientific discovery. Without the faculty of memory, mammals cannot experience nor function in the world; among homosapiens specifically, language, relationships, and personal identity cannot be developed (Eysenck, 2012). After all, some philosophers such as John Locke argued we are nothing but a collection of past memories in which we have developed and improved upon (Nimbalkar, 2011). In John Locke's own words: "For as far as any intelligent being can repeat the idea of any past action with the same consciousness it had of it at first, and with the same consciousness it has of any present action; so far it is the same personal self" (Locke, 1694). By definition, memory is the faculty of the mind by which information is encoded, stored, and retrieved (Atkinson & Shiffrin, 1968).

Despite the philosophical developments that have established the importance of memory in the mammalian and human lives, on a molecular level, the understanding of learning and memory is still being unraveled. There is a critical need to develop treatments that will help patients suffering from learning and memory related disorders such as Alzheimer's disease, depression, autism, schizophrenia, and post-traumatic stress disorders. Alzheimer's disease alone is estimated to affect 5.1 million Americans aged 65 years and older and to have annual costs exceeding $214 billion (NIH Fact Sheet

2014: Alzheimer's disease). Dysfunction of synaptic plasticity plays a unifying role in these disorders (Luscher et al., 2009; Bliss et al., 2014) and our research sheds light on the molecular mechanisms that regulate learning-related plasticity in the brain. This research has broad relevance for neurological disease and may thus reveal novel targets for the development of clinical treatments, which continue to remain costly and largely ineffective.

Excitability of Cells

The formation of memory is dependent on the plasticity of neurons, which on an extremely simplified level, involves the ability of neurons to generate synchronized action potentials (Fell & Axmacher, 2011). This synchronization is dependent on molecular changes that occur which facilitate the ability of the cell to generate and transmit an action potential. An action potential is the mechanism by which a cell can communicate with a neighboring cell and involves changes that occur to membrane potential. When a depolarization has breached a certain membrane potential threshold, (measured in mV), a cell will undergo a chain-reaction that induces a positive feedback loop that exponentially accelerates the depolarization and propagates the action potential to neighboring cells. This ability to depolarize and subsequently propagate an electrical signal, and thus information, is the initial mechanism that can lead to the formation of synaptic plasticity that can induce the creation of memories.

Resting membrane potential

The membrane potential of a cell is dependent on a concentration gradient that is formed by the unequal separation of cations and anions. This concentration gradient forms across a living capacitor, the plasma membrane, in which the extracellular and intracellular fluids serve as plates and the cell membrane serves as a dielectric that separates these plates and prevents the free flowing of ions into and out of the cell. In a neuron, the presence of K+ cations is most concentrated intracellularly while the presence of Na+ cations is most concentrated extracellularly. This concentration gradient is kept in a specific balance by Na+/K+ pumps, which utilize energy in the form of ATP to maintain the concentration gradient. In addition to the Na+/K+ pumps, the cell membrane contains leak channels that allow for passive movement of Na+ and K+ cations across the membrane. The membrane potential of a cell at rest, known as the resting membrane potential, is dependent on numerous factors, the most important being the presence of leak channels. Due to the presence of a 40:1 concentration of K+ to Na+ leak channels, the resting membrane potential is most dependent on the equilibrium potential of K+ cations, which is the voltage gradient needed to counteract the concentration gradient so that there is no net movement of an ion through open ion channels across the membrane. In a neuron, this is generally -70 mV, while the equilibrium potential of K+ is -95 mV. Therefore, the increased presence of K+ channels on a cells surface can further hyperpolarize the cell.

Neuronal depolarization

While leak channels are continuously open and allow for the passive influx and efflux of ions, voltage-gated ion channels require a specific voltage threshold to be reached in order to open and permit more influx or efflux of ions. Voltage-gated Na+ channels open when a stimulus depolarizes the neuron above a specific voltage membrane threshold and thus triggers a domino effect of voltage-gated Na+ channel openings. These openings, if sufficient for the action potential to propagate, will generally depolarize a neuron towards the equilibrium potential of Na+. At such a time when driving force of Na+ reaches 0, that is, when the membrane voltage of the cell is equal to the equilibrium potential of Na+, Na+ influx into the cell will cease. Simultaneously, voltage-gated K+ channels open and result in K+ efflux from the cell, repolarizing the cell and eventually hyperpolarizing the cell below its original resting membrane potential. As these channels close, the Na+/K+ ATPase pump will restore the proper concentration gradient of the cations and the cell will return to its original resting potential. When an action potential propagates, it will reach an axon terminal that involves either an electrical or chemical synapse. In an electrical synapse, gap junctions are formed that allow the cytoplasm of two cells to be continuous, through the use of specialized transmembrane proteins called connexins that form a hemichannel. Such transmission can occur rapidly and thus found primarily in cardiac myocytes, but is not often found in neurons (Connors & Long, 2004; Dere & Zlomuzica, 2012).

Neural synapses are primarily composed of chemical junctions; in this case the propagating electrical signal is in turn converted into a chemical signal that diffuses across the synapse. Whereas the action potential is a large all-or-none signal, the signal at the synapse, the synaptic potential, is graded. The production of current by the action potential in the presynaptic cell cannot jump directly across the cleft of a chemical synapse to activate the postsynaptic target. It instead undergoes a major transformation that allows the release of a neurotransmitter to spill into the synaptic cleft and act as a signal to the target cell. Common neurotransmitters include glutamate, gamma-aminobutyric acid (GABA), acetylcholine, epinephrine, norepinephrine, serotonin, and dopamine. When an action potential reaches the presynaptic terminal, membrane channels for Ca2+ ions open and allow a rapid increase of Ca2+ into the cell. This Ca2+ increase causes neurotransmitter vesicles to fuse with the membrane and leads to the release of the chemical transmitter. These neurotransmitters then bind receptors which open and allow Na+ to enter the postsynaptic cell and propagate the action potential.

Potassium channels

One family of ion channels that are important for regulating membrane potentials, and thus have implications for action potential propagation, are potassium channels. Potassium channels are a diverse class of ion channels that in the simplest form only consist of two transmembrane domains and one pore (Perney & Kaczmarek 1991; Choe 2002). Diversity within these channels allow for channel regulation, as some channels have intracellular regions with sites of modification as well as binding sites for other molecules

which can affect their structure and function. In the simplest form of potassium channels that consist of two transmembrane domains and one pore, consists of inward rectifier potassium channels, KATP channels, as well as G-protein coupled inward rectifier potassium channels; such channels are critical for resting membrane potential. In addition to this class, more complex potassium channels form another family involving four transmembrane domains and two pores, known as leak potassium channels. Another even more complex form involves six transmembrane domains and 1 pore; the additional 4 transmembrane domains harbor a voltage sensor and include the voltage-gated potassium channels (Kv) as well as other smaller calcium-gated potassium channels; such channels can not only be responsible for resting membrane potential, but also for action potential propagation. Specifically, Kv1, Kv2, Kv3, and Kv4 are voltage gated potassium ion channel alpha subunits, each with different biophysical properties; in mammalians, voltage gated potassium ion channels consist of genetic subfamilies that for example are numerically labeled Kv1.1 through Kv1.12 (Salkoff et al., 1992). Such channels can form tetramers in a cell transmembrane that result in a specific set of functions. These tetramers can be either heterotetramers with different alphasubunits or homotetramers; for example, voltage-gated potassium channels Kv1, Kv10, and Kv7 are homotetramers (Gutman et al., 2005). One example of a heterotetramer is Kv1.2 which may heteromultermize with Kv1.1. Kv1.1 itself on the other hand can also form a homomultimeric channel composed of only Kv1.1 alpha subunits (Shen & Pfaffinger, 1995). Furthermore, Kv channel alpha subunits have several functional domains; in the case of Kv1.2, such domains include the formation of the T1 tetramer within the N-terminus, S1-S6 transmembrane domains which includes

an S4 voltage-sensing domain, and a potassium selective pore found between transmembrane domains S5 and S6, as well as extracellular domains and a C-terminus. In some circumstances, the alpha subunits may associate with another family for necessary function; for example Kv8.2 cannot function as a homotetramer, but functions when it is a heterotetramer with other Kv2 alpha subunits (Ottschytsch et al., 2002).

Voltage-gated potassium channel subfamily A member 2 (Kv1.2)

While the function of potassium channels can vary widely, and some are indeed involved in repolarization of the cell after depolarization, some potassium channels serve to alter the resting membrane potential through a mechanism known as prepulse potentiation. This unique regulatory mechanism that results in a decreased or increased resting membrane potential is attributed to Kv1.2 (Grissmer et al., 1994; Rezazadeh et al., 2007, Baronas, et al., 2015). Part of Kv1.2's contribution to resting membrane potential is through its activation at membrane potentials below the critical threshold for action potential generation (Bekkers & Delaney, 2001; Dodson, Barker, & Forsythe, 2002; Shen et al., 2004). Such activation can occur in a wide range of membrane potentials that range from +30 mV to -40 mV (Rezazadeh et al., 2007). Due to this ability as well as their ability to rapidly activate, Kv1.2 channels have been characterized as low-voltage activated channels that only need a small depolarization, if any at all, to open at rest (Al-Sabi et al., 2013). Their functionality depends on their localization within the cells (Southan & Robertson 1998); in the brain, Kv1.2's expression can be found in many critical parts of neurons such as dendrites, the soma, the initial axon segment as well as axons and their

terminals. In the cerebellar region of the brain for example, Kv1.2 may oppose dendritic depolarization in cerebellar Purkinje cell dendrites (Khavandgar et al., 2005).

Post-Translational Modifications and Kv1.2

Post-translational modification (PTM) is a mechanism of protein function regulation that can regulate protein trafficking, survival, and function as well as assist in cell signaling and migration; furthermore, PTMs can help in cell proliferation and interaction with other cells. (Doerig et al., 2015). After a protein has been translated, PTM can aid in folding and trafficking of the protein (Dunham et al., 2012). Beyond these stages, PTM can also affect the biological activity of the protein and eventually tag for its degradation. Enzymes that can perform PTMs include phosphatases, transferases, kinases, ligases, and numerous others that can either remove, add, or transfer modification groups.

One type of PTM which I will be focusing on in this manuscript is phosphorylation; protein phosphorylation is the most understood and prevalent PTM and involves the attachment of a phosphate group to a protein. This process may occur on either tyrosine, serine, or threonine and less frequently, histidine or aspartate (Thomason & Kay, 2000). Phosphorylation results in changes to protein properties that often result in a change of protein confirmation, which can alter its activity. Phosphorylation can affect ion channel biophysical properties, biosynthesis and endocytic trafficking, resulting in profound effects on ion channel function. Such effects can vary, but the combination of these modifications on a single protein can lead to numerous permutations of complex outcomes (Barford et al., 1998; Pawson & Nash, 2003; Pellicena & Kuriyan, 2006).

One method of Kv1.2 PTM regulation is through N-linked glycosylation on the first extracellular loop of Kv1.2, which is involved in trafficking of Kv1.2 after translation (Shi & Trimmer, 1999; Zhu et al., 2003). After Kv1.2 has been trafficked, the channel may undergo another PTM such as phosphorylation. For example, tyrosine phosphorylation of the channel, through m1 mAchR activation and subsequent phospholipase C (PLC) activation, has been shown to down regulate the channel and suppress its current (X. Y. Huang, Morielli, & Peralta, 1993; Nesti, Everill, & Morielli, 2004). In addition, Morielli and colleagues have shown that tyrosine phosphorylation plays a role in the interaction between an actin binding molecule, cortactin, and Kv.1.2. Particularly, activation of m1 mAchRs reduce interaction between cortactin and Kv1.2, and resulted in Kv1.2 ion current suppression (Hattan et al., 2002; Williams et al., 2007). Another type of phosphorylation of Kv1.2, on serine residues, has been shown to also affect Kv1.2 trafficking (J.W. Yang et al., 2007; Connors et al., 2008). Specifically, it was shown that decreasing levels of the second messenger cyclic adenosine monophosphate (cAMP) would increase Kv1.2 surface expression while increasing levels of cAMP would decrease Kv1.2 surface expression (Connors et al., 2008). This pathway has been determined to occur primarily through protein kinase A (PKA), a common protein kinase dependent on cAMP. It was also shown that increasing levels of adenylyl cyclase (AC), an enzyme that generates cAMP from ATP, also increased Kv1.2 surface expression (Connors et al., 2008).

Another type of PTM, ubiquitination has been recently identified as the next most common PTM after phosphorylation (W. Kim et al., 2011; Chen & Sun 2009; Komander,

2009). Similar to phosphorylation, ubiquitination involves attachment of ubiquitin to a substrate such as lysine. Also similarly, to phosphorylation, ubiquitination is a reversible process (Clague, Coulson, & Urbe, 2012). However, unlike phosphorylation, ubiquitination can involve the addition of just one ubiquitin to a substrate, known as monoubiquitination, or it can involve the addition of a chain of ubiquitin known as polyubiquitination. Ubiquitination, like phosphorylation, can have varying effects on Kv1.2 surface expression. Our lab has recently shown that monoubiquitination is important for non-degradative trafficking of Kv1.2 to the cell surface (Cilento et al., 2015). For example, monoubiquitin expressed with Kv1.2 in HEK 293 cells had determined that interaction of Kv1.2 with Kvβ2, an auxiliary subunit which interacts with the Kv1.2α subunit (Coleman et al., 1999), had important and differing effects on Kv1.2 surface expression (Cilento et al.; 2015). In addition, several studies have determined that Kvβ2 plays an important role in Kv1.2 forward surface trafficking (Shi et al., 1996; Campomanes et al., 2002; Gu et al., 2003). In one such study, association of Kv1.2 and Kvβ2 in COS1 cells was found to occur early, during biosynthesis in the endoplasmic reticulum (ER) (Shi et al., 1996). Furthermore, Kvβ2 was found to increase the efficiency of N-Linked Glycosylation of Kv1.2 in the ER, a type of co-translational modification. Such an association suggests that the Kvβ2 subunit may have a modulatory role in post-translational modifications. In the same study, Kvβ2 co-expression increased the cell surface expression of Kv1.2 in a dose-dependent manner with the amount of co-transfected Kvβ2 cDNA. While virtually all of the cells (>95%) in co-transfected dishes expressed both Kvβ2 and Kv1.2, the percentage of Kv1.2-expressing cells exhibiting surface expression peaked at

only 60%. These findings suggest that while Kvβ2 appears to be important for Kv1.2 forward surface trafficking, Kv1.2 is also regulated by other factors. In one study, monoubiquitin expressed with Kv1.2 in HEK 293 cells decreased surface expression of Kv1.2 as analyzed by flow cytometry, but did not have any effect on Kv1.2 when Kvβ2 was not expressed. In another series of experiments, monoubiquitin was expressed with and without Kvβ2 resulting in no effect on Kv1.2 surface expression. When N-termini and C-termini lysines were mutated to arginine in a mutant version of Kv1.2 (referred to as Kv1.2-KR), opposing effects on Kv1.2 regulation were found. These results suggested that lysines are important for Kv1.2 regulation, and henceforth indicated a possible importance for monoubiquitination in regulating Kv1.2 surface expression.

Such PTMs have important roles in trafficking; In particular, early experiments done in Xenopus oocytes and mammalian cell lines suggested that Kv1.2 ionic current can be reduced by activation of Gq protein couple M1 muscarinic receptor activation (Huang, Morielli, et al., 1993; Tsai, Morielli, et al., 1997). In addition, stimulation of the endothelin G-protein coupled receptor (GPCR) in Xenopus oocytes can inhibit Kv1.2 function (Murakoshi et al., 1994) as does the stimulation of the beta-2 adrenergic receptor, which couples to Gs (Huang, Morielli, et al., 1994). Furthermore, over-expression of a GPCR effector, RhoA, was shown to suppress Kv1.2 current while inhibiting RhoA blocked M1 receptor mediated suppression of Kv1.2 current (Cachero, Morielli, et al., 1998). Stemming from these experiments, it has been shown through the use of the human embryonic kidney (HEK293) cell line that GPCR activation can change Kv1.2 function by regulating its trafficking. In particular, it was shown that Gq coupled M1 receptor stimulation resulted in

endocytosis of the channel from the plasma membrane (Nesti et al., 2004; William et al., 2007) as well as Rhoa stimulation (Stirling et al., 2009). Several studies have highlighted the possible importance of Kv1.2 regulation in the mammalian brain; in one such study, Kv1.2 mRNA in the hippocampus was significantly reduced after a chemical or electrically induced seizure (Tsaur et al., 1992; Pei et al., 1997). In addition, Kv1.2 levels are elevated in the brain after ischemia (Chung et al., 2001); further experiments have shown that Kv1.2 undergoes PTM after such ischemia (Qiu et al., 2003). In pathological diseases such as amyotrophic lateral sclerosis and diabetes, Kv1.2 levels are significantly altered (Shibuya et al., 2011; Zenker et a l., 2012).

Aplysia, Classical Conditioning, Ion Channels and Neural Plasticity

Through regulation of ion channel activation or trafficking, cell excitability can be regulated. Before we introduce the molecular basis behind such plasticity, it is important to understand and detail the types of behavior that have been studied in regards to basic plasticity. While early work on behavior involved the simple instances of habituation and sensitization, these types of learning are considered non-associative as the individual learns about the properties of only a single type of stimulus. Classical conditioning is a complex form of learning in which an organism associates at least two stimuli. Classical conditioning was first delineated by Ivan Pavlov while studying the digestive reflex of dogs. In his early work, Pavlov noted that a dog started to salivate at the sight of an attendant who had fed the dog in the past. This salivation was triggered by an apparently neutral stimulus, the attendant (Douglas et al., 2009).

In this example, the attendant was the conditioned stimulus (CS) and the attendant was associated/paired with the dog's food, the unconditioned stimulus (US). The dog's salivation to the food is the unconditioned response (UR). After repeated pairing, the dogs had learned to associate that the attendant, the CS, would present them with food, the US, and began to salivate in response to the attendant. This salivation in response to the CS is known as the conditioned response (CR) and is a learned response. Since Pavlov's dogs, classical conditioning has been used as the simplest and clearest example of the rules we learn to associate two events. Two fundamental rules that must be met for this conditioning to occur. The first rule, temporal contiguity, is when the organism learns that one event, the CS, preceded by some critical interval of time, a reinforcing event, the US (Douglas et al., 2009). The second rule, contingency, requires that the organism learns that the CS predicts the occurrence of the US; this rule specifically requires that the CS provide novel information about the US, of which more will be discussed about later in this introduction (Kim et al., 1998). In other words, the organism must be able to recognize a predictive relationship between events within their environment.

One of the most primitive organisms that has been studied in regards to classical conditioning is the sea slug, Aplysia. In this case, a mild touch or weak electric shock to the siphon of the slug is the CS and stronger electric current applied to the tail is the US (Carew, Kandel, et al., 1983). When the two are paired for 10 trials, the mild stimulation of the siphon elicits a measurably strong withdrawal of both the gill and the siphon. This withdrawal is significantly larger than if the two stimuli are presented in an unpaired or random fashion during training. Knowing this, the question is, how do Aplysia elicit a

larger spurt of neurotransmitter that elicits the large withdrawal when the timed stimuli are learned? The components to this involve presynaptic and postsynaptic plasticity.

The presynaptic component of this regulation can occur through numerous mechanisms, the most common requiring a protein kinase. A protein kinase transfers a phosphate group to a protein substrate and is responsible for coordinating nearly every cell process. To do so, a protein kinase can deliver a phosphoryl group from ATP to the hydroxyl group oxygen on a serine, threonine, or tyrosine side chain of a protein. Counter-acting a kinase, a phosphatase can rapidly reverse this reaction by removing the phosphate group and can regulate the homeostasis of a protein's phosphorylation state (Cheng et al, 2011). One common protein kinase PKA, comes from a family of enzymes dependent on cyclic AMP (cAMP), a second messenger.

To understand PKA in light of ion channel regulation, we have to go back to Ca2+ signaling as discussed previously in this manuscript. As previously discussed, each action potential leads to an influx of Ca2+ into the presynaptic terminals which led to neurotransmitter release across the synapse. However, in addition to acting directly on neurotransmitters, Ca2+ flows into the presynaptic neuron and binds to a protein known as calmodulin. Calmodulin in-turn binds to AC, an enzyme that generates cAMP from ATP; the way it does so involves a metabotropic GPCR pathway; in such a pathway in Aplysia, GPCRs are activated by serotonin; their activation triggers a cascade of events necessary for chemical signaling to occur (Rahman et al., 2013). This activation stimulates the Gs alpha subunit of the G protein complex to exchange GDP for GTP; the activated Gs alpha subunit then binds to and activates AC, which as discussed before, generates cAMP and

thus activates PKA. Increased cAMP levels then activate PKA, with four cAMP molecules required to activate a single PKA enzyme; and thus the activation of PKA results in more neurotransmitter release.

In other words, pre-synaptically, the neuron is activated by the CS and fires an action potential just before the US. Ca2+ influx induces the cascade described above which in turn results in making the enzyme, PKA, more readably activated by the serotonin released by the US. In response, more cAMP is generated during classical conditioning than during sensitization, in which no preceding activity occurs.

Post-synaptically, the cell, in addition to its own synaptic plasticity, can signal the presynaptic cell. This occurs through the release of glutamate which activate two types of ionotropic receptors, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) type, and the N-methyl-D-aspartic acid (NMDA) type, glutamate receptors. During synaptic transmission as well as habituation and sensitization in Aplysia, only AMPA receptors are activated by glutamate as NMDA receptors are blocked by Mg2+ ions (Kandel, 2012). However, during CS and US pairing, reduced electrical potential of the cell membrane results in the removal of the Mg2+ from the NMDA receptor and as a result Ca2+ rushes into the postsynaptic cell and acts like a second messenger, activating many molecular downstream steps. This Ca2+ release results in the production of a retrograde signal that feeds back to the presynaptic cells, indicating to them to release even more neurotransmitter.

Another enzyme known as protein kinase C (PKC) is a common kinase involved in modulation of synaptic plasticity and maintenance of long-term memory. Its activation

involves the activation of GPCRs; primarily, the activation of GPCRs activate an enzyme, phospholipase C (PLC). PLC in turn from PIP2 forms IP3 and diacylglycerol (DAG), two common second messengers. DAG recruits PKC while IP3 binds IP3 receptors in the endoplasmic reticulum (ER). This IP3 receptor is a Ca2+ channel that releases Ca2+ from the ER which in turn bind PKC and thus activate it (Kandel, 2012). PKC has been associated with regulating neural plasticity involving AMPA and NMDA receptors; AMPA receptors are the most studied and established in the field of neural plasticity and trafficking of such receptors have been implicated in both hippocampal and cerebellar plasticity among other neural structures. Most work involving PKC regulation of AMPA and NMDA receptors have been in the hippocampus involving long-term potentiation (LTP) and long-term depression (LTD) (Malinow, Schulman, Tsien, 1989). Similar to how AMPA and NMDA receptors function in Aplysia, in mammalian hippocampal models, NMDA channels are activated when the Mg2+ block is expelled after high frequency (tetanus) firing of the presynaptic neuron during LTP. Ca2+ rushing into the cell activates several protein kinases in the postsynaptic cell, specifically, calcium-calmodulin-dependent protein kinase II (CaM kinase II), PKC, and the tyrosine kinase, fyn. In the case of CaM kinase II, it can phosphorylate the non-NMDA receptor in the postsynaptic cell which in turn enhances the ability of the receptor respond to glutamate release by the presynaptic neuron (Chang, Mukherji, and Soderling, 1998). Furthermore, it can influence the subsynaptic localization of AMPA receptors and even results in new AMPA receptors being delivered to the synaptic membrane of the postsynaptic cell (Malinow & Malenka, 2002). As previously discussed, a retrograde signal such as nitric oxide (NO) can diffuse

back across the synaptic cleft and enhance presynaptic neurotransmitter release to strengthen the synaptic firing.

This mechanism of synaptic plasticity that strengthens the synapse through enhanced neurotransmitter release and AMPA receptor insertion is known as Early-LTP. After learning has consolidated at this synapse, Late-LTP takes place; in such an example, repeated stimulation activate dopaminergic modulatory inputs which release dopamine on GPCRs, resulting in further cAMP production and PKA activation. In this example, PKA can translocate to the nucleus of the postsynaptic cell to phosphorylate CREB, which in turn activates targets for gene growth that lead to entirely new synaptic growth for long-term consolidation of learning (Abel et al., 1997).

Kv1.2 Regulation in the Cerebellum

Kv1.2 presynaptic localization has been shown to occur in cerebellar inhibitory interneuron basket cell (BC) axon terminals known as pinceaus (McNamara et al., 1993). As seen in Figure 1.1, basket cells form a GABAergic synapse around the soma and

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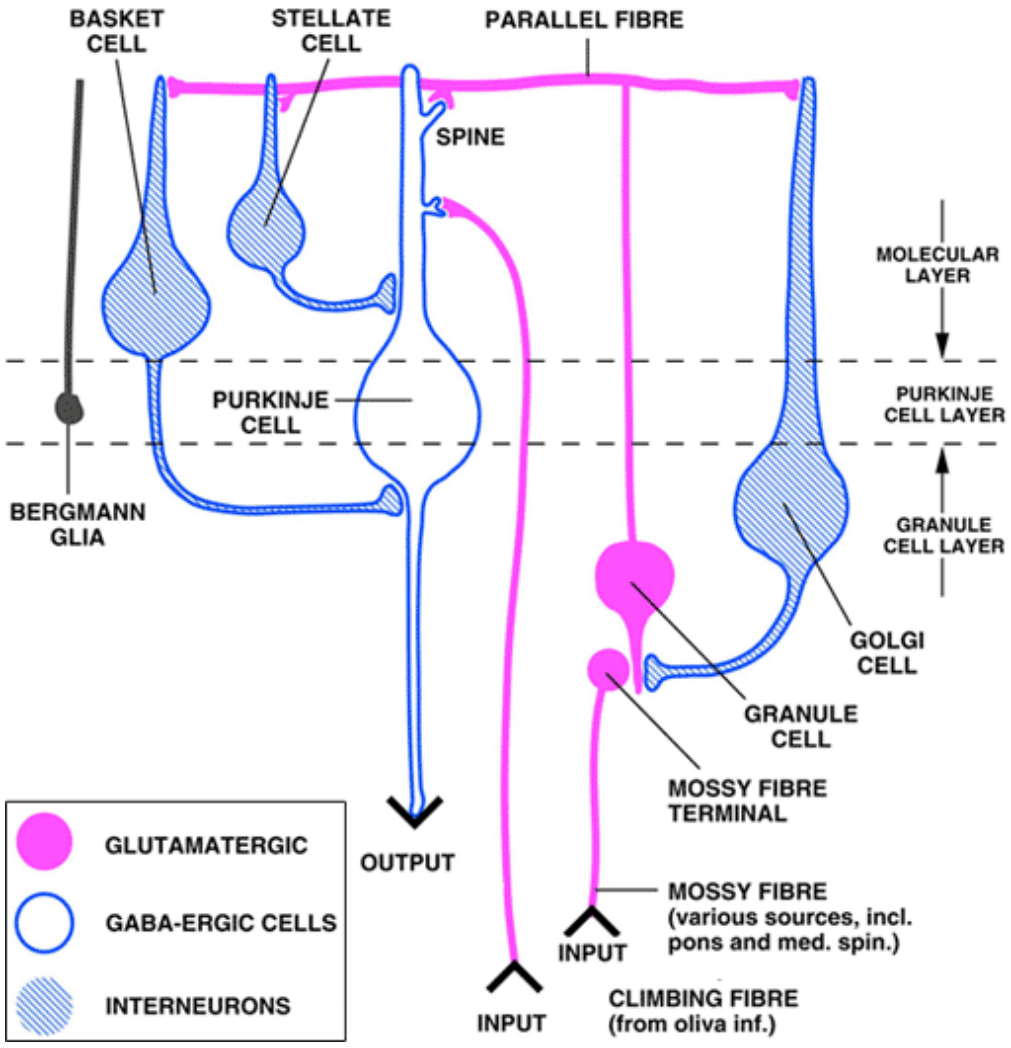


Figure 1.1. Cerebellar Cortex Model.

initial axon segment of PCs, which are the sole output of the cerebellar cortex (Napper et al., 1988; Harvey et al., 1991). In addition to Kv1.2, Kv1.1 is also found in BC pinceaus, possibly as a heterotetramer with Kv1.2; however, the expression of K1.2 is much more significant. (Chung et al., 2005; Chung, Shin et al. 2001). In fact, a quantitative study using autoradiography reported that Kv1.2 on BC axon terminals represents roughly 50% of cerebellar Kv1.2, with the remaining expressed throughout the molecular layer (Koch et al., 1997). In particular, the heteromultimers of Kv1.2 and Kv1.1 form 80% of the cerebellar Kv1.2, while the other 20% are Kv1.2 homomultimers (Koch et al., 1997). In BC axon terminals, Kv1.2 is found where axon collaterals branch and coalesce with axon collaterals from other basket cells, known as septate-like junctions (Laube et al., 1996). In addition to the BC pinceaus, Kv1.2 is found in PC dendrites (Khavandgar et al., 2005) and is sparsely found in deep cerebellar nuclei, but not in the granule cell layer (McNamara et al., 1996; Chung et al., 2001).

The role of Kv1.2 in basket cell axon terminals has been best shown using a toxin inhibitor of Kv1.2 and Kv1.1, alpha-dendrotoxin (alpha-DTX); in such experiments, application of alpha-DTX increases the frequency of inhibitory post-synaptic currents (IPSC's) recorded in PC cells (Southan & Robertson, 1998; Tan & Llano, 1999). Evidence that these IPSC's in PCs originate by a presynaptic mechanism is given by the lack of alpha-DTX sensitive channels in the PC soma; furthermore, alpha-DTX sensitive channels are not found in BC soma, but only in BC axon terminals (Southan & Robertson, 2000). If Kv1.2 is blocked in the BC axon terminals, it is possible that such a blockage increases the frequency of GABA release of BC pre-synaptically; such a mechanism could occur through

control of calcium influx by altering axon terminal excitability or through a decrease of synaptic failures (Southan & Robertson, 1998; Tan & Llano, 1999). This second possible mechanism has strong support; in an experiment involving mutant mice with reduced function of Kv1.1, IPSC frequency was found to increase in PC's without a change in BC firing nor a change in mini IPSCs (Herson et al., 2003). In another experiment involving Kv1.1 knockout mice, PC IPSC frequency increased also without a change in BC firing rate, IPSC amplitude, nor mini IPSCs (Zhang et al., 1999). Furthermore, application of alpha-DTX increased PC IPSC frequency similar to the Kv1.1 knockout and did not alter BC firing rate (Zhang et al., 1999). Given that Kv1.2 inhibition does not affect BC firing, but presence of tetrodotoxin prevents alpha-DTX mediated enhancement of IPSCs in PCs, it was deduced that the function of Kv1.2 in BC pinceaus is likely to increase the failure rate at the axon branch points where these channels are found in high density (Southan & Robertson, 1998).

While 50% of Kv1.2 is present on BC axon terminals, the remaining 50% is expressed throughout the molecular layer (Koch et al., 1997). The molecular layer is primarily composed of parallel fibers (PFs) and climbing fibers (CFs) which both synapse on PC dendrites (Figure 1.1). When CFs become active, PCs display a complex spike that is indicative of calcium (Ca^{2+}) influx. Furthermore, CFs have been shown to reduce excitatory post-synaptic potentials (EPSPs) in the cerebellum following a tetanus of stimulation (Eccles, Llinas, Sasaki, & Voorhoeve, 1966). Initially, CF stimulation increased EPSPs, which declined after repeated stimulation, indicative of longer-term changes taking place (Eccles et al., 1966; Ito et al., 1982). Long-term depression (LTD) is

known to occur when parallel fibers (PFs) and climbing fibers (CFs) are co-stimulated and results in reduction of postsynaptic AMPA receptors (Ito et al., 1982; Crepel & Krupa, 1988; Linden & Connor, 1991; Linden et al., 1991). This process occurs when the PFs and CFs release glutamate on Purkinje cell (PC) dendrites, and is a well-documented form of synaptic plasticity. This glutamate release in turn down-regulates AMPA receptors, and thus reduces excitability of the PC dendrites from PF inputs. Specifically, it has been shown that LTD requires both the CF and PF to stimulate the PC dendrites; CFs or PFs stimulated in isolation stimulation had little effect on PC excitability (Kano & Kato, 1988).

Induction of cerebellar LTD has been shown to rely on calcium influx as a signaling molecule (Linden et al., 1991). Activation of L-type voltage-gated calcium channels (VGCCs), T-type and P/Q-type VGCCs after dendritic depolarization, and internal cascades such as IP3 mediated calcium release, all serve to increase PC dendrite intercellular concentration (Liljelund et al., 2000; Womack et al., 2004; Womack & Khodakhah, 2004; Ross et al., 2005). This IP3 mediated calcium release is activated through Group I glutamergic activation of metabotropic glutamate receptors (mGluRs) (Finch & Augustine, 1998; Takechi et al., 1998). It has been suggested that mGluRs may have a role in LTD induction (Aiba et al., 1994; Kishimoto et al., 2002; Linden et al., 1991; Miyata et al., 2001; Ohtani et al., 2014). MGlurRs are post-synaptic targets for glutamate for PFs and CFs and knocking down mGluRs led to deficiencies in ataxic movements as well as impaired LTD and cerebellar learning (Aiba et al., 1994; Kishimoto et al., 2002). The LTD-dependent decrease of AMPA receptors was found to occur through a mechanism of endocytosis, and it was suggested that activation of PKC, through mGluR

activation, may induce phosphorylation of AMPA receptors to induce endocytosis (Wang & Linden, 2000; Xia et al., 2000). Specifically, AMPA receptors which contain GluR,2/3 subunits have been suggested to be phosphorylated on serine residue 880 by PKC, targeting them for internalization (Schonewille et al., 2011).

Yet, despite the potential role of LTD in cerebellar learning, several studies have raised questions regarding whether it is the only mechanism. For example, pharmacological inhibition of PF-PC LTD with T-588 in rats found no impairment in cerebellar learning (Welsh et al., 2005). Furthermore, mice with GluR2 mutants blocking AMPA receptor internalization targeting PF-PC expression found no impairment in cerebellar learning (Schonewille et al., 2011). These data amongst others have weakened the case of cerebellar LTD as the only mechanism behind cerebellar learning and suggest an alternative mechanism might also be occurring (Hesslow et al., 2013). One of these pathways that our lab has studied is Kv1.2

To delineate the role of Kv1.2 in cerebellar function, tityustoxin (TsTx) has been used in several experiments. TsTx- Kα has been shown to be sensitive to Kv1.2 but not other Kv channel homomers, and its selective action on voltage-gated noninactivating K+ current is also described in hippocampal and cerebellar neurons (Eccles et al., 1994; Hopkins, 1998; Rodrigues et al., 2003). Pharmacological inhibition of Kv1.2 in cerebellar PC dendrites increases excitability (Khavandgar et al., 2005), while its inhibition in BC axon terminals increases inhibition to PCs (Southan & Robertson, 1998). These seemingly opposing effects may provide for a regulatory mechanism of learning at two key inputs to

the PC. While BCs provide inhibitory output onto the PC, the PC dendrites receive excitatory input from parallel fibers (PF). However facilitation at the PC dendrites by PFs and climbing fibers (CFs) enhances long-term depression (LTD); thus Kv1.2 inhibition in PC dendrites, by modulation of membrane excitability and thus calcium influx, may in fact also inhibit the PC through the induction of LTD.

Eye-Blink Conditioning

Direct infusion of TsTx in the lobolus simplex of the cerebellum can enhance the acquisition of delay eye-blink conditioning (EBC) in rats, a form of cerebellar learning (Williams et al., 2012). EBC, a type of classical conditioning, is an established mammalian model for cerebellar-dependent learning and memory (Ito et al., 2002). In the simplest form of EBC, an auditory stimulus, the conditioned stimulus (CS), is paired with a mild stimulus to the eye, the unconditioned stimulus (US). Learning is expressed when the rat blinks to the auditory stimulus, the conditioned response (CR).

EBC is a powerful experimental paradigm for mammalian learning as the neural circuit is well delineated and understood. The tone (CS) is processed through the pons, which activates mossy fibers that in turn activate PFs, which synapse on PC dendrites (Figure 1.2). The shock (US) is processed through the inferior olive (IO), which activates

CFs, which also synapse on PC dendrites. PC output in this region typically inhibits one of the deep cerebellar nuclei, the interpositus nucleus (IN), and thus LTD at PF-PC synapses subsequently leads to disinhibition of the IN. The IN is the sole output of cerebellum that carries information about the eye blink CR and lesions of the IN of 1 cubic millimeter abolishes all previously learned and future CR responses (Gluck et al., 2007).

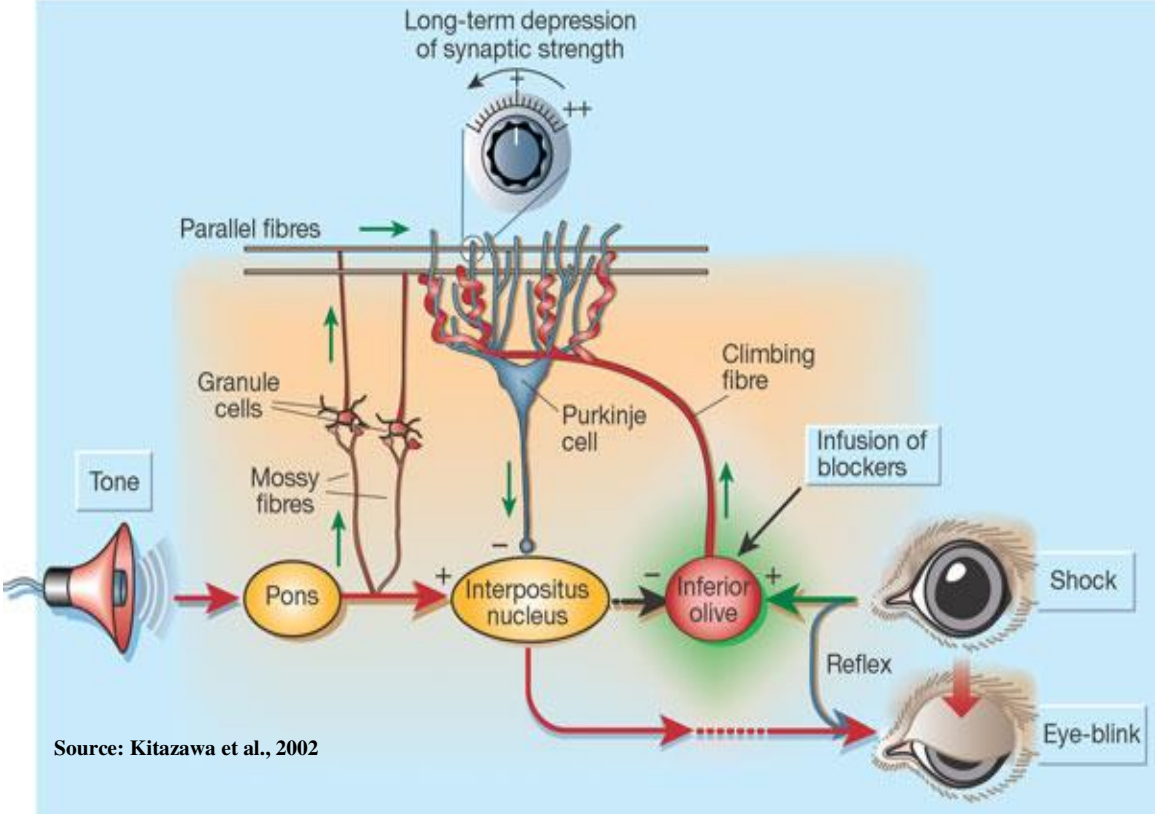


Figure 1.2. Model of eyeblink conditioning.

Fine-tuning of this circuit at PC dendrites regulates learning, as the PC through the IN regulates the learned eye-blink. LTD is a well-documented form of synaptic

plasticity that occurs at PC dendrites, when simultaneous input from PFs and CFs release glutamate on PC dendrites. This glutamate release in turn down-regulates AMPA receptors, and thus reduces excitability of the PC dendrites from PF inputs. LTD at these synapses disinhibits the IN and leads to learning in an EBC paradigm. In addition, the pons can also directly activate the IN, which can directly inhibit the IO, thus modulating this circuit through a feedback loop (Sears & Steinmetz, 1991). This can occur when CS input to the IN is strengthened through new connections between pontine nuclei and IN neurons, and thus results in CR expression through downstream activation of the red nucleus and subsequently the facial nucleus. Feedback inhibition of the IN to the IO limits the strength of the incoming US input to the PCs and the IN, and thus acts as an error correction signal, in which the US is no longer necessary when the system has learned the CS predicts the US.

To understand what is occurring on a molecular level during CS-US conditioning, we must go back to the early theoretical models that dealt with this paradigm. In 1969 Allen Wagner theorized that incremental effects of a behavior are assumed to be linear functions of the composite signal resulting from all stimuli present on that trial, something he referred to as a the “signal cue” (Wagner, 1969 a, b). In 1968 Leon Kamin further expended upon Wagner’s initial model by accounting for what he called a blocking effect, when the relationship of the information provided by the CS and US are essential for conditioning to occur. In his argument, Kamin suggested that conditioning only occurs when the US is a surprise to the organism (Kamin, 1968). This

surprise is reduced if that US is preceded by a CS previously paired with it. In other words, it is reasonable to conclude from this that an organism will only learn when events violate their expectations (Rescorla & Wagner, 1972). Evidence for this was found such experiments that tested blocking in which a CS is paired with US while a secondary CS is further compound conditioned with that same US. In this experiment, the secondary CS was found to not in of itself elicit the US, but it does when the first CS was no conditioned. In other words, the first CS "blocks" any conditioning from occurring in the second CS unless the second CS provided new information about the US, preventing redundancy of learning. Eyeblink conditioning in itself is an experimental paradigm that can explain how the neural network mediates blocking; for example, when the IO was prevented from being inhibited in rabbit cerebellums, blocking did not occur (Kim et al., 1968). This suggests that during conditioning, plasticity that inhibits the IO (presumably through the IN), prevents further conditioning from occurring, unless that secondary CS provides novel information about the US.

In a model now known as the Rescorla-Wagner model of behavioral pavlovian learning, Robert Rescorla pointed to the importance of CS-US correlations in Pavlovian fear conditioning (Rescorla, 1969). In developing this model, Rescorla employed a conditional emotional response model, a type of classical conditioning involving a fear-inducing stimulus. In this experiment, rats received brief electric shocks randomly dispersed, the US; furthermore, a tone, the CS, was presented irregularly without regard to the occurrence of the shocks. In this example, the US may occur in both the presence

and absence of the CS, and there is no correlation between the two; hence the tone and shock would be considered "unpaired" and a "truly random" control (Rescorla, 1968). In addition, Rescorla ran a second group in which the shocks and tones always were paired. In this experiment, the paired, but not the unpaired, groups of rats showed fear of the CS. Several conclusions were made from this experiment; first, the experiment suggested that a correlation between the CS-US can be formed during "paired" conditioning. In other words, the rats could reasonably learn that the two events are positively correlated and the probability of the US occurring in the presence of the CS is higher than in its absence. Second, when the probability of the US is higher in the absence of the CS, as in the unpaired group, these two events can be considered negatively correlated. In other words, these group of rats had undergone a form of behavioral inhibition, which in of itself is still a type of learning, but a negative correlation rather than a positive correlation. In further experiments, Rescorla provided evidence that such probabilities are important for conditioning to occur (Rescorla, 1969).

Knowing this about pavlovian conditioning, and knowing that Kv1.2 is involved in this cirtcuitry, what mechanisms might be occuring on a molecular level to regulate this behavior? Since TsTx can enhance EBC, it therefore predicts that Kv1.2's modulation in cerebellar basket cell terminals may also affect the output of PCs. Yet, given that Kv1.2 may be increasing failure rate at the BC axon terminals, how does such a model fit in a system of feedback regulation that might involve learning? In this example, one proposed mechanism at the BC-PC synapse is known as depolarization

induced potentiation of inhibition (DPI) (Diana & Marty, 2003). Here, PC depolarization might release retrograde factors that diffuse back to enhance GABA release pre-synaptically. While still debated, glutamate is a proposed model for that retrograde factor. In this case, glutamate released from the depolarized PC soma may reach presynaptic BC AMPA and NMDA receptors, which in turn would increase calcium release to enhance neurotransmitter release (Duguid & Smart, 2004). Despite this model, the physiological evidence in mammals for this mechanism is still lacking (Tanimura et al., 2009). However, there is a second retrograde factor believed to also be released from the PC that could regulate such a mechanism, and that factor is the peptide secretin. Secretin is a hormone that has the highest concentration of binding sites in the cerebellum (Fremeau et al., 1983; Nozaki et al., 2002). In the cerebellum, secretin is expressed in PC while its receptor is in the presynaptic BC and the PC, suggesting it might be a retrograde factor (Yung et al., 2001). Research has shown that the endogenous peptide secretin decreases cell-surface expression of cerebellar Kv1.2 through a process involving AC/PKA-dependent channel endocytosis (Williams et al., 2012). Indeed, infusion of secretin enhances EBC in rats similarly to TsTx (Williams et al., 2012), and infusion of a secretin receptor antagonist, 5-27 secretin, disrupted EBC (Fuchs et al., 2014). Furthermore, studies have shown that secretin increases IPSCs recorded from PCs (Yung et al. 2001; Lee et al. 2005). In addition to modulation of Kv1.2 in BCs, regulation of PC dendritic Kv1.2 may also inhibit the PC through induction of LTD, by modulating membrane excitability and thus calcium influx. This secretin-mediated decrease in Kv1.2 surface expression may lead to depolarization of BCs and facilitation of LTD of PCs, which

results in net inhibition of PC output, thus the similar behavioral effects of secretin and TsTx. In order to better understand this complex learning model, we must delve further into how Kv1.2 may be regulated. As we have discussed, PKA is one mechanism of Kv1.2 regulation, however there is evidence to suggest that Kv1.2 might also be regulated by PKCs, which we have described earlier as being involved in maintenace of longer-term forms of memory.

Protein Kinase C isotypes

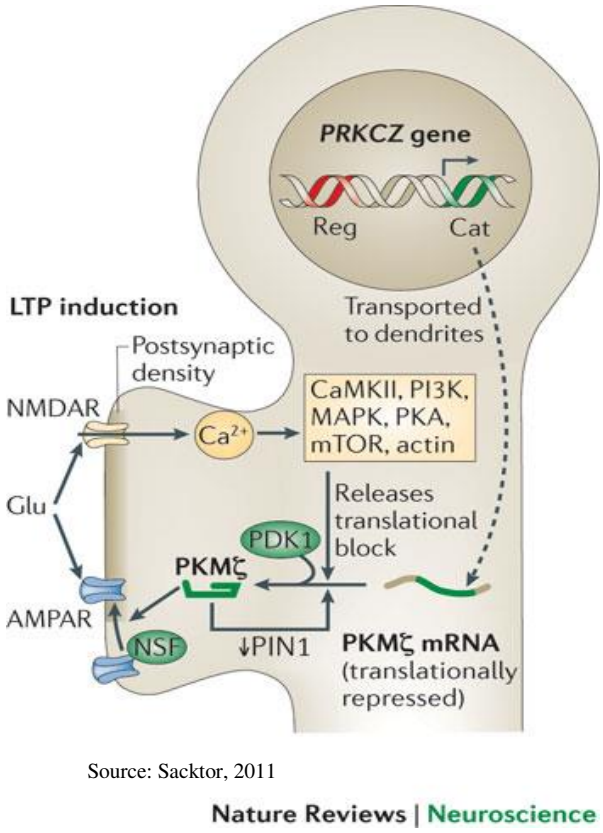
While we have briefly touched upon several mechanisms of PKC induced synaptic plasticity, PKC is a more-complex kinase than initially understood. In mammals, there are at least 10 PKC isotypes encoded by 9 genes and multiple isotypes are expressed in a single cell (Ohno & Nishizuka, 2002). These 10 isotypes are further divided into three classes based on second messenger requirements: conventional PKC isotypes (cPKC), novel PKC isotypes (nPKC), and atypical PKC isotypes (aPKC). All isotypes share a characteristic sequence motif C1 in addition to a serine/threonine protein kinase domain. Amongst the cPKCs, PKC-α, PKC-β1, PKC-β2, and PKC-γ share structural motifs C1 and C2. Amongst nPKCs, PKC-δ, PKC-ε, PKC-η, and PKC-θ share structural motifs C1 and C2. In clear contrast to cPKCs, nPKCs do not require calcium to activate DAG. The last class of PKCs, aPKCs, include PKC-ζ and PKC-λ/ι. These aPKCs share structural motifs C1 and OPR; interestingly, aPKCs lack critical residues required for interaction with DAG and do not bind nor are activated by DAG. The N-terminal of aPKC isotypes contain the

OPR motif and constitute a binding site for PAR-6, and ZIP/p62 also known as Zeta-interacting protein.

Protein Kinase C, Zeta, Kvβ2, and Behavior

Interestingly, two prior studies have demonstrated that the atypical PKC-ζ is able to phosphorylate and bind cerebellar Kvβ2. (Gong et al., 1999; Croci et al., 2003). However, no further studies were undertaken to determine the function of such phosphorylation nor have any phosphorylation sites been identified. PKC-ζ and its N-terminal truncated form, PKM-ζ, have long been implicated in the regulation of hippocampal AMPA receptors (Pastalkova et al 2006). Sacktor and colleagues provided the first evidence that PKM-ζ played an important role in maintaining learning-related synaptic change by showing that interruption of the activity of PKM-ζ disrupted the maintenance of long-term potentiation (LTP), a cellular mechanism that underlies learning and memory (Serrano et al., 2005; Ling et al., 2006). PKM-ζ in the hippocampus has been shown to enhance AMPA receptor trafficking to the synapse (Ling et al., 2002; Yao et al., 2008), which increases synaptic conductivity and is characteristic of LTP (Figure 1.3). Both PKC-ζ and PKM-ζ are highly expressed in the cerebellum (Oster et al., 2004), however nothing is known about their role in this region of the brain. Having been implicated in the regulation of Kv1.2's β subunit, it is possible that PKC-ζ and PKM-ζ may have regulatory effects on Kv1.2, and that this may have implications for cerebellar learning. While on the cellular level PTMs may play an important role in Kvβ2

regulation of Kv1.2, little is known about how regulating Kvβ2 can affect behavior. One study has shown that genetic knockouts of



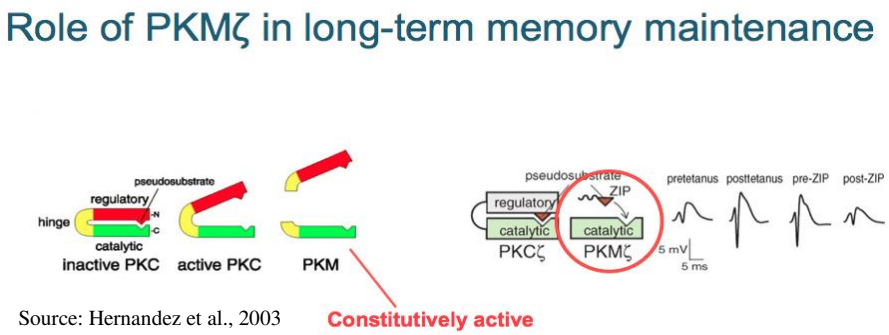
Source: Sacktor, 2011

Figure 1.3. PKM-ζ AMPAR Maintenance Model.

the Kvβ2 gene (KCNAB2) in mice produce deficits in fear conditioning (Perkowski and Murphy, 2011), which like EBC, is a form of associative learning and memory. The study also showed that the knockout mice had a reduction in the slow after-hyperpolarization following a burst of action potentials in the lateral nucleus of the amygdala, suggesting involvement of Kv1.2. While the underlying neurological structures are different, this key study demonstrates the ability of Kvβ2 to impact associative learning and memory,

suggesting that regulation of Kv1.2 may have brain-wide implications for learning and memory.

ZIP, also known as PKC-ζ pseudosubstrate inhibitor (not to be confused with Zeta-interacting protein), effectively inhibits both PKC-ζ and PKM-ζ and subsequently inhibits the late phase or maintenance of LTP in the hippocampus (Figure 1.4; Lee et al., 2013*)*. ZIP has been shown over many studies and in many mammalian models to



Source: Hernandez et al., 2003

Figure 1.4. ZIP and PKM-ζ Structures.

effectively disrupt learning in the hippocampus, even weeks after the learning has occurred (Shema et al., 2007, 2009, 2011; Serrano P et al., 2008; Hardt et al., 2010; Parsons et al., 2011; Gamiz et al., 2011). Studies have also shown that blocking endocytosis of the AMPA receptor subunit, Glutamate Receptor 2 (GluR2), was sufficient to prevent impairments in LTP maintenance caused by ZIP (Migues et al., 2010). Since PKM-ζ in the hippocampus has been shown to maintain surface expression of AMPA receptors, it is possible it also maintains AMPA receptors in the cerebellum.

Therefore, ZIP-mediated inhibition of PKM-ζ may disrupt the maintenance of surface AMPA receptors in PC dendrites, and thus would enhance LTD. This would hyperpolarize the PC and result in enhanced acquisition of EBC. This may provide insight

on PKM-ζ's role in the cerebellum, possibly being involved in preventing cerebellar LTD rather than enhancement of LTP as is shown in the hippocampus. Furthermore, prevention of LTD through maintenance of AMPA receptors may occur in parallel with a change in Kv1.2 surface expression, further depolarizing the PC and disrupting learning through a mechanism dependent on membrane excitability. Therefore, inhibition of PKM-ζ with ZIP, which may regulate Kv1.2 endocytosis, may also in cause a change in cell surface Kv1.2 expression. Given the localization and expression of PKM-ζ in the cerebellum, we are presented with a unique opportunity to investigate whether PKM-ζ is involved in Kv1.2-dependent cerebellar learning.

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CHAPTER 2: INTRA-CEREBELLAR INFUSION OF THE PROTEIN KINASE MZETA (PKMζ) INHIBITOR ZIP DISRUPTS EYEBLINK CLASSICAL CONDITIONING.

Abstract

PKM-ζ, a constitutively active N-terminal truncated form of PKC-ζ, has long been implicated in a cellular correlate of learning, long-term potentiation (LTP). Inhibition of PKM-ζ with Zeta-inhibitory peptide (ZIP) has been shown in many brain structures to disrupt maintenance of AMPA receptors, irreversibly disrupting numerous forms of learning and memory that have been maintained for weeks. Delay eyeblink conditioning (EBC) is an established model for the assessment of cerebellar learning; here, we show that PKC-ζ and PKM-ζ are highly expressed in the cerebellar cortex, with highest expression found in Purkinje cell (PC) nuclei. Despite being highly expressed in the cerebellum, no studies have examined how regulation of cerebellar PKM-ζ may affect cerebellar-dependent learning and memory. Given its disruption of learning in other brain structures, we hypothesized that ZIP would also disrupt delay EBC. We have shown that infusion of ZIP into the lobulus simplex of the rat cerebellar cortex can indeed significantly disrupt delay EBC.

Introduction

Understanding the cellular mechanisms behind learning, and the subsequent formation of memory, has been a topic that has garnered scientific interest for many decades. One particular kinase that has been at the center of scientific attention in the last decade is the serine/threonine kinase PKM-ζ, an N-terminal truncated form of PKC-ζ

that renders it constitutively active (Hernandez et al., 2003). Its role in trafficking AMPA receptors into the postsynaptic membrane is key to formation of long-term potentiation (LTP), a cellular mechanism that underlies learning and memory (Kessels et al., 2009). Sacktor and colleagues provided the first evidence that PKM-ζ has an important role in maintaining learning-related synaptic changes, by showing that interruption of PKM-ζ activity is sufficient to disrupt maintenance of hippocampal LTP (Serrano et al., 2005; Ling et al 2006, Pastalkova et al., 2006).

The role of PKM-ζ in learning has been studied through the use of Zeta inhibitory peptide (ZIP), a selective PKC-ζ pseudosubstrate inhibitor that effectively inhibits both PKC-ζ and PKM-ζ. Primarily, ZIP has been studied in regards to hippocampal-dependent memory in rats; for example, ZIP infusion into dorsal hippocampus after training impaired object location retention (Hardt et al., 2010), place avoidance retention (Pastalkova et al., 2006; Serrano et al., 2008), spatial location retention in the water maze (Serrano et al., 2008), reference memory in the 8-arm radial maze (Serrano et al., 2008) and expression/retrieval of CRs in trace eyeblink conditioning (Madronal et al., 2010). In other brain structures, including medial prefrontal cortex (mPFC), sensorimotor cortex, insular cortex, basolateral amygdala (BLA), nucleus accumbens (NAc), and dorsal striatum, ZIP has also been found to impair memory retention (Crespo et al., 2012; Evuarherhe et al., 2014; Gamiz & Gallo, 2011; He et al., 2011; Kwapis et al., 2009; Li et al., 2011; Migues et al., 2010; Pauli et al., 2012; Shabashov et al., 2012; Shema et al., 2007; Shema et al., 2009; von Kraus et al. 2010).

Unlike other forms of learning that rely on LTP, cerebellar learning is believed to primarily occur through a mechanism of long-term depression (LTD) in which AMPA receptors undergo endocytosis (Linden 1996). While no studies have investigated the role of PKM-ζ in cerebellar learning, measurement of mRNA expression of PKC-ζ and PKM-ζ using *in situ* hybridization has shown that the kinases are highly expressed in the cerebellar cortex (Oster et al., 2004). However, that study did not resolve the expression pattern of PKC-ζ and PKM-ζ with a higher level of spatial resolution at the protein level. Given the complexity of the cerebellar circuitry, we wanted to show where in the cerebellar cortex PKC-ζ and PKM-ζ are expressed. In the current study, we stained parasagittal slices of rat cerebellar cortex with a c-terminal specific α-PKC-ζ and revealed both PKC-ζ and PKM-ζ's high expression pattern throughout the cortex, primarily localized in Purkinje cell (PC) nuclei.

Delay eyeblink conditioning (EBC) is an established model for the assessment of cerebellar learning. As first elucidated by Richard F. Thompson and colleagues in the 1980's, EBC is critically dependent upon one of the deep cerebellar nuclei, the interpositus nucleus (IN), with modulation of learning by the cerebellar cortex (Lincoln et al., 1982; Lavond et al., 1984, 1987; Lavond & Steinmetz, 1989; McCormick et al., 1981; McCormick & Thompson, 1984; Perrett et al., 1993). In EBC, an auditory stimulus, the conditioned stimulus (CS), is paired with a mild stimulation to the eye, the unconditioned stimulus (US). Learning is expressed when the subject blinks to the auditory stimulus, resulting in a conditioned response (CR). In the standard model for

EBC, LTD at PF-PC synapses in the lobulus simplex of the cerebellar cortex leads to disinhibition of the IN (Thompson & Steinmetz, 2009). The IN is the sole output of the cerebellum that carries information about the eyeblink CR (Thompson & Steinmetz, 2009). In the hippocampus, LTD induction in rats has been shown to produce a down-regulation of PKM-ζ that is reversed with high-frequency afferent stimulation, suggesting that LTD may be inversely correlated with PKM-ζ expression (Osten et al., 1996; Hrabetova & Sacktor 1996).

While the established model of cerebellar EBC has been understood to occur through a mechanism of LTD, this model has been recently challenged; surprisingly, disrupting AMPA receptor regulation and cerebellar LTD did not impair EBC (Schonewille et al., 2011). However PF-PC LTD may be one of a variety of plasticity mechanisms that support cerebellar-dependent learning (Gao et al., 2012). Voltage-gated potassium channel 1.2 (Kv1.2) is an ion channel known to regulate neuronal excitability (Khavandgar et al., 2005; Southan & Robertson, 1998). Kv1.2 is most abundantly expressed in cerebellar basket cell (BC) axon terminals (pinceaus) and in PC dendrites (Wang et al., 1993; Laube et al., 1996; Koch et al., 1997; Chung et al., 2001). Indeed, inhibition of Kv1.2 with Tityustoxin-Kα (TsTx) in cerebellar PC dendrites increases PC excitability (Khavandgar et al., 2005), while its inhibition in BC axon terminals increases inhibition of PCs (Southan & Robertson, 1998). Furthermore, we have previously shown that intra-cerebellar infusion of tityustoxin (TsTx), a Kv1.2 inhibitor, enhances EBC (Williams et al., 2012). Two prior studies have demonstrated that PKC-ζ associates with

and is able to phosphorylate cerebellar Kvβ2 (Gong et al., 1999; Croci et al., 2003), an auxiliary subunit that interacts with Kv1.2 (Coleman et al., 1999). Given the high levels of PKM-ζ expression in the cerebellum and its possible interaction with Kv1.2, we hypothesized that ZIP would disrupt cerebellar-dependent learning.

Materials and Methods

Immunohistochemistry

A 4 month old male Wistar rat was euthanized according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Vermont and cerebellar tissue was harvested. Cerebellar tissue underwent fixation with a transcardial perfusion of PBS followed by 4% paraformaldehyde, both chilled to 4°C. 400-μm-thick parasagittal sections were cut on on a vibratome and collected in PBS. Fixed cerebellar slices were briefly acetone extracted and then incubated in blocking buffer (3% normal goat serum in PBS). Slices were then incubated for 24 hours each in primary and secondary antibodies. Primary antibodies were mouse monoclonal α-Kv1.2 (1:200) (Neuromab, Clone K14/16, Cat. #75-008) and rabbit polyclonal α-PKC-ζ C-Terminal (1:50) (Sigma-Aldrich, Cat. #SAB4502380). The secondary antibodies were goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 568 (ThermoFisher Scientific, Cat. #A-11001 and A-11036). Slices were mounted with Prolong Gold anti-fade with DAPI (ThermoFisher Scientific, Cat. #P-36931) and imaged using a Nikon C2+ Confocal. Images were processed with ImageJ software.

EBC Subjects

Male Wistar rats (59-63 days old) were purchased from Harlan (Indianapolis, IN) or Charles River (Quebec, Canada) and housed in pairs upon arrival with access to food and water ad libitum. Rats were single housed after surgery. The colony room was maintained on a 12-h light–dark cycle (lights on at 7:00 a.m. and off at 7:00 p.m.). Rats weighed 300–400 g prior to surgery. All testing took place during the light phase of the schedule and the IACUC of the University of Vermont approved all procedures.

EBC Surgery

Surgeries took place 4–6 d after arrival. Surgeries were performed under aseptic conditions. Rats were anesthetized with 3% isoflurane in oxygen. During the surgery, a 7-mm stainless steel 22-gauge guide cannula (Plastics One) was implanted into the lobulus simplex of the cerebellar cortex (AP: -11.0 from bregma; ML: -3.0; DV: -3.2; infusion at DV: -4.2).

After securing the cannula to the skull with dental cement, an 8-mm dummy cannula was placed in the guide cannula and secured with screw threads. The dummy cannula served to seal the guide cannula to prevent infection and to prevent the guide cannula from becoming obstructed prior to infusions. A bipolar stimulation electrode (Plastics One) was positioned subdermally immediately dorsocaudal to the ipsilateral eye. Two EMG wires for recording activity of the external muscles of the eyelid, the orbicularis oculi, were constructed from two strands of 75-mm Teflon coated stainless steel wire soldered at one end to a round threaded pedestal connector (Plastics One). The

other end of the wire was passed subdermally to penetrate the skin of the upper eyelid of the eye, ipsilateral to the guide cannula. A ground wire was wrapped around two skull screws at one end and the other end was soldered to the pedestal connector. The cannula, pedestal connector, and stimulation bipolar electrode were cemented to the skull with dental cement. The wound was numbed with a local injection of 0.1 mL bupivacaine, spread out over three points around the wound. The wound was salved with antibiotic ointment (Povidone), and an analgesic (buprenorphine) and fluids (lactated Ringers) were administered (s.c.) immediately after surgery. Analgesic was administered twice the following day. Rats were given 5–6 d to recover prior to eyeblink conditioning.

EBC Apparatus

Eyeblink conditioning took place in one of four identical testing boxes (30.5 × 24.1 × 29.2 cm; Med-Associates), each with a grid floor. The top of the box was altered so that a 25-channel tether/commutator could be mounted to it. Each testing box was kept within a separate electrically shielded, sound-attenuating chamber (45.7 × 91.4 × 50.8 cm; BRS-LVE, Laurel, MD). A fan in each sound-attenuating chamber provided background noise of approximately 60 dB sound pressure level. A speaker was mounted in each corner of the rear wall and a light (off during testing) was mounted in the center of the rear wall of each chamber. The sound-attenuating chambers were housed within a walk-in soundproof room.

Stimulus delivery was controlled by a computer running Spike2 software (CED). A 2.8 kHz, 80 dB tone, delivered through the left speaker of the sound-attenuating

chamber, served as the CS. The CS was 365-msec in duration. A 15-msec, 4.0-mA uniphasic periorbital stimulation, delivered from a constant current stimulator (model A365D; World Precision Instruments), served as the US during conditioning. Recording of the eyelid EMG activity was controlled by a computer interfaced with a Power 1401 high-speed data acquisition unit and running Spike2 software (CED). Eyelid EMG signals were amplified (10K) and bandpass filtered (100 – 1000 Hz) prior to being passed to the Power 1401 and from there to the computer running Spike2. Sampling rate was 2 kHz for EMG activity. The Spike2 software was used to full-wave rectify, smooth (10 msec time constant), and time shift (10 msec, to compensate for smoothing) the amplified EMG signal to facilitate behavioral data analysis.

Eyeblink conditioning procedure

At the beginning of each session, each rat was plugged in, via the connectors cemented to its head, to the 25-channel tether/commutator, which carried leads to and from peripheral equipment and allowed the rat to move freely within the testing box. On day one (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 (i.e., 2 sec samples with an average intertrial interval (ITI) of 30 sec and a range of 20 – 40 sec). On days 2 – 7 (conditioning), rats received 100 trials per day, with an average ITI 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 hours prior to the first session of conditioning, rats received an intra-cerebellar infusion of 0.50 μL of 20 mM myristoylated-ZIP (AnaSpec, Cat. #AS-63361), 20 mM myristoylated-scrambled-ZIP (AnaSpec, Cat. #AS-63695), 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, making the final infusion depth 4.2 mm below bregma. Infusions were made with a 10-mL Hamilton syringe loaded onto an infusion pump (KD Scientific) set to deliver 0.5 μL of solution over 2 min. At the end of the infusion period, the internal cannula remained in place an additional 1 min to allow diffusion of the infused solution away from the cannula tip. Subsequently, the internal cannula was removed, the dummy cannula was replaced, and the rats were placed back in their home cage for 2 hours. Thereafter the rats were plugged in, and the EBC session began. Rats were infused and tested in groups of four.

EBC Histology

Within approximately 24 h after the final session, rats were overdosed with sodium pentobarbital (150 mg/kg) and transcardially perfused with 0.9% saline followed by 10% buffered formalin. A small DC electrolytic lesion (100 μA, approximately 10 sec) was made by passing current through an electrode made from a 000 gauge insect pin insulated (except for 0.5 mm on the tip) with nail polish, that was placed into the guide

cannula, with the tip extending out of the guide cannula by approximately 1.0 mm. The brain was removed and stored in 10% buffered formalin. Four or five days prior to sectioning, the brain was transferred to a 30% sucrose/10% buffered formalin solution. Before sectioning, cerebella were embedded in albumin–gelatin. Frozen sections of the cerebellum were taken at 60 μm. Tissue was mounted on gelatin-coated glass slides, stained with Prussian blue (for iron deposits left by the marking lesions) and cresyl violet (for cell bodies) and cover slipped with Permount. Slides were examined under a microscope by an observer blind to group membership to confirm cannula placement.

Behavior analysis

For the conditioning sessions, CS–US trials were subdivided into four time periods: (1) a "baseline" period, 280 msec prior to CS onset; (2) a nonassociative "startle" period, 0 – 80 msec after CS onset; (3) a "CR" period, 81–350 msec after CS onset; and (4) a "UR period," 65–165 msec after US onset (the first 65 msec is obscured by the stimulation artifact). On CS-alone trials, the "CR" period extended for 150 msec after CS offset to capture CRs that may normally be masked by the US. In order for a response to be scored as a CR, eyeblinks had to exceed the mean baseline activity for that trial by 0.5 arbitrary units (where these units had a range of 0.0 – 5.0) during the CR period. Eyeblinks that met this threshold during the startle period were scored as startle responses and were analyzed separately. Trials in which eyeblinks exceeded 1.0 arbitrary unit during the baseline period were discarded. Comparable scoring intervals and criteria were used to evaluate spontaneous blink rate during the initial adaptation day when no

stimuli were administered. The primary dependent measure for all experiments was the percentage of CRs across all CS – US (acquisition) trials of each session.

Data were analyzed using repeated-measures ANOVAs and post-hoc analyses consisted of independent-samples t-tests. We computed all statistical analyses using SPSS 23.0. A p-value of 0.05 was set as the rejection criterion for all statistical tests.

Results

Identification of PKC-ζ/PKM-ζ expression in rat cerebellar cortex

In order to determine the expression of PKC-ζ and PKM-ζ in the rat cerebellar cortex, we used immunohistochemistry in parasagittal cerebellar sections. Using a Nikon C2+ Confocal, we identified dense expression of PKC-ζ/ PKM-ζ (red) throughout the cerebellar cortex in the molecular (ML) and granular layers (GL), as well as the pinceaus of BC axon terminals as shown in Figure 1. The highest expression was found in the nuclei of PCs as well as throughout the ML. We used Kv1.2 (green) as a marker for BC axon terminals, which form "pinceaus" around the PC soma. No expression of PKC-ζ/ PKM-ζ was identified in the deep cerebellar nuclei.

PKM-ζ function in cerebellar cortex affects cerebellar-dependent learning in rats

In order to determine the role of endogenous PKM-ζ in cerebellar-dependent learning, we infused rats with the selective PKM-ζ pseudosubstrate inhibitor, myristoylated-ZIP, (0.50 μL; 20 nmol/μl; Anaspec), myristoylated-scrambled-ZIP, (0.50 μL; 20 nmol/μl; Anaspec) or vehicle phosphate-buffered saline (0.50 μL). The day before

infusion, rats were placed in experimental chambers and received 100 no-stimulus trials to measure spontaneous eyeblink activity. Infusions were made into the lobulus simplex of the cerebellar cortex ipsilateral to the conditioned eye two hours prior to conditioning session 1 of six acquisition sessions of EBC. The six conditioning sessions consisted of 80 CS–US paired trials (350-msec delay paradigm), and 10 CS-alone trials and 10 US-alone trials evenly spaced within the CS–US paired trials, so that every fifth trial alternated between a CS-alone and a US-alone trial.

Prior to analysis, we verified all cannula placements to ensure placement was in the lobulus simplex of the cerebellum ipsilateral to the conditioned eye. A total of 31 rats (13 ZIP; 10 Scr-ZIP, 8 Veh) were included in the analysis. Three rats were removed prior to data analysis due to poor electromyographic (EMG) signals (n=2) or illness (n=1). None were removed because of incorrect cannula placement.

As shown in Figure 2, the results suggest that while all 3 groups learned, the vehicle-infused group exhibited significantly more eyeblink CRs than the ZIP-infused group. A 3 (group: ZIP; Scr-ZIP; Veh) by 6 (session) repeated-measures ANOVA on the percentage of CRs confirmed this. A main effect of session ($F(5,140) = 36.30$, $p < 0.05$) and a group x session interaction ($F(10,140) = 1.99$, $p < 0.05$) were revealed. The main effect of group approached, but did not attain, statistical significance ($p = 0.067$). Post-hoc independent-samples t-tests of the significant interaction revealed that the percentage of CRs in Group ZIP were significantly lower than Group Veh in sessions 2 ($p = 0.008$), 3 ($p = 0.016$), 4 ($p = 0.042$), and 6 ($p = 0.016$). The same analyses comparing Group Scr-

ZIP to Group Veh revealed that the percentage of CRs did not differ significantly (p's > 0.08); furthermore, Group Scr-ZIP showed a greater percentage of CRs than Group ZIP in sessions 5 ($p = 0.020$) and 6 ($p = 0.021$).

To confirm that intra-cerebellar infusions of ZIP or Scr-ZIP prior to acquisition session 1 did not change the reflexive startle response to the CS, we analyzed the percentage of startle responses to the CS (eyeblinks with an onset latency of 80 msec or less after CS onset). A 3 (group: ZIP; Scr-ZIP; Veh) by 6 (session) repeated-measures ANOVA yielded no significant effects (F's < 1.2, p's > 0.34). To confirm that intra-cerebellar infusions of ZIP or Scr-ZIP prior to acquisition session 1 did not change the reflexive eyeblink to the US, we analyzed the amplitude of URs on US-alone trials. A 3 (group: ZIP; Scr-ZIP; Veh) by 6 (session) repeated-measures ANOVA also yielded no significant effects (F's < 0.9, p's > 0.47). Thus, infusion-related changes in reflexive responding to either the CS or the US are unlikely to explain our results. Finally, intra-cerebellar infusions of ZIP or Scr-ZIP did not affect measures of the learned response itself. This was confirmed for measures of CR topography from CS-US trials (CR amplitude; CR onset latency) and from CS-alone trials (CR peak latency) with a series of 3 (group: ZIP; Scr-ZIP; Veh) by 6 (session) repeated-measures ANOVAs. For these analyses, rats without any CRs in one or more sessions were removed from the analysis, since CR topography values are undefined in these cases. A significant effect of session was revealed for CR amplitude ($F(5,95) = 12.44$, $p < 0.01$) and for CR peak latency ($F(5,90) = 4.81$, $p = 0.001$); the session effect for CR onset latency approached, but did

not attain, statistical significance ($F(5,95) = 2.25$, $p = 0.056$). No other effects were significant (F's < 1.6, p's > 0.23).

Histological analysis

Figure 3 shows approximate locations of confirmed cannula tip placements in the lobulus simplex of cerebellar cortex. Cannula tip placements of rats included in the analyses were confirmed to be within the lobulus simplex of the cerebellar cortex. They were slightly more dorsal than the cannula tip placements reported in rats to be the most effective for impairment of EBC by muscimol infusion (Steinmetz & Freeman, 2014). Importantly, however, they were clearly distant from the deep cerebellar nuclei.

Discussion

We have shown that a single infusion of the PKM-ζ inhibitor, ZIP, into the lobulus simplex of the cerebellar cortex, two hours prior to the first acquisition session, impairs EBC. This is the first demonstration that PKM-ζ may be important for cerebellar-dependent memory, and is consistent with the established literature showing ZIP-mediated impairment of other forms of learning mediated by other brain structures.

In order to study PKM-ζ in rats, we relied on the use of the inhibitor ZIP as previously described. One particular concern with the use of ZIP is its specificity for PKM-ζ; in a mouse knockout line for PRKCZ, the gene for PKC-ζ and PKM-ζ, either constitutively or conditionally, ZIP was reported to still block maintenance of hippocampal LTP and disrupt nucleus accumbens-dependent CPP (Volk et al., 2013; Lee

et al., 2013). This suggests that pathways independent of PKM-ζ and PKC-ζ were involved in memory formation.

One interpretation for these results is that knocking out the gene for PKC-ζ/PKM-ζ causes compensatory, abnormal upregulation, or enhanced phosphorylation, of a closely related PKC isoform, PKC-iota, and that ZIP also blocks the activity of this kinase (Selbie et al., 1993; Lee et al., 2013; Jalil et al., 2015). In addition, others have shown that PKM-ζ conditional knockout mice show disrupted late-phase LTP, and that infusion of PKM-ζ antisense oligonucleotides into the hippocampus could also function similar to ZIP (Tsokas et al., 2012). Late-phase LTP is a protein synthesis-dependent form of LTP that is understood to be a key component of long-term memory (Frey et al., 1988; Abel et al., 1997). Furthermore, it is important to note that PKM-ζ has been shown to play a role in memory using techniques other than ZIP infusion; Sacktor and colleagues have shown that PKM-ζ lentiviral over-expression in rat insular cortex 5 days before acquisition enhances CTA, while lentiviral over-expression of dominant-negative PKM-ζ 5 days before acquisition disrupts CTA (Shema et al., 2011).

While the specificity of ZIP is debated, it remains the most specific inhibitor for PKC-ζ/PKM-ζ. However, another concern with ZIP is its use with a proper control; several studies using Scr-ZIP have shown that it behaves similar to ZIP and could also disrupt both LTP and memory. In one study, Scr-ZIP was found to be equally effective as ZIP at reversing LTP in mouse hippocampal slices (Volk et al., 2013). This same study also found that ZIP and Scr-ZIP can both inhibit purified PKC-ζ and PKM-ζ and had

overlapping inhibition curves. In order to rule out an effect due to the myristoylation group present on both Scr-ZIP and ZIP, Volk and colleagues demonstrated that myr-PKI, a PKA inhibitor, was without effect and not responsible for disrupting LTP maintenance. In another study examining the BLA, rats that received Scr-ZIP infusions into the BLA showed a slight, but non-significant, decrease in freezing behavior compared to the saline controls (Kwapis et al., 2009). The authors suggested that Scr-ZIP may weakly bind and inactivate some PKM-ζ due to its nearly palindromic basic sequence, and recommended use of vehicle as the proper control (Kwapis et al., 2009). Using an in-vitro kinase assay, Lee and colleagues demonstrated that in fact both Scr-ZIP and ZIP inhibit PKM-ζ and PKC-ζ with only a 7.3-fold difference in their K_i values (Lee et al., 2013).

In defense of Scr-ZIP, Yao and colleagues recently demonstrated in hippocampal slices that a 5 μM concentration of Scr-ZIP had no effect on PKM-ζ-mediated potentiation of postsynaptic CA1 pyramidal cell AMPA receptor responses (Yao et al., 2013). However, they did acknowledge and demonstrate that in higher doses, Scr-ZIP inhibited PKM-ζ and therefore may not be a proper control. This poses potential problems with its use in behavioral studies, as the concentration of initial drug infusion must be greater than the expected target dose, in order to account for loss of the drug during diffusion. In a letter to the editor of *Hippocampus*, Sacktor and Fenton addressed the concerns surrounding the high dosage of ZIP, but not Scr-ZIP, in hippocampal infusions, by using immunocytochemistry to show biotin-labeled ZIP diffusion (Sacktor & Fenton 2012). However, it remains to be shown what the final deliverable

concentration of ZIP would be in behavioral infusions, especially in other brain structures. Given that Scr-ZIP can inhibit PKM-ζ, we suggest a cautious approach be taken when Scr-ZIP is used as the only control for ZIP in behavioral infusion studies.

In order to explain our ZIP-mediated disruption of EBC, we suggest three possible models. One well-studied mechanism of EBC involves LTD at PF-PC synapses; cerebellar PKM-ζ may induce endocytosis of these AMPA receptors rather than maintain them on the surface, and thus may maintain LTD as opposed to LTP. It is believed that LTD at PF-PC synapses in the cerebellar lobulus simplex during EBC leads to disinhibition of the IN, the sole output of cerebellum that carries information about the eyeblink CR (Thompson & Steinmetz, 2009). This would suggest that PKM-ζ acts to enhance cerebellar-dependent learning, similar to its function in other brain structures. Therefore infusion of ZIP may prevent LTD facilitation and lead to an increase in PC AMPA receptors at the PC-PF synapse, resulting in disruption of EBC. However, Schonewille and colleagues have also shown that disrupting AMPA receptor regulation and cerebellar LTD does not impair EBC (Schonewille et al., 2011).

While LTD is the most well-studied mechanism of EBC, recent work suggests that LTP may play a more important role in cerebellar learning than previously thought. To study the role of LTP in EBC, Schonewille and colleagues generated mice with knockouts of calcium/calmodulin-activated protein-phosphatase-2B (PP2B) that abolished LTP in PCs and their ability to increase their intrinsic excitability (Schonewille et al., 2010). These mutant mice demonstrated impaired acquisition of delay EBC;

therefore it is possible that PKM-ζ disruption by ZIP may prevent LTP in PCs as it does in other neural structures. A follow-up study found further evidence of cerebellar LTP in mossy fiber-granule cell (MF-GC) synapses, involving activation of a specific subtype of acetylcholine receptors, α7-nAChR (Prestori et al., 2013). This may disrupt GC transmission of CS information to PCs. Disruption of GC inputs to PCs has been shown to impair EBC (Wada et al., 2007), and given the high expression of PKM-ζ that we have shown in the granular layer of the cerebellar cortex, disruption of MF-GC LTP is a plausible mechanism to explain our results.

Our prior work has provided evidence for another mechanism by which EBC may occur, through modulation of voltage-gated potassium channels, particularly Kv1.2. We have shown that intra-cerebellar infusion of both tityustoxin (TsTx), a Kv1.2 inhibitor, and secretin, an endogenous Kv1.2 suppressor, enhances EBC (Williams et al., 2012). Furthermore, we have demonstrated that the secretin receptor antagonist, 5-27 secretin, disrupts EBC (Fuchs et al., 2014). Kv1.2 is most abundantly expressed in cerebellar BC axon terminals (pinceaus) and in PC dendrites (Wang et al., 1993; Laube et al., 1996; Koch et al., 1997; Chung et al., 2001). Through its ability to alter neuronal excitability (Khavandgar et al., 2005; Southan & Robertson, 1998), Kv1.2 is in a unique position to ultimately affect the excitability of PCs and thus be able to modulate learning through mechanisms other than LTD/LTP. Interestingly, our preliminary data in HEK 293 cells show that PKM-ζ induces a significant decrease in Kv1.2 surface expression (Chihabi et

al., 2015). Thus we propose that the current results may be partially explained by disruption of a PKM-ζ induced decrease in Kv1.2 surface expression in cerebellar cortex.

Furthermore, we have shown that PKC-ζ/ PKM-ζ is densely expressed throughout the cerebellar cortex with highest expression in PC nuclei. We did not identify any expression of PKC-ζ/ PKM-ζ in the deep cerebellar nuclei, including the IN. Thus, PKM-ζ may be involved in EBC only through modulation of the cerebellar cortex. This finding extends a previous experiment by Oster and colleagues in which no evidence of PKC-ζ/ PKM-ζ mRNA expression was found outside the cortex in the cerebellum (Oster et al., 2004). These results, together with the fact that our cannula placements were fairly dorsal to the IN, strongly suggest that the impairment in EBC produced by ZIP infusion in the current study was due to effects on the cerebellar cortex.

It is important to note that our study is one of a few to look at ZIP's involvement in early acquisition of memory. Our reasoning for beginning with this time point for infusion comes from our previous data showing that blocking Kv1.2 in cerebellar cortex in the first session of EBC can facilitate learning (Williams et al., 2012) and from data showing that PKM-ζ can modulate cell surface expression of Kv1.2 (Chihabi et al., 2015). Our infusion of ZIP two hours prior to the first acquisition session is different from most studies that looked at the effect of ZIP after learning has already occurred. Notably, in our experiment the impairment produced by infusion of ZIP into the cerebellar cortex persisted through days 2-6 despite the fact that ZIP infusion occurred only before session 1. However, this is likely consistent with what is known about PKM-

ζ; its temporary ZIP-mediated disruption causes permanent memory loss and learning deficits (Sacktor, 2011). Thus, ZIP infusion into cerebellar cortex prior to day one may have prevented consolidation of EBC from that first day of acquisition, effectively putting these rats one day behind the vehicle control rats in acquisition. The difference in apparent asymptote is, however, more difficult to explain. While we cannot directly comment on how ZIP may affect EBC if infused after learning has reached asymptote, or if ZIP was infused after every session, it is quite reasonable to hypothesize from the data that consolidation of day one acquisition may have been completely disrupted. This would be a direction for future work.

Interestingly, one study that looked at associative recognition memory found that ZIP disrupts memory in both the hippocampus and mPFC when infused post-acquisition; however, ZIP infusion pre-acquisition disrupted memory in the mPFC but not the hippocampus (Evuarherhe et al., 2014). Evuarherhe and colleagues postulated a possible role for the glutamate receptor 2 (GluR2) subunit of AMPA receptors in this temporal difference; they found that blocking GluR2-dependent removal of AMPA receptors through the co-infusion of peptide $GluR2_{3Y}$ did not affect the impairment caused by ZIP in the mPFC, but did disrupt the impairment of ZIP in the hippocampus. Trafficking of GluR2 is critical for maintenance of hippocampal late-phase LTP (Yao et al., 2008) and past studies have shown that blocking endocytosis of GluR2 is sufficient to prevent impairments in LTP maintenance caused by ZIP (Migues et al., 2010). This suggested that impairment by ZIP in the mPFC was independent of GluR2 receptors, indicating an

alternative pathway in PKM-ζ dependent mPFC memory formation (Evuarherhe et al., 2014). One possibility in the cerebellum is regulation of Kv1.2 by PKM-ζ. In conclusion, we have shown that PKC-ζ and PKM-ζ are highly expressed in the cerebellar cortex and that intra-cerebellar infusion of a PKM-ζ inhibitor, ZIP, prior to conditioning disrupts cerebellar-dependent delay EBC. We propose that the effect of ZIP on cerebellar EBC may be through disruption of both AMPA receptor and Kv1.2 channel maintenance in the cerebellar cortex.

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Figure Captions

Figure 2.1. Expression of PKC-ζ and Kv1.2 in cerebellar cortex. Overnight incubation of fixed rat cerebellar slices with α-PKC-ζ (red) gives positive signal throughout the cortex in the molecular (ML) and granular layers (GL) as well as the pinceaus of basket cell (BC) axon terminals. Its highest expression was found in the nuclei of Purkinje cells (PC) and throughout the molecular layer. Overnight incubation with α-Kv1.2 (green) gives positive signal throughout cortex with highest expression in the pinceaus of BC axon terminals. Blue signal is indicative of DAPI nuclear stain.

Figure 2.2. Cerebellar infusion of ZIP significantly disrupts EBC. Error bars indicate SEM. Rats received either 0.50 μL intra-cerebellar infusions of ZIP (20 nmol/μl) or Scr-ZIP (20 nmol/μl) or PBS vehicle into cerebellar lobulus simplex prior to the first session of acquisition (80 CS–US trials per session with 350 ms delay, with interspersed CS and US probe trials). A 3 (Group) x 6 (Session) repeated-measures ANOVA on the percentage of CRs on CS–US trials revealed a significant main effect of session ($F(5,140) = 36.30$, $p < 0.05$) and a group x session interaction ($F(10,140) = 1.99$, $p < 0.05$). The main effect of group approached, but did not attain, statistical significance ($p = 0.067$). Follow-up independent-samples t-tests of the significant interaction revealed that the percentage of CRs in Group ZIP were significantly lower than Group Veh in sessions 2 ($p = 0.008$), 3 ($p = 0.016$), 4 ($p = 0.042$), and 6 ($p = 0.016$). The same analyses comparing Group Scr-ZIP to Group Veh revealed that the percentage of CRs did not differ significantly (p's > 0.08); furthermore, Group Scr-ZIP

showed a greater percentage of CRs than Group ZIP in sessions 5 ($p = 0.020$) and 6 ($p = 0.021$). Stars (* and **) indicate $p<0.05$ and $p<0.01$, respectively for Group ZIP vs Group Veh. Pound (#) indicates $p<0.05$ for Group Scr-ZIP vs Group ZIP.

Figure 2.3. Cannula placements in cerebellar lobulus simplex. Numbers on the right are posterior from bregma. (Adapted from Paxinos and Watson [2007] with permission from Elsevier © 2007.)

Figure 2.1. Expression of PKC-ζ and Kv1.2 in cerebellar cortex.

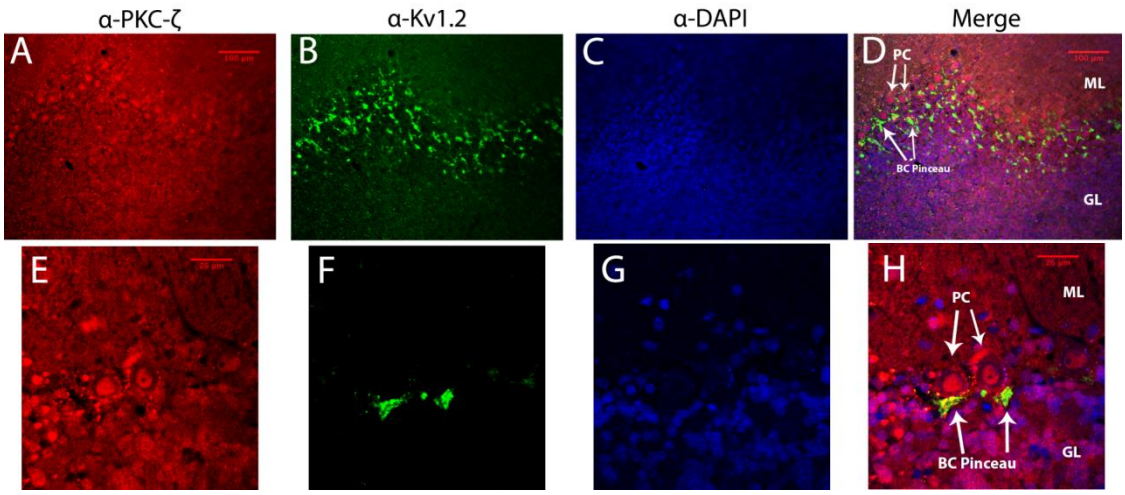


Figure 2.2. Cerebellar infusion of ZIP significantly disrupts EBC.

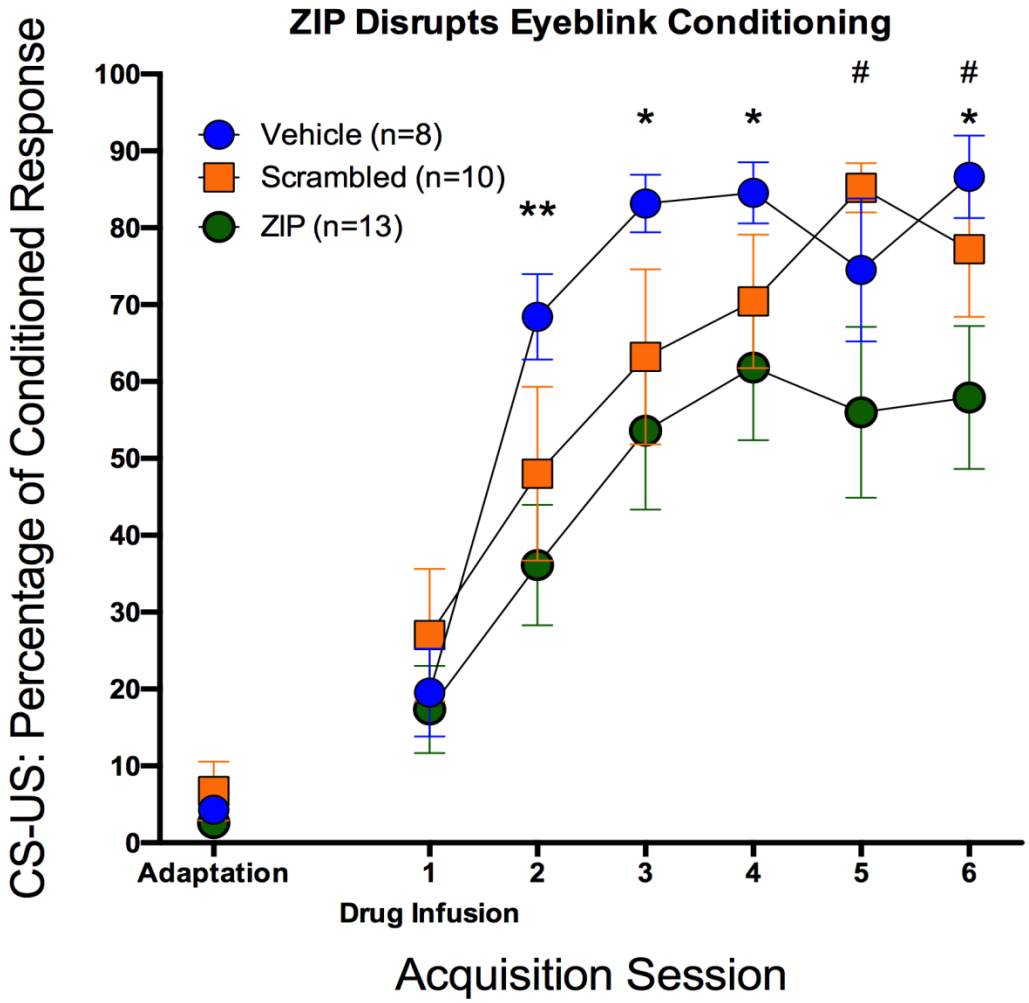
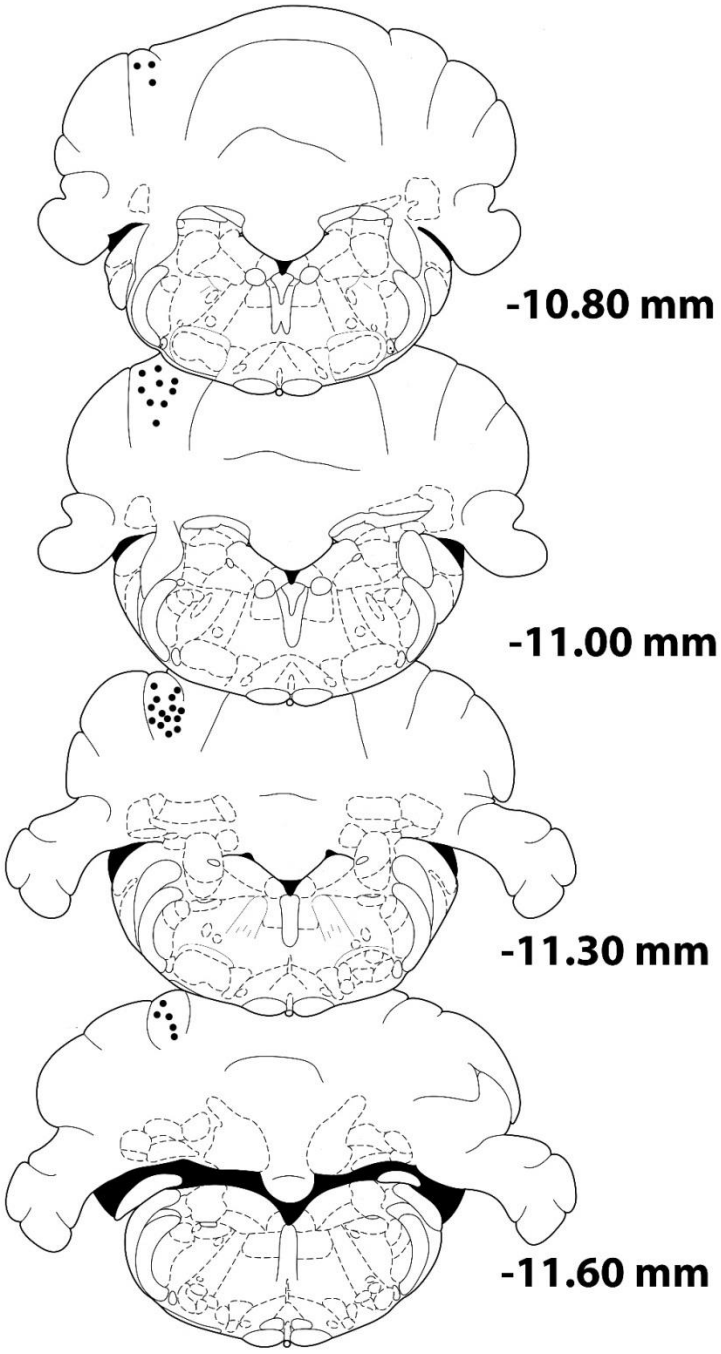


Figure 2.3. Cannula placements in cerebellar lobulus simplex.



CHAPTER 3: PROTEIN KINASE MZETA (PKM-ζ) REGULATES KV1.2 DEPENDENT CEREBELLAR EYEBLINK CLASSICAL CONDITIONING

Abstract

PKM-ζ has long been implicated in a hippocampal cellular correlate of learning, long-term potentiation (LTP), through its regulation of hippocampal AMPA receptors. Disruption of PKM-ζ with Zeta-inhibitory peptide (ZIP) in many brain structures can irreversibly disrupt learning and memory that has been maintained for many weeks. Furthermore, disruption of PKM-ζ with cerebellar ZIP infusion has been shown to disrupt delay eyeblink conditioning (EBC), an established model for the assessment of cerebellar learning (Chihabi et al., 2016). Here, we show that *in vivo* PKM-ζ disruption with ZIP can induce an upregulation of Kv1.2 surface expression in live cerebellar slices, and that PKM-ζ decreases Kv1.2 surface expression *in-vitro*. Furthermore, we show that PKM-ζ protein expression is significantly decreased in rats receiving unpaired tones and eyelid stimulation, but not paired stimuli (i.e. EBC), when compared to no stimulus controls. This is the first demonstration that PKM-ζ regulates a voltage-gated channel and opens up an entirely new approach for studying the kinase's effects on regulating learning.

Introduction

PKM-ζ, a kinase that has been extensively studied in the last decade by researchers investigating learning and memory, has been shown to disrupt maintenance of AMPA receptors and subsequently, irreversibly disrupt consolidated long-term

memory. Due to its permanent nature, researchers have been enamored in attempting to understand how PKM-ζ may work to maintain memory in coordination with other lesser-studied kinases such as PKC-λ/ι. The significance of the serine/threonine kinase PKM-ζ is due to its truncation of the PKC-ζ N-terminal that renders it constitutively active (Hernandez et al., 2003). Being constitutively active and brain-specific, PKM-ζ is well suited to maintain learning until it is degraded. Despite its widespread attention, PKM-ζ has not been well studied in the cerebellum. Our lab has previously shown that disruption of PKM-ζ with cerebellar Zeta-inhibitory peptide (ZIP) infusion disrupts delay eyeblink conditioning (EBC), an established model for the assessment of cerebellar learning (Chihabi et al., 2016). Furthermore, we were the first to show that PKC-ζ and PKM-ζ are highly expressed in the cerebellar cortex, with highest expression found in Purkinje cell (PC) nuclei.

The role of PKM-ζ in trafficking AMPA receptors into the postsynaptic membrane is key to formation of long-term potentiation (LTP), a cellular mechanism that underlies learning and memory (Kessels et al., 2009). Sacktor and colleagues provided the first evidence that PKM-ζ has an important role in maintaining learning-related synaptic changes, by showing that interruption of PKM-ζ activity is sufficient to disrupt maintenance of hippocampal LTP (Serrano et al., 2005; Ling et al 2006, Pastalkova et al., 2006). The role of PKM-ζ in learning has been primarily studied with the use of ZIP, a selective PKC-ζ pseudosubstrate inhibitor that effectively inhibits both PKC-ζ and PKM-ζ. While the literature has focused on PKM-ζ's effect on AMPA receptors, our lab

has taken a different approach and identified a novel interaction between PKM-ζ and Kv1.2, a voltage-gated potassium ion channel, in the cerebellum.

Cerebellar EBC has been understood to rely upon a mechanism of long-term depression (LTD) in the cerebellar cortex, in addition to changes to interpositus nucleus plasticity. Yet, this model has been challenged in the past few years and surprisingly, disrupting AMPA receptor regulation and cerebellar LTD did not impair EBC (Schonewille et al., 2011). However PF-PC LTD may be one of a variety of plasticity mechanisms in cerebellar cortex that support cerebellar-dependent learning (Gao et al., 2012); another mechanism may involve regulation of voltage-gated potassium channel 1.2 (Kv1.2). Kv1.2 is an ion channel known to regulate neuronal excitability and is most abundantly expressed in cerebellar basket cell (BC) axon terminals (pinceaus) and in PC dendrites (Khavandgar et al., 2005; Southan & Robertson, 1998; Wang et al., 1993; Laube et al., 1996; Koch et al., 1997; Chung et al., 2001). Indeed, inhibition of Kv1.2 with Tityustoxin-Kα (TsTx) in cerebellar PC dendrites increases PC excitability (Khavandgar et al., 2005), while its inhibition in BC axon terminals increases inhibition of PCs (Southan & Robertson, 1998).

Furthermore, we have previously shown that intra-cerebellar infusion of tityustoxin (TsTx), a Kv1.2 inhibitor, enhances EBC while the secretin receptor antagonist, 5–27 secretin, disrupts EBC (Fuchs et al., 2014; Williams et al., 2012). Two prior studies have demonstrated that PKC-ζ associates with and is able to phosphorylate cerebellar Kvβ2 (Gong et al., 1999; Croci et al., 2003), an auxiliary subunit that interacts with Kv1.2 (Coleman et al., 1999). Given the high levels of PKM-ζ expression in the

cerebellar cortex and its possible interaction with Kv1.2, we hypothesized that PKM-ζ may be able to regulate Kv1.2 in a model of cerebellar learning.

Results

PKM-ζ expression in unpaired rat cerebellar cortex significantly differed from control

Because PKM-ζ is primarily activated through expression and degradation, any effect of PKM-ζ in learning would have a direct impact on protein expression levels; anti-PKC-ζ antibody, which binds both PKC-ζ and PKM-ζ, allows for identification of respective protein expression levels from the lobulus simplex resolved on a Western Blot. EBC (paired tones and eyelid stimulation) did not appear to have a significant effect on PKM-ζ expression levels (Figure 3.5). Unpaired stimuli did however produce a significant decrease in PKM-ζ expression levels; statistical analysis using a student's t-test found a $p<0.001$ between the Unpaired and No Stimulus groups (Figure 3.5). Analysis of Phospho-410 PKC-ζ, the active-form of PKC-ζ, did not reveal a significant difference between the groups (Figure 3.6). Analysis of PKC-ζ expression also revealed no significant difference between the groups, but the Paired group showed a slight trend of an increase in PKC-ζ expression levels as compared to the No Stimulus group, $p = 0.095$. (Figure 3.7).

Paired vs Unpaired EBC Rats differ in acquisition

After analyzing the molecular state of the cerebellum in regards to PKC-ζ and PKM-ζ after 3 days of EBC, we wanted to understand if the group receiving unpaired stimuli learned differently than the sit groups. In this experiment, we would give two

groups of rats either unpaired or no stimulus trials for 3 days as previously described, and then on the 3rd day we would condition both groups with paired trials for an additional 6 sessions. 2 (Sit, Unpaired) x 6 (EBC session) repeated-measures ANOVA revealed significant main effects of both group and session, as well as a significant group x session interaction effect (Figure 3.8). Follow-up independent-samples t-tests comparing the two groups in each session revealed significant group differences in EBC sessions 1-3, and no group differences in EBC sessions 4-6 between the unpaired and "sit" groups, $F\_Acq1 = 0.431$ and $p<0.0001$, $F\_Acq2 = 9.104$ and $p<0.0001$, $F\_Acq3 = 8.937$ and $p<0.0001$, $F\_Acq4 = 0.167$ and $p = 0.234$, $F\_Acq5 = 0.119$ and $p = 0.80$, $F\_Acq6 = 0.083$ and $p = 0.842$.

Cell Culture Analysis of Kv1.2 Surface Expression (Kv1.2 KR, Kvβ2)

In order to understand if PKC-ζ or PKM-ζ can alter Kv1.2 surface expression, HEK293/T cells were overexpressed with either PKM-ζ + Kv1.2 + Kvβ2 or PKC-ζ T410E (Active-PKC-ζ) + Kv1.2 + Kvβ2 and compared with controls overexpressed with Kv1.2 + Kvβ2 + Vector control. Further experiments were conducted to analyze the effect of ubiquitination on PKM-ζ regulation of Kv1.2 with overexpression of PKM-ζ + Kv1.2-KR + Kvβ2 or PKC-ζ T410E (Active-PKC-ζ) + Kv1.2-KR + Kvβ2 and compared with controls overexpressed with Kv1.2-KR + Kvβ2 + Vector control. Kv1.2-KR is a specific mutant of Kv1.2 where N-termini and C-termini lysines were mutated to arginine. Analysis of Kv1.2 and Kv1.2-KR surface expression in HEK 293/T cells was performed by measuring the median fluorescence of transfected cells. Statistical analysis

was performed using GraphPad Prism 7 with a student's t-test and revealed a $p<0.0001$ between PKC-ζ T410E + Kv1.2 + Kvβ2 and Kv1.2 + Kvβ2 controls and between PKM-ζ + Kv1.2 + Kvβ2 and Kv1.2 + Kvβ2 controls. (Figure 1). Statistical analysis with a student's t-test revealed a $p<0.05$ between PKM-ζ + Kv1.2 KR + Kvβ2 and Kv1.2-KR + Kvβ2 and $p<0.01$ between PKC-ζ T410E + Kv1.2-KR + Kvβ2 and Kv1.2-KR + Kvβ2 (Figure 2). Follow-up post-hoc tests revealed a $p<0.0001$ between PKC-ζ T410E + Kv1.2 + Kvβ2 and PKC-ζ T410E + Kv1.2KR + Kvβ2 and a $p<0.001$ between PKM-ζ + Kv1.2 + Kvβ2 and PKM-ζ + Kv1.2-KR + Kvβ2 (Figure 2). Statistical analysis with a student's t-test revealed a $p<0.0001$ between Kv1.2 + PKM-ζ and Kv1.2 and revealed a $p<0.0001$ between Kv1.2 + PKM-ζ + Kvβ2 and Kv1.2 + Kvβ2 (Figure 3). No significant difference was found between Kv1.2 + PKM-ζ + Kvβ2 and Kv1.2 + PKM-ζ.

Biotinylated Cerebellar Slices

In order to determine if cerebellar PKM-ζ can alter Kv1.2 surface expression, use of the PKM-ζ inhibiter ZIP allowed us to use biotinylation to measure the effect of the kinase on Kv1.2 surface expression. The results of the biotinylation were measured via immunoblot after normalization to GAPDH in live slices with a ZIP or control bath. Statistical analysis was performed using GraphPad Prism 7 with a student's t-test and revealed a significant difference in Kv1.2 surface expression between the ZIP applied cerebellar slices as compared to the control cerebellar slices, $p<0.05$.

Discussion

We have shown that PKM-ζ decreases Kv1.2 surface expression *in-vitro* (Figure 3.1) and that *in vivo* bath application of the PKM-ζ inhibitor ZIP can cause a direct up-regulation of Kv1.2 surface expression in rat cerebellar slices (Figure 3.4). This is the first demonstration that PKM-ζ regulates a voltage-gated channel and opens up an entirely new approach for studying the kinase's effects on regulating learning. Kv1.2 has been implicated in regulation of cerebellar eyeblink conditioning (EBC) by its ability to alter cell excitability (Williams et al., 2012, Fuchs et al., 2014).

In a behavioral model involving EBC, three sets of rats received either paired, explicitly unpaired, or no stimuli for 3 days and tissue from the cerebellar lobulus simplex of these rats was used to resolve protein expression on a western blot. We have shown that PKM-ζ protein expression is down-regulated during a paradigm involving behavioral inhibition in which rats received explicitly unpaired stimuli (Figure 3.5). While unexpected, the novel results suggest that expression changes in the unpaired group may be in part due to a larger mechanism that involves spatio-temporal learning. In other words, the rats in the unpaired group may have learned something about the CS differently than the paired group, and this learning was measured in the significant reduction in PKM-ζ expression. In order to further behaviorally analyze the changes that occurred in the unpaired group, another set of naive rats received explicitly unpaired or no stimuli for 3 days, and then were switched to receive paired stimuli to determine what, if anything, the unpaired rats had learned. The second set of rats revealed that the

unpaired group had undergone inhibitory learning about the CS and essentially, learned that the CS predicted that they would not receive eyelid stimulation. This behavioral inhibition was shown to be significant for 3 days after the unpaired rats were switched to receive paired stimuli (Figure 3.8). The inference from this data suggests that PKM-ζ in the case of the unpaired group is down-regulated, and thus possibly inhibiting further learning from occurring. Interestingly, Fuchs and colleagues found that during a similar experiment, Kv1.2 surface expression significantly decreased in BC pinceaus but increased in PC dendrites, also in the unpaired but not the paired group (Fuchs et al., 2015). More importantly, this result matches what we would expect to have found if PKM-ζ is responsible for regulating Kv1.2 surface expression, as it is consistent with our finding that PKM-ζ is expressed in the PC dendrites (Chihabi et al., 2016).

One interpretation for these results is that PKM-ζ is downregulated in the unpaired group, and behaviorally, this group developed conditioned inhibition to the tone. Behavioral inhibition has been previously observed in models of eyeblink conditioning in which acquisition of the eyeblink will be prolonged in unpaired animals as compared to naïve animals (Ricart et al., 2011; Rush, Robinette, & Stanton, 2001; Stanton, Fox, & Carter, 1998). In the unpaired group, it is possible that the rats are learning that the conditioned stimulus predicts the absence of the unconditioned stimulus, and therefore, through inhibition of PKM-ζ expression, and a subsequent increase in Kv1.2 surface expression, the explicitly unpaired group is possibly experiencing a decreased activation of PCs and subsequent activation of the IN. While in such a case one would expect a reflexive eyeblink to be conditioned due to the activation of the IN, it is possible that this

behavior is altering the feedback inhibition of the IN to the IO, and thus strengthening the incoming US input to the PCs. Therefore, it is a plausible to suggest that the rats are learning that the tone is a safety signal, knowing they will not receive a shock when the tone is on. However, unlike the rats in the paired group who have learned that the tone predicts the shock, this type of behavioral inhibition is consistent with what is expected of the Rescorla-Wagner model of learning. Particularly, the rats receiving the explicitly unpaired stimuli, while understanding something about the CS, have not learned to positively correlate the CS-US as the groups receiving the paired stimuli have. Instead, they have learned a negative correlation between the CS-US; therefore, it is reasonable to suggest that this snapshot into the cerebellum of the explicitly unpaired rats is exposing the large molecular changes that are occurring in response to this negative correlative learning. The rats in the explicitly unpaired group are at the critical point in time where a sizeable decrease in PKM-ζ and increase in Kv1.2 is measureable, and are at the point in time of the Rescorla-Wagner model just prior to the point of acquisition. In particular, the Rescorla-Wagner model describes association formation but is silent on how these associations are translated into behavior. In other words, molecular changes have occurred to facilitate learning, but the negative correlation between the CS-US is preventing this learning from translating into any down-stream plasticity. Therefore we speculate that the paired group which has likely relied on PKM-ζ and Kv1.2 to acquire the CS and has then strengthened pons input to the IN and subsequent inhibition of the IO, the unpaired group has not made any downstream plasticity changes, and therefore the changes in the PC dendrites are still measurable. Therefore the rats that have received

the paired stimuli are no longer reliant on the US when the CS is elicited, due to inhibition of the IO. Therefore, it is hypothesized, that the paired group by this 3rd day of learning is no longer reliant on this early-acquisition model of learning dependent on Kv1.2.

While a trend suggests that PKC-ζ may increase in the paired group ($p = 0.095$), this did not attain conventional statistical significance (Figure 3.7). Furthermore, Phospho410 PKC-ζ (Threonine 410 is the well-documented site of PKC-ζ activation) was not significantly different between groups (Figure 3.6); in addition, PKM-ζ had no measureable increase in the paired group (Figure 3.5). While this was not what we expected, our experiment was a snapshot into the state of the cerebellar cortex after 3 days of conditioning. It is possible that the effect of PKM-ζ in the paired group may be earlier on in learning or much later than 3 days when the learning has consolidated. Most studies on PKM-ζ involve looking at the effect of the kinase in maintaining consolidated memory and therefore the effect on PKM-ζ protein expression may not be visible until weeks after EBC has occurred (Pastalkova et al., 2006; Sacktor, 2011; Shema et al., 2011). Furthermore, we cannot definitively rule out the effect of PKM-ζ in the paired group, as the kinase is dispersed widely throughout the cerebellar cortex. Thus, PKM-ζ may be increasing in expression in some cell types such as the PC while decreasing in expression in other cell types responsible for LTP such as MF's or GC's, resulting in a zero sum net effect on PKM-ζ expression levels (Chihabi et al., 2016; Grasselli & Hansel, 2014; Schonewille et al., 2010, cf. Hansel, 2005). Given the expression of PKM-ζ that we have shown in the granular layer of the cerebellar cortex, disruption of MF-GC LTP is a plausible mechanism. Furthermore, if PKM-ζ is affecting both AMPA and Kv1.2

channels, its effect may not be detectable in overall protein expression as it might be overexpressed in one cerebellar region and degraded in another, upregulated for example in the PC while downregulated in the BC.

While our results from this data set suggested that only the unpaired group had a measureable change in PKM-ζ expression, there are numerous reasons why we may not have seen an effect in the paired group. First, it is important to mention that our infusion of ZIP as described in Chapter 2 occurred prior to the first day of EBC. This may have impaired consolidation of session 1 learning, which is why the rats may have performed more poorly than vehicle controls on subsequent days and might suggest that changes in PKM-ζ expression are most important early in EBC, prior to the emergence of CRs. This is further evidenced when one considers the possible role of PKC-ζ. Given the trend we observed in PKC-ζ expression in the paired group, it is possible that early consolidation of cerebellar learning is dependent on PKC-ζ while later-term consolidation of cerebellar learning is dependent on PKM-ζ. On day three, the paired group has already achieved asymptote and may have already begun a transition to consolidate their learning (Fuchs et al., 2015); such a transition may possibly explain the PKC-ζ trend we observed. Interestingly, several studies in the hippocampus have suggested that a closely related PKC isoform, PKC-λ/ι, is responsible for early-LTP while PKM-ζ is responsible for the consolidation of long-term late-LTP. These studies have found that by knocking out the gene for PKC-ζ/PKM-ζ, they have induced compensatory, abnormal upregulation, or enhanced phosphorylation, of PKC-λ/ι (Selbie

et al., 1993; Lee et al., 2013; Jalil et al., 2015). However even more interestingly, while PKC-λ/ι is highly expressed in the hippocampus and PKC-ζ is sparsely expressed, cerebellar expression of these two isoforms appear to be reversed; that is, expression of PKC-λ/ι has been observed to be extremely sparse while expression of PKC-ζ has been observed to be highly expressed in the cerebellar cortex (Oster et al., 2004). Given that PKC-ζ behaves similarly to PKM-ζ once activated, and given that PKC-ζ and PKC-λ/ι are very similar in molecular composition, this interesting dichotomy presents a framework for the potential that PKC-ζ acts in place of PKC-λ/ι and provides for early learning acquisition in the cerebellar cortex. Such an occurrence would explain our previously shown data in which ZIP infusions into the cerebellar cortex prior to EBC acquisition disrupts learning (Chihabi et al., 2016); our disruption of EBC may have been through disruption of PKC-ζ in early-phase acquisition of learning rather than through late-phase PKM-ζ maintenance of learning.

Given that the data suggests complex and differentiated roles of PKC-ζ and PKM-ζ regulation of Kv1.2, we decided to look at an *in-vitro* model that allows us to look at the molecular interactions between the kinase and the channel. One of the plasmids we used in addition to PKM-ζ is an always-active PKC-ζ mutant T410E. Threonine 410 is the well-documented site of PKC-ζ activation and its mutation to Glutamine induces a constitutively active PKC-ζ (Chou et al., 1998). Research suggests that PKC-ζ T410E functions similarly to PKM-ζ, which is a constitutively active catalytic domain of PKCζ (Smith et al., 2002). Despite the possible interaction between PKC-ζ and Kvβ2 (Gong et al., 1999; Croci et al., 2003), our *in-vitro* data suggests that Kvβ2 is not necessary for

PKC-ζ and PKM-ζ to affect Kv1.2 surface expression (Figure 3.3). Kvβ2 is an auxiliary subunit which interacts with the Kv1.2α subunit (Coleman et al., 1999) and plays an important role in Kv1.2 forward surface trafficking (Shi et al., 1996; Campomanes et al., 2002; Gu et al., 2003). The possible mechanisms for such an interaction are numerous and were outside the scope of this study, however preliminary data suggests to us that the effect of PKC-ζ and PKM-ζ on Kv1.2 surface expression may be dependent on the physical presence of Kv1.2 in lipid rafts, and Kvβ2 may be part of the answer. We arrived at this preliminary hypothesis due to a number of observations we have made. Our lab has recently shown that monoubiquitination is important for non-degradative trafficking of Kv1.2 to the cell surface; particularly, Cilento and colleagues found that monoubiquitination of Kv1.2 can regulate lipid raft dynamics, and the mutation of lysines to arginines on the channel's N and C termini (Kv1.2-KR) may move the channel outside of lipid rafts (Cilento et al., 2015). However, as it is suspected Kvβ2 is responsible for clathrin-dependent endocytosis mechanisms, monoubiquitin expressed with and without Kvβ2 had opposing effects on Kv1.2-KR regulation. These findings suggested that indeed, Kv1.2 is in a delicate balance between lipid rafts which are dependent on cholesterol-dependent endocytosis mechanisms.

Interestingly, prior studies have shown that PKC-ζ targets to lipid rafts and acts as a critical adaptor molecule to regulate lipid raft dynamics (Kanzaki et al., 2004; Shin et al., 2008). Our observations suggest but do not prove that PKC-ζ/PKM-ζ may only activate a cholesterol-dependent endocytosis mechanism. In investigating the possible

effect of Kvβ2, this observation is strengthened by our data which show that PKM-ζ in Kv1.2-KR has a significantly diminished effect in comparison to PKM-ζ in Kv1.2-wt in the presence vs the absence of Kvβ2 (Figure 3.2). Our preliminary reasoning for this data suggests that the presence of Kv1.2 in lipid rafts, which may be dependent on mechanisms of ubiquitination, can be altered, thus the perceived effect of PKM-ζ on Kv1.2 surface expression appears to be diminished.

In conclusion, our data suggests that PKM-ζ may be an important regulator of EBC in the cerebellar cortex. More importantly, our data is the first of its kind to suggest that PKM-ζ may be involved with maintenance of learning independent of ligand-gated receptors. The literature on voltage-gated channel modulation of learning is still in its early prime, and our data link an important kinase known to be involved with maintenance of learning with the maintenance of voltage-gated potassium channel Kv1.2. Furthermore, regulation of Kv1.2 by PKM-ζ suggests that ligand-gated channels such as AMPA receptors may not be solely responsible for maintenance of learning. Indeed, studies have shown EBC left intact with AMPA receptor knockouts or inhibitors (Schonewille et al., 2011). While PKM-ζ is primarily known for its ability to maintain AMPA receptors in regions such as the hippocampus and amygdala, it may also be maintaining a secondary process of learning through voltage-gated ion channels. Outside the cerebellum, Kv1.2 is present in high density in the hippocampus and its function is not well-understood (Grosse et al., 2000; Park et al., 2001). If a symbiotic relationship between Kv1.2 and AMPA receptor maintenance of learning is discovered in the

cerebellum, it is possible that such a learning mechanism may be occurring in other regions as well. Certainly, if this learning mechanism was time-dependent, where Kv1.2 regulation may induce acquisition of short-term learning while AMPA receptors maintain long-term learning, the inter-dependency of the two linked by the constitutively active PKM-ζ and PKC-ζ provide for an interesting endeavor to be investigated further.

Methods

Subjects

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

Surgery

Surgeries took place 4-6 days after arrival. Surgeries were performed under aseptic conditions. Rats were anesthetized with 3% isoflurane in oxygen. A midline scalp incision was made and four skull screw holes were drilled and skull screws were placed as anchors for the head stage. A bipolar stimulating electrode (Plastics One, Roanoke, VA) was positioned subdermally immediately dorsocaudal to the left eye. Two electromyogram (EMG) wires for recording activity of the orbicularis oculi muscle were

each constructed of a 75-μm Teflon coated stainless steel wire soldered at one end to a gold pin fitted into a plastic threaded pedestal connector (Plastics One). The other end of each wire was passed subdermally to penetrate the skin of the upper eyelid of the left eye and a small amount of insulation was removed so that the bare electrodes made contact with the orbicularis oculi muscle. A ground wire was wrapped around two skull screws at one end and the other end was also soldered to a gold pin fitted into the pedestal connector. The ground wire, bipolar and EMG electrodes were cemented to the skull with dental cement. Rats were given 5-6 days to recover prior to eyeblink conditioning.

Apparatus

Eyeblink conditioning took place in one of four identical testing boxes (30.5 x 24.1 x 29.2 cm; Med-Associates, St. Albans, VT), each with a grid floor. The top of each box was altered so that a 25-channel tether/commutator could be mounted to it. Each testing box was kept within a separate electrically-shielded, sound attenuating chamber (45/7 x 91.4 x 50.8 cm; BRS-LVE, Laurel, MD). A fan in each sound-attenuating chamber provided background noise of approximately 60 dB sound pressure level. A speaker was mounted in each corner of the rear wall and a light (off during testing) was mounted in the center of the rear wall of each chamber. The sound-attenuating chambers were housed within a walk-in sound-proof room.

Stimulus delivery was controlled by a computer running Spike2 software (CED, Cambridge, UK). A 2.8 kHz, 80 dB tone, delivered through the left speaker of the sound-attenuating chamber, served as the conditioned stimulus (CS). The CS was 365-ms in

duration. A 15-ms, 4.0 mA uniphasic periorbital stimulation, delivered from a constant current stimulator (model A365D; World Precision Instruments, Sarasota, FL), served as the unconditioned stimulus (US) during conditioning. Recording of the eyelid EMG activity was controlled by a computer interfaced with a Power 1401 high-speed data acquisition unit and running Spike2 software (CED, Cambridge, UK). Eyelid EMG signals were amplified (10k) and bandpass filtered (100-1000 Hz) prior to being passed to the Power 1401 and from there to the computer running Spike2. Sampling rate was 2 kHz for EMG activity. The Spike2 software was used to full-wave rectify, smooth (10 ms time constant), and time shift (10 ms, to compensate for smoothing) the amplified EMG signal to facilitate behavioral data analysis.

Eyeblink Conditioning Procedure, 1st set of 3 day EBC rats

At the beginning of each session, each rat was connected 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. Rats received 3 days of training. For Group Paired, each day of training consisted of 100 CS-US trials with an average inter-trial interval (ITI) of 30 sec (range = 20-40 sec) in which a 365 ms tone CS and a 15 ms periorbital stimulation US offset co-terminated (350-ms delay procedure). For Group Unpaired, each day of training consisted of 100 tone and 100 periorbital stimulation trials with an average ITI of 15 sec (range = 10-20 sec) with trial type intermixed such that no more than 3 consecutive trials were of the same type. The unpaired stimuli were explicitly unpaired and were never presented together. For Group No Stimulus, each day of

training consisted of 100 “blank” trials with an average ITI of 30 sec (range = 20-40 sec) with no stimulus presentation. Each session lasted approximately 50 minutes regardless of group.

Behavior Analysis

For Group Paired and Group Unpaired, CS-US or CS-alone trials were subdivided into three time periods: (1) a “baseline” period, 280 ms prior to CS onset; (2) a non-associative “startle” period, 0-80 ms after CS onset; and (3) a “CR” period, 81-350 ms after CS onset. For Group No Stimulus, the same time periods were used, although there was no CS. In order for a response to be scored as a CR, eye blinks had to exceed the mean baseline activity for that trial by 0.5 arbitrary units during the CR period. Eye blinks that met this threshold during the startle period were scored as startle responses and were analyzed separately. The primary behavioral dependent measure for all experiments was the percentage of trials with an eye blink response during the “CR period” across all 100 CS-US Paired trials (Paired group), 100 CS-alone trials (Unpaired group), or comparable “trial periods” for the No Stimulus group for each session.

Sample blinding

All subsequent tissue processing and data collection was performed blind with respect to the behavioral studies; fixed cerebellar hemispheres were assigned random code numbers which were revealed only after data collection was complete.

Generation of Parasagittal Cerebellar Sections

Immediately after training, rats were deeply anaesthetized using isoflurane. The brain was fixed by transcardial perfusion of ~100 ml of 1X PBS (Life Technologies) followed by ~100ml of 4% paraformaldehyde (Thermo Scientific). The left hemisphere (ipsilateral to the eye receiving the US) of the cerebellum was harvested and stored in ice cold PBS in 50ml conical tubes for about 60-90 minutes prior to sectioning. In order to isolate the region around the base of the primary fissure, 1,200 μm of tissue from the lateral portions of the hemisphere was first sectioned off. Subsequently, four 400 μm sections were collected and placed into individual wells in a 24 well plate on ice.

Western Blot Analysis of EBC

Sample buffer (50 μl Laemmli buffer + 100 mM DTT) was added to each tube and the beads were suspended by gentle mixing. 50 μl of each sample was transferred to individual new centrifuge tubes and 50 μl of sample buffer (200 mM DTT plus 5X sample buffer) was added to each tube. All of the samples were incubated for 10 minutes at 55°C. After the incubation, the tubes were spun at 16Kxg for ~15 seconds, mixed, and spun again. The samples were resolved using polyacrylamide gel electrophoresis and transferred onto nitrocellulose.

The nitrocellulose blots were placed in 3% bovine serum albumin (BSA; Sigma) for one hour and then incubated overnight at 4°C with the following primary antibodies: 1:1000 rabbit polyclonal C-Terminal PKC-ζ (SAB4502380; Sigma-Aldrich) and 1:4000 mouse monoclonal anti-GAPDH antibody (ThermoScientific). After rinsing, the

nitrocellulose blots were incubated for one hour in goat anti-mouse-700 (GAM-700) and goat anti-rabbit-800 (GAR-800) secondary antibodies both diluted 1:5000 (Rockland). Blots were then rinsed and imaged on an Odyssey Infrared Imager (LI-COR, Lincoln, NE, USA) and quantified LI-COR analysis software. Rectangular regions of interest (ROI) were drawn around the PKM-ζ, PKC-ζ and GAPDH within each lane. Background was determined from signal-free regions of each lane and subtracted from the ROI measurement. The data are presented by using the inverse of the raw ratios normalized to GAPDH. Ratio values were compiled in Graph Pad Prism 6 (Graph Pad Software, La Jolla, CA, USA) and group values are presented using the mean ±SEM.

Eyeblink Conditioning Procedure, 2nd set of 6 days EBC rats, Behavioral Analysis Only

At the beginning of each session, each rat was connected 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. Rats initially received 3 days of training as follows: For Group Unpaired, each day of training consisted of 100 tone and 100 periorbital stimulation trials with an average ITI of 15 sec (range = 10-20 sec) with trial type intermixed such that no more than 3 consecutive trials were of the same type. For Group No Stimulus, each day of training consisted of 100 "blank" trials with an average ITI of 30 sec (range = 20-40 sec) with no stimulus presentation. Each session lasted approximately 50 minutes regardless of group. On the 3rd training day, after the initial 100 trials, both groups received a 2nd set of 100 paired tone and eyelid stimulation trials. Both groups underwent EBC for a total of 6 sessions of 100 trials each day. EBC

consisted of 100 CS-US trials with an average inter-trial interval (ITI) of 30 sec (range = 20-40 sec) in which a 365 ms tone CS and a 15 ms periorbital stimulation US offset co-terminated (350-ms delay procedure). Behavioral analysis was conducted as previously described in this chapter.

Biotinylation

Surface Kv1.2 in formaldehyde fixed slices was detected using cell-surface biotinylation. Each section assigned to the biotinylation and western blot (WB) analysis of surface Kv1.2 was transferred into its own well in a new 12-well plate containing 1ml of 1X PBS in each well. The well plate was kept in an ice bath unless otherwise noted. Mature (surface) Kv1.2 is N-glycosylated within its first intrahelical extracellular loop. Detection of surface Kv1.2 was performed by oxidizing surface polysaccharides with 1 ml of 1 mM sodium meta-periodate (SMP, Thermofisher Scientific) for 30 minutes at 4°C, rinsing with ice-cold PBS and then biotinylating the oxidized polysaccharides by incubating with 50 mM alkoxyamine-biotin- PEG4 (Biotin, Thermofisher Scientific) for two hours at room temperature. 3 ml of either 5 μM ZIP or buffer was applied in a bath application for 2 hours.

The biotin reaction was quenched with ice-cold 50 mM TRIS in PBS. Sections were transferred into individual 1.5 ml centrifuge tubes containing RIPA buffer plus 10% HALT protease inhibitor (Life Technologies). Sections were disrupted by sonication using a Branson Digital Sonifier at 25% amplitude, twice for 2-4 seconds. The samples were then centrifuged at 16Kxg for 20 minutes at 4°C. 200 μl of the supernatant was

transferred to separate tubes marked as "Total" and the remaining ~800 µl was transferred to new centrifuge tubes marked "Beads" containing 40 µl of rinsed Neutravidin beads (Sigma). After a one-hour incubation at 4°C on a rotating centrifuge tube holder the beads were pelleted by centrifugation (2,000 RPM at 4°C for two minutes) and the supernatant was transferred to tubes marked as the "Post" fraction. The beads were rinsed with RIPA buffer three times for five minutes per rinse. Each rinse consisted of replacing the RIPA, allowing the centrifuge tubes to spin on the rocker, and collection by centrifugation. Following the last rinse, the remaining liquid was removed by aspiration using a 27G needle with the beveled end pressed against the side of the tube to prevent aspiration of the beads. Samples were then resolved on Western Blot as previously described in this chapter.

Cell Culture

The following primary antibodies were used in this study: A rabbit polyclonal antibody directed against the first extracellular loop of Kv1.2 (α-Kv1.2e) developed in conjunction with BioSource International (Camarillo, CA) (Stirling et al., 2009), mouse monoclonal Kv1.2 (K14/16; Neuromab), rabbit polyclonal C-Terminal PKC-ζ (SAB4502380; Sigma-Aldrich), Anti-Rabbit Alexa Fluor 488 (Invitrogen), and Alexa Fluor 568 (Invitrogen).

Human embryonic kidney 293T cells were used and cultured as previously reported (Nesti et al., 2004). Cells were plated onto 60 mm petri dishes pre-coated in polyethylenimine and allowed to grow to ~60-80% confluence. Experimental plasmids

we transfected into cells using a PEI transfection method: Diluted DNA (5µg) was added to PEI (1µg/µL) at a 1:1 ratio and immediately mixed by pipetting (FLAG.PKCzeta.T410E was a gift from Alex Toker Addgene plasmid # 10804, FLAG.PKCzeta.T410A was a gift from Alex Toker Addgene plasmid # 10801). Mixture was allowed to sit at room temperature for 15 minutes before being added drop wise to cells. Transfections were checked manually with an arc-lamp microscope prior to proceeding. For immunoblotting, transfected cells were lysed 48 hours later. For flow cytometry, 24 hours following transfection cells were re-plated to a density of 3.3×10^4 cells/cm^2 onto 35 mm petri dishes. Once cells adhered, media was aspirated, plates were rinsed with PBS, and media and new serum-free media was given for overnight serum starvation (10-16 hours). In all experiments comparing multiple cells lines, each were maintained at similar passage and treated equally from plating to treatment. Results comparing the cell lines were analyzed blindly.

Surface Detection of Kv1.2 and Flow Cytometry

Cells were given 154 mM sodium azide as described previously (Nesti et al., 2004). Cells were then lifted, put in a microcentrifuge tube, labeled for surface Kv1.2 by application of 0.33 µg/ml α-Kv1.2e, and antibody binding detected with fluorescently conjugated anti-rabbit immunoglobulin G (IgG) (0.1 µg/ml). Whole cell fluorescence was detected by a single laser flow cytometer and distribution selection and background subtraction was performed as previously described (Stirling et al., 2009).

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Figure Captions

Figure 3.1 PKM-ζ significantly decreases Kv1.2 Surface Expression. HEK 293T cells were cultured with either Kv1.2+Kvβ2 plasmids or PKC-ζ T410 + Kv1.2 + Kvβ2 plasmids or PKM-ζ + Kv1.2 + Kvβ2 plasmids. Cells were tagged with a rabbit polyclonal antibody directed against the first extracellular loop of Kv1.2 (α-Kv1.2e) and run through a flow cytometer. Statistical analysis was performed using GraphPad Prism 7 with a student's t-test and revealed a $p<0.0001$ significance between PKC-ζ T410E + Kv1.2 + Kvβ2 and Kv1.2 + Kvβ2 controls and between PKM-ζ + Kv1.2 + Kvβ2 and Kv1.2 + Kvβ2 controls. N = 12 Kv1.2 + Kvβ2, 6 PKC-ζ T410E + Kv1.2 + Kvβ2, 12 PKM-ζ + Kv1.2 + Kvβ2

Figure 3.2 PKM-ζ's and Active-PKC-ζ's effects on Kv1.2 are partially lysine-dependent. HEK 293 cells were cultured with either: Kv1.2 + Kvβ2 plasmids, PKC-ζ T410E + Kv1.2 + Kvβ2 plasmids, PKM-ζ + Kv1.2 + Kvβ2 plasmids, Kv1.2-KR + Kvβ2 plasmids, PKC-ζ T410E + Kv1.2-KR + Kvβ2 plasmids, or PKM-ζ + Kv1.2 KR + Kvβ2 plasmids. Cells were tagged with a rabbit polyclonal antibody directed against the first extracellular loop of Kv1.2 (α-Kv1.2e) and run through a flow cytometer. Statistical analysis was performed using GraphPad Prism 7 with a student's t-test and revealed a $p<0.0001$ significance between PKC-ζ T410E + Kv1.2 + Kvβ2 and Kv1.2 + Kvβ2 controls and between PKM-ζ + Kv1.2 + Kvβ2 and Kv1.2 + Kvβ2 controls. Statistical analysis with a student's t-test also revealed a $p<0.05$ between PKM-ζ + Kv1.2 KR + Kvβ2 and Kv1.2-KR + Kvβ2 and $p<0.01$ significance between PKC-ζ

T410E + Kv1.2-KR + Kvβ2 and Kv1.2-KR + Kvβ2. Follow-up adhoc tests revealed a $p<0.0001$ significance between PKC-ζ T410E + Kv1.2 + Kvβ2 and PKC-ζ T410E + Kv1.2KR + Kvβ2 and a $p<0.001$ significance between PKM-ζ + Kv1.2 + Kvβ2 and PKM-ζ + Kv1.2-KR + Kvβ2. N = 84 Kv1.2, 84 PKC-ζ T410E "Active" + Kv1.2, 6 PKM-ζ + Kv1.2, 42 Kv1.2-KR, 42 PKC-ζ T410E "Active" + Kv1.2-KR, 6 PKM-ζ + Kv1.2-KR

Figure 3.3 PKM-ζ's effect on Kv1.2 is independent of Kvβ2. HEK 293T cells were cultured with either: Kv1.2 plasmids, PKM-ζ + Kv1.2 plasmids, Kv1.2 + Kvβ2 plasmids, or PKM-ζ + Kv1.2 + Kvβ2 plasmids. Statistical analysis was performed using GraphPad Prism 7 with a student's t-test revealed a $p<0.0001$ significance between PKM-ζ + Kv1.2 and Kv1.2 and revealed a $p<0.0001$ significance between PKM-ζ + Kv1.2 + Kvβ2 and Kv1.2 + Kvβ2. No significance was found between PKM-ζ + Kv1.2 + Kvβ2 and PKM-ζ + Kv1.2. N = 6 Kv1.2, 6 PKM-ζ + Kv1.2, 6 Kv1.2 + Kvβ2, 6 Kv1.2 + Kvβ2 + PKM-ζ.

Figure 3.4 ZIP increases surface Kv1.2 in cerebellar live slices. Statistical analysis was performed using GraphPad Prism 7 with a student's t-test and revealed a significance of $p<0.05$ in Kv1.2 surface expression between the ZIP applied cerebellar slices as compared to the control cerebellar slices. N = 4 rats; 7 slices ZIP, 10 slices control

Figure 3.5 PKM-ζ protein expression significantly reduced in the unpaired group. Statistical analysis was performed using GraphPad Prism 7 with a student's t-

test and found a $p<0.001$ between the Unpaired and No Stimulus groups. The paired group did not appear to have a significant effect on PKM-ζ expression levels. N = 39 slices Paired from 20 rats, 31 slices Unpaired from 16 rats, 41 slices No Stim from 21 rats.

Figure 3.6 Phospho-410 PKC-ζ expression did not change between the groups. Statistical analysis was performed using GraphPad Prism 7 with a student's t-test and found no significance between the three groups. N = 22 slices Paired from 11 rats, 22 slices Unpaired from 11 rats, 28 slices No Stim from 14 rats.

Figure 3.7 PKC-ζ expression did not change between the groups. Statistical analysis was performed using GraphPad Prism 7 with a student's t-test and found no significance between the three groups, but the Paired group had a slight trend with an increase in PKM-ζ expression levels with a p value of 0.095 as compared to the No Stimulus group. N = 35 slices Paired from 18 rats, 32 slices Unpaired from 16 rats, 40 slices No Stim from 20 rats.

Figure 3.8 The Unpaired groups appears to exhibit behavioral inhibition. 2 (Sit, Unpaired) x 6 (EBC session) repeated-measures ANOVA revealed significant main effects of both group and session, as well as a significant group x session interaction effect. Follow-up independent-samples t-tests comparing the two groups in each session revealed significant group differences in EBC sessions 1-3, and no group differences in EBC sessions 4-6. N = 7 rats sit, 9 rats unpaired.

Figure 3.1. PKM-ζ significantly decreases Kv1.2 Surface Expression.

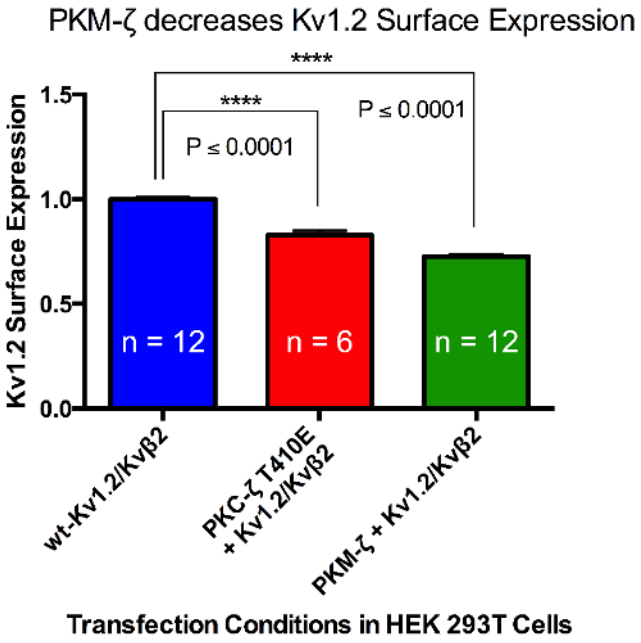


Figure 3.2. PKM-ζ's and Active-PKC-ζ's effects on Kv1.2 are partially lysine-dependent.

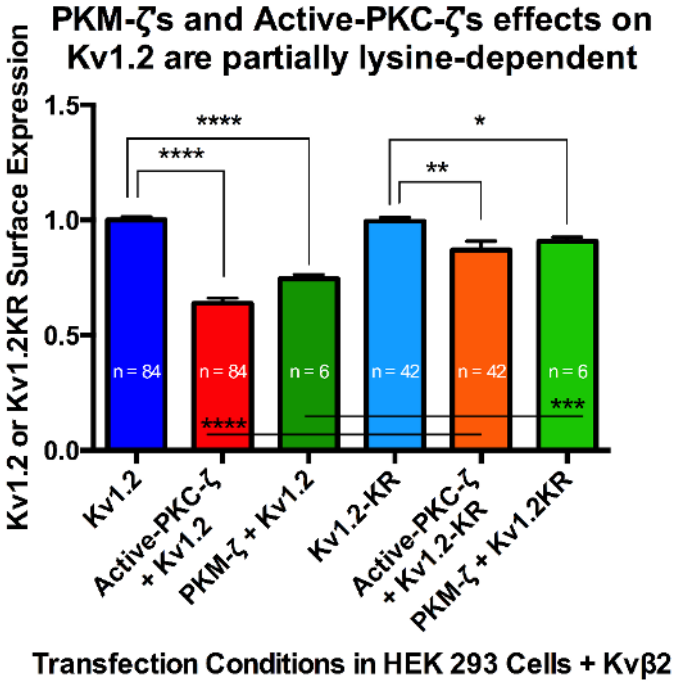


Figure 3.3. PKM-ζ's effect on Kv1.2 is independent of Kvβ2.

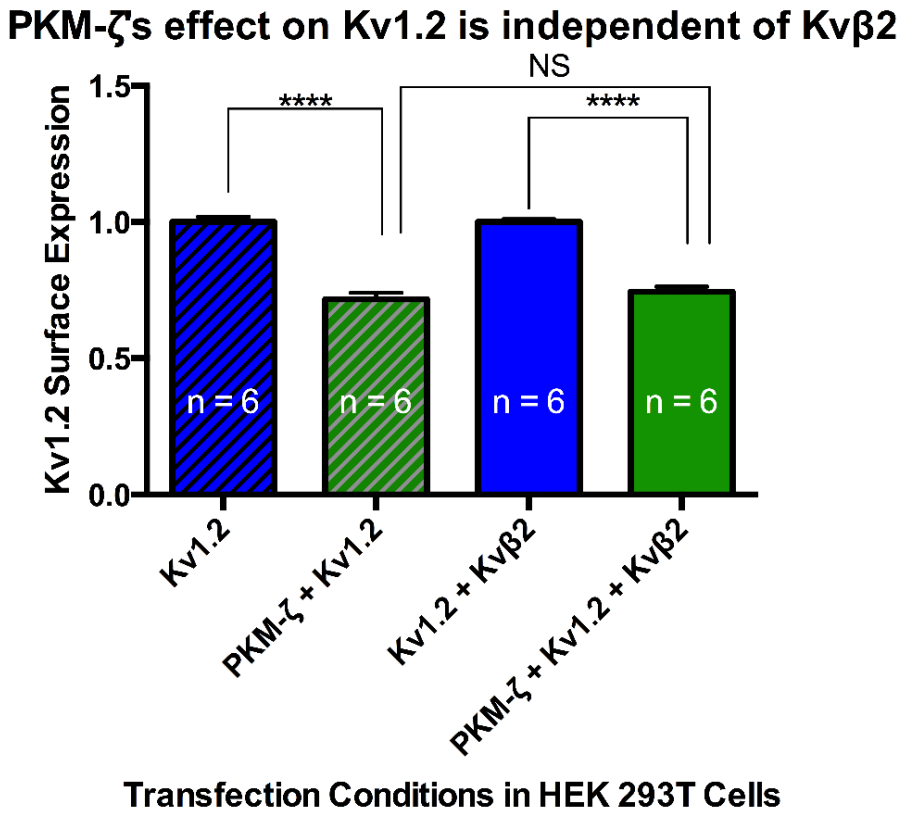


Figure 3.4. ZIP increases surface Kv1.2 in cerebellar live slices.

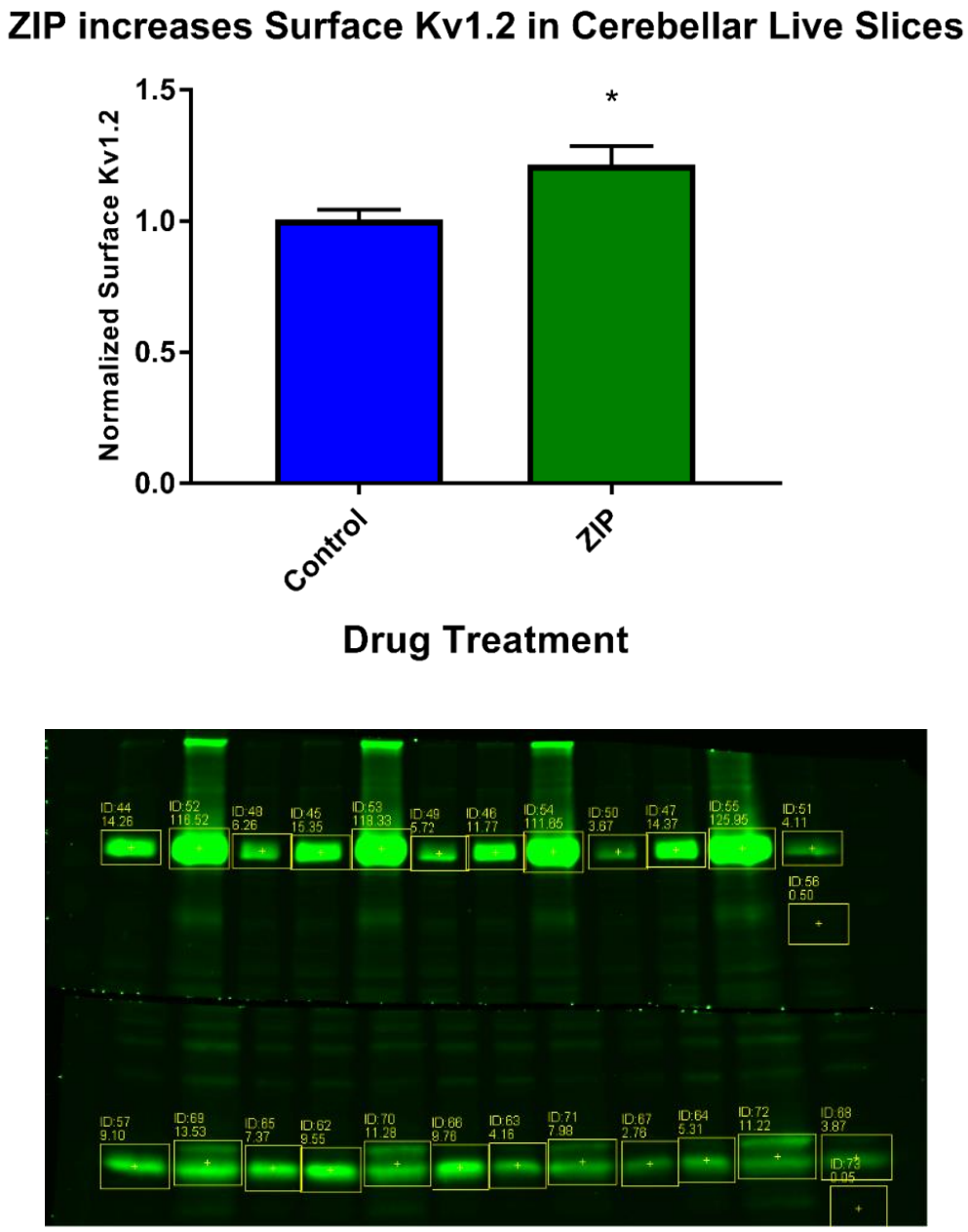
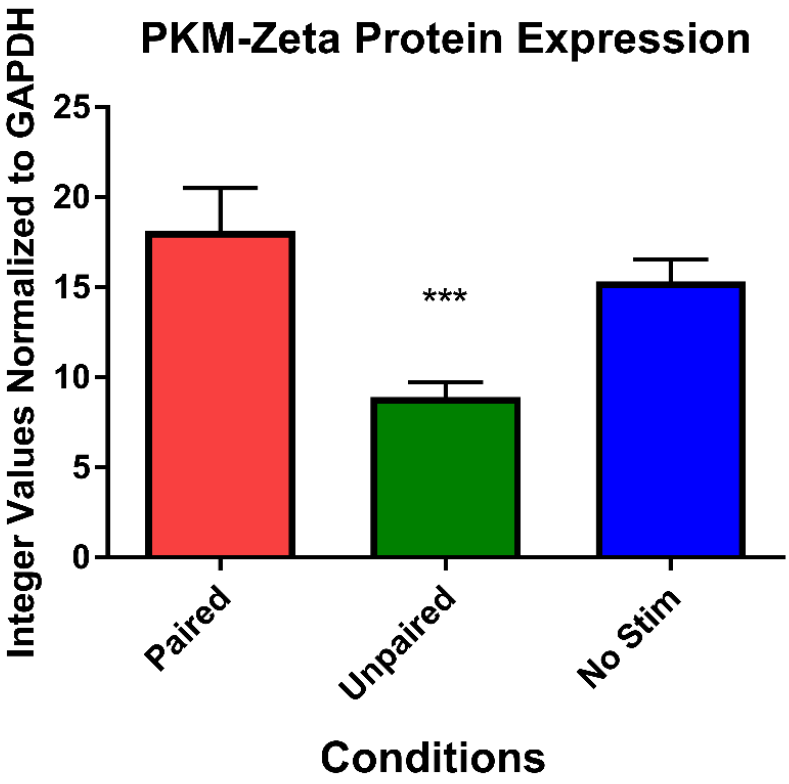


Figure 3.5. PKM-ζ protein expression significantly reduced in the unpaired group.



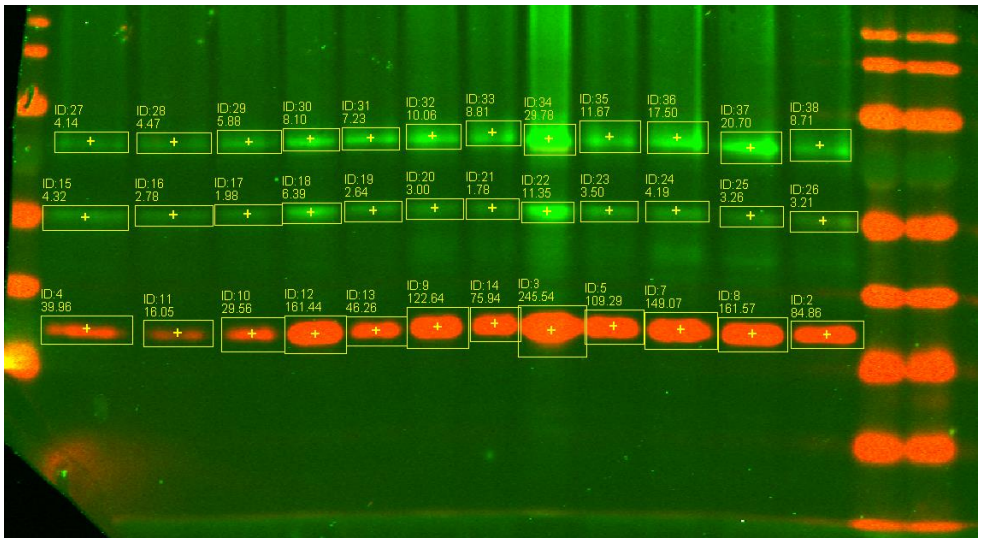


Figure 3.6. Phospho-410 PKC-ζ expression did not change between the groups.

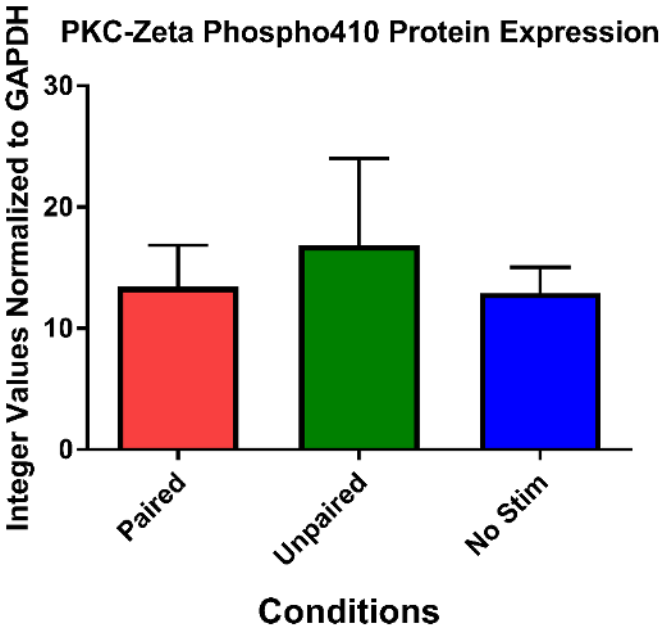


Figure 3.7. PKC-ζ expression did not change between the groups.

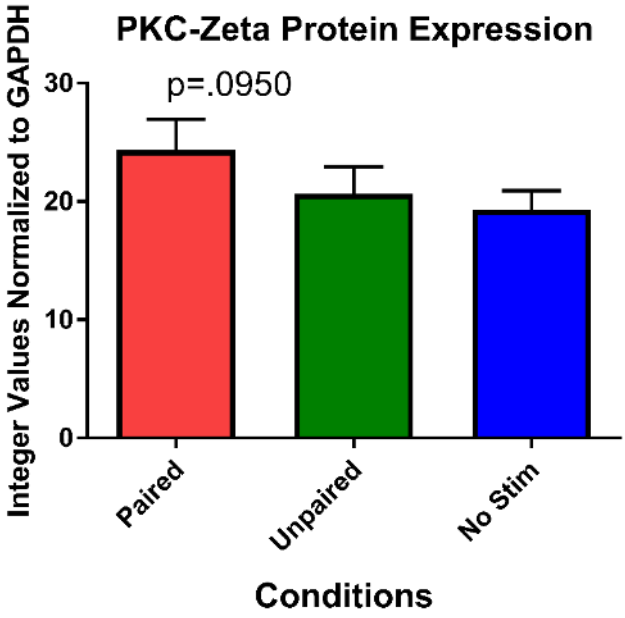
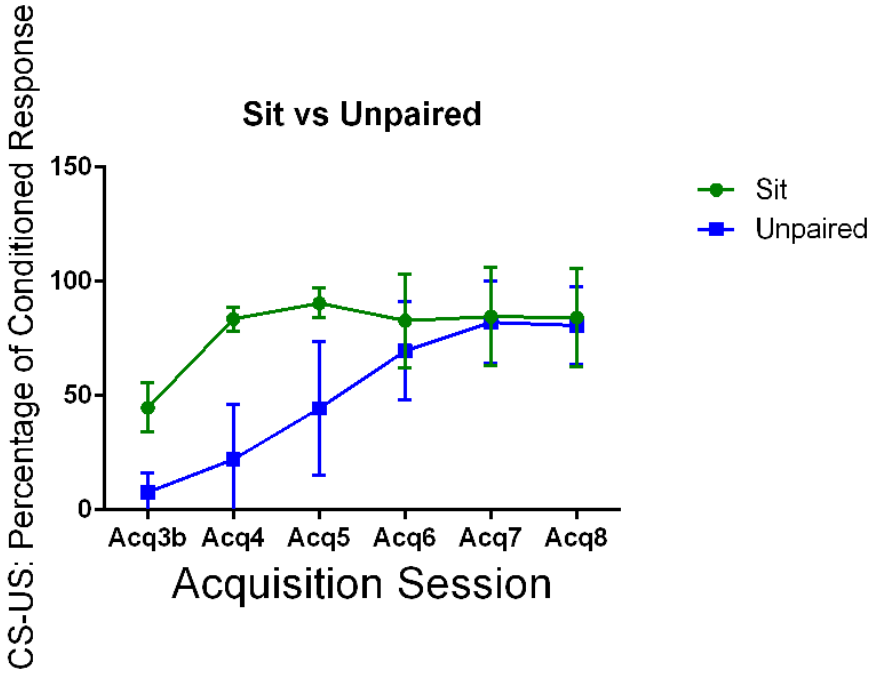


Figure 3.8. The Unpaired groups appears to exhibit behavioral inhibition.



CHAPTER 4: COMPREHENSIVE DISCUSSION

Overview

In the preceding papers, cerebellar eyeblink conditioning was linked to PKM-ζ dependent regulation of Kv1.2. Use of microscopy techniques determined that PKM-ζ is highly expressed in the cerebellar cortex, and by the use of pharmacological manipulations, it was found that PKM-ζ has an important role in regulating the acquisition of EBC. Through the use of biotinylation, flow cytometry, and behavioral manipulations, it was determined that PKM-ζ regulates Kv1.2 during eyeblink conditioning. In Chapter 2, the hypothesis that PKM-ζ was a regulator of eyeblink conditioning was tested. Using the PKM-ζ inhibitor ZIP in rats undergoing eyeblink conditioning, a model was developed that suggested the importance of PKM-ζ in eyeblink conditioning. In Chapter 3, the hypothesis that Kv1.2 regulation by PKM-ζ in eyeblink conditioning was tested. Collectively, this work demonstrates the first evidence of a role for PKM-ζ in the cerebellar cortex and demonstrates the first example that PKM-ζ regulates a voltage-gated ion channel, Kv1.2 in the mammalian nervous system. This discovery raises several questions as to the role of PKM-ζ in regulating Kv1.2 in eyeblink conditioning.

Regulation of PKC-ζ in EBC

A main question that arose out of this research concerns the role of PKC-ζ. While this research investigated the role of PKM-ζ primarily, the role of PKC-ζ was not

as easy to discern. We were not able to investigate the impact of only PKC-ζ on eyeblink conditioning as the pharmacological inhibitor ZIP can bind and inhibit both PKC-ζ and PKM-ζ. Due to the nature that PKM-ζ is a truncated form of PKC-ζ, it is not possible to pharmacologically inhibit one kinase without affecting the other.

Approaches that investigate PKC-ζ vs PKM-ζ must involve the use of knockout mice, something that was beyond the scope of this dissertation and our lab's capabilities. One technique we employed to gain a limited understanding of the role of PKC-ζ was through protein quantification of both PKC-ζ and Phospho-PKC-ζT410. As the regulation of PKM-ζ primarily occurs through upregulation or degradation, quantification of PKM-ζ protein expression is the logical choice for analyzing the downstream impacts of eyeblink conditioning. However, PKC-ζ is primarily regulated through phosphorylation, not protein expression. Therefore as expected, we did not see an impact on PKC-ζ protein expression level. While we did quantify phospho-PKC-ζT410, we could not determine any effect of eyeblink conditioning on this protein. Threonine 410 is the well-documented site of PKC-ζ activation and its mutation to Glutamate induces a constitutively active PKC-ζ (Chou et al., 1998). Research suggests that PKC-ζ T410E functions similarly to PKM-ζ, which is a constitutively active catalytic domain of PKCζ (Smith et al., 2002). One of the plasmids we used *in-vitro* in addition to PKM-ζ is an always-active PKC-ζ mutant T410E and we did find that PKC-ζ T410E behaved similarly to PKM-ζ in our *in-vitro* model.

One possibility to explain our inability to detect a change in PKC-ζ *in-vivo* is that PKC-ζ may be involved in a different, and possibly early, acquisition of EBC.

Therefore, by day three, when EBC had reached a plateau, it may be that the impact of PKC-ζ is no longer detectable and PKM-ζ had been substituted for PKC-ζ in maintenance of the memory trace. It is likely that an effect on PKC-ζ protein expression or phosphorylation of Thr-410 would be detectable earlier in the memory phase. It has been proposed that Thr-410 phosphorylation causes the release of the inhibitory pseudosubstrate domain from the active site and possibly facilitates the binding of the substrate proteins; substitution of Thr-410 with an alanine caused a substantial reduction of the PKC-ζ kinase activity, although it did not completely abolish the enzyme activity. (Standaert et al 2001)

Involvement of PKC-λ/ι

Our deductive reasoning suggested that PKC-ζ might be involved in early conditioning; this reasoning comes from the impact of a closely related PKC isoform, PKC-λ/ι, and its involvement in early LTP in the hippocampus. Studies have shown that in the hippocampus, PKC-λ/ι works to acquire early-phase LTP while PKM-ζ is responsible for maintenance of late-phase LTP. Knockouts of PKC-ζ/PKM-ζ have shown that PKC-λ/ι can upregulate and substitute for PKM-ζ (Selbie et al., 1993; Lee et al., 2013; Jalil et al., 2015). However, unlike the hippocampus, which has high expression of PKC-λ/ι and sparse expression of PKC-ζ, the cerebellum has high expression of PKC-ζ and sparse expression of PKC-λ/ι (Oster et al., 2004; Chihabi et al., 2016). This interesting yet important difference suggests to us a model in which we may consider

that PKC-ζ acts as a replacement for PKC-λ/ι in the cerebellum in regards to acquisition of early forms of cerebellar learning.

While PKM-ζ can compensate for PKC-λ/ι in early-LTP when PKC-λ/ι have been knocked out, the compensation is not perfect and fails when considering complex learning paradigms such as behaviorally intensive tasks involving fear conditioning or a more difficult Y-maze (Sheng et al., 2017 under review). This compensation has also been shown to work in reverse; knockouts of PKM-ζ have shown undisturbed memory consolidation and maintenance, possibly through compensation by PKC-λ/ι (Volk et al., 2013; Lee et al., 2013). To test this compensation further, recent studies have targeted PKM-ζ directly with PKM-ζ antisense oligodeoxynucleotides, and through a specific antagonist of PKC-λ/ι, ICAP (Tsokas et al., 2016). PKM-ζ antisense in hippocampus was shown to block late-LTP and spatial long-term memory in wild-type mice, but not in PKM-ζ-null mice without the target mRNA. In addition, use of the PKCι/λ-antagonist, ICAP, disrupted late-LTP and spatial memory in PKM-ζ-null mice but not in wild-type mice. As previously described, unlike the hippocampus which has relatively high expression of PKC-λ/ι and virtually no expression of PKC-ζ, expression of PKC-λ/ι in the cerebellum is virtually non-existent (Oster et al., 2004), and rather expression of PKC-ζ is very high. Therefore, in the case of the cerebellum, it is certainly plausible that PKC-ζ takes the role of PKC-λ/ι in early LTP maintenance in cerebellar learning. More interestingly, no studies on PKC-ζ in regards to learning exist, possibly owing to its relatively sparse expression in more prominently studied learning regions such as the hippocampus. However, given that PKC-ζ behaves similarly to PKM-ζ once activated,

and given that PKC-ζ and PKC-λ/ι are very similar in molecular composition, it would not be far-fetched to consider this hypothesis as plausible. Such an occurrence would explain our previously shown data in which ZIP infusions into the cerebellar cortex prior to EBC acquisition disrupts learning (Chihabi et al., 2016); our disruption of EBC may have been through disruption of PKC-ζ in early-phase acquisition of learning rather than through late-phase PKM-ζ maintenance of learning.

Kv1.2 and PKM-ζ

Through the advent of our recent work that provides for a mechanism of EBC dependent on PKM-ζ regulation of Kv1.2, it may be suggested that this kinase acts in a special role to properly maintain EBC in both an AMPA and Kv1.2 dependent manner. This inter-dependency of mechanisms is critical for the maintenance of complex memory models and it may be difficult to tease apart. Through the possibility of PKC-ζ maintaining early-phase learning and PKM-ζ maintain late-phase learning, several questions are posed. What is the inter-dependency between AMPA and Kv1.2 in regulation of EBC? If cerebellar PKM-ζ induces endocytosis of AMPA receptors at PC dendrites, it would provide for disinhibition of the IN and thus facilitation of EBC. Interestingly, our lab has suggested another possible role for Kv1.2 in PC dendrites; it may be that Kv1.2 suppression at PC dendrites itself facilitates LTD through an increase of dendritic excitability. Therefore PKM-ζ may have a dual role in facilitation of LTD through both Kv1.2 PC endocytosis and AMPA receptor endocytosis. In addition to LTD, another proposed mechanism for EBC suggests that LTP in the cerebellar cortex may be

crucial for learning (Grasselli & Hansel, 2014). Furthermore, knockouts of calcium/calmodulin-activated protein-phosphatase-2B (PP2B) in mice, which abolished LTP in PCs and their ability to increase their intrinsic excitability, resulted in impaired acquisition of delay EBC and impaired adaptation of vestibular-ocular reflex (Schonewille et al., 2010). Therefore, it is possible that PKM-ζ has a role in both cerebellar LTP and LTD in different locations of the cerebellar cortex. Furthermore, some observations in our lab have suggested that indeed, both regulation of AMPA receptors and Kv1.2, are occurring simultaneously (Madasu et al., 2015). If PKM-ζ indeed has a role in both AMPA receptor and Kv1.2 regulation, one might conceive of a temporal hypothesis for this mechanism.

Such a mechanism may be explained in part from our data in Chapter 3 in which we observed a reduction in cerebellar PKM-ζ in the unpaired group of rats. If indeed PKM-ζ is involved in regulating learning, we would have expected PKM-ζ expression to increase in the paired group, and the no stimulus control group. As we have noted, we saw no significant difference in PKM-ζ expression in the paired group, which might be due to the timing of when we were looking for changes (Figure 3.5). In this case, the experiment may have looked into the protein expression too soon in the learning paradigm and in which there may have not been enough time for memory consolidation to occur. Therefore, what we witnessed may have been a transition point of time in which PKC-ζ expression was essentially handing off maintenance of learning to PKM-ζ. Such a reasoning is plausible given our observed trend in which PKC-ζ expression increased in the paired group ($p = 0.095$) (Figure 3.7). An experimenter may wish to look earlier

and later than the current time point to shed light as to whether this was truly occurring, and given unlimited time and resources, it would be prudent to pursue such a hypothesis.

Yet, despite not seeing the intended effect in this snapshot of time, we did uncover some other unexpected findings that may provide us with an even deeper understanding of how PKM-ζ may be acting in the cerebellum during learning. As we previously described, we unexpectedly may have discovered a point in time in which behavioral inhibition was transitioning in the cerebellar cortex; that is, when the unpaired group had received new information about the CS when we switched the group to the paired paradigm, its learning was inhibited by the prior unpaired learning (Figure 3.8). Such a reference point in time would explain the results we have observed in Figures 3.5-3.8 in which we saw a reduction in PKM-ζ expression only in the unpaired group. For example, this reduction in PKM-ζ expression in the unpaired group coincided with a decrease in Kv1.2 in BC pinceaus and an increase in Kv1.2 in PC dendrites also in the unpaired group and not in the paired group (Fuchs et al., 2017., 2015). Such a finding would be consistent with our understanding of PKM-ζ's ability to reduce surface expression of Kv1.2, and therefore a reduction in PKM-ζ in the unpaired group would have led us to hypothesize an increase in Kv1.2 surface expression in PC dendrites, given that PKM-ζ is well expressed in the PC (Chihabi et al., 2016).

Furthermore, an increase in Kv1.2 in PC dendrites would be consistent with what is expected to occur in this model of behavioral inhibition; in this situation, it is a plausible to suggest that the rats are learning that the tone is a safety signal, knowing they will not receive a shock when the tone is on. However, unlike the rats in the paired group

who have learned that the tone predicts the shock, this type of behavioral inhibition is consistent with what is expected of the Rescorla-Wagner model of learning. Particularly, the rats receiving the explicitly unpaired stimuli, while understanding something about the CS, have not learned to positively correlate the CS-US as the groups receiving the paired stimuli have. Instead, they have learned a negative correlation between the CS-US; therefore, it is reasonable to suggest that this snapshot into the cerebellum of the explicitly unpaired rats is exposing the large molecular changes that are occurring in response to this negative correlative learning. The rats in the explicitly unpaired group are at the critical point in time where a sizeable decrease in PKM-ζ and increase in Kv1.2 is measureable, and are at the point in time of the Rescorla-Wagner model just prior to the point of acquisition. In particular, the Rescorla-Wagner model describes association formation but is silent on how these associations are translated into behavior. In other words, molecular changes have occurred to facilitate learning, but the negative correlation between the CS-US is preventing this learning from translating into any down-stream plasticity. Therefore we speculate that the paired group which has likely relied on PKM-ζ and Kv1.2 to acquire the CS and has then strengthened pons input to the IN and subsequent inhibition of the IO, the unpaired group has not made any downstream plasticity changes, and therefore the changes in the PC dendrites are still measurable. Therefore the rats that have received the paired stimuli are no longer reliant on the US when the CS is elicited, due to inhibition of the IO. Therefore, it is hypothesized, that the paired group by this 3rd day of learning is no longer reliant on this early-acquisition model of learning dependent on Kv1.2.

Involvement of AMPA and Kv1.2

One molecular mechanism that has dominated the field of cerebellar EBC for several decades is the involvement of AMPA receptors. The proposed mechanism for AMPA receptors in EBC involves LTD at the PC dendrites. Through stimulation of parallel fibers and climbing fibers, LTD through endocytosis of AMPA receptors decreases PC output, which allows for disinhibition of the interpositus nucleus (Freeman, 2015; Thompson & Steinmetz, 2009). However, this established model of cerebellar EBC has recently been challenged; disrupting AMPA receptor regulation and cerebellar LTD did not impair EBC (Schonewille et al., 2011). Undoubtedly, AMPA receptors are important for EBC; however, the established model needs revision in order to understand what compensation is occurring independently of AMPA receptors in maintaining and acquiring EBC. While our lab has focused efforts on Kv1.2, the kinase PKM-ζ may play an important role in connecting these two intricate models of learning.

Given this, it would be prudent for a future examiner to re-run such experiments with the addition of a 4th group of rats; in this case, a group of unpaired rats that are not explicitly unpaired, a truly random control. In other words, in this case the probability of the CS and US occurring together is roughly equivalent to the probability of them not occurring together. It would be predicted that in such an experiment, the "truly random" group of rats would not have undergone behavioral inhibition, and would not have any measurable change in PKM-ζ nor Kv1.2. Furthermore, future experiments should intricately examine loss of function vs gain of function; inhibiting PKM-ζ in cerebellar slices is different than over-expressing PKM-ζ in HEK 293 cells. Over-expression of

PKM-ζ in the cerebellar cortex for example my shed more light about to its functions in EBC when done in comparison with a PKM-ζ antisense infusion. While these gaps are certainly something to investigate, due to the preponderance of data, our conclusions in this work are reasonably consistent with our hypothesis.

Lipid Raft Hypothesis

Given the financial and experimental limitations of understanding the molecular basis of learning in an *in-vivo* model, our work pursued an *in-vitro* model that allowed us to look at the molecular interactions between the kinase and the channel. Our findings revealed that the Kvβ2 subunit is not necessary for PKC-ζ and PKM-ζ to affect Kv1.2 surface expression (Figure 3.3), despite the possible interaction between PKC-ζ and Kvβ2 (Gong et al., 1999; Croci et al., 2003). Therefore the mechanism of PKM-ζ on Kv1.2 must involve another interaction, possibly on the α subunit of Kv1.2 itself. In investigating this possibility, we discovered a potential mechanism that was beyond the scope of this dissertation, yet warrants further investigation. Prior studies have shown that PKC-ζ targets to lipid rafts and acts as a critical adaptor molecule to regulate lipid raft dynamics (Kanzaki et al., 2004; Shin et al., 2008). Our observations suggest that PKC-ζ/PKM-ζ might only induce endocytosis of Kv1.2 in a cholesterol-dependent manner when Kv1.2 is present in lipid rafts. In investigating the possible effect of Kvβ2, this observation was strengthened by our data which show that PKM-ζ in Kv1.2-KR has a significantly diminished effect in comparison to PKM-ζ in Kv1.2-wt in the presence vs the absence of Kvβ2 (Figure 3.2). Our preliminary reasoning for this data suggests that

the presence of Kv1.2 in lipid rafts, which may be dependent on mechanisms of ubiquitination (Cilento et al., 2015), can be altered, thus the perceived effect of PKM-ζ on Kv1.2 surface expression appears to be diminished.

Given more time and resources, such an investigation could compare the effect of PKM-ζ on Kv1.2 in the presence and absence of Kvβ2 with the use of the clathrin endocytosis inhibitor Pitstop-2, and the caveolae/cholesterol endocytosis inhibitor Filipin. For example, in one set of experiments, PKM-ζ can be tested with and without Pitstop-2. In such an experiment, we would hypothesize that to observe a larger decrease in Kv1.2 surface expression as compared without Pitstop-2 as the channel would be more likely to be present in lipid rafts. In another set of experiments, we would use Filipin and expect to see an abolished effect of PKM-ζ as the channel would not be able to be endocytosed by PKM-ζ when it is present outside lipid rafts. Such experiments can be repeated with and without Kvβ2 in order to determine if Kvβ2 is necessary for clathrin-dependent endocytosis as predicted previously. Furthermore, experiments that study the specific endocytosis pathway should be pursued; for example known sites of PKM-ζ phosphorylation on Kv1.2 can be used to generate a lenti-viral mutant Kv1.2 for such sites. Expression of this lenti-viral mutant Kv1.2 in the cerebellum, by using a promotor to target dendrites specifically if one existed for example, followed by a PKM-ζ antisense infusion and behavioral EBC paradigm can help answer and tease apart the importance of PKM-ζ on Kv1.2 from AMPA receptors. Further experiments can also study the effect of PKM-ζ overexpression in HEK293 cells on Kv1.2 degradation or recycling. Such

experiments would shed more light on the molecular mechanisms that might be occurring in the cerebellum, and therefore, may provide another regulatory pathway for which PKM-ζ may maintain learning without an observable measure in protein expression.

Beyond PKM-ζ

In this dissertation, the majority of our work was focused on what is occurring downstream of PKM-ζ. It would be prudent to also investigate what may be occurring upstream of this kinase. It has been well-established that LTD is dependent on mGluR1 but this mechanism is not well understood. The general understanding is that mGluR1 activates PDK1 which induces Ca2+ influx and subsequently activated PKC's that in turn phosphorylate S880 on AMPA receptors (Hou and Klann, 2004). Interestingly, DHPG, an mGluR1 agonist, is shown to increase hippocampal PKM-ζ mRNA expression (Sajikumar and Korte, 2011; Panaccione et al., 2013). Even further, DHPG-induced allodynia has been shown to be reversed by spinal administration of ZIP, further linking DHPG to PKM-ζ (Price et al., 2013). As previously stated, in hippocampal slices, DHPG application activates second messengers and PDK1 (Hou and Klann, 2004). Such a finding is critical for the link to PKM-ζ, as PKM-ζ mRNA requires second messengers to release its translational block (Kelly, Yao, Sondhi, and Sacktor, 2007; Muslimov et al., 2004). Furthermore, phosphorylation of PKM-ζ by PKD1 has been shown to convert the kinase into a conformation with high constitutive activity (Kelly, Crary, and Sacktor, 2007). Therefore, it is possible that PKM-ζ is the missing link between mGluR1 and AMPA receptor phosphorylation of S880. Such evidence that makes a hypothesis such

as this plausible is through work from our lab that has shown that DHPG enhances EBC, opposite of ZIP (Shipman, Madasu, Morielli, & Green, 2015). Furthermore, DHPG causes a reduction in Kv1.2 surface expression in HEK 293 cells, similar to PKM-ζ (Madasu et al., 2015). More interestingly, DHPG also decreases both Kv1.2 and AMPA receptor surface expression in biotinylated slices, further raising the question as to whether mGluR1 is upstream of PKM-ζ in the cerebellar cortex (Madasu et al., 2015). Such an investigation into the role of mGluR1 in relation to PKM-ζ would likely answer many unresolved questions.

Discussion Summary

By providing evidence that PKM-ζ regulates cerebellar Kv1.2, this manuscript provides support for the novel contention that cerebellar EBC may be involved in mechanisms independent of AMPA receptor regulation. In order to further assess the molecular mechanism(s) behind how PKM-ζ and PKC-ζ can reduce surface expression of Kv1.2, and the neurobehavioral temporal, and spatial implications in which this occurs, requires experiments that will supplement the pharmacological approaches utilized here. Manipulation of PKM-ζ and PKC-ζ with ZIP, while a substantiated method of pharmacologically inhibiting the kinase, raises multiple concerns as previously discussed in Chapter 2 of the limitations of the ZIP inhibitor. Direct manipulation of PKM-ζ and PKC-ζ with an optogenetic approach or with the new PKM-ζ antisense oligodeoxynucleotides, would allow for a fine-tuned and more direct approach to answering such questions. Another approach that may be taken involves specifically

looking at how cerebellar AMPA receptors may or may not be regulated by PKM-ζ and PKC-ζ during EBC, and more specifically, if such receptors are regulated by the kinase, asking if there is a spatial-temporal correlation between AMPA and Kv1.2 regulation by the kinase. Finally, the undertaking of work investigating the role of mGluR1 in relation to PKM-ζ would likely answer many unresolved questions about cerebellar learning.

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