The role and regulation of Protein Kinase Ms (PKMs) during the maintenance of synaptic facilitation in *Aplysia*

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ABSTRACT

Memory-induced changes in synaptic strength must be actively maintained in order to persist for long periods of time. Isoforms of the constitutively active protein kinase M (PKM)—the truncated form of protein kinase Cs (PKCs)—have been implicated in the maintenance of long-term changes in synaptic strength. Interfering with the activity of the mammalian zeta (ζ) isoform of PKM through pharmacological inhibition or dominant negative expression abolishes previously established memories. However, knockout of PKMζ does not impair memory. It has been proposed that other PKM isoforms may compensate for the loss of PKMζ in knockout animals, suggesting a possible role for additional PKM isoforms in memory maintenance. Indeed, different PKM isoforms are required for the maintenance of different forms of memory in the marine mollusc, *Aplysia californica*. Our work identifies a possible downstream target of PKMs in memory maintenance and characterizes the kidney-brain adaptor protein, KIBRA, as a stabilizer of PKMs crucial for determining PKM isoform specificity at synapses during different forms of memory in *Aplysia*.

At glutamatergic synapses, potentiation results in increased postsynaptic trafficking of AMPA receptors to the synapse. Evidence suggests a possible role for PKMs in preventing AMPA receptor endocytosis following synaptic potentiation, although substrates for PKM in this process remain elusive. Our first line of research utilizes a pH-sensitive GFP to quantify AMPA receptor trafficking in *Aplysia* sensory-motor neuron synaptic cultures. We identify the endocytic adaptor protein, Numb, as a candidate phosphorylation target of PKMs during the maintenance of long-term changes in synaptic strength and propose that PKMs maintain increases in synaptic strength through Numb-mediated inhibition of AMPA receptor endocytosis.

Our second line of research focusses on KIBRA as an upstream regulator of PKMs in *Aplysia*. We show that KIBRA stabilizes PKMs in an isoform-specific and activity-independent manner and identify a new domain on KIBRA important for this interaction. We identify a region on PKMs required for the differentiation of PKM isoforms by KIBRA. We propose that isoform specificity of PKMs in *Aplysia* synapses during different forms of synaptic facilitation is determined through selective stabilization by KIBRA and KIBRA splice variants.

The work presented in this thesis addresses important questions regarding both the role of PKMs during memory maintenance as well as the regulation of PKM activity, bringing us closer to understanding and defining the molecular processes underlying memory.

RÉSUMÉ

Les modifications de la force synaptique induites par la mémoire doivent être activement maintenues afin de persister pendant de longues périodes. Les isoformes de la protéine kinase M (PKM) constitutivement active - les formes tronquées de la protéine kinase C (PKC) - ont été impliquées dans le maintien de modifications à long terme de la force synaptique. Interférer avec l'activité de l'isoforme zêta (ζ) du PKM chez les mammifères par le biais d'une inhibition pharmacologique ou d'une expression négative dominante supprime les mémoires déjà établies. Cependant, la knock-out de PKMζ ne nuit pas à la mémoire. Les preuves suggèrent que d'autres isoformes de PKM pourraient compenser la perte de PKMζ chez les animaux knock-out, en proposant un rôle pour d'autres isoformes de PKM dans la maintenance de la mémoire. En effet, différentes isoformes de PKM sont nécessaires pour le maintien de différentes formes de mémoire dans le mollusque marin, *Aplysia californica*. Notre travail identifie une cible possible en aval des PKM dans la maintenance de la mémoire et caractérise la protéine adaptatrice rein-cerveau, KIBRA, en tant que stabilisateur des PKM essentiel pour déterminer la spécificité de l'isoforme de la PKM au niveau des synapses au cours des différentes formes de mémoire dans l'*Aplysia*.

Au niveau des synapses glutamatergiques, la potentialisation entraîne une augmentation du trafic post-synaptique des récepteurs AMPA vers la synapse. Les preuves suggèrent que les PKM pourraient jouer un rôle dans la prévention de l'endocytose des récepteurs AMPA après une potentialisation synaptique, bien que les substrats de la PKM dans ce processus restent insaisissables. Notre premier axe de recherche utilise une GFP sensible au pH pour quantifier le trafic de récepteurs AMPA dans les cultures synaptiques d'*Aplysia*. Nous identifions la protéine adaptatrice endocytaire, Numb, en tant que cible de phosphorylation candidate des PKM lors du

maintien de modifications à long terme de la force synaptique, et proposons que les PKM maintiennent des augmentations de la force synaptique par le biais d'une inhibition de l'endocytose des récepteurs AMPA induite par Numb.

Notre deuxième axe de recherche se concentre sur KIBRA en tant que régulateur en amont des PKM en *Aplysia*. Nous montrons que KIBRA stabilise les PKM d'une manière dépendante de l'isoforme et indépendante de l'activité et identifions un nouveau domaine sur KIBRA important pour cette interaction. Nous identifions une région sur les PKM requise pour la différenciation des isoformes de PKM par KIBRA. Nous proposons que la spécificité isoforme des PKM dans les synapses de l'*Aplysia* au cours de différentes formes de facilitation synaptique soit déterminée par stabilisation sélective par KIBRA et les variants d'épissure KIBRA.

Le travail présenté dans cette thèse aborde des questions importantes concernant à la fois le rôle des PKM lors de la maintenance de la mémoire et la régulation de l'activité des PKM, ce qui nous rapproche de la compréhension et de la définition des processus moléculaires de la mémoire.

PUBLICATIONS ARISING FROM THIS WORK AND CONTRIBUTION OF AUTHORS

Chapter Three:

DNA construct for ApGluR1-pHluorin was a gift from Robert D. Hawkins and Eric Kandel (Columbia University, New York). I optimized the pHluorin assay for use in *Aplysia* sensory-motor neuron cocultures and performed experiments contributing to Figure 3.3. Dr. Farah performed experiments contributing to Figure 3.3, performed the statistics, wrote methods pertaining to Figure 3.3, and assembled the figure and legend. Dr. Farah designed the model figure (Figure 3.4). Dr. Sossin quantified puncta fluorescence for all experiments. Figures 3.3 and 3.4 have been published in *A role for Numb in protein kinase M (PKM)-mediated increase in surface AMPA receptors during facilitation in* Aplysia, by Carole A. Farah, Tyler W. Dunn, Margaret H. Hastings, Larissa Ferguson, Cherry Gao, Katrina Gong, and Wayne S. Sossin (Journal of Neurochemistry, 2019).

Chapter Four:

Dr. Sossin performed all KIBRA and PKM bioinformatics contributing to Figures 4.1, 4.7, and 4.8. Xiaotang Fan made all PKM, PKC, and KIBRA constructs. I purified the KIBRA antibody and confirmed expression of KIBRA and KIBRA-AAA constructs in *Aplysia* sensory neurons. I performed all PKM stabilization assays and immunocytochemistry. Dr. Sossin performed image analysis and quantification. I performed electrophysiology experiments with technical help from Dr. Dunn.

Data contributing to Figures 4.1 and 4.2 has been published in *Selective erasure of distinct forms of long-term synaptic plasticity underlying different forms of memory in the same*

postsynaptic neuron, by Jiangyuan Hu, Larissa Ferguson, Kerry Adler, Carole A. Farah, Margaret H. Hastings, Wayne S. Sossin, and Samuel Schacher (Current Biology, 2017).

Figure 4.4 has been published in *The zinc fingers of the small optic lobes calpain bind polyubiquitin*, by Margaret H. Hastings, Alvin Qui, Congyao Zha, Carole A. Farah, Yacine Mahdid, Larissa Ferguson, and Wayne S. Sossin (Journal of Neurochemistry, 2018).

Data contributing to Figures 4.3, 4.5, 4.6, 4.7, and 4.8 will be published in *Isoform specificity of PKMs during long-term facilitation in* Aplysia *is mediated through stabilization by KIBRA*, by Larissa Ferguson, Jiangyuan Hu, Diancai Cai, Shanping Chen, Tyler W. Dunn, Kaycey Pearce, David L. Glanzman, Samuel Schacher, and Wayne S. Sossin (Journal of Neuroscience, in press).

LIST OF ABBREVIATIONS

5HT – serotonin (5-hydroxytryptamine)

AMPA receptor – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ApGluR1 – *Aplysia* Glutamate Receptor 1 (AMPA receptor subunit)

aPKC – atypical protein kinase C

ASW – artificial sea water

cAMP – cyclic adenosine monophosphate

DAG – diacylglycerol

DLC1 – dynein light chain 1

DN – dominant negative

DPF motif – aspartate-proline-phenylalanine motif

eGFP – enhanced green fluorescent protein

E-LTP – early LTP

EPSP – excitatory postsynaptic potential

GluA1 – glutamate receptor 1 subunit

GluA2 – glutamate receptor 2 subunit

His tag – polyhistidine tag

ICA-1 – 4-(5-amino-4-carbamoylimidazol-1-yl)-2,3-dihydroxycyclopentyl methyl dihydrogen phosphate

ITF – intermediate-term facilitation

KIBRA – Kidney Brain Protein

KSD – KIBRA Specific Domain

L-LTP – late LTP

Lats – large tumor suppressor protein

LTF – long-term facilitation

LTP – long-term potentiation

LTD – long-term depression

mITF – massed intermediate term facilitation

mRFP – monomeric red fluorescent protein

NMDA receptor – N-methyl-D-aspartate receptor

NPF motif – asparagine-proline-phenylalanine motif

Par3 – partitioning defective protein 3

PATJ – PALS1-associated tight junction protein

PBS – phosphate-buffered saline

pHluorin – superecliptic pH-sensitive GFP

PKM Apl I – Protein kinase M Aplysia I

PKM Apl II – Protein kinase M Aplysia II

PKM Apl III – Protein kinase M Aplysia III

PKMζ – Protein kinase M zeta (mammalian atypical)

PKC – Protein kinase C

PTB – phosphotyrosine-binding domain

ROCK – Rho-associated protein kinase

Sf9 – *Spodoptera frugiperda*

SNX4 – sorting nexin 4

SOL calpain – small optic lobe calpain

WWC1 – WW-domain containing protein 1 (KIBRA)

WWC2 – WW-domain containing protein 2

WWC3 – WW-domain containing protein 3

ζ-stat – 8-hydroxy-1,3,6-naphthalenetrisulfonic acid

ZIP – myristoylated PKCζ pseudosubstrate inhibitor

CHAPTER ONE

General Introduction

1.1 The molecular memory trace

Understanding the molecular mechanisms underlying memory formation and storage is one of the key problems facing modern neurobiologists. The process of learning involves the induction of distinct physical and biochemical changes within neurons that affects both the strength of their synaptic connections and their pattern of connectivity. The idea that enhanced communication between neurons could be a mechanism of memory storage was first proposed by Donald Hebb over fifty years ago, a postulate that was later epitomized by the famous phrase, "neurons that fire together, wire together" (Hebb, 1949; Lowel and Singer, 1992). Building on this fundamental idea, researchers have focused on identifying and characterizing the neuronal circuits implicated in memory storage. Artificial activation of the neuronal assemblies underlying specific memories is sufficient to induce recall of that memory in the absence of recall-inducing stimuli (Liu et al., 2012). Similarly, disrupting the activity of a memory's underlying circuitry is sufficient to disrupt the memory (Denny et al., 2014; Tanaka et al., 2014). The neuronal assemblies that make up a memory are commonly called *memory engrams* within the field. Although this term implies permanence, the molecular mechanisms underling memory storage within these ensembles remains unknown. What changes occur within neurons to allocate them to a neuronal ensemble during memory formation? Are these molecular changes permanent, or do they require constant maintenance? Certainly, the most convincing argument for an active mechanism underlying the long-term maintenance of memory comes from the fact that pharmacological agents targeting specific proteins upregulated during memory formation are able to erase said memories (Li et al., 2011; Madronal et al., 2010; Pastalkova et al., 2006;

Sanhueza et al., 2007; Serrano et al., 2005). But the identity of the physical substrates of memory—the molecular traces that differentiate neurons incorporated into memory ensembles from those that are not—remains unclear. The lability of memory has allowed us to speculate on the nature of these molecular memory traces and identify possible candidate proteins that fill this role to allow for the long-term storage of a memory.

1.1.1 The reconsolidation theory of memory

Memories must be stabilized in a process known as consolidation in order to be converted into long-term storage. The time-dependency of this process was established early in the 20th century when it was demonstrated that acquisition of a second memory retroactively interferes with consolidation of the first memory if presented within a short time period (Dewar et al., 2007; McGaugh, 1966; Muller and Pilzecker, 1900). The short-term retrograde amnesia observed in humans following electroconvulsive shock therapy was similarly shown to be due to interference with the consolidation of recently acquired memories (Duncan, 1949). Protein synthesis inhibitors can also disrupt memory consolidation (Flexner et al., 1962; Hernandez and Abel, 2008)—indeed, a dependence on protein synthesis is one of the key differences between short-term and long-term memory mechanisms. While short-term memories are inherently labile and transient, memories that have undergone consolidation are resilient to disruption and can persist for long periods of time (McGaugh, 1966). Because protein synthesis inhibitors are not able to disrupt memory after it has been consolidated, it was originally assumed that the process of consolidation resulted in a permanent change within the neurons encoding the memory. However, treatments that disrupt memory when applied before consolidation also disrupt memory when applied during memory retrieval (Misanin et al., 1968; Sara, 2000), suggesting that previously stable memories become unstable during recall and must be reconsolidated to

regain their previous stability. Infusion of the protein synthesis inhibitor anisomysin into the amygdala during retrieval of a fear memory is sufficient to disrupt the memory, despite the fact that the memory is resistant to such manipulations prior to recall (Nader et al., 2000). The process of reconsolidation required for memories to be converted back into a stable state after recall is similar to the process underlying initial consolidation. The reconsolidation theory of memory also provides a mechanism for updating previously established memories with new information (Jones et al., 2012), and provides an explanation for the phenomenon of "false memories" observed in humans (Hupbach et al., 2007).

Because even stable memories are converted to a labile state during reactivation, it suggests that the protein synthesis-dependent changes that occur during consolidation and reconsolidation do not produce permanent alterations in neuronal structure. Rather, consolidation of a memory into long-term storage involves the activation of cellular processes that actively maintain memory-induced changes in synaptic strength throughout the course of the memory's lifetime. Francis Crick originally proposed that the ability of memories to outlast the rate of molecular turnover could be due to a mechanism of constant maintenance, likely by an unknown protein positioned at the memory synapse (Crick, 1984). In order to understand how these potential memory traces might function to maintain memory at the synapse, it's necessary to first understand the cellular and molecular processes that underly memory storage.

1.1.2 Mechanisms of synaptic plasticity

While neurogenesis (Jobe and Zhao, 2017; Kitabatake et al., 2007) and synaptogenesis (Maletic-Savatic et al., 1999; Parajuli et al., 2017) are viable theories for how new memories are formed in the brain, the prevailing hypothesis for the mechanisms of memory storage in the field today involves memory-induced changes in the strength of synapses. Specifically, synaptic

strength can increase or decrease in response to specific patterns of input, a change that is thought to occur during learning and to be maintained for the lifetime of the memory. The strength of chemical synapses can be persistently increased [resulting in a long-term potentiation (LTP) of the synapse] or persistently decreased [resulting in a long-term depression (LTD) of the synapse]. These opposing mechanisms of synaptic plasticity usually depend on presynaptic input (in the case of homosynaptic plasticity), but can also occur via heterosynaptic mechanisms—indeed, homosynaptic and heterosynaptic plasticity appear to be complementary processes (Chistiakova and Volgushev, 2009). LTP in particular is considered one of the main cellular mechanisms underlying learning and memory and involves a persistent strengthening of excitatory synapses.

Early evidence demonstrating LTP in rabbits showed that repetitive stimulation of perforant path fibres is sufficient to increase response from dentate gyrus granule cells (Bliss and Lomo, 1973). Two possible mechanisms for this increased efficiency in synaptic transmission were described—increased perforant path activity, and/or increased excitability of the downstream granule cells. The relative importance of presynaptic and postsynaptic mechanisms in LTP is a widely discussed issue (Lisman, 2009), although it is likely that they work in tandem to increase synaptic strength from both sides of the synapse.

While synchronous pre- and postsynaptic firing at glutamatergic synapses can lead to transcription-dependent, persistent increases in synaptic strength, the mechanisms for how this occurs are still not fully understood. NMDA receptors act as coincidence detectors on the postsynaptic neuron, allowing calcium influx only when the receptor detects presynaptic neurotransmitter and postsynaptic depolarization (Mayer et al., 1984; Nowak et al., 1984). This calcium influx is required for LTP induction (Brocher et al., 1992; Lynch et al., 1983). Increased

intracellular calcium also plays a role in LTD induction, likely through voltage-dependent calcium channels, transient receptor potential (TRP) channels, and/or the release of intracellular calcium stores signalled through the activation of metabotropic glutamate receptors or NMDA receptors (Brocher et al., 1992; Jo et al., 2008). Because LTP induction relies on calcium increases postsynaptically, any presynaptic contribution would likely rely on retrograde signalling to trigger persistent increases in presynaptic neurotransmitter release (Regehr et al., 2009).

Postsynaptically, increased intracellular calcium activates a variety of signalling cascades through the activity of calcium binding proteins, such as calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) (Lisman et al., 2012). There are two temporally distinct forms of LTP—early LTP (E-LTP) and late LTP (L-LTP). E-LTP is protein-synthesis independent and decays within 1-2 hours, while L-LTP requires protein synthesis and persists for at least eight hours (Huang, 1998). The short-term potentiation characterizing E-LTP is often associated with short-term memory and can be induced with weaker induction protocols. Induction of LTP at glutamatergic synapses results in an upregulation of postsynaptic α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors (Hayashi et al., 2000), increasing the neuron's ability to respond to presynaptic glutamate release. However, while LTP induction involves the synaptic insertion of AMPA receptors containing glutamate receptor 1 (GluA1) subunits, LTP maintenance requires a shift to predominantly glutamate receptor 2 (GluA2) subunit-containing AMPA receptors in the synapse (Plant et al., 2006). Inhibition of proteins upregulated during L-LTP that are believed to be responsible for LTP maintenance is sufficient to convert L-LTP into the decaying E-LTP (Hardt et al., 2014; Sacktor, 2012). As such, while E-

LTP is the root of short-term memory, L-LTP is considered the underlying synaptic mechanism of long-term memory.

The mechanisms of AMPA receptor localization to the synapse during LTP induction are not yet clear, nor is it understood how this increased excitability of the synapse is maintained for long periods of time. As mentioned previously, it is likely that the changes induced during potentiation of the synapse require an active mechanism of maintenance in order to persist, and persistently active kinases are one of the most promising candidate mechanisms for how this might occur.

The ability to induce long-term changes in synaptic strength is a fundamental neurological process that can be observed throughout evolution, from the simple nervous system of the roundworm to the complex brain of a human. While the synapses in human brains are only now beginning to be able to be studied with our current level of technology, the synapses of less complex organisms are much more accessible and provide researchers with a convenient means of studying the molecular processes underlying memory.

1.2 Using the invertebrate Aplysia californica to study memory

From an evolutionary standpoint, it is beneficial for animals to be able to change their behaviour based on past experiences—indeed, learning and memory are highly conserved processes that can be observed in nearly every organism that contains a nervous system. Even organisms lacking nervous systems (for example, sponges) express homologs of proteins that are found in mammalian synapses and express rudimentary forms of learning and behavioural coordination via non-neuronal cell-cell communication (Sakarya et al., 2007). However, the

human brain—the organ that all neuroscientists ultimately strive to understand—is orders of magnitude more complex than the nervous systems of lower organisms. But while the complexity differs, the underlying mechanisms share fundamental similarities. The nervous systems of all animals contain neurons, and those neurons communicate with one another via synapses. In studying the processes that influence this communication, it is often beneficial to utilize reductionist model systems due to their accessibility, the ease with which they can be manipulated, and their relative simplicity, allowing for a more focused investigation into fundamental processes like learning and memory.

1.2.1 Advantages of the Aplysia system

The marine mollusk *Aplysia californica* is ideally suited to studying synapses at the molecular level for a number of reasons. Firstly, *Aplysia* neurons are exceptionally large (up to 1 mm in diameter), which makes them suitable for a variety of physical and biochemical manipulations. It has been speculated that this large size may be a result of their polyploid nature or that, like the giant squid axon, large neuron size may facilitate information transfer, helping to improve the animal's response time to stressful stimuli (Moroz, 2011). The nervous system of *Aplysia* is also relatively small, containing around 20 000 neurons within five pairs of ganglia. Most of these neurons are easily accessible and—due to their differing colours, sizes, and location within the animal—easily identifiable. *Aplysia* also have the capacity to form memories, another feature that makes them attractive model organisms in neuroscience. Specifically, the *Aplysia* defensive reflex of gill withdrawal can form both short- and long-term memories through habituation, sensitization, and classical conditioning (Hawkins et al., 1998). Dissociating the synapses involved in this reflex and reconstituting them in culture provides a convenient avenue for studying the synaptic mechanisms underlying these types of learning.

1.2.2 The Aplysia gill-withdrawal reflex

The defensive gill-withdrawal reflex of *Aplysia* offers a unique system for studying synaptic plasticity. Tactile stimulation of the animal's siphon activates a sensory neuron which directly innervates a motor neuron that causes the gill to defensively contract and withdraw. The reflex can be habituated by repeated sensory stimulation of the siphon, resulting in decreased postsynaptic firing and a less robust defensive response (Castellucci et al., 1970). Application of a strong stimulation (such as a shock) to the animal's tail will sensitize the reflex, producing a stronger gill withdrawal even in response to previously innocuous sensory stimulation. A single shock to the tail results in sensitization that lasts for minutes—repeated tail shocks result in sensitization that can persist for weeks (Frost et al., 1985; Pinsker et al., 1973). Additionally, classical conditioning can be observed when a weak stimulus to the siphon that doesn't normally induce a response (conditioned stimulus) is paired with a strong tail shock (unconditioned stimulus) (Hawkins, 1984). Each of these types of learning are mediated by the change in strength of the synapse between the sensory and motor neuron underlying the reflex. As mentioned previously, these neurons can be dissociated from the animal and, when allowed to grow in culture, will reform synapses with one another (Rayport and Schacher, 1986). *Aplysia* neurons have synapse specificity and will preferentially form synapses with specific partners—this precludes the possibility that the cultured sensory neurons will form synapses with themselves, with other nearby sensory neurons, or with non-specific motor neurons, making these synaptic cultures ideal mediums for studying synaptic plasticity *in vitro* (Glanzman et al., 1989a).

While the gill-withdrawal reflex is largely controlled by this monosynaptic circuit, the various forms of learning discussed above involve the activity of interneurons that release factors

onto the synapse. The majority of learning within this reflex is mediated by serotonin (5-HT) released in the vicinity of the synapse (Glanzman et al., 1989b)—correspondingly, application of 5-HT to sensory motor neuron synapses in culture can mimic these forms of learning *in vitro*. The sensitization response seen following tail shock can be replicated in synaptic cocultures with 5-HT application: a single burst of 5-HT produces a short-term increase in synaptic strength lasting minutes [called short-term facilitation (STF)], while repeated applications of 5-HT produce an increase in synaptic strength that can persist for up to 1.5 hours [intermediate-term facilitation (ITF)] (Mauelshagen et al., 1996) or more than 24 hours [long-term facilitation (LTF)] (Montarolo et al., 1986; Schacher et al., 1990). STF occurs independent of protein synthesis and involves presynaptic mechanisms of potentiation (Byrne and Kandel, 1996; Klein, 1994). 5-HT increases presynaptic cyclic AMP (cAMP) levels, which transiently activates cAMP-dependent protein kinase A (PKA) and results in an increase in presynaptic neurotransmitter release (Byrne and Kandel, 1996; Castellucci et al., 1980; Castellucci et al., 1982; Klein, 1994). Postsynaptically, 5-HT increases intracellular calcium levels, ultimately affecting the concentration of neurotransmitter receptors at the synapse (Chitwood et al., 2001). Unlike STF, both ITF and LTF require the synthesis of new proteins. Translational blockers prevent the induction of ITF (Ghirardi et al., 1995; Mauelshagen et al., 1996), while inhibition of transcription has no effect. LTF, on the other hand, is sensitive to both transcriptional and translational inhibition (Montarolo et al., 1986).

Presynaptic activity can influence the expression of both ITF and LTF. Since the activity of both PKA and PKC is increased by the presence of calcium (Abrams et al., 1991; Sossin and Schwartz, 1992), adding sensory neuron firing (which increases calcium levels) to the protocol of 5-HT application prolongs facilitation, resulting in a type of facilitation called activity-

dependent ITF (a-ITF) (Sutton and Carew, 2000). a-ITF can be induced, therefore, with only a single pulse of 5-HT, which alone is not sufficient to induce ITF, and does not require protein synthesis. On the other hand, activity-independent ITF can be induced with spaced applications of 5-HT and does require the synthesis of new proteins (Sutton and Carew, 2000). Massed application of 5-HT can also induce a persistent form of facilitation called massed ITF (m-ITF). m-ITF requires protein synthesis and results in an enhanced excitability of the postsynaptic neuron (Villareal et al., 2007). While the mechanisms inducing and underlying these types of ITF differ, the resulting facilitation is temporally indistinct. LTF can be similarly categorized into two different types: associative LTF and non-associative LTF, both of which are transcription-dependent, persist for more than 24 hours, and require activation of mitogen-activated protein kinase (MAPK), PKA, and PKC for induction (Hu et al., 2004; Liu et al., 2004; Villareal et al., 2009). Associative LTF can be induced by 5-HT application in combination with presynaptic activity and is a cellular analogue of classical conditioning (Carew et al., 1981). Non-associative LTF can be induced by spaced 5-HT application in the absence of presynaptic action potential firing and is analogous to long-term sensitization (Castellucci et al., 1978; Montarolo et al., 1986). Applying these stimuli on two consecutive days results in LTF that persists for more than a week in culture (Hu et al., 2011).

While the mechanisms of induction for each of these types of facilitation have been extensively studied, the mechanisms involved in the maintenance of facilitation in the *Aplysia* gill withdrawal reflex are still not fully understood. However, due to the ease with which synapses can be cultured and studied using this model system, research into potential maintenance mechanisms has been making strides in recent years, particularly concerning the role of persistently active kinases.

1.3 The role of persistently active kinases in memory maintenance

Protein phosphorylation plays an important role in LTP. However, while inhibitors of kinases such as PKA (Blitzer et al., 1995; Otmakhova et al., 2000), PKC (Huang et al., 1992; Malinow et al., 1989), and CaMKII (Buard et al., 2010; Chang et al., 2017; Chen et al., 2001; Malinow et al., 1989) are sufficient to block LTP induction, they have no effect on established LTP (Yang et al., 2004). This suggests that the mechanisms of LTP induction are distinct from those of LTP maintenance. Long-lasting memories are upheld through a dynamic process of ongoing maintenance that involves the regular turnover of synaptic receptors (Wang et al., 2006), and persistently active kinases provide one possible mechanism for how this may be achieved (Crick, 1984). Autophosphorylation of CaMKII produces a persistently active form of the kinase and the activation of this autophosphorylation cascade was one of the first theories for how LTP maintenance may occur (Lisman, 1985). A similar theory involving production of a persistently active form of PKA through degradation of PKA's autoinhibitory region has also been described and appears to be important for the maintenance of ITF in *Aplysia* (Hegde et al., 1993; Schwartz and Greenberg, 1987). Since the discovery in the early 1990s that levels of the persistently active kinase, protein kinase M (PKM), are upregulated during the maintenance phase of LTP (Sacktor et al., 1993), recent work has focused on these kinases and their potential role in maintaining the persistent increases in synaptic strength induced during LTP.

1.3.1 PKMs, the persistently active catalytic fragments of PKCs

All isoforms of PKC—from which the various PKM isoforms are derived—contain an N-terminal regulatory domain connected via a hinge region to the C-terminal catalytic domain. The pseudosubstrate region on the regulatory domain controls the activity of the PKCs—when the pseudosubstrate is blocking the active site of the catalytic domain, the PKC is inactive and is

unable to phosphorylate its substrates. The kinase undergoes a conformational change via the hinge region in response to the appropriate signals, removing the pseudosubstrate block and freeing the active site to interact with its substrates (Sacktor, 2012). PKCs are classified into three families—conventional, novel, and atypical (PKC Apl I, PKC Apl II, and PKC Apl III in *Aplysia*)—which differ mainly through the structure of their regulatory domain (Sossin, 2007). Conventional PKCs are sensitive to the second messenger activators calcium and diacylglycerol (DAG); novel PKCs are insensitive to calcium, but sensitive to DAG; atypical PKCs are insensitive to both calcium and DAG (Zhang et al., 2016). However, this regulation is not present in PKMs.

PKMs are the truncated forms of PKCs—they contain the catalytic domain and part of the hinge region, but not the regulatory domain (Hernandez et al., 2003). Consequently, because they are not sensitive to the same activation/inactivation triggers as their parent PKCs and do not have a pseudosubstrate sequence, they have no means of autoinhibition and are enzymatically active upon formation. The gene encoding the vertebrate zeta (ζ) isoform of PKC contains an internal promoter that allows for transcription of an mRNA encoding only the catalytic domain of the PKC. The catalytic fragment produced by translation of this mRNA is the constitutively active PKM. Levels of this PKM isoform can be regulated in vertebrates by translational repression of the alternatively transcribed mRNA, a block that is removed following LTP to allow for PKM upregulation (Hernandez et al., 2003). This internal promoter is not present in other PKC genes and seems to be specific for the zeta isoform. While direct evidence for the endogenous formation of other PKM isoforms has not yet been found, they are likely formed through cleavage of their parent PKC. In *Ayplsia*, PKMs can be formed through cleavage of fully formed PKC proteins. Invertebrate atypical PKC genes do not contain the alternative promoter or

the conserved methionine required to begin translation of the PKM protein—invertebrate PKMs are instead produced through cleavage by the protein calpain (Bougie et al., 2009). The PKMs produced through cleavage by calpains seem to induce activation of additional calpains, resulting in a positive feedback loop (Bougie et al., 2012). While calpain-mediated cleavage of PKCs into PKMs has not yet been shown to occur in vertebrates during the induction of synaptic plasticity, it is possible that this method of PKM formation occurs in tandem with translation of PKMζ mRNA in vertebrates.

While there are many isoforms of PKMs—classified based on the family of PKC they are derived from—most of the focus in the memory field has been on the role of the mammalian atypical PKMζ.

1.3.2 The putative memory maintenance molecule, PKMζ

The ζ isoform is from the atypical family of PKMs, derived from the atypical PKCζ. Early research identified this isoform in particular as a candidate molecule required for the maintenance of LTP, and subsequent research has both supported and contested this assertion.

PKMζ levels are elevated shortly after learning in various brain areas, including the prefrontal cortex (Wang et al., 2014), sensorimotor cortex (Gao et al., 2018), insular cortex (Shema et al., 2011), and hippocampus (Hsieh et al., 2017; Li et al., 2014). PKMζ levels are increased in hippocampal slices after LTP induction (Osten et al., 1996; Sacktor et al., 1993) as well as in cultured neurons following chemically-induced LTP (Palida et al., 2015). It has also been shown that PKMζ is highly expressed in the dendritic postsynaptic region (Hernandez et al., 2014). Correspondingly, overexpression of PKMζ *in vivo* is sufficient to improve memory in rodents. For example, hippocampal overexpression of PKMζ improves contextual fear memory

(Schuette et al., 2016). Overexpression in the insular cortex improves performance in a conditioned taste aversion task (Shema et al., 2011). Auditory fear memory can be improved by overexpressing PKMζ in the prelimbic cortex (Xue et al., 2015). This memory enhancement effect was also observed in the fruit fly, *Drosophila melanogaster*, where expression of the rodent PKMζ gene enhanced memory in an olfactory learning task (Drier et al., 2002). At the level of the synapse, injection of PKMζ into CA1 hippocampal neurons is sufficient to enhance synaptic strength (Ling et al., 2006; Ling et al., 2002).

An upregulation of PKMζ is observed during LTP maintenance but not during LTP induction (Sacktor et al., 1993), suggesting that PKMζ is specifically involved in the maintenance of LTP. Similarly, blocking PKMζ activity negatively affects both synaptic potentiation as well as memory maintenance and retention *in vivo*. Dominant-negative PKMζ reverses established LTP when overexpressed in rodent hippocampal slices (Ling et al., 2002), as well as abolishing established memory when overexpressed *in vivo* in the insular cortex (Shema et al., 2011). Additionally, pharmacological inhibition of PKMζ interferes with memory and hippocampal LTP. The myristoylated PKCζ pseudosubstrate inhibitor (ZIP) mimics the pseudosubstrate of the atypical PKC (aPKC) regulatory domain and is able to block the active site of PKMζ, inhibiting its kinase activity. Injection of ZIP into the rodent hippocampus abolishes established spatial memories *in vivo* (Pastalkova et al., 2006). Similar effects are observed with injection of ZIP into the insular cortex in conditioned taste aversion (Shema et al., 2007), the sensorimotor cortex in procedural memory tasks (von Kraus et al., 2010), the amygdala in contextual fear conditioning (Kwapis et al., 2009), and even the nucleus accumbens core in the case of drug memory (Crespo et al., 2012). Furthermore, bath application of ZIP to hippocampal slices is sufficient to abolish established increases in synaptic strength (Ling et al.,

2002; Sajikumar et al., 2005; Serrano et al., 2005). Indeed, most evidence for PKMζ's role in memory maintenance stems from research showing that pharmacological inhibition by ZIP abolishes memory, which is why ZIP's ability to abolish memory in PKMζ knockout animals sparked debate on whether or not previous evidence for PKMζ's role in memory maintenance could be trusted.

By excising exon 11 of the PKCζ catalytic domain, Volk et al. (2013) produced complete PKCζ/PKMζ knockout animals that were both viable, fertile, and anatomically normal. Behaviourally, knockout animals performed just as well as wild-type controls in tasks testing spatial reference memory and those testing classical associative conditioning, both of which are hippocampal-related memories that have previously been shown to require PKMζ activity for their maintenance (Pastalkova et al., 2006; Serrano et al., 2008). Similarly, PKCζ/PKMζ knockout had no effect on the induction or maintenance of LTP in hippocampal slices. Neither LTP induced via theta-burst stimulation nor that induced by high-frequency stimulation was affected by the knockout. To limit compensatory effects, Volk et al. (2013) also generated conditional knockout animals in which PKMζ knockout could be induced during adulthood. Although this conditional knockout resulted in ~80% reduction in PKMζ in the hippocampus, LTP remained unaffected. The final blow against PKMζ came when they showed that application of ZIP reversed LTP in acute hippocampal slices from both wildtype and PKCζ/PKMζ knockouts, despite the fact that ZIP was previously asserted to be a specific inhibitor for PKMζ (Ling et al., 2002). While this certainly sheds doubt on the validity of previous experiments that drew conclusions based on ZIP's effects on LTP and memory, it does not necessarily preclude the role of PKMζ in memory maintenance.

Further exploration revealed that compensation by a closely related *iota* isoform accounts for the lack of memory deficits and the sensitivity to ZIP observed in the PKMζ knockout animals (Tsokas et al., 2016). They found that translational repression of PKMζ mRNA (through the use of a PKMζ-antisense oligodeoxynucleotide that specifically disrupts the translation start site of PKMζ mRNA) results in reversal of LTP in wild-type hippocampal slices but not in slices from PKMζ knockout animals. PKMζ-antisense also disrupts long-term memory in a spatial conditioning task in wild-type animals, but not PKMζ knockouts. Levels of PKCι/λ (an isoform closely related to PKCζ/PKMζ) are transiently increased following tetanisation in wild-type hippocampal slices (Osten et al., 1996)—since ZIP was shown to also inhibit activity of PKCι/λ (Tsokas et al., 2016), they next investigated whether PKC ι/λ may be upregulated to compensate for the loss of PKMζ in the knockout. Indeed, in PKMζ knockout mice, the increase in PKC ι/λ observed after tetanisation is no longer transient and instead persists up to 3 h (as opposed to 30 min), although this increase was only observed in the hippocampus. Volk et al. (2013) did not detect this upregulation 2 hours after LTP induction in whole-brain slices from PKMζ knockout animals, suggesting that compensation for PKMζ knockout in brain regions outside of the hippocampus likely don't rely on PKC ι/λ. Indeed, PKMζ is present in numerous brain regions (Naik et al., 2000), and ZIP targeted to these regions is sufficient to impair memory (Chihabi et al., 2016; Evuarherhe et al., 2014; Migues et al., 2010; Shema et al., 2007), suggesting that there must be some alternative means of compensation occurring in PKMζ knockouts if PKMζ is required for memory maintenance in these regions. While the precise target for ZIP is unknown, evidence suggests compensation by PKC ι/λ accounts at least in part for the normal phenotype observed in PKMζ knockout animals. Using an inhibitor specific for PKC ι/λ that is confirmed to have no effect on PKMζ kinase activity (Pillai et al., 2011), Tsokas et al. (2016) were able to

reverse LTP and spatial memory in the PKMζ knockout animals. Because this inhibitor has no effect on wild-type LTP, it highly suggests that PKC ι/λ is indeed compensating for the loss of PKMζ in knockout animals. While compensation has not been confirmed to occur in the conditional PKMζ knockdown animals used in Volk et al. (2013), shRNA-mediated PKMζ knockdown does not induce compensatory mechanisms and is sufficient to cause premature decay of LTP and disrupt memory (Dong et al., 2015). Additionally, while much of the evidence for PKMζ as the putative memory maintenance molecule comes from the effects of ZIP, other means of PKMζ inhibition (including the cell-permeable PKC inhibitor, chelerythrine, as well as shRNA described above) have also been shown to disrupt memory storage (Cai et al., 2011; Pastalkova et al., 2006; Serrano et al., 2008; Serrano et al., 2005; Shema et al., 2011; Villareal et al., 2009).

Confirming that closely related isoforms of PKMζ compensate in the PKMζ knockout not only helped assuage some of controversy, it also suggested that the atypical zeta isoform may not be the only PKM isoform important for memory maintenance.

1.3.3 Isoform specificity of PKMs

As described previously, PKMs are classified into three families based on their parent PKC despite not containing the regulatory domain that is the key distinguishing feature between these families. Because their catalytic domains are similar, and because it has already been shown that compensation in PKMζ knockout can occur by a closely related isoform, it is likely that conventional and novel PKM isoforms may perform similar functions to those of the mammalian atypical PKMζ in regard to maintaining persistent increases in synaptic strength.

The advantages provided by the *Aplysia* model system have allowed researchers to investigate the isoform specificity of PKMs. PKMs in *Aplysia* are formed through isoform specific calpain-mediated cleavage (Bougie et al., 2009; Sutton and Carew, 2000). Classical calpain, for example, is required for cleavage of PKC Apl I into PKM Apl I in the presynaptic neuron during a-ITF, while the same calpain is required for cleavage of PKC Apl III into PKM Apl III in the postsynaptic neuron during m-ITF (Farah et al., 2017). Calpain activity is also required for the expression of LTF—blocking classical calpain activity prevents expression of associative LTF, while blocking the small optic lobe (SOL) calpain prevents expression of non-associative LTF (Hu et al., 2017a), suggesting an isoform specific role for the cleavage products of calpains: PKMs.

While both associative and non-associative persistent LTF are protein-synthesis dependent, it has recently been shown that they depend on different mechanisms for their maintenance (Hu et al., 2017a). Overexpression of dominant negative (DN) PKM isoforms pre- and postsynaptically has different effects on the persistence of each type of LTF. In these experiments, presynaptic overexpression of either DN PKM Apl II or DN PKM Apl III reversed non-associative LTF. Similarly, non-associative LTF was reversed by postsynaptic overexpression of DN PKM Apl I. The reverse was true for associative LTF—presynaptic overexpression of DN PKM Apl I or postsynaptic overexpression of either DN PKM Apl II or DN PKM Apl III reversed associative LTF. These dominant negative constructs were injected into neurons during the maintenance phase of LTF (specifically, two days following LTF induction), distinguishing the observed effects from those arising from interference with LTF induction.

While these experiments demonstrate the differing roles for PKMs during unique forms of memory, the factors determining the isoform specificity of PKMs remain elusive. One hypothesis is that PKMs engage in isoform-specific protein-protein interactions upon formation, resulting in upregulation and localization to specific synapses (Sossin, 2007). PKMs may also achieve isoform specificity based on the availability of the substrates they interact with, determined by differences in the substrate binding pockets between PKM isoforms. To address this second hypothesis, we first need to identify potential synaptic substrates for PKMs that play a role in the maintenance of persistent changes in synaptic strength.

1.3.4 Postsynaptic targets of PKMs

As mentioned previously, one underlying consequence of synaptic potentiation is an increased concentration of AMPA receptors in the postsynaptic membrane, resulting in increased postsynaptic excitability (Hardt et al., 2014; Lu et al., 2001). AMPA receptors inserted into the membrane do not remain there permanently but are instead thought to undergo constant recycling into and out of extrasynaptic and internal AMPA receptor pools (Huganir and Nicoll, 2013; Malinow and Malenka, 2002). In *Aplysia*, both ITF and LTF result from increased trafficking of AMPA receptors to the potentiated synapse in the postsynaptic neuron (Glanzman, 2008; Jin et al., 2012). AMPA receptor endocytosis must be prevented in order for this increased excitability to persist, which has been proposed to be achieved through the activity of persistently active kinases like PKMs (Sacktor, 2011). Indeed, overexpression of PKMζ increases synaptic AMPA receptor levels (Yao et al., 2008; Yu et al., 2017). A peptide inhibitor that blocks the endocytosis of GluA2-containing AMPA receptors prevents the LTP decay observed following application of PKMζ inhibitors like ZIP (Dong et al., 2015; Migues et al., 2010). Interestingly, the GluA1 AMPA receptor subunit contains a PKC lambda (λ)

phosphorylation site that is required for incorporation of GluA1-containing AMPA receptors into the synapse during the induction of LTP (Ren et al., 2013). PKM is thought to affect AMPA receptor endocytosis indirectly, despite the fact that many kinases (such as PKA and CaMKII) can directly phosphorylate AMPA receptors (Wang et al., 2005). Mammalian PKMζ is believed to facilitate the dissociation of protein interacting with C-kinase 1 (PICK1) from the GluA2 subunit of GluA2-containing extrasynaptic AMPA receptors (Yao et al., 2008), allowing them to diffuse into the membrane and acting as a possible mechanism through which endocytosed AMPA receptors can be replenished during memory maintenance. Mammalian AMPA receptors contain tyrosine residues that are required for AMPA receptor endocytosis (Ahmadian et al., 2004; Yu et al., 2008), but these residues are not highly conserved. In *Aplysia*, for example, there are six different AMPA receptor isoforms (Greer et al., 2017; Sossin and Abrams, 2018), yet the effect of PKMs on AMPA receptor trafficking appears to be conserved. However, while the evidence suggests that PKM's role in memory maintenance is to prevent AMPA receptor endocytosis, the kinase's precise phosphorylation targets remain elusive.

The isoform specificity of PKMs may not extend to their substrates but instead may result from their upstream protein-protein interactions. PKMs may be stabilized and localized to synapses by upstream effectors that selectively interact with specific PKM isoforms over others. While there is evidence for the rapid and local production of PKM at synapses (Doyle and Kiebler, 2011; Muslimov et al., 2004), it is possible that mechanisms of PKM capture and stabilization may help upregulate PKM isoforms at specific synapses. One candidate PKM-stabilizing protein that may act as a synaptic tag during memory is the kidney-brain expressed protein, KIBRA.

1.4 KIBRA, a potential PKM stabilizing protein

Also known as WW-domain and C2 containing protein 1 (WWC1), KIBRA is a 125 kDA protein expressed predominantly in the kidneys and the brain (Kremerskothen et al., 2003). The evolutionarily conserved WWC family of proteins has three members—WWC1, WWC2, and WWC3—although these appear to be a vertebrate-specific duplication and many invertebrates and lower organisms do not express all three homologues (Yoshihama et al., 2012). Whether or not there is functional similarity between the WWC family proteins is unknown—however, in KIBRA knockout animals, an upregulation of WWC2 has been observed, suggesting that compensation may be possible (Makuch et al., 2011).

KIBRA has a number of highly conserved domains. There are two WW-domains of approximately 40 amino acids each near the N-terminal end of the protein that recognize proline-rich sequences via the two highly conserved tryptophan residues contained within (Zhang et al., 2014). KIBRA's C2-like domain is centrally located and contains two groups of β-sheets important for calcium-regulated phospholipid interaction (Duning et al., 2013). In addition to these domains (from which KIBRA gets its WWC namesake), KIBRA also contains various coiled-coil structures, a glutamic acid-rich domain, and a PDZ binding motif near the C-terminal end (Zhang et al., 2014). Due to these various domains, KIBRA has been implicated in a variety of cellular pathways, which will be discussed in the following sections.

1.4.1 KIBRA's roles in non-memory related pathways

Initial characterization of KIBRA linked it to cell polarity and motility due to its interactions with cytoskeleton-associated proteins Dendrin (Ji et al., 2019; Kremerskothen et al., 2003) and synaptopodin (Duning et al., 2008), which occur via KIBRA's WW-domain. Interaction with other polarity proteins, such as the PDZ domain-containing protein, PATJ

(PALS1-assocated tight junction protein), occurs via KIBRA's PDZ binding motif (Duning et al., 2008). In epithelial cells, these interactions are thought to play a role in vesicular trafficking of membrane proteins that influence membrane polarity (Yoshihama et al., 2012). KIBRA also interacts with dynein light chain 1 (DLC1) (via an unknown site) and histone H3 (via KIBRA's glutamic acid-rich region) in the regulation of gene expression in breast cancer cells (Rayala et al., 2006). Because KIBRA has also been shown to interact with sorting nexin 4 (SNX4), it is thought to mediate the interaction between SNX4 and DLC1 during endosome recycling (Traer et al., 2007).

Genetic analysis in *Drosophila* revealed that KIBRA acts as an upstream component of the Hippo tumour suppression pathway, which is involved in managing epithelial cell growth and organ size (Baumgartner et al., 2010). Inactivation of this pathway results in increased cell proliferation and decreased apoptosis—indeed, inactivation of this pathway has been linked with a variety of cancers (Genevet and Tapon, 2011). The Hippo pathway is a phosphorylation cascade that ultimately results in the inactivation of effector molecules, thereby inhibiting the activation of genes involved in proliferation and anti-apoptosis (Zhang et al., 2014). KIBRA is thought to act as a scaffolding protein in this pathway due to its variety of interaction domains, promoting interaction between kinases involved in the phosphorylation cascade. In *Drosophila*, KIBRA interacts with tumor suppressors Merlin (Mer) and Expanded (Ex). Both the N-terminal and C-terminal ends of KIBRA are sufficient to facilitate the KIBRA-Mer interaction, while Ex interacts with KIBRA's WW-domains (Genevet et al., 2010). The KIBRA-Mer-Ex complex then homodimerizes with Salvador (Sal)'s WW domain, facilitating initiation of the Hippo pathway's phosphorylation cascade (Ohnishi et al., 2007; Yu et al., 2010). Human KIBRA interacts with

orthologs of many of the previously mentioned proteins to facilitate activation of the pathway (Genevet and Tapon, 2011; Yu et al., 2010).

Posttranslational modification of KIBRA through phosphorylation may also play a role in the Hippo pathway (Buther et al., 2004). Phosphorylation of KIBRA at sites near the C-terminal end of the protein are thought to downregulate the Hippo pathway by preventing KIBRA's dimerization (Genevet and Tapon, 2011). Posttranslational modification of KIBRA through phosphorylation can be achieved by aPKCs, as well as a variety of other kinases (see Zhang et al., 2014 for review). KIBRA has also been shown to interact with aPKCs. Non-neuronally, this interaction is thought to be important for bridging aPKCs with Sec3 (an exocyst protein) during migration of normal rat kidney cells (Rosse et al., 2009). KIBRA interacts with the catalytic region of aPKCs (Buther et al., 2004), which suggests that KIBRA may also have a role in memory through a possible interaction with PKMs in neurons.

1.4.2 KIBRA's role in memory

KIBRA is expressed in a number of brain regions, including the cerebellum and hypothalamus, as well as memory-related areas like the hippocampus and cortex (Papassotiropoulos et al., 2006). KIBRA is distributed in the somatodendric region of both neuronal and non-neuronal cells (Duning et al., 2008; Johannsen et al., 2008; Kremerskothen et al., 2003), but in hippocampal primary cultures, KIBRA was found to be enriched in the postsynaptic density (Johannsen et al., 2008). The most striking early evidence for KIBRA's role in memory comes from the fact that a single nucleotide polymorphism (SNP) [a thymine (T) to cytosine (C) substitution] within the 9th intron of the KIBRA gene is associated with differences in human cognition. Humans with at least one T allele perform better in tasks testing episodic memory (Papassotiropoulos et al., 2006), possibly due to increased hippocampal activation

(Kauppi et al., 2011). The reproducibility of this result varies based on genetic background, gender, age, lifestyle, and disease status, but meta-analysis confirms the likelihood of a link between this KIBRA SNP and human episodic and working memory (Milnik et al., 2012). Two SNPs within exon 15 have also been linked with human cognitive performance—these SNPs have been shown to affect the calcium sensitivity of KIBRA's C2-like domain, altering KIBRA's lipid binding preferences (Duning et al., 2013). But while KIBRA seems to play some role in memory, research into what this role may be is still ongoing.

The interaction between KIBRA and Dendrin has recently been shown to be necessary for learning and memory. While the role of Dendrin in neurons is poorly understood, disruption of the strong KIBRA-Dendrin interaction reduces synaptic AMPA receptor expression, blocks LTP induction in hippocampal slices, decreases the number of excitatory synapses *in vivo*, and impairs learning and memory in mice (Ji et al., 2019). KIBRA has been directly linked to AMPA receptor trafficking through an interaction with PICK1 mediated by KIBRA's PDZ binding motif (Makuch et al., 2011). KIBRA-PICK1 was found to form a complex with AMPA receptors *in vivo*—indeed, KIBRA knockdown results in an accelerated rate of AMPA receptor recycling and a decreased number of membrane-bound AMPA receptors (Heitz et al., 2016; Makuch et al., 2011). Consistent with this finding, KIBRA overexpression was shown to increase hippocampal LTP and prevent induction of LTD (Heitz et al., 2016), while KIBRA knockout in rodents results in reduced learning and memory in both spatial (Vogt-Eisele et al., 2014) and contextual fear tasks (Makuch et al., 2011). Genetic deletion of KIBRA also impairs LTD and LTP in hippocampal synapses (Makuch et al., 2011) and increases the ratio of filopodial-like elongated dendritic spines in the hippocampus and neocortex (Blanque et al., 2015).

Investigation into KIBRA's role at the synapse in relation to memory maintenance is still in its infancy. Due to KIBRA's various domains, it is possible that KIBRA acts as a scaffolding protein at the potentiated synapse. Recent evidence suggests that PKMζ is one of KIBRA's neuronal interaction partners, making KIBRA an even more attractive candidate in the search for the molecular memory trace.

1.5 Objectives

The work contributing to this thesis is focused on elucidating the role of PKMs in memory maintenance through investigation of both upstream and downstream components of the PKM-memory pathway using the *Aplysia* model system.

The experiments described in Chapter three investigate endocytic adaptor proteins as candidate PKM substrates in the regulation of AMPA receptor trafficking. We hypothesize that phosphorylation of the endocytic adaptor protein Numb by PKM is important for preventing AMPA receptor endocytosis during the long-term maintenance of increases in synaptic strength. We first seek to characterize a technique for quantifying changes in membrane-bound AMPA receptors by overexpressing a pH-sensitive GFP-tagged AMPA receptor in cultured *Aplysia* neurons, and then utilize this technique to measure changes in AMPA receptor membrane concentration in response to 5HT application.

Chapter four of this thesis looks at the role of KIBRA as an upstream regulator of PKMs. We investigate the interaction between KIBRA and the three *Aplysia* PKM isoforms and show that KIBRA stabilizes PKMs in an isoform-specific manner. We also generate a dominant negative form of KIBRA that exhibits PKM stabilization patterns opposite to that of the wildtype

KIBRA. We identify attributes that are required for KIBRA mediated stabilization of PKMs and show that KIBRA stabilizes PKMs independent of kinase activity. Additionally, we identify a new domain on KIBRA and characterize the PKM-stabilization ability of a KIBRA splice isoform. We identify a region on PKMs that may be important for isoform differentiation and propose that selective stabilization by KIBRA and KIBRA splice isoforms is important for determining PKM isoform specificity at synapses.

Determining the cellular pathway through which PKMs maintain memory-induced increases in synaptic strength is critical to understanding how the brain works at a fundamental level. *Aplysia* is a powerful tool for studying the molecular interactions of PKMs at the synapse, especially since the role of PKM in memory maintenance appears to be highly conserved throughout evolution. Our work here helps address some important questions about how PKM works and how PKM activity is regulated, bringing us one step closer to understanding and defining the molecular memory trace.

CHAPTER TWO

Methodology

This chapter describes the methods and the materials used for the experiments performed in Chapters 3 and 4.

2.1 Bioinformatics

Orthologues of KIBRA were determined using the reverse BLAST method. Organisms with established genomes on NCBI were BLAST searched to identify proteins with homology to KIBRA. To distinguish between true orthologues and proteins with similar WW and C2 domains, these proteins were then used as a query in a BLAST search and if they were much more homologous to other proteins with WW and C2 domains than to KIBRA, they were rejected as orthologues. Based on this, no KIBRA orthologues exist in sponge (*Amphimedon queenslandia*), Trichoplax (*Trichoplax adhaerens*), Ctenophores (*Mnemiopsis*) and choanoflagellate (*Monosiga bervicolis; Salpingoeca rosetta*). KIBRA orthologues were found in other Cnidaria (Coral, *Styklophora postillata* and *Acropora digitefara*; jellyfish, *Hydra vulgari*s), but the strongest homology was to Anemone, *Nemostella vectensis*, and this Cnidarian was used in the comparison studies. An *Aplysia* orthologue of Numb (ApNumb) was similarly identified using NCBI BLAST and the *Aplysia* transcriptosome [as described in (Farah et al., 2019)].

2.2 Cloning

All constructs were made in the pNEX3 vector (Kaang, 1996). All PKM constructs (including dominant negatives) were made as fusion proteins with monomeric red fluorescent protein (mRFP). The mRFP has been removed from the construct names for clarity. Dominant negative constructs (DN PKM Apl I, DN PKM Apl II, and DN PKM Apl III) and PKMs (PKM Apl I, PKM Apl II and PKM Apl III) used for overexpression and stabilization studies were previously described (Bougie et al., 2012; Farah et al., 2017; Hu et al., 2017a). The DN PKM Apl III K-R was generated by cutting out this region from the plasmid encoding PKC Apl III K-R (Bougie et al, 2012) with AarI and SalI and inserting it into the plasmid encoding PKM Apl III at the same sites. For the chimeras, GBLOCKS (Integrated DNA technology, IO, USA) were purchased with the PKM Apl III sequences [carboxy-terminus or handle] replaced by PKM Apl I sequences. These were then cut out with either BsmBI and Kpn I (C-terminus) or Sal I and Kpn I (handle) and inserted into the plasmid encoding PKM Apl III at the same sites. The chimeras were sequenced for confirmation.

ApNumb was identified using bioinformatics from NCBI and the *Aplysia* transcriptome (Aplysiagenetools.org). The ApNumb sequence on NCBI (XP_012939143.1) contains an additional 15 amino acids (MERKGSKRFGRYRNI) preceding the initiating methionine that was not found in the transcriptome. We could not PCR this alternative 5' sequence from *Aplysia* nervous system cDNA. We cloned ApNumb from nervous system cDNA with forward primers TAATGCAAAGCATACGTCGAAGGTTC and reverse primer CACCACTACAACTGGACTTCAAAC and inserted the PCR product into pJET2 (ThermoFisher, Waltham, MA USA). The ApNumb coding sequence was then transferred to the Baculovirus transfer vector FBHA (THermoFisher) and serine 9 was converted to alanine with

the forward primer GGGGGATCCCATGCAAAGCATACGTCGAAGGTTCGCATTACGTAAGAAAAAGGACC ATGC and the reverse primer GCCAGGAACCATGGCACATC with the PCR product inserted into the FBHA-ApNumb plasmid with BamHI and NdeI. The coding sequence of ApNumb and $ApNumb_{S9A}$ were then inserted into the *Aplysia* expression vector pNEX3 (Kaang et al., 1992) by cutting the FBHA vector with BamHI and ScaI and inserting into pNEX3 cut with BamHI and SmaI. The remaining two conserved atypical PKC serines were mutated to alanine using PCR overlap as has been described (Nakhost et al., 1999). The resultant construct is called pNEX3 $ApNumb_{S3\text{-}A3}$. The DNA construct for ApGluR1-pHluorin was a kind gift from Robert D. Hawkins and Eric Kandel (Columbia University, New York) and has been previously described (Jin et al., 2012). This construct was sequenced and consists of superecliptic pHluorin (Kopec et al., 2006) inserted immediately after the first transmembrane domain of ApGluR1. This receptor is also called Aplysia GluR4 (Greer et al., 2017).

KIBRA was cloned using primers derived from National Center for Biotechnology Information (NCBI) XP_012936697.1 with XBA and BamHI sites at their ends and cloned into the pNEX3 vector cut with XBA and BamHI. Insertion of KIBRA into the vector was confirmed via sequencing. The KIBRA-AAA dominant negative mutant was generated using overlap PCR. Mutations correspond to *Aplysia* homologues of human R965, S967, and R969, all mutated to alanine. Mutations were inserted into a C-terminal fragment of KIBRA cloned in the pJET vector (Thermo Fisher Scientific) using StuI and PaeI sites surrounding the mutated region. The KIBRA splice form, KIBRA SPL, was generated similarly to KIBRA but from a separate PCR clone that fortuitously encoded the splice form. This region was then inserted into the full length KIBRA

with Drd1 and XbaI. KIBRA, KIBRA-AAA, and KIBRA SPL are not fusion proteins with a fluorescent protein.

2.3 Aplysia neuronal cultures

Adult *Aplysia californica* (60-100g) obtained from Miami *Aplysia* Resource Facility (RSMAS) were used for isolation of sensory and motor neurons. Animals were anesthetized via injection of 50-60 mL of 400 mM isotonic $MgCl_2$ and abdominal and/or pleuropedal ganglia were removed. Ganglia were digested at 19°C in L15 media containing 10 mg/ml Dispase II (Roche Diagnostics) for 18-19 h. L15 medium (Sigma-Aldrich) was supplemented with 0.2 M NaCl, 26 mM $MgSO_4$, 35 mM dextrose, 27 mM $MgCl_2$, 4.7 mM KCl, 2 mM $NaHCO_3$, 9.7 mM $CaCl_2$, and 15 mM HEPES, with pH 7.4. Glass bottom culture dishes were coated with 0.05% poly-L-lysine for 1-2 h and washed with ddH_2O prior to use. Sensory neurons and LFS motor neurons were isolated from pleural and abdominal ganglia respectively. Neurons were cultured in 50% hemolymph/50% L15 media supplemented with L-glutamine. For electrophysiology experiments, motor neurons were removed from the abdominal ganglia and allowed to adhere to the culture dish for 1-24 h before pairing with a sensory neuron from pleural ganglia as previously described (Zhao et al., 2006). Note that each coculture comprised a single presynaptic sensory neuron paired with a single postsynaptic LFS motor neuron. Cells were incubated for 48 h at 19°C to allow time for them to adhere to the dish and form stable synapses prior to injection (Hu et al., 2017a; Hu and Schacher, 2015). All plating, injections, and electrophysiology experiments were performed at 19°C.

2.4 Microinjection

For stabilization experiments, molar equivalent levels of plasmids encoding KIBRA/KIBRA SPL/KIBRA-AAA and the PKMs/DN PKMs were used. A solution containing the desired constructs (max 0.4 ug/ul of DNA in ultrapure water) with 0.2% fast green were microinjected into the nuclei of neurons using back-filled glass micropipettes. A short pressure pulse was delivered to release plasmid solution into the nucleus. KIBRA and enhanced green fluorescent protein (eGFP) were injected into the LFS neuron of sensorimotor cocultures for ITF experiments. KIBRA was co-expressed with an eGFP construct so that expression could be confirmed by detecting eGFP fluorescence. Stabilization experiments were performed by injecting constructs into isolated sensory neurons. For pHluorin experiments, ApGluR1-pHluorin was injected into the motor neuron with or without ApNumb/ApNumb$_{S9A}$ and either mRFP or red dextran dye was injected into the sensory neuron of sensorimotor cocultures. Cultures were incubated at 19 ºC for 24 h to allow sufficient time for expression of injected plasmids prior to imaging.

2.5 Antibody production

A C-terminal peptide of KIBRA (N-terminal LESFFHDDRIGEEV C-terminal) was synthesized for production of an antigen and for purification of KIBRA C-terminal antibody. The peptide was coupled to a bovine serum albumin-Maleimide and Sulfo-link (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. After conjugation to BSA-Maleimide, rabbits were injected and final serum (after three boosts) was affinity purified on the Sulfo-link column. KIBRA C-terminal antibody (~1.32 µg/µL) was used for immunocytochemistry at a

concentration of 1:1000. Production of rabbit anti-PKC Apl III C-terminus antibody was previously described (Farah et al., 2017).

2.6 Immunocytochemistry

Cells were fixed 24 hours following microinjection with 4% paraformaldehyde with 30% sucrose in PBS for 30 min and washed with PBS. Fixed cells were permeabilized with 0.1% Triton X-100 with 30% sucrose in PBS for 10 minutes and washed briefly with PBS. Free aldehydes were quenched with 50 mM ammonium chloride for 10-15 min followed by brief PBS wash. To block nonspecific antibody binding, cells were incubated with 10% normal goat serum (Sigma-Aldrich) plus 0.5% Triton X-1000 in PBS for 30 min. Cells were incubated with KIBRA C-terminal antibody (1:1000) or rabbit anti-PKC Apl III (1:5000) in blocking solution for 1 hour, followed by 4 PBS washes of 5-10 minutes each. Cells were then incubated in the dark with Alexa Fluor 647-conjugated goat anti-rabbit (1:200, Invitrogen) or donkey anti-rabbit (1:500, Invitrogen) antibody or Alexa Fluor 488 conjugated goat anti-rabbit (1:1000) in blocking solution for 1 hour and washed with PBS as described above.

2.7 Electrophysiology

Sensorimotor cocultures were incubated in culture media at room temperature for 24 h following microinjection to allow sufficient time for expression of injected plasmids before electrophysiological recordings. Prior to recording, culture media was replaced with a recording saline [NaCl (460 mM); MgCl2 (55 mM); CaCl2 (10 mM); KCl (10 mM); D-Glucose (10 mM); HEPES (10 mM); pH 7.6]. Membrane potentials were recorded and controlled in current clamp

mode with sharp intracellular electrodes attached to an Axoclamp 2B amplifier (Molecular Devices, Palo Alto, California). Microelectrodes (15-30 MΩ) were backfilled with 2 M potassium acetate and bridge-balanced before and after membrane penetration. The postsynaptic cell was impaled first, so that if entry into the presynaptic cell resulted in generation of an action potential, the resultant postsynaptic potential (PSP) would be recorded. Injection of hyperpolarizing current was used to keep both presynaptic and postsynaptic neurons at -80 mV during recording. Postsynaptic input resistance was measured with 500 msec, -0.5 nA pulses. For ITF experiments, electrodes were removed from cells after initial recording and 5HT (10 µM) was added for 10 min before being washed out with 25 ml recording saline solution. Cultures were left at 19ºC for 2 hours, at which time the second recording was performed. Initial PSP rise-rate was measured as previously described (Dunn and Sossin, 2013).

2.8 Imaging

Images were captured by an LSM 710 (Zeiss) laser confocal scanning microscope equipped with an Axiovert 100 inverted microscope (Zeiss) and a 40x, NA 1.4 objective. The eGFP/A488, mRFP, and/or A647 images were acquired sequentially. Laser power was kept constant within each experiment.

For image analysis of surface AMPA receptors using pHluorin assay, puncta were selected as regions of interest if they were in close proximity to the presynaptic neuron (red) and decreased in fluorescence intensity at pH 6.0 by at least 25%. Puncta that did not satisfy these criteria were not included in the quantification. The ΔF/F value for each region of interest was calculated as the change in fluorescence after treatment with 5HT divided by the initial

fluorescence (pre-5HT). A single value for the change in fluorescence per sensory-motor neuron pair was calculated as the average of the ΔF/F values for all regions of interest on that pair. Quantification was performed by blind observer.

2.9 PKM stabilization assay

To measure stabilization by KIBRA, we express mRFP-tagged PKMs in the sensory neuron in the presence or absence of KIBRA and measure the intensity of mRFP in processes (rather than the cell soma, to avoid red pigment granules that interfere with the measurement) normalized to the expression of eGFP (plasmid encoding eGFP is co-injected with plasmid expressing mRFP-tagged PKMs). A blind observer chooses one region of interest per neuron representing a sensory neuron neurite of intermediate thickness for the measurement. The level of expression is measured at 24 h after injection. Low levels of plasmids are used to minimize toxicity and to enable expression of the plasmid expressing KIBRA at equimolar levels to the PKMs. For analysis of DN PKM and PKM stabilization, single processes for each neuron are outlined using NIH Image J. Background fluorescence is subtracted from the fluorescence values measured. The red/green or cyan/green ratio for all neurons is normalized to the average ratio seen in vector-injected neurons from the same experiment. All quantification of stabilization was done blindly.

CHAPTER THREE

PKM-mediated regulation of synaptic AMPA receptor trafficking

3.1 Introduction

Overexpression of mammalian PKMζ has been associated with increased levels of membrane-bound AMPA receptors (Ling et al., 2006; Yao et al., 2008; Yu et al., 2017)—similarly, postsynaptic overexpression of all three *Aplysia* PKM isoforms is sufficient to increase synaptic strength in *Aplysia* sensory motor neuron cocultures (Ferguson et al., 2019). However, despite a clear link between PKMs and AMPA receptor trafficking, substrates for phosphorylation in this pathway have not yet been defined. One hypothesis is that PKMs directly phosphorylate AMPA receptors to prevent their internalization during the maintenance of LTP. Mutation of the conserved serine 818 PKC phosphorylation site on the mammalian GluA1 AMPA receptor subunit blocks LTP (Boehm et al., 2006), and PKC-mediated phosphorylation of serine 880 of GluA2 is important for AMPA receptor interaction with PDZ-containing protein PICK1 (Chung et al., 2000). Another hypothesis is that AMPA receptor trafficking is regulated through PKM-mediated phosphorylation of the endocytic machinery associated with AMPA receptors. The endocytic adaptor protein Numb is a candidate substrate for PKM in this model.

Numb regulates the endocytosis of a number of neuronal and non-neuronal transmembrane proteins and is itself regulated through phosphorylation by atypical PKCs. Numb was first discovered as a determinant of neuronal cell fate when it was shown that loss of Numb function in *Drosophila* promotes the formation of non-neuronal support cells from precursors that normally form sensory neurons (Uemura et al., 1989). Further investigation revealed that Numb regulates the endocytosis of Notch, a transmembrane protein that signals non-neuronal cell development in *Drosophila* embryos (Guo et al., 1996). Cell division in precursor cells with

asymmetrically localized Numb results in one daughter cell that contains Numb (neuronal cell fate due to increased Numb-mediated endocytosis of Notch) and one that does not (non-neuronal cell fate due to decreased endocytosis of Notch). Numb also colocalizes with endosomes and vesicles containing internalizing receptors (Santolini et al., 2000). During cell motility, Numb regulates endocytosis of the cell adhesion molecule, integrin, on the leading edge of the cell, while asymmetrically localized aPKCs at the trailing edge phosphorylate Numb to prevent integrin endocytosis (Nishimura and Kaibuchi, 2007). Numb is similarly involved in the trafficking of cadherins in determining epithelial cell polarity (Sato et al., 2011), metabotropic glutamate receptors in cerebellar Purkinje cells (Zhou et al., 2015), tropomysin receptor kinase B (TrkB) in regulating cell polarity of cerebellar granule cell precursors (Zhou et al., 2011), and membrane transporters in rodent hepatoma cells (Su et al., 2016).

Highly conserved regions within Numb are important for localization, target selection, and interaction with the cell's endocytic machinery. The C-terminal region contains an aspartate-proline-phenylalanine (DPF) motif that is required for interaction with clathrin-coated pits and an asparagine-proline-phenylalanine (NPF) motif required for interaction with endocytic machinery (Santolini et al., 2000). The phosphotyrosine-binding domain (PTB) near the N-terminal region of the protein is important for localization to the plasma membrane (Dho et al., 1999) as well as for selecting membrane targets for endocytosis (Traub, 2003). Phosphorylation of Numb by aPKC inhibits binding to endocytic clathrin adaptor proteins by promoting the interaction of Numb with the regulatory protein 14-3-3 via the Numb-specific Numbf domain (Chen et al., 2018). Because PKMs share the catalytic domain of their parent PKCs, it is possible PKMs phosphorylate Numb to the same effect in the regulation of AMPA receptor endocytosis. In our

model, PKM-mediated phosphorylation of Numb prevents interaction with the cell's endocytic machinery, thereby inhibiting AMPA receptor endocytosis during the maintenance of LTF.

In this chapter, we adapt a live imaging technique that utilizes a pH-sensitive GFP for use in measuring AMPA receptor trafficking in *Aplysia* sensorimotor cocultures. We demonstrate an increase in AMPA receptors at synapses following 5HT treatment using this assay and show that overexpression of a non-phosphorylatable Numb blocks this increase. These results support our model that AMPA receptor trafficking is regulated by Numb and establishes Numb as a potential target for PKM during the maintenance of facilitation-induced increases in synaptic strength.

3.2 Results

3.2.1 Characterization of the pHluorin-AMPA receptor assay

To determine the effect of PKM-mediated phosphorylation on AMPA receptor membrane trafficking, we utilize a superecliptic pH-sensitive green fluorescent protein (pHluorin) tagged to the GluR1 subunit of *Aplysia* AMPA receptors (ApGluR1-pHluorin) to visualize AMPA receptor surface dynamics in *Aplysia* neuronal cultures via live imaging. Similar pHluorin-tagged AMPA receptor constructs have been used in rodent hippocampal neuronal cultures (Ashby et al., 2004a). The DNA for the pHluorin construct used in our experiments was a gift from Robert D. Hawkins and Eric Kandel (Columbia University, New York) and has been previously described and used to measure AMPA receptor membrane localization in *Aplysia* cell cultures (Jin et al., 2012). The pHluorin is tagged to the N-terminus of the *Aplysia* GluR1 subunit—when the receptor is bound to the cell membrane, the pHluorin is exposed to the extracellular space. If the culture is exposed to media with a neutral pH, the extracellularly facing pHluorin will fluoresce

[pHluorin pK_a is ~7.1 (Ashby et al., 2004b)]. Internal AMPA receptors exposed to the relatively acidic environment of the vesicle lumen (pH ~5.5-6.0, a pH that quenches fluorescence of the pHluorin) will not fluoresce (Ashby et al., 2004b). In this way, trafficking of AMPA receptors to and from the synaptic membrane following facilitation can be visualized and quantified.

We first attempted to replicate the results observed in Jin et al. (2012) using our pHluorin construct. Cultured Aplysia sensory neurons overexpressing ApGluR1-pHluorin were exposed to artificial sea water (ASW) solutions with varying pH as described in **Figure 3.1**. Cells were exposed to an ASW solution at pH 7.6 and pH 6.0 sequentially, followed by a pH 7.6 ASW solution containing 10mM ammonium chloride (NH_4Cl). At pH 6.0, the fluorescence of the membrane bound ApGluR1-pHluorin is quenched, resulting in decreased fluorescence intensity. Any residual fluorescence at this pH is believed to be from internalized receptors in non-acidic cellular compartments, such as the endoplasmic reticulum (Ashby et al., 2006; Paroutis et al., 2004). NH_4Cl diffuses across cell membranes and alkalizes the cytosol and internal organelles, equalizing the internal and external pH (Roos and Boron, 1981), allowing visualization of internal and external receptor pools and quantification of the pHluorin surface fraction (Sankaranarayanan et al., 2000).

In isolated *Aplysia* sensory neurons expressing ApGluR1-pHluorin, exposure to 5HT did not result in a detectable change in fluorescence as would be expected from increased AMPA receptor membrane localization (**data not shown**). A small increase in diffuse surface expression was observed in isolated motor neurons following 10 minutes 5HT application at pH 7.6 (**Figure 3.2A**). In order to visualize synaptic AMPA receptor dynamics during 5HT-mediated facilitation, we needed to optimize the pHluorin assay for use in *Aplysia* sensory-motor neuron cocultures. Sensory neurons were injected with mRFP or a red dextran dye to help visualize areas of contact

between the two neurons to assist in identifying potential synapses. As shown in **Figure 3.2B**, clusters of ApGluR1-pHluorin can be observed on the motor neuron (green) as fluorescent puncta and likely represent synapses (Li et al., 2009). Puncta whose fluorescence decreased by at least 25% after exposure to pH 6.0 (see methods) were considered representative of externally facing AMPA receptors on synapses and were included in the quantification. Exposure to 10 minutes 5HT resulted in increased fluorescence intensity of puncta, indicative of increased localization of ApGluR1-pHluorin to the synaptic membrane in the postsynaptic neuron, validating use of this assay in determining factors that affect AMPA receptor trafficking dynamics during facilitation.

3.2.2 Phosphorylation of Numb is required to mediate AMPA receptor endocytosis

Aplysia Numb (ApNumb) contains all of the characteristic regions mentioned in the introduction. Notably, all three of the known PKC phosphorylation sites are conserved in ApNumb, and both PKM Apl I and Apl III phosphorylate ApNumb *in vitro* (Farah et al., 2019). Mutation of these three residues ($ApNumb_{S3\text{-}A3}$) produces a Numb that cannot be phosphorylated by PKMs (Farah et al., 2019). Based on our model, this mutant Numb will be unable to be inhibited by phosphorylation during the maintenance of synaptic facilitation—therefore, we expect overexpression of $ApNumb_{S3\text{-}A3}$ to result in increased AMPA receptor endocytosis and decreased pHluorin fluorescence.

The postsynaptic motor neurons of *Aplysia* sensory motor neuron cocultures were injected with ApGluR1-pHluorin and exposed to either 5HT or ASW control for 10 minutes. As mentioned in Chapter 1, 10 minutes application of 5HT is sufficient to induce m-ITF in *Aplysia* synaptic cultures, a type of facilitation that induces formation of PKM Apl III and is reversed by interfering with PKM Apl III activity (Bougie et al., 2012). As shown in **Figure 3.3**, induction of

m-ITF results in an increase in ApGluR1-pHluorin fluorescence in puncta on the postsynaptic neuron compared to controls exposed to 10 minutes ASW. This was expected, given that m-ITF has been linked with increased postsynaptic localization of AMPA receptors in cultured *Aplysia* synapses (Jin et al., 2012). However, this increase in fluorescence is prevented by postsynaptic overexpression of our non-phosphorylatable Numb mutant. Overexpression of $ApNumb_{S3\text{-}A3}$ in the motor neuron [overexpression confirmed using antibody against ApNumb (Farah et al., 2019)] blocks the 5HT-induced increase in puncta fluorescence, suggesting that phosphorylation of Numb is required for the increase in synaptic AMPA receptor levels observed following induction of m-ITF. This fits with our model (**Figure 3.4**) wherein phosphorylation is required to inhibit Numb's ability to interact with the cell's endocytic machinery, thereby preventing AMPA receptor endocytosis following increases in synaptic strength at glutamatergic synapses.

3.3 Discussion

3.3.1 Optimization of the pHluorin assay

A major issue with utilizing our pHluorin assay to measure surface dynamics of AMPA receptors in *Aplysia* neurons is the substantial amount of internal fluorescence observed. While the theory is that internalized ApGluR1-pHluorin should not fluoresce, the reality is that not all internalized pHluorin is quenched. One major contributor to this residual fluorescence in the endoplasmic reticulum (ER), which has a pH around 7.1 (Paroutis et al., 2004). In *Aplysia*, the ER is often located in neurites in close proximity to the cell membrane (Rathje et al., 2013), making it difficult to distinguish between pHluorins fluorescing on the cell surface from those fluorescing on the nearby ER membrane. We accounted for this by applying a pH 6.0 wash,

which quenches extracellularly facing pHluorins but does not affect internal pHluorin fluorescence (Fox-Loe et al., 2017). While our images were taken within 5 minutes of applying the pH 6.0 wash, it should be noted that prolonged exposure (e.g. 15 minutes) to pH 6.0 acidifies internal compartments and invalidates the use of a pH 6.0 wash as a control (Wilkinson et al., 2014). Another means of bypassing the issue of internal pHluorin fluorescence is to use total internal reflection fluorescence (TIRF) microscopy, which would allow for imaging of a very thin region of the cell (e.g. the membrane) while excluding regions outside of our areas of interest (e.g. internal cell components like the ER) (Ashby et al., 2006). However, the sensory and motor neurons of Aplysia cultured synapses often grow on top of one another, forming synapses within multiple planes of focus. As such, while TIRF microscopy may be possible for imaging and quantifying AMPA receptor surface dynamics in single-cell cultures, it is not feasible for investigating AMPA receptor trafficking in synapses during facilitation in *Aplysia* neuronal cocultures.

3.3.2 Facilitation induced increase in synaptic AMPA receptors requires Numb phosphorylation

We've shown that the increased trafficking of AMPA receptors to the postsynaptic membrane during m-ITF requires phosphorylation of Numb. Overexpression of a non-phosphorylatable form of Numb prevents the increase in fluorescence of synaptic puncta following 10-minute application of 5HT. While this indicates a decrease in synaptic AMPA receptor levels, overexpression of this mutant Numb has no effect on synaptic strength after induction of m-ITF (Farah et al., 2019). There is also no change in basal synaptic strength in synapses expressing $ApNumb_{S3\text{-}A3}$ in the postsynaptic neuron (Farah et al., 2019), suggesting that phosphorylation of Numb may not play a role in regulating the endocytosis of AMPA receptors at non-potentiated synapses. Similar to our m-ITF experiments, postsynaptic overexpression of

$ApNumb_{S3\text{-}A3}$ prevents the increase in pHluorin fluorescence intensity at puncta measured 24 hours after induction of LTF (Farah et al., 2019). However, $ApNumb_{S3\text{-}A3}$ postsynaptic overexpression also blocks the increase in EPSP amplitude observed 24 hours after LTF induction, indicating that phosphorylation of Numb is required for the expression of LTF but not the expression of m-ITF. Overexpression of wildtype Numb in the postsynaptic neuron also blocks LTF, which may be due to saturation of endogenous PKM (Farah et al., 2019).

One explanation for the discrepancy between Numb's role in the expression of m-ITF and LTF may be that Numb may be involved in regulating endocytosis of membrane components other than AMPA receptors. Indeed, there is evidence showing that Numb regulates the endocytosis of metabotropic glutamate receptors (mGluRs) (Wang et al., 2018; Zhou et al., 2015), suggesting that overexpression of non-phosphorylatable Numb may have consequences on synaptic strength apart from an effect on AMPA receptor trafficking. Furthermore, while Numb usually interacts with membrane-bound receptors via its PTB domain in order to regulate endocytosis, there is no evidence in vertebrates or *Aplysia* that a similar interaction occurs between Numb and AMPA receptors. Indeed, Numb may regulate AMPA receptor endocytosis through an indirect interaction, such as through an unknown AMPA receptor-binding protein (Farah et al., 2019; Jackson and Nicoll, 2011).

Aplysia AMPA receptors have 6 types of subunits, each of which diverged from a common ancestor after the divergence of *Aplysia* and vertebrate AMPA receptors (Greer et al., 2017). Although our pHluorin tag is on the GluR1 *Aplysia* AMPA receptor subunit, ApGluR1 is not any more conserved than the other five *Aplysia* AMPA receptor subunits (Greer et al., 2017). While AMPA receptor subunit composition has been shown to change across different stages of LTP in mammals (Plant et al., 2006), less is known about subunit dynamics of *Aplysia* AMPA

receptors. As such, we may be overlooking important aspects of AMPA receptor trafficking dynamics that involve AMPA receptors with subunits other than ApGluR1. For example, the decrease in pHluorin intensity in synaptic puncta is not necessarily indicative of a decrease in total synaptic AMPA receptors—rather, it may instead indicate a shift in the predominant subunit composition of membrane bound AMPA receptors. It is also possible that Numb only interacts with AMPA receptors with specific subunit compositions, providing another possible explanation for why overexpression of non-phosphorylatable Numb affected puncta fluorescence during m-ITF but had no effect on synaptic strength. However, another possibility is that synaptic strength during m-ITF is mediated through presynaptic mechanisms and that the increase in membrane-bound AMPA receptors in the postsynaptic neuron does not influence synaptic strength (for example, an increase in extrasynaptic AMPA receptors).

3.4 Conclusions

Utilizing the pH-sensitivity of the pHluorin construct, we were able to show that Numb is a candidate substrate for PKM in the PKM-mediated increase in synaptic AMPA receptors observed after induction of facilitation in *Aplysia* synaptic cultures. This assay also provides a means of measuring colocalization with synaptic AMPA receptors in live neurons as well as providing a means of investigating other pathways that affect AMPA receptor trafficking.

3.5 Figures

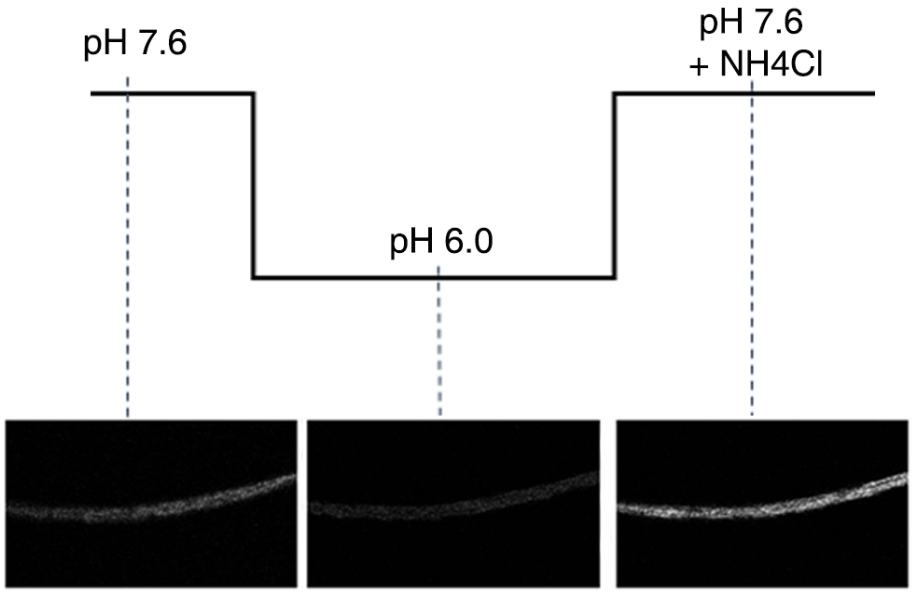


Figure 3.1 pHluorin fluorescence indicates AMPA receptor surface expression in *Aplysia* sensory neurons.

Experimental paradigm using pH changes to visualize changes in pHluorin fluorescence. Cultured sensory neurons expressing ApGluR1-pHluorin were exposed to extracellular media containing ASW at pH 7.6, followed by a wash with ASW at pH 6.0 to quench surface fluorescence of membrane-bound ApGluR1-pHluorin. Cells were then exposed to extracellular environment containing 10mM NH4Cl at pH 7.6. Sample images show fluorescence change at each pH wash.

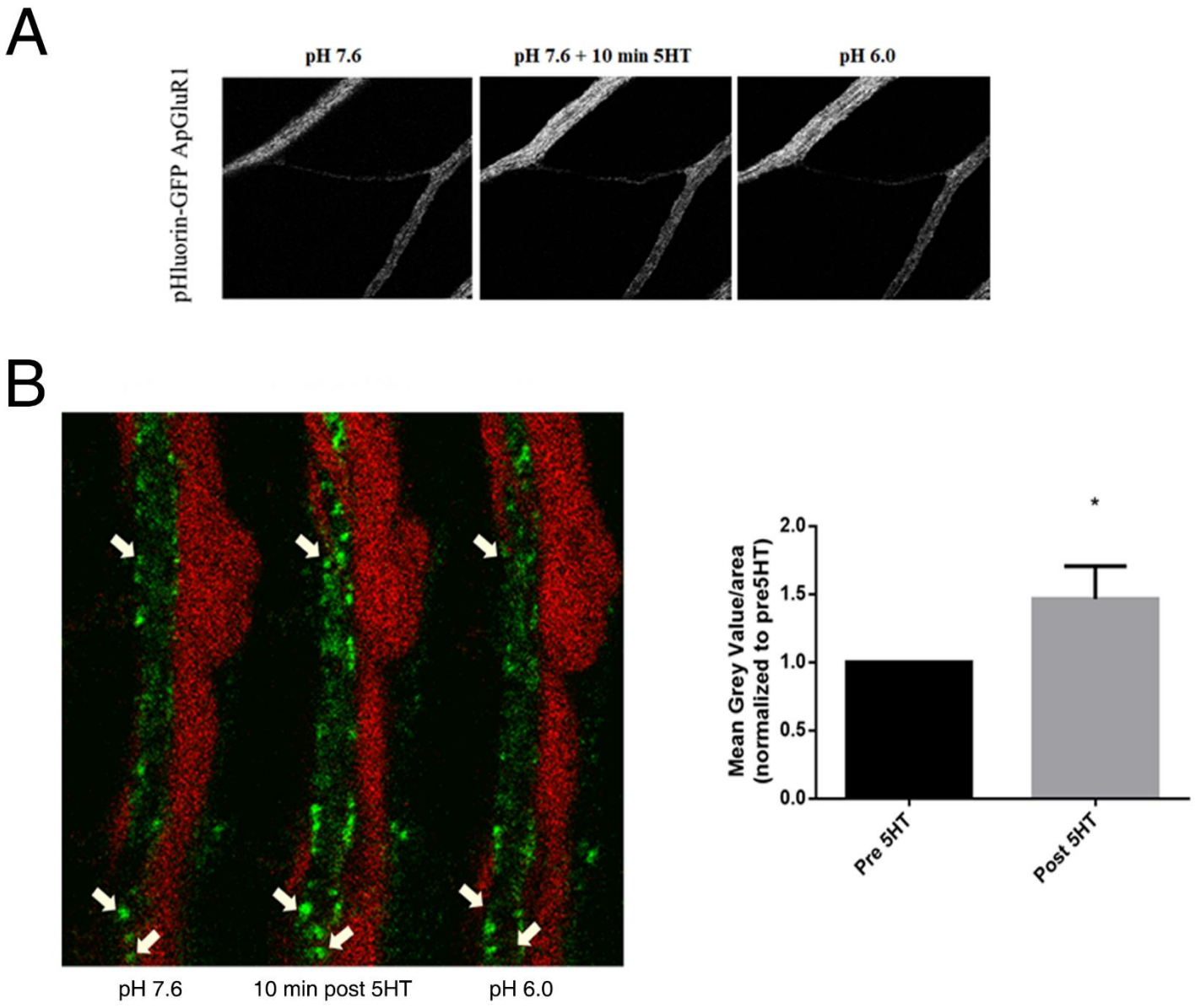


Figure 3.2 Changes in synaptic AMPA receptor levels following 5HT treatment

A) Isolated motor neurons expressing ApGluR1-pHluorin. Exposure to 10 minutes 5HT results in a slight increase in diffuse pHluorin fluorescence, which is quenched by exposure to acidic

media (pH 6.0). **B)** Sensory neurons expressing mRFP (red) and motor neurons expressing ApGluR1-pHluorin (green). Images are taken at pH 7.6 before and after 10 min 5HT treatment, followed by a third image taken after media is changed to pH 6.0. Arrowheads identify puncta that increase in fluorescence intensity after 5HT treatment and decrease in intensity after pH 6.0 wash. Right, quantification of puncta fluorescence intensity after 5HT treatment normalized to initial intensity. Only puncta whose fluorescence decreased by at least 25% after pH 6.0 wash compared to pre-5HT were included in quantification. *, $p<0.05$ two-tailed t-test (n=12 puncta from three synaptic co-cultures).

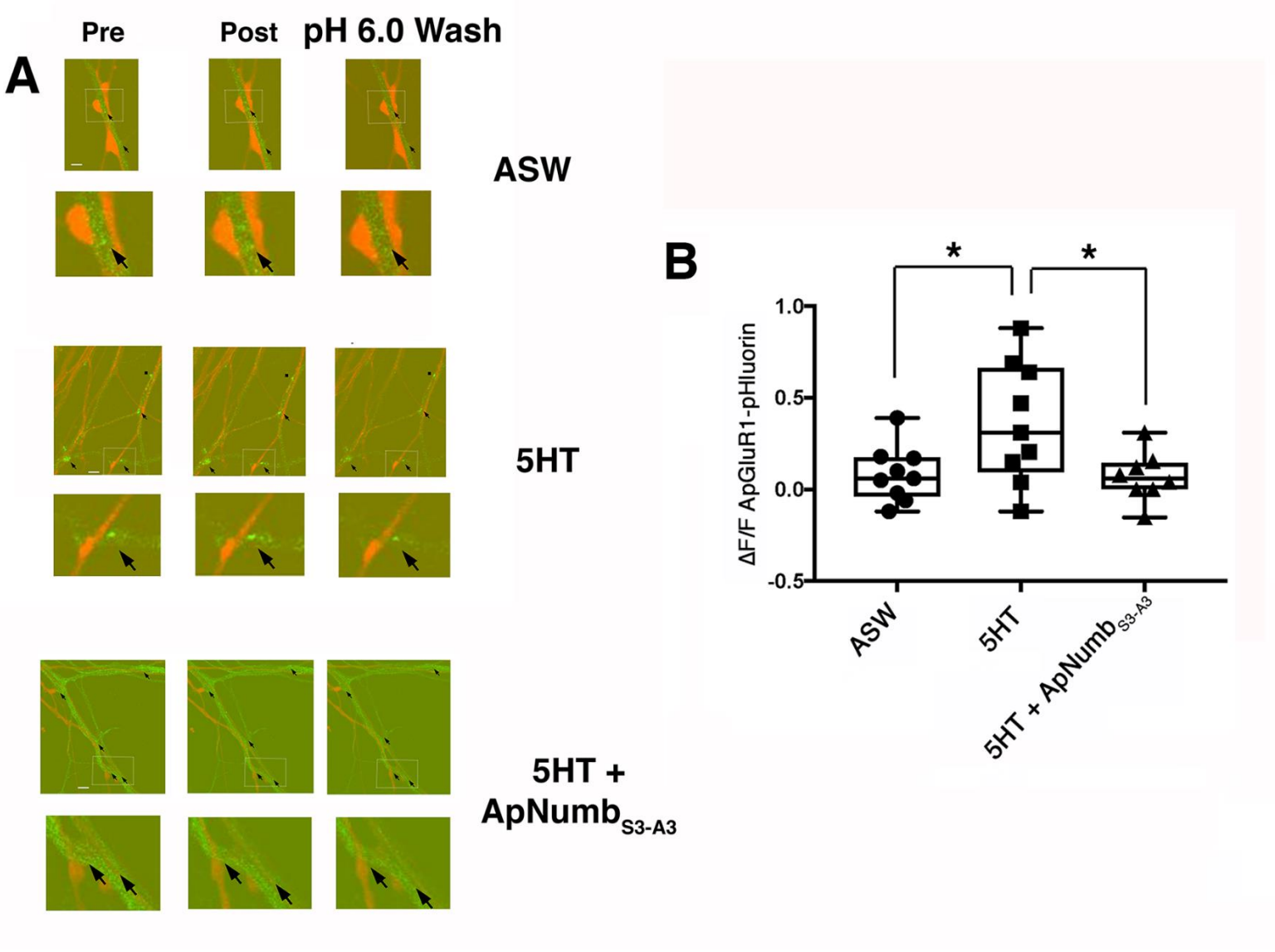


Figure 3.3 ApNumb$_{S3-A3}$ blocks the 5HT-mediated increase in the intensity of ApGluR1-pHluorin

A) Representiative images of sensory motor neuron synases before treatment, immediately after a 10 min treatment (5HT or ASW control), and post-treatment after exposure to pH 6.0 media. Motor neurons are overexpressing ApGluR1-pHluorin (green); sensory neurons are filled with dextran dye (red). Bottom, motor neurons are co-expressing ApNumb$_{S3-A3}$. Arrows point to regions of interest quantified for changes in ΔF/F. Scale bar is 13 μm for 5HT and 20 μm for

other figures. **B**) Box and Whisker plot with an overlay of the individual data points for changes in fluorescence in the three conditions. *, $p<0.05$ Tukey's Post-Hoc test after one-way ANOVA. (n=9 synapses ASW, n=9 synapses 5HT, n=8 synapses 5HT $ApNumb_{S3\text{-}A3}$)

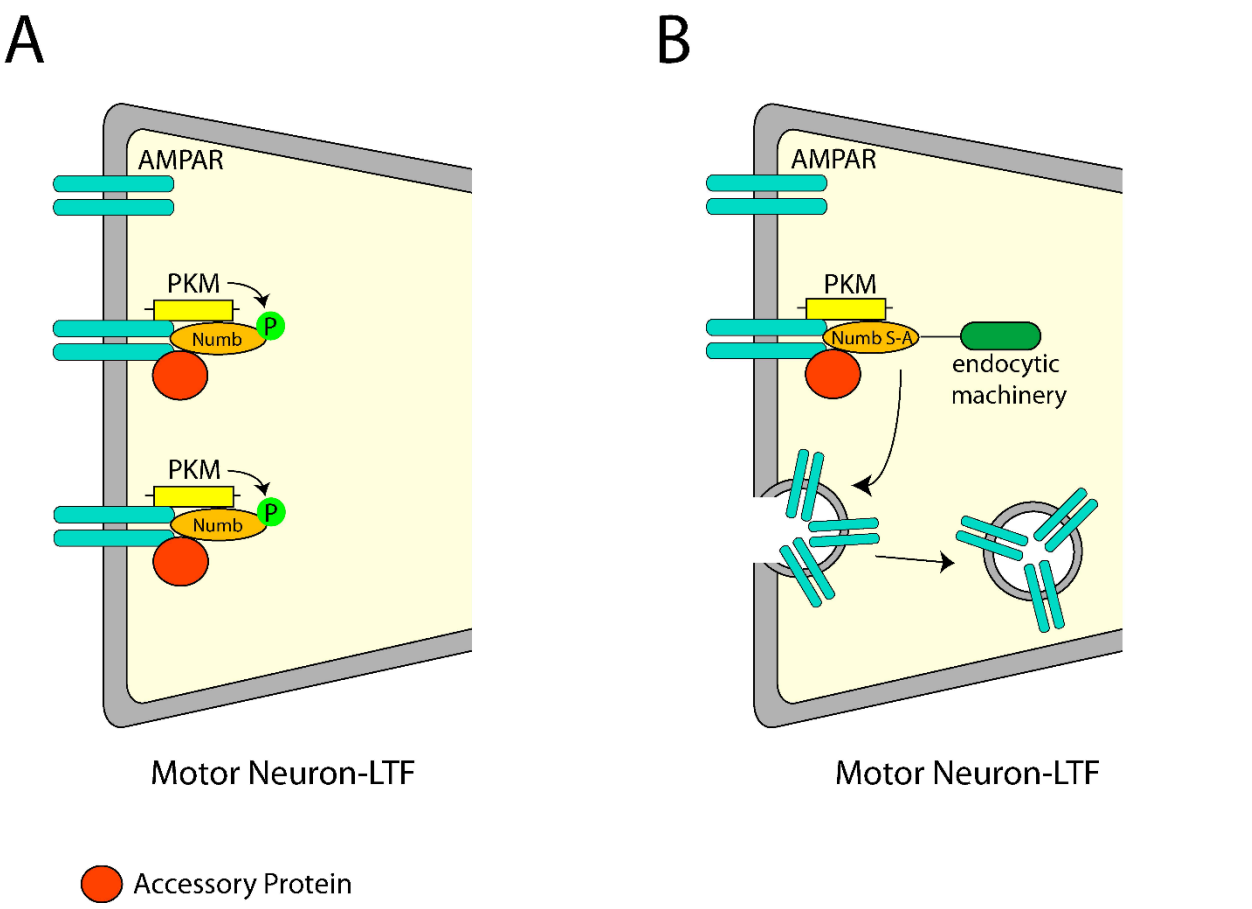


Figure 3.4 The role of PKM-mediated Numb phosphorylation in maintaining synaptic facilitation.

A) A model of postsynaptic AMPA receptors in association with Numb during LTF. Phosphorylation of Numb by PKM prevents Numb from interacting with the cell's endocytic machinery, thereby inhibiting AMPA receptor endocytosis. Numb is likely associated with both PKM and a putative accessory protein at the synapse during LTF. **B**) When Numb is not phosphorylated (for example, when $ApNumb_{S3\text{-}A3}$ mutant is overexpressed), it is unable to be inhibited by PKM-mediated phosphorylation and is able to facilitate AMPA receptor endocytosis through interaction with the cell's endocytic machinery. AMPA receptors are endocytosed and synaptic strength decreases.

CHAPTER FOUR

Stabilization by KIBRA mediates isoform specificity of PKMs in *Aplysia*

4.1 Introduction

Persistently active kinases, such as PKMs, maintain the long-term changes in synaptic strength that underlie memory (Sacktor, 2011; Sossin, 2018). PKMs are truncated forms of PKCs and are classified into three families: conventional, novel, and atypical [represented in *Aplysia* as Apl I, Apl II, and Apl III, respectively (Bougie et al., 2009)]. Because the catalytic domains of the different PKC families are similar—and because PKMs are made up solely of the catalytic domain of their parent PKC—it is plausible that different PKM isoforms may perform similar functions. The ability of the iota isoform to compensate for the loss of PKMζ in PKMζ knockout mice (Tsokas et al., 2016) supports this hypothesis. However, while evidence for the importance of the mammalian atypical PKM ζ isoform in memory maintenance is abundant (see Chapter 1 for review), it is only recently that the isoform specificity of PKMs has begun to be explored.

As described by the synaptic tagging and capture hypothesis, synapses are "tagged" for sustained potentiation during the induction of LTP. Plasticity-related proteins are then captured by these marked synapses, allocating the memory specifically to these synapses (Frey and Morris, 1998; Rogerson et al., 2014). It is hypothesized that different types of memory induce synapses to express different tags, thereby recruiting different plasticity-related proteins and distinguishing one memory synapse from another on a given neuron. PKMs are potential plasticity-related proteins, and indeed, different PKM isoforms are required to maintain different types of LTF in *Aplysia* (see **Table 4.1** for summary). It has been shown that different types of transcription-dependent persistent LTF can be induced simultaneously on the same postsynaptic neuron, and that their maintenance depends on the activity of different PKM isoforms at each

synapse (Hu et al., 2017b). This suggests that the synapses involved in each type of LTF are able to selectively recruit different PKM isoforms. This chapter will explore the kidney-brain adaptor protein, KIBRA, as a possible tag at these synapses that allows synapse-specific isoform specificity of PKMs to be achieved.

KIBRA has been implicated in human episodic memory, is expressed in memory-related brain areas, and is enriched in the postsynaptic density (see Chapter One for review). Evidence that KIBRA colocalizes with PKMζ (Vogt-Eisele et al., 2014; Yoshihama et al., 2009) suggests that KIBRA's role in memory may be related to PKMs. While PKMζ phosphorylates KIBRA (Buther et al., 2004), mutation of the two serine residues required for this phosphorylation does not affect colocalization (Vogt-Eisele et al., 2014), suggesting that the interaction between KIBRA and PKMζ in neurons is not a typical kinase-substrate interaction. Indeed, overexpression of KIBRA prevents the rapid decrease in PKMζ levels seen after inhibition of protein translation, providing evidence that KIBRA stabilizes PKMζ by preventing proteasomal degradation (Vogt-Eisele et al., 2014). Impairment in spatial memory performance is observed after conditional KIBRA knockout in mice (Vogt-Eisele et al., 2014), supporting our hypothesis that KIBRA upregulation may be the transcription dependent step that links PKM activity to transcription dependent LTF in *Aplysia*.

In order to investigate KIBRA's role in stabilizing PKMs in *Aplysia*, we first cloned *Aplysia* KIBRA and confirmed that KIBRA's characteristic domains were conserved. We found that *Aplysia* KIBRA and a mutated form of KIBRA [in which the three residues crucial for PKM interaction (Vogt-Eisele et al., 2014) were mutated] stabilize PKMs in an isoform-specific manner, suggesting that stabilization by KIBRA could be the mechanism that allows isoform-specific capture of PKMs at tagged synapses during the maintenance of LTF. Mutated KIBRA

also acts as a dominant negative, disrupting KIBRA-mediated stabilization of endogenously formed PKM Apl III. Although KIBRA mRNA is upregulated following behavioural sensitization in *Aplysia* (Ferguson et al., 2019), we found that overexpression of KIBRA alone is not sufficient to increase basal synaptic strength nor to prolong a PKM-dependent type of transcription-independent intermediate facilitation (m-ITF). Despite evidence in the literature that PKMζ kinase activity is required for stabilization by KIBRA (Vogt-Eisele et al., 2014), we found that KIBRA stabilizes PKMs independent of catalytic activity in *Aplysia* by assessing the stabilization of two different kinase-dead dominant negative forms of the kinase. We identify a region in PKM that allows KIBRA to differentiate and selectively stabilize specific PKM isoforms. While the region on KIBRA that is required for stabilization of PKMs remains inconclusive, we characterize a new conserved domain within KIBRA that contains both the PKM-binding residues identified by Vogt-Eisele et al. (2014) as well as a splice site. We show that a splice variant of KIBRA also stabilizes PKMs in an isoform-specific manner, suggesting a possible mechanism through which isoform specificity of PKMs can be achieved in the postsynaptic neuron. Because of KIBRA's isoform-specific interaction with PKMs, stabilization by KIBRA remains a promising potential mechanism through which isoform specificity of PKMs during LTF is established.

4.2 Results

4.2.1 Cloning of Aplysia KIBRA

We first looked at the conservation of KIBRA's functional domains in our model organism, *Aplysia californica.* The domains present in mammalian KIBRA are present in *Aplysia*

KIBRA with high sequence homology, including the characteristic WW-domains and the C2-domain (**Figure 4.1A**). *Aplysia* KIBRA also has a PKC phosphorylation site near the C-terminal end of the protein, similar to vertebrate KIBRA. Of particular interest is the conservation within the PKM binding region identified in mammalian KIBRA, especially the three residues that have been shown to be required for stabilization of the mammalian atypical PKM ζ (Vogt-Eisele et al., 2014). As these three residues (identified with an asterisk in **Figure 4.1B**) are conserved in *Aplysia* KIBRA, it supports the hypothesis that KIBRA's ability to stabilize PKMs is similarly conserved in this system. This PKM binding region is described in more detail in **Figure 4.7**. We cloned *Aplysia* KIBRA into a pNEX3 vector for microinjection and overexpression in cultured *Aplysia* neurons. Additionally, we generated a mutant version of KIBRA in which these three residues were converted to alanine. This KIBRA-AAA mutant was similarly cloned into a pNEX3 vector for expression in *Aplysia* neurons.

4.2.2 Generation of *Aplysia* KIBRA antibody

To confirm overexpression of these constructs, we generated an antibody specific for the C-terminal region of KIBRA. As this region is unchanged in the KIBRA-AAA mutant, we expected the antibody to be able to recognize the overexpression of both the KIBRA and KIBRA-AAA constructs. Indeed, antibody staining in cells overexpressing these constructs was much stronger than in controls expressing empty pNEX vector alone (**Figure 4.1C**). Antibody staining also confirmed that both KIBRA and KIBRA-AAA were expressed at equivalent levels when injected into cells with equal molar ratios of plasmid. While the antibody had sufficient affinity to detect the overexpressed constructs, it was not sensitive enough to detect endogenous KIBRA. Western blot of homogenized *Aplysia* ganglia did not reveal any bands corresponding to

KIBRA (expected size of the intact protein is ~190 kDa) after probing with our KIBRA antibody (data not shown).

4.2.3 KIBRA stabilizes PKMs in cultured *Aplysia* sensory neurons

We overexpressed each of the mRFP-tagged PKM isoforms (PKM Apl I, PKM Apl II, and PKM Apl III) in cultured sensory neurons along with plasmids containing eGFP and either KIBRA, KIBRA-AAA mutant, or empty pNEX vector. As all plasmid constructs share the same promoter, we overexpressed them in 1:1 molar ratios in an attempt to produce an equal number of proteins for each construct. We quantified the mRFP/eGFP ratio to determine PKM levels relative to the total amount of plasmid injected per cell. As expected based on previous findings in vertebrates (Vogt-Eisele et al., 2014), the mRFP/eGFP ratio in PKM Apl III expressing cells was significantly higher in the presence of KIBRA compared to pNEX control (**Figure 4.2**). This increase was not observed when the atypical PKM Apl III-interaction site on KIBRA was mutated (KIBRA-AAA), suggesting that these residues are indeed required for stabilization of the PKM Apl III isoform. Stabilization by KIBRA seems to be isoform specific, as the mRFP/eGFP ratio for the PKM Apl I isoform was not significantly different from control in the group overexpressing KIBRA. Unexpectedly, this ratio was increased with overexpression of KIBRA-AAA, suggesting that mutation of the three PKM-binding residues not only disrupted the protein's ability to stabilize the PKM Apl III isoform, but also generated a new function that is not exhibited by the unmutated KIBRA. The mutation likely generates a conformational change in KIBRA that reveals an otherwise unavailable PKM Apl I interaction site. It is possible that the specificity of KIBRA for different PKM isoforms may be regulated within the cell through post-translational modifications or alternative splice variants and that our mutation inadvertently mimics one of these endogenous forms of the protein.

Expression of both KIBRA and KIBRA-AAA was sufficient to increase the mRFP/eGFP ratio in cells overexpressing PKM Apl II. This may suggest that the site required for stabilization of PKM Apl II is independent of the mutated site and not obscured by the conformational change induced by the mutation. Alternatively, PKM Apl II may interact with KIBRA nonspecifically through whichever PKM interaction site is available.

4.2.4 KIBRA overexpression does not prolong ITF into LTF

Intermediate-term facilitation (ITF) in *Aplysia* is a form of facilitation that lasts longer than short-term facilitation but, unlike LTF, does not require transcription and weakens after approximately 1 hour. Massed ITF (m-ITF) requires cleavage of PKC Apl III into PKM Apl III (Farah et al., 2017) and can be blocked by postsynaptic overexpression of DN PKM Apl III (Bougie et al., 2012). If KIBRA upregulation in the postsynaptic neuron is indeed the transcription-dependent step required for PKM Apl III-mediated LTF, we hypothesized that overexpression of KIBRA postsynaptically may convert PKM Apl III-dependent m-ITF into persistent LTF. However, as shown in **Figure 4.3**, postsynaptic overexpression of KIBRA was not sufficient to increase basal synaptic strength nor to prolong m-ITF in *Aplysia* sensory motor cocultures.

4.2.5 KIBRA-AAA acts as a dominant negative

While we have shown that KIBRA stabilizes overexpressed PKMs, we haven't addressed whether KIBRA stabilizes endogenously formed PKMs. In *Aplysia*, PKMs are formed through calpain-mediated cleavage of PKCs—we therefore investigated whether KIBRA could stabilize the PKM that forms following overexpression of mRFP-tagged PKC Apl III. This PKM can be considered "endogenously formed" given that its formation depends on and is limited by the

activity and availability of the cell's endogenously produced calpains (Bougie et al., 2009). The mRFP tag is located on the N-terminal end of the PKC, which is cleaved off during PKM formation. Our PKC Apl III antibody is specific to the C-terminal end of the kinase—therefore, it will recognize both PKC Apl III as well as PKM Apl III. Uncleaved PKC Apl III is shuttled in and out of the nucleus, while cleaved PKM Apl III is present exclusively in the cytoplasm (Bougie et al., 2009). Therefore, the levels of PKM Apl III produced from overexpressed PKC Apl III can be quantified as the cytoplasmic ratio of green (C-terminal antibody staining) to red (N-terminal mRFP tag). Overexpression of KIBRA did not have an effect on this ratio compared to control, indicating that endogenous KIBRA is sufficient to stabilize the PKM produced through the cell's endogenous cleavage mechanisms (**Figure 4.4**). However, we found that the cytoplasmic green-red ratio was decreased when KIBRA-AAA was overexpressed, indicating that it acts as a dominant negative and interferes with the endogenous stabilization of PKM Apl III produced through cleavage.

4.2.6 PKM stabilization by KIBRA occurs independent of kinase activity

Overexpression of KIBRA-AAA in the postsynaptic motor neuron of *Aplysia* sensorimotor synaptic cultures has no effect on the maintenance of non-associative LTF (Hu et al., 2017b). This is expected, since non-associative LTF requires PKM Apl I (Hu et al., 2017a) and we have already shown that KIBRA-AAA stabilizes PKM Apl I (**Figure 4.2**). However, postsynaptic overexpression of KIBRA-AAA does reverse associative LTF (Hu et al., 2017b), a finding that is a bit more difficult to resolve. As summarized in **Table 4.1**, associative LTF can be reversed by postsynaptic overexpression of either DN PKM Apl II or DN PKM Apl III—this originally led us to suppose that the maintenance of associative LTF requires the activity of either PKM Apl II or PKM Apl III. Because KIBRA-AAA stabilizes PKM Apl II (**Figure 4.2**),

one might expect that KIBRA-AAA would have no effect on associative LTF, but this is not the case. One explanation for this discrepancy is that overexpression of DN PKM Apl II has an off-target effect—instead of outcompeting endogenous PKM Apl II, it competes with endogenous PKM Apl III for stabilization by KIBRA, resulting in a decrease in synaptic strength due to the destabilization of endogenous PKM Apl III. To investigate this hypothesis, we needed to assess whether KIBRA can stabilize kinase dead PKMs. In mammals, PKMζ catalytic activity is required for interaction with KIBRA (Vogt-Eisele et al., 2014). However, overexpression of dominant negative (DN) PKM isoforms along with either KIBRA, KIBRA-AAA, or empty pNEX vector in cultured *Aplysia* sensory neurons reveals that KIBRA can stabilize catalytically inactive PKMs in this system (**Figure 4.5A-B**). The pattern of stabilization between KIBRA and KIBRA-AAA was similar to that seen with catalytically active PKMs—KIBRA stabilizes DN-PKM Apl II and DN-PKM Apl III, whereas KIBRA-AAA stabilizes DN-PKM Apl I. These dominant negatives were generated by an aspartate (D) to alanine (A) mutation in the catalytic aspartic acid (D392A) (Cameron et al., 2009). While this mutation prevents catalytic activity, the kinase is still able to receive the priming phosphorylation required for proper folding of the kinase (Bougie et al., 2012; Cameron et al., 2009). The PKMs used in Vogt-Eisele et al. (2014) were rendered catalytically inactive through mutations that prevent priming phosphorylation. Mammalian PKMζ is activated by phosphorylation by PDK1 (Kelly et al., 2007), and mutations that affect PDK1 binding prevent KIBRA interaction (Vogt-Eisele et al., 2014). Additionally, they found that mutation of the lysine in the ATP binding pocket [known to prevent priming phosphorylation (Cameron et al., 2009)] also prevented KIBRA interaction. We hypothesized that it is proper protein folding mediated through priming phosphorylation, rather than catalytic activity, that is required for stabilization by KIBRA. However, PKM Apl III with a similar lysine

mutation (K279R, numbering from PKC Apl III), which does not receive priming phosphorylation (Bougie et al., 2012), was stabilized by KIBRA (**Figure 4.5C-D**). Therefore, in *Aplysia*, PKMs do not require catalytic activity or priming phosphorylation in order for KIBRA-mediated stabilization to occur.

4.2.7 DN PKM Apl II interferes with KIBRA-mediated PKM Apl III stabilization

To confirm whether the reversal of associative LTF seen after overexpression of DN PKM Apl II is due to competition for stabilization by KIBRA, we overexpressed PKM Apl III with either KIBRA or empty pNEX vector and looked at whether concurrent overexpression of DN PKM Apl II interfered with KIBRA-mediated PKM Apl III stabilization. Since DN PKM Apl I is not stabilized by KIBRA (**Figure 4.5**), we also overexpressed DN PKM Apl I to confirm that this competition for KIBRA binding is specific for DN PKM Apl II. We quantified PKM Apl III levels using a PKC Apl III C-terminal antibody as both the dominant negative constructs and the PKM Apl III construct were tagged with mRFP. Overexpression of DN PKM Apl I did not affect KIBRA's stabilization of PKM Apl III, whereas the stabilization of PKM Apl III in the presence of DN PKM Apl II was significantly reduced and, indeed, not significantly different from vector control (**Figure 4.6**). This demonstrates that DN PKM Apl II is able to interfere with KIBRA-mediated stabilization of PKM Apl III, supporting the hypothesis that the reversal of associative LTF seen when DN PKM Apl II is overexpressed in the motor neuron (Hu et al., 2017b) is due to competition with PKM Apl III for stabilization by KIBRA and not the dominant negative's influence on endogenous PKM Apl II.

4.2.8 Splice variant of KIBRA stabilizes PKM Apl I, but not PKM Apl III

The KIBRA-AAA mutant is able to stabilize PKM Apl I when both are overexpressed in cultured sensory neurons, a stabilization that is not seen with the unmutated KIBRA. We hypothesized that this mutation led to a conformational change within the KIBRA protein that opened up a binding site for PKM Apl I. If this conformational change reflects an endogenous form of the protein, it indicates a mechanism through which isoform specificity of PKMs can be achieved at synapses. In order to investigate whether or not such KIBRA variants are possible, we first examined KIBRA's structure and compared regions of high homology across evolution. In addition to the WW domains and the C2 domain already identified in the literature, we identified a new region in KIBRA that we called the KIBRA Specific Domain (KSD) (**Figure 4.8A**). The KSD is approximately 200 amino acids in length and is not similar to any other defined domain in any database. The percent identity of the KSD is similar to the percent identity of the other two conserved KIBRA domains (**Figure 4.8C**), supporting our assertion that the KSD is indeed its own domain. The sequence conservation of the KSD across evolution is depicted in **Figure 4.8B**. KIBRA is not found in prebilaterians that lack a nervous system, but KIBRA containing the KSD can be found (albeit with poor conservation) in prebilaterians like the sea anemone, Nematostella. The amino acids required for PKMζ binding [as identified in Vogt-Eisele et al. (2014)] are highly conserved across all species.

Because there is an alternative splice site within the *Aplysia* KSD, we hypothesized that upregulation of KIBRA splice variants that selectively stabilize specific PKM isoforms is how isoform specificity of PKMs may be achieved at synapses. The splice site seen in *Aplysia* is conserved in other molluscs (**Figure 4.8B**), while the Human KIBRA gene contains two distinct splice acceptor sites within the KSD (purple in **Figure 4.8B**). We hypothesized that our mutation

in KIBRA-AAA mimics a conformational change induced endogenously through production of a KIBRA splice variant. In order to test this, we cloned the KIBRA Splice (SPL) into a pNEX vector and overexpressed it in cultured *Aplysia* sensory neurons along with either mRFP-tagged PKM Apl I or PKM Apl III and assessed whether or not the splice form was able to stabilize these constructs. We found that KIBRA SPL stabilizes PKM Apl I and does not stabilize PKM Apl III (**Figure 4.8D**)—a pattern of stabilization identical to that of the KIBRA-AAA mutant (**Figure 4.2**). An upregulation of KIBRA SPL may be one mechanism through which PKM Apl I is selectively stabilized during the maintenance of non-associative LTF.

4.2.9 The "handle" region of PKMs is important for conferring isoform identity and stabilization profile

We next sought to determine how different PKM isoforms may be distinguished from one another by KIBRA. The catalytic region of classical and atypical PKCs from both *Aplysia* and mammals is depicted in **Figure 4.9A**, illustrating regions of homology between the two isoforms as well as conserved isoform specific sequences. To determine which regions are important for determining isoform specificity for KIBRA stabilization, we generated chimeras of the two isoforms by exchanging one of two select isoform specific regions: the C-terminal (CT) region important for interaction with PDZ-containing proteins (Wan et al., 2012) and an alpha-helical region that looks like a "handle" in the crystal structure of the PKMs (**Figure 4.9B**). We then assessed whether exchanging these regions had an effect on KIBRA-mediated stabilization. Exchanging the CT from PKM Apl III with the CT from PKM Apl I (PKM Apl III-CT PKM Apl I) did not affect stabilization compared to wildtype PKM Apl III (**Figure 4.9C-D**). The chimera generated by exchanging the handle region (PKM Apl III-handle PKM Apl I), on the other hand, resulted in a stabilization profile similar to PKM Apl I. Unlike PKM Apl III, the PKM Apl III-

handle PKM Apl I chimera was not stabilized by KIBRA and was instead stabilized by KIBRA SPL (**Figure 4.9C-D**). This pattern of stabilization was apparent despite the fact that the chimeras were expressed at lower levels in the absence of KIBRA compared to wildtype PKM Apl III (**Figure 4.9E**), indicating that the isoform-specific stabilization by KIBRA splice forms is mediated by the handle region of their target PKM.

4.3 Discussion

4.3.1 KIBRA may be involved in the capture of plasticity related proteins at LTF synapses

Disrupting PKM Apl III through postsynaptic overexpression of a dominant negative form of the kinase during the maintenance phase of LTF is sufficient to reverse associative LTF but has no effect on non-associative LTF (Hu et al., 2017a). The reverse is true when dominant negative PKM Apl I is overexpressed postsynaptically—interrupting PKM Apl I activity reverses non-associative LTF but has no effect on associative LTF. This is evidence that PKMs act as plasticity-related proteins during these different forms of LTF, and that different plasticity-related proteins are required for and must be captured by synapses involved in different forms of LTF. Our results showing isoform-specific stabilization of PKMs by KIBRA suggest a possible mechanism through which the postsynaptic neuron can selectively upregulate PKMs at specific synapses during LTF.

The induction of LTF initiates the production of plasticity-related proteins, such as PKMs, in the postsynaptic neuron. In *Aplysia*, this upregulation occurs through cleavage of PKCs by calpains, although whether this cleavage occurs locally at the affected synapse or globally in the cell soma is unclear. Different calpains are responsible for generating distinct

PKMs in *Aplysia*—for example, overexpression of dominant negative small optic lobe (SOL) calpain disrupts the formation of PKM Apl III from overexpressed PKC Apl III (Hastings et al., 2018). It is unknown whether this cleavage occurs at the synapse after LTF induction, or whether PKMs are formed in the cell soma and subsequently shuttled to the affected synapse through an unknown mechanism. However, it is unlikely that calpains themselves act as synaptic tags—interfering with calpain activity prevents the induction of LTF, but not LTF maintenance (Hu et al., 2017a). Even if cleavage occurs locally, the affected synapse must still be tagged in some way in order to recruit the calpains required for cleavage of specific PKM isoforms. This becomes apparent when there is one postsynaptic cell receiving input from multiple presynaptic sources. Different synapses within one postsynaptic neuron rely on the activity of different PKM isoforms (Hu et al., 2017b). Our results indicate that KIBRA could be one mechanism through which the cell achieves this isoform specificity. Following induction of associative LTF, for example, KIBRA may be upregulated and localized to the affected synapses. KIBRA then selectively upregulates PKM Apl III at these synapses by preventing its degradation and allowing the activity of the kinase to persist during the maintenance of associative LTF. A similar mechanism likely occurs at synapses encoding non-associative LTF—the synaptic tag in non-associative LTF is likely a variant of KIBRA (such as KIBRA SPL) that selectively stabilizes PKM Apl I. The production of synapse-specific tags and the subsequent capture of their corresponding plasticity-related proteins is one potential mechanism through which the postsynaptic neuron is able to distinguish inputs received from different presynaptic sources.

4.3.2 KIBRA-mediated stabilization of PKMs is required for the maintenance of LTF

We've shown that the KIBRA-AAA construct generated through the mutation of three crucial PKM ζ binding residues acts as a dominant negative in regard to the stabilization of

endogenously formed PKM Apl III. Because KIBRA overexpression did not stabilize the PKM Apl III formed through overexpression of PKC Apl III in these experiments, we hypothesize that PKC Apl III overexpression induces production of endogenous KIBRA and that this endogenous KIBRA is sufficient to stabilize the PKM Apl III generated from overexpressed PKC Apl III. Unfortunately, our KIBRA antibody is not specific enough to detect endogenous KIBRA levels and confirm this hypothesis. Although this model suggests that KIBRA-AAA overexpression interferes with the ability of endogenously produced KIBRA to stabilize PKM Apl III, how this interference occurs is not clear. We have shown that KIBRA-AAA cannot stabilize PKM Apl III, so it is unlikely that KIBRA-AAA acts in competition with KIBRA for binding to PKM Apl III. We do not yet fully understand how KIBRA stabilizes PKMs, but it is likely that KIBRA-AAA's dominant negative effect stems from its interference with this unknown stabilization mechanism rather than through competition for interaction with PKM Apl III.

Our mutant KIBRA construct was overexpressed postsynaptically by our collaborators during the maintenance phase of two different forms of LTF in *Aplysia* sensory-motor neuron cultures (Hu et al., 2017b). Overexpression of KIBRA-AAA had no effect on the persistence of PKM Apl I-dependent non-associative LTF. This is in line with our results showing that KIBRA-AAA stabilizes PKM Apl I. However, KIBRA-AAA overexpression was sufficient to reverse PKM Apl III-dependent associative LTF. Since we've shown that KIBRA-AAA disrupts the stabilization of endogenous PKM Apl III, this result supports the hypothesis that stabilization of PKM Apl III is required for the maintenance of associative LTF. When this stabilization is disrupted, endogenous PKM Apl III is presumably degraded, removing the synapse's mechanism of maintaining facilitation-induced increase in synaptic strength. A similar mechanism is likely

in place for PKM Apl I-dependent synapses—by interfering with the stabilization of PKM Apl I, we would expect to disrupt the maintenance of non-associative LTF.

4.3.3 Isoform specificity of PKMs is determined by KIBRA splice variants

As summarized in **Table 4.2**, KIBRA stabilizes PKMs in an isoform-specific and activity-independent manner. The presence of endogenous KIBRA splice variants suggests a possible mechanism through which PKM isoform specificity at synapses can occur. We've shown that a KIBRA splice variant present in *Aplysia* stabilizes different PKM isoforms compared to wildtype KIBRA, presenting a transcriptional means of regulating PKM isoform levels at synapses. Based on this model, one might expect an upregulation of KIBRA SPL mRNA following induction of non-associative LTF to increase levels of PKM Apl I at the affected synapse. Similarly, an upregulation of wildtype KIBRA mRNA is expected following induction of PKM Apl III-dependent associative LTF.

As mentioned previously, it is not yet known whether PKMs are formed locally at memory synapses or if they are formed globally and must be localized to specific synapses by additional mechanisms. If PKMs are produced outside of the synapse, KIBRA may act as a scaffolding protein in localizing PKMs to the appropriate synapses. Although the new domain we've identified within KIBRA contains the PKMζ-binding region described by Vogt-Eisele et al. (2013) as well as the alternative splice site we've shown to be important in determining isoform specificity for PKM stabilization, the region has also been implicated in other pathways. For example, the KIBRA-Ex-Mer complex required for activation of the Hippo pathway is formed through interactions localized to this region (Baumgartner et al., 2010; Genevet et al., 2010). KIBRA often acts as a scaffold in nonneuronal cell processes due to interactions with its WW and C2 domains (see Chapter 1)—it is possible that similar mechanisms occur in neurons

during memory, linking KSD-interacting proteins (like PKMs) with proteins that interact with KIBRA's other domains [e.g. the postsynaptic density protein, Dendrin, which interacts with KIBRA's WW-domain (Ji et al., 2019)]. KIBRA has been shown to associate with AMPA receptors (Makuch et al., 2011), which may be important in bringing PKMs in close proximity to endocytic adaptor proteins or other substrates affecting AMPA receptor trafficking. Alternatively, distinct calpains may be present at synapses to selectively cleave only the required PKMs. In this case, all splice isoforms of KIBRA may be present uniformly across synapses and may only be important for stabilization, not for localization. Because our KIBRA antibody is not specific enough to detect endogenous KIBRA, future experiments looking at KIBRA localization within neurons during different forms of LTF are necessary.

4.3.4 PKM Apl II stabilization by KIBRA and KIBRA-AAA

We have shown that the reversal of associative LTF seen after postsynaptic overexpression of DN PKM Apl II (Hu et al., 2017a) is due to an interference with the stabilization of endogenous PKM Apl III. While PKM Apl II does not seem to be required for the maintenance of associative LTF, we've shown that KIBRA is able to stabilize PKM Apl II and that this stabilization occurs even after mutation of the three serine residues important for PKMζ stabilization. This may indicate that PKM Apl II stabilization can occur at both of KIBRA's stabilization sites: the PKM Apl III stabilization site and the cryptic PKM Apl I stabilization site that is revealed in the mutated KIBRA-AAA. Alternatively, it may indicate a PKM Apl II-specific stabilization site on KIBRA, a site that remains available even after the conformational change we propose is induced by the mutation. Interestingly, the ability of KIBRA-AAA—but not KIBRA—to stabilize PKM Apl II depends on kinase activity, a level of specificity that may be influenced by KIBRA's protein conformation. While we did not assess

the ability of KIBRA SPL to stabilize PKM Apl II, it is likely that the pattern of stabilization we observed by KIBRA and KIBRA-AAA has physiological significance. Given that DN PKM Apl II overexpression in the presynaptic neuron is sufficient to reverse both associative and non-associative LTF (Hu et al., 2017a), it is possible that KIBRA-mediated stabilization of PKM Apl II serves a purpose in the presynaptic neuron. However, the precise role of PKM Apl II and other members of the novel family of PKMs in memory is still largely unstudied.

4.3.5 KIBRA overexpression does not prolong m-ITF

Given that overexpression of PKM Apl III in the postsynaptic neuron is sufficient to increase synaptic strength in the absence of stimulation (Ferguson et al., 2019), we had expected that upregulating PKM Apl III levels through KIBRA-mediated stabilization would similarly produce an increase in synaptic strength and/or prolong m-ITF. However, its failure to do so is not completely surprising. While PKM Apl III is produced during m-ITF and is required for the formation of m-ITF, it is likely that the function of the kinase in this process is very different from its function in LTF. The postsynaptic release of neurotrophins required for the induction of m-ITF, for example, may require PKM activity (Farah et al., 2019). Additionally, m-ITF may not rely on the constitutive activity of the kinase—rather, PKM Apl III may be important during the induction of m-ITF but not necessarily its maintenance. Our lab has shown that, although induction of m-ITF results in an increased localization of AMPA receptors to the membrane, increased synaptic AMPA receptor levels are not required for the expression of m-ITF (Farah et al., 2019). This may explain why prolonging the half life of the PKM by overexpressing KIBRA did not have the effect we predicted. Another explanation could be that the amount of PKM Apl III produced during m-ITF is simply not comparable to the amount generated through overexpression to produce an increase in synaptic strength. In this case, even if KIBRA is

present and is stabilizing endogenous PKM Apl III, PKM levels may not be high enough to produce a noticeable effect. It is also likely that the expression of LTF requires the activation of synaptic pathways independent of PKM Apl III activity that are simply not engaged by the induction of m-ITF. While overexpression of KIBRA was not sufficient to prolong m-ITF, this result does highlight some potential differences between the mechanisms governing ITF and LTF.

4.3.6 What is required for KIBRA-mediated stabilization of PKMs to occur?

We have determined that catalytic activity of PKMs is not required for KIBRA-mediated stabilization to occur. Additionally, the presence of the priming phosphorylation that is necessary for kinase activation is not required for stabilization by KIBRA. Analysis of the 3D crystal structure of PKMs reveals an alpha helical "handle" region as a possible interaction site for KIBRA—indeed, the amino acid sequences of this region across PKM isoforms are distinct, and we've shown that the isoform specific stabilization of PKMs by KIBRA and KIBRA SPL depends on the identity of this handle region. This handle is the target of isoform-specific PKC inhibitors like ICA-1 and ζ-stat (Pillai et al., 2011; Ratnayake et al., 2018) and may be important for determining isoform-specific substrate interactions of PKCs (Soriano et al., 2016). Since switching the handle region from one isoform to another was sufficient to cause KIBRA and KIBRA SPL to misidentify the chimeric PKM, it is very likely that the handle is the key feature of PKMs that determines their isoform specificity at synapses during LTF.

4.4 Conclusions

We've shown that KIBRA is not only conserved in *Aplysia*, but that the protein's ability to stabilize PKMs is conserved as well. We've indicated that KIBRA is not only a potentially crucial memory-related protein but that it may also play a role in differentiating synapses within neurons involved in multiple memory networks. We've begun to narrow down the requirements for KIBRA-mediated stabilization of PKMs to occur in an attempt to explain how neurons can differentiate and regulate PKM isoforms during LTF. KIBRA-mediated isoform-specific stabilization of PKMs provides a possible mechanism through which synapses in the brain can be allocated to different memory circuits, helping us understand how the brain is organized at a cellular and molecular level.

4.5 Tables and Figures

	Presynaptic			Postsynaptic		
	DN PKM Apl I	DN PKM Apl II	DN PKM Apl III	DN PKM Apl I	DN PKM Apl II	DN PKM Apl III
Non-Associative LTF	No effect	Reverses	Reverses	Reverses	No effect	No effect
Associative LTF	No effect	Reverses	No effect	No effect	Reverses	Reverses

Table 4.1 The effect of different dominant negative PKM isoforms on LTF when expressed in the presynaptic sensory neuron or the postsynaptic motor neuron of *Aplysia* sensory motor neuron cocultures, as demonstrated in Hu et al., 2017a. Dominant negative constructs were expressed during the maintenance phase of LTF (2 days after LTF induction).

	PKM Apl I		PKM Apl II		PKM Apl III		PKM Apl III CT-PKM Apl I	PKM Apl III handle-PKM Apl I
	WT	**DN**	**WT**	**DN**	**WT**	**DN**		
KIBRA	X	X	Stabilizes	Stabilizes	Stabilizes	Stabilizes	Stabilizes	X
KIBRA-AAA	Stabilizes	Stabilizes	Stabilizes	X	X	X	-	-
KIBRA-Spl	Stabilizes	-	-	-	X	-	X	Stabilizes

Table 4.2 Stabilization of wildtype (WT) and dominant negative (DN) PKM isoforms by each of our KIBRA constructs. X, no stabilization. Stabilization of dominant negative isoforms and PKM Apl II by KIBRA-Spl was not explored. Stabilization of chimeras (PKM Apl III CT-PKM Apl I and PKM Apl III handle-PKM Apl I) by KIBRA-AAA was not explored.

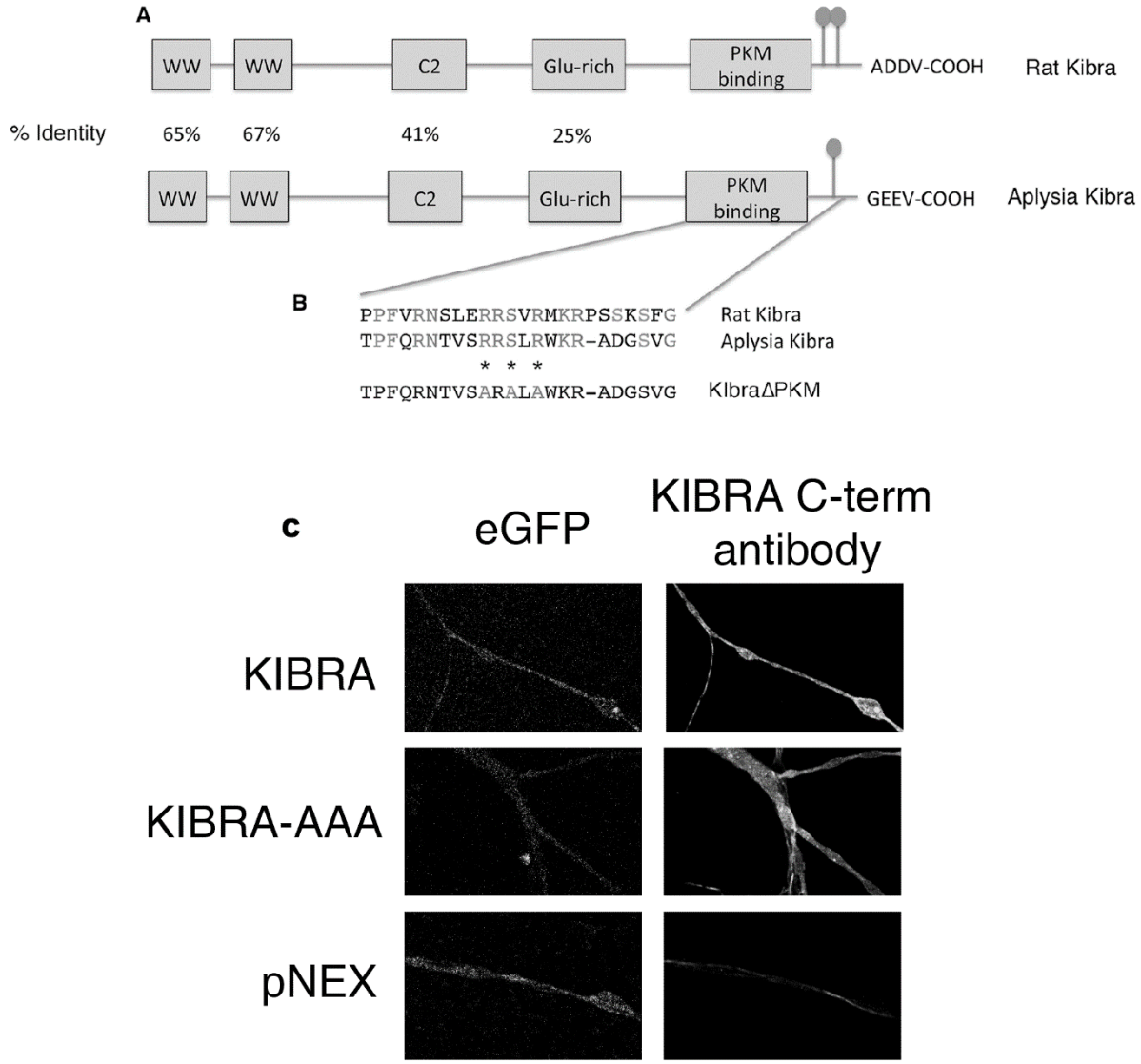
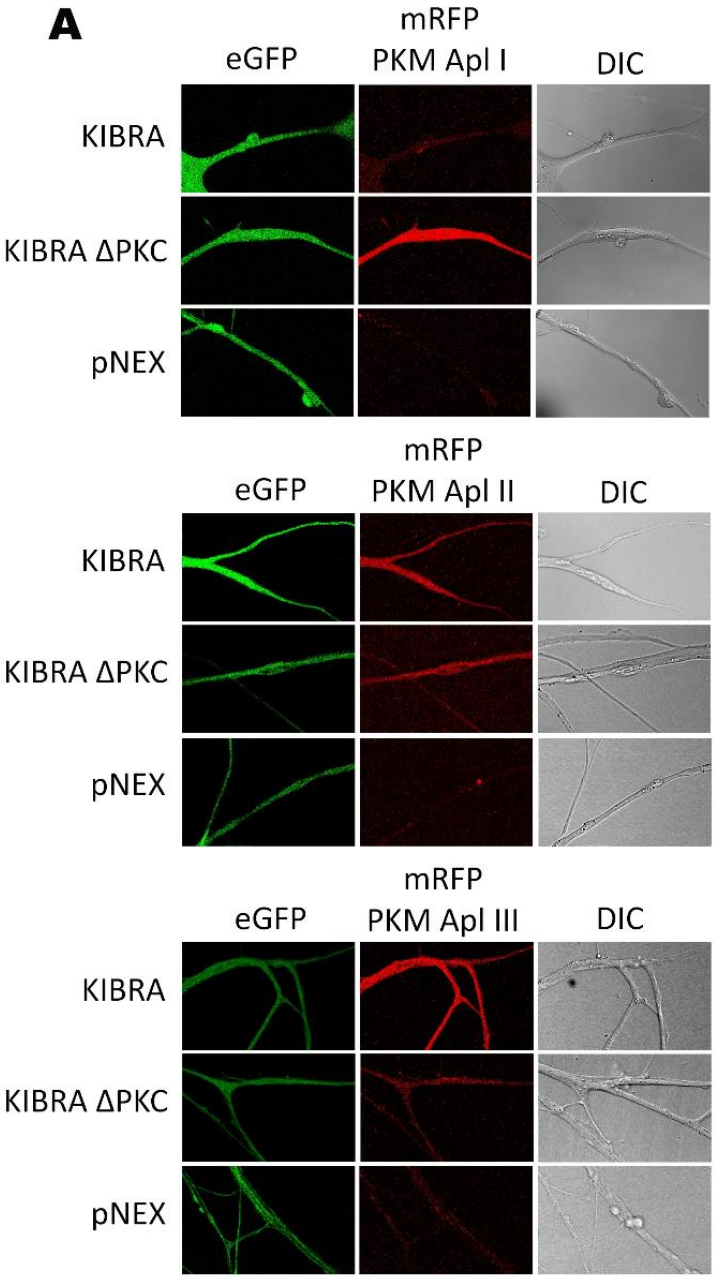


Figure 4.1 Characterization of Aplysia KIBRA

A) Description of KIBRA domains and conservation between vertebrate and *Aplysia* KIBRA. Sequence homology presented as percentages within each of KIBRA's characterized domains. PKC-phosphorylation sites are identified with markers near the C-terminal end. **B**) Within the atypical PKC binding domain ("PKM binding"), conserved residues are highlighted. The three residues marked with an asterisk are mutated to alanine in our mutant construct (KIBRA-AAA,

or KIBRAΔPKM). **C**) Representative images of *Aplysia* sensory neurons overexpressing KIBRA, KIBRA-AAA, or empty pNEX vector in combination with eGFP (to confirm successful injection and expression of constructs). Cells were fixed and immunostained with KIBRA C-terminal antibody followed by Alexa Fluor 647 goat anti-rabbit secondary antibody. Antibody staining was increased in KIBRA expressing cells (4.2 ± 1.3 fold, N=15) and KIBRA-AAA expressing cells (4.8 ± 2.1 fold, N=18) to a similar extent ($p > 0.1$, two-tailed Student's t-test between the increase in KIBRA and KIBRA-AAA expressing cells) compared to pNEX control cells.


A
eGFP
mRFP
PKM Apl I
DIC
KIBRA
KIBRA ΔPKC
pNEX
eGFP
mRFP
PKM Apl II
DIC
KIBRA
KIBRA ΔPKC
pNEX
eGFP
mRFP
PKM Apl III
DIC
KIBRA
KIBRA ΔPKC
pNEX

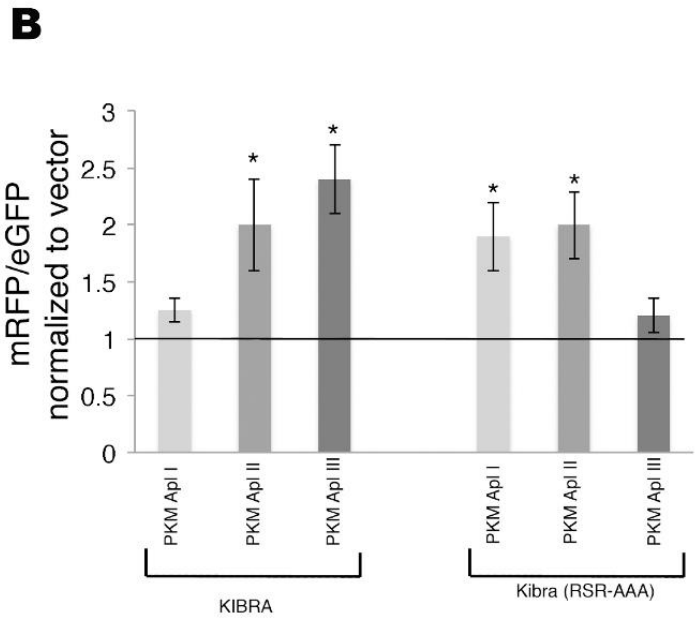

B
mRFP/eGFP
normalized to vector
3
2.5
2
1.5
1
0.5
0
*
*
*
*
PKM Apl I
PKM Apl II
PKM Apl III
PKM Apl I
PKM Apl II
PKM Apl III
KIBRA
Kibra (RSR-AAA)

Figure 4.2 KIBRA stabilizes PKMs in an isoform-specific manner

A) Representative images showing expression of eGFP (green) and mRFP-tagged PKM Apl I (top), PKM Apl II (middle), and PKM Apl III (bottom) with either empty vector (pNEX), KIBRA, or KIBRA-AAA in neurites of isolated *Aplysia* sensory neurons. Images were taken in live neurons approximately 24h following injection. **B**) Quantification of stabilization. All results are normalized in each experiment to the average mRFP/eGFP ratio of the vector alone (pNEX control group, shown with a solid horizontal line). ANOVAs were performed separately for PKM Apl I (pNEX, N=29; KIBRA, N=43; KIBRA-AAA, N=26; $F_{2,97} = 6.7$, $p < 0.001$), PKM Apl II (pNEX, N=18; KIBRA, N=15; KIBRA-AAA, N=22; $F_{2,54} = 4.04$, $p < 0.05$), and PKM Apl III (pNEX, N=16; KIBRA, N=31; KIBRA-AAA, N=31; $F_{2,77} = 12.1$, $p < 0.0001$). N= number of neurons. *p<0.05 for a post-hoc comparison between this group and the control pNEX group (Bonferroni post-hoc test). All experiments were repeated in a minimum of three separate preparations of sensory neurons. Error bars are SEM.

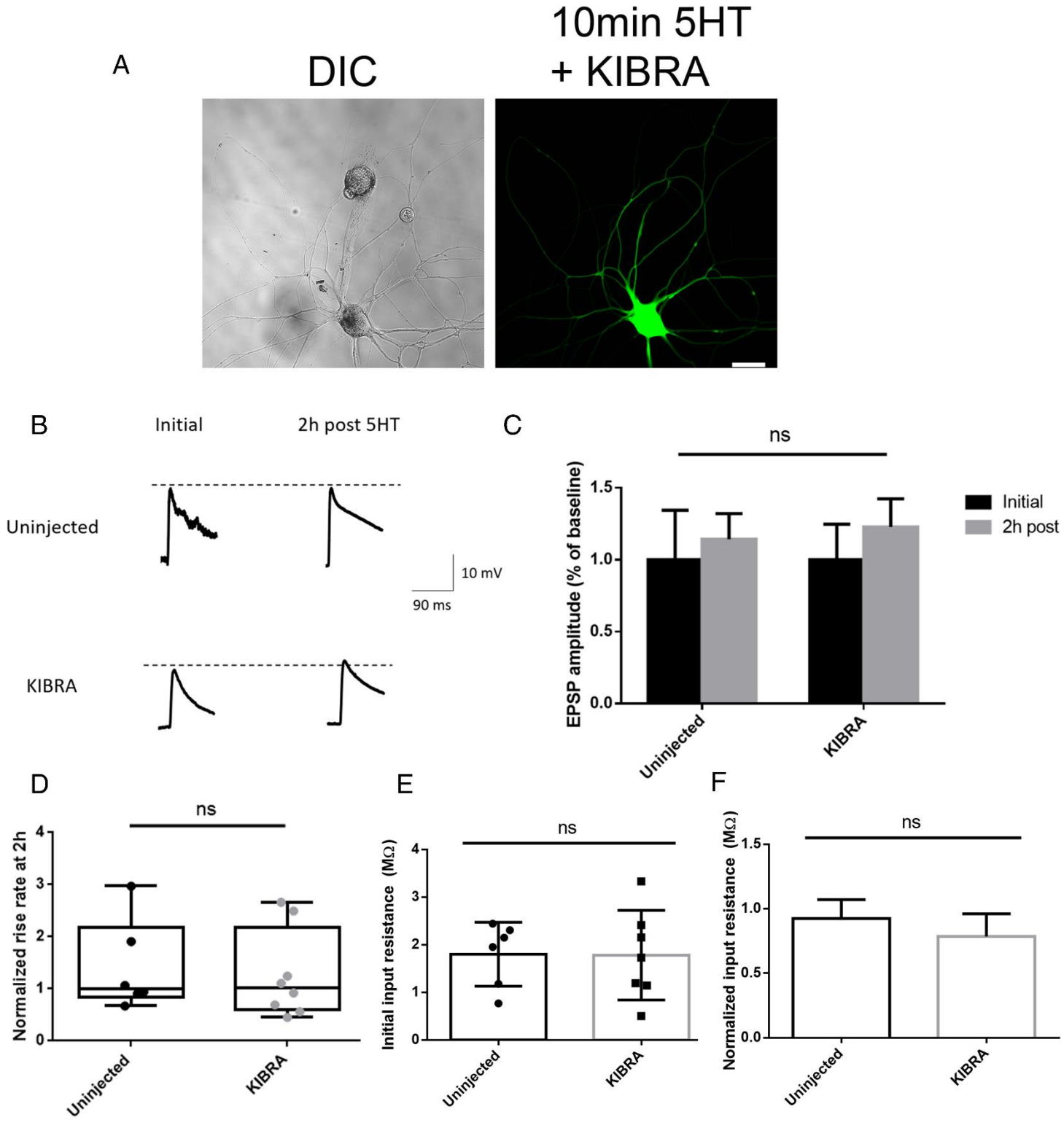
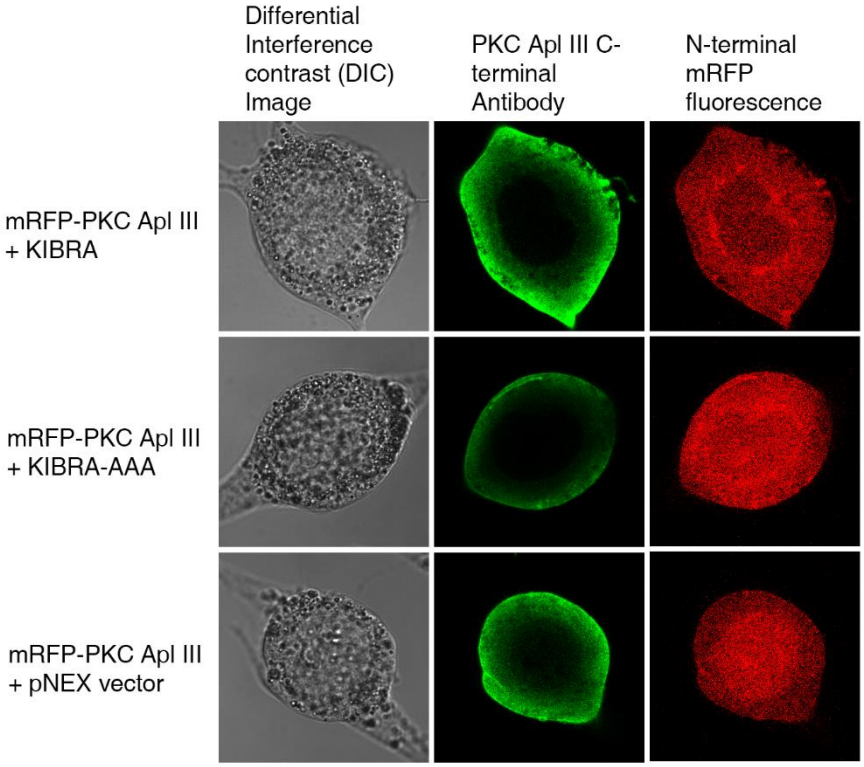

A
DIC
10min 5HT
+ KIBRA
B
Initial
2h post 5HT
Uninjected
KIBRA
10 mV
90 ms
C
ns
EPSP amplitude (% of baseline)
1.5
1.0
0.5
0.0
Initial
2h post
Uninjected
KIBRA
D
ns
Normalized rise rate at 2h
4
3
2
1
0
Uninjected
KIBRA
E
ns
Initial input resistance (MΩ)
4
3
2
1
0
Uninjected
KIBRA
F
ns
Normalized input resistance (MΩ)
1.5
1.0
0.5
0.0
Uninjected
KIBRA

Figure 4.3 KIBRA overexpression is not sufficient to prolong ITF.

A) Representative confocal and differential interference contrast (DIC) image of *Aplysia* sensorimotor synaptic culture. Motor neurons were injected with plasmids containing KIBRA and eGFP (to confirm expression) 24 hours prior to electrophysiological recordings. Scale bar is 50 μm. **B**) Representative traces before and 2 hours following 10 min 10μM 5HT treatment in synapses overexpressing KIBRA in the postsynaptic neuron or uninjected controls. Scale bar is 10mV/90ms. **C**) Summary of the change in EPSP amplitude 2 hours post 5HT treatment in postsynaptic neurons expressing KIBRA (n=8) and uninjected controls (n=5). The change in PSP at 2 h was similar between groups via unpaired t-test (ns, p = 0.4380). **D**) Box and whisker graph with overlaid individual data points (uninjected controls, n = 6; KIBRA, n = 8) showing the change in rise-rate 2 hours post-5HT treatment as a percentage of the initial rise rate of the synapses measured. Rise-rate was not significantly different between groups (one-way unpaired t-test [ns, p = 0.7613]). The change in PSP at 2 h was similar between groups via unpaired t-test (ns, p = 0.4380). Initial synaptic strength was similar for both groups (Uninjected 35.99 ± 12.8 mV; KIBRA 30.31 ± 7.4 mV; unpaired t-test p = 0.6919). Initial postsynaptic input resistance (**E**) and the change in postsynaptic input resistance (**F**) was similar between groups (180.0 ± 27.4 MΩ Uninjected and 177.9 ± 35.6 MΩ KIBRA, P=0.9661 that changed to 92.4 ± 6.0 % and 78.6 ± 6.2 % respectively at 2h, p = 0.1448 compared with unpaired t-tests). Membrane potential of the LFS motor neuron was not significantly different before and after 5HT application in either group (unpaired t-tests: uninjected, p = 0.8910; KIBRA, p = 0.5881). LFS holding potential was similarly unchanged (unpaired t-tests: uninjected, p = 0.6187; KIBRA, p = 0.3021).

A


Differential Interference contrast (DIC) Image
PKC Apl III C-terminal Antibody
N-terminal mRFP fluorescence
mRFP-PKC Apl III + KIBRA
mRFP-PKC Apl III + KIBRA-AAA
mRFP-PKC Apl III + pNEX vector

B

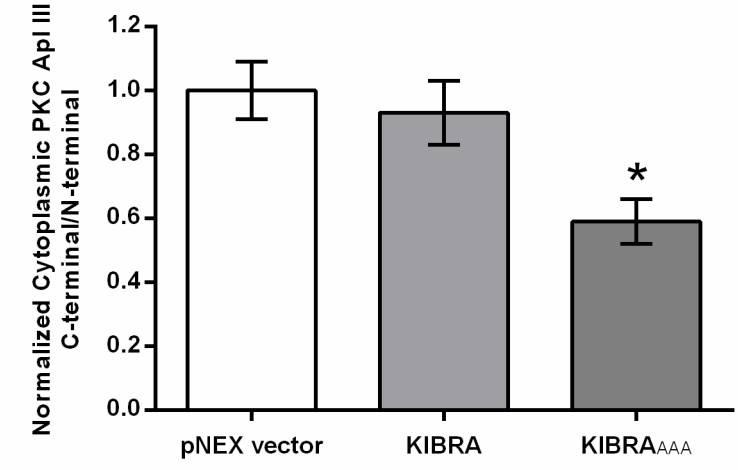

Normalized Cytoplasmic PKC Apl III C-terminal/N-terminal
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
pNEX vector
KIBRA
KIBRAAAA

Figure 4.4 KIBRA-AAA acts as a dominant negative and destabilizes cleaved PKM Apl III

A) Representative confocal fluorescence images of cell bodies from fixed *Aplysia* sensory neurons expressing mRFP-tagged PKC Apl III along with either KIBRA, KIBRA-AAA, or empty pNEX vector. Cells were fixed and immunostained with rabbit anti-PKC Apl III C-terminal antibody followed by Alexa 488 goat anti-rabbit secondary antibody. **B**) Quantification of the cytoplasmic green/red ratio. One-way ANOVA for the three groups (pNEX, KIBRA, and KIBRA-AAA) [$F(57,2) = 31.8$, $p < 0.01$] with Tukey's test post-hoc ($p < 0.01$) revealed a significant difference in the green/red ratio in the KIBRA-AAA group compared to KIBRA and pNEX. Results are from three independent experiments (60 total sensory neurons: pNEX, N=28; KIBRA, N=14; KIBRA-AAA, N=18). Error bars are SEM.

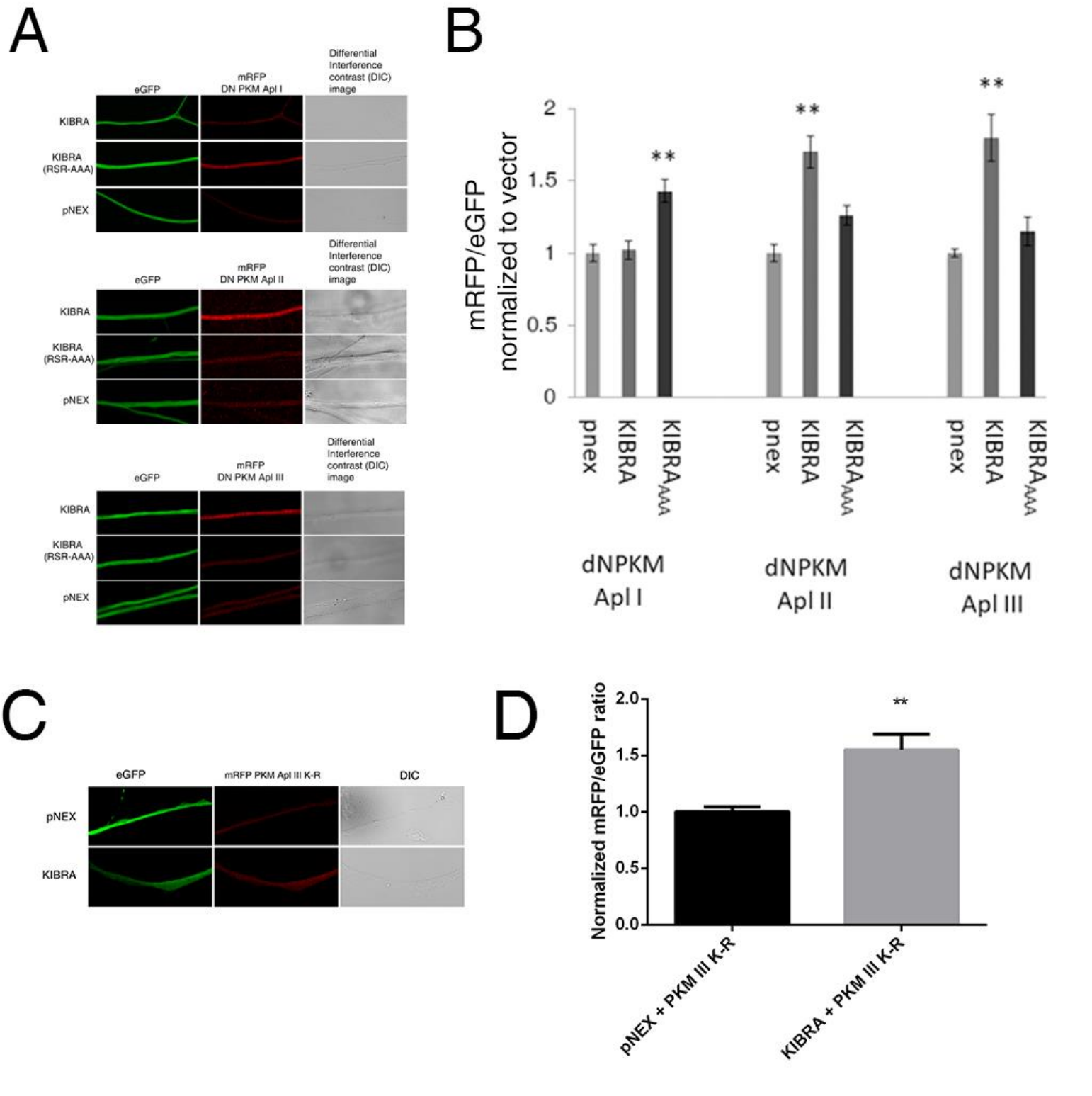

A
eGFP
mRFP
DN PKM Apl I
Differential
Interference
contrast (DIC)
image
KIBRA
KIBRA
(RSR-AAA)
pNEX
eGFP
mRFP
DN PKM Apl II
Differential
Interference
contrast (DIC)
image
KIBRA
KIBRA
(RSR-AAA)
pNEX
eGFP
mRFP
DN PKM Apl III
Differential
Interference
contrast (DIC)
image
KIBRA
KIBRA
(RSR-AAA)
pNEX
B
mRFP/eGFP
normalized to vector
2
1.5
1
0.5
0
**
**
**
pnex
KIBRA
KIBRA$_{AAA}$
pnex
KIBRA
KIBRA$_{AAA}$
pnex
KIBRA
KIBRA$_{AAA}$
dNPKM
Apl I
dNPKM
Apl II
dNPKM
Apl III
C
eGFP
mRFP PKM Apl III K-R
DIC
pNEX
KIBRA
D
Normalized mRFP/eGFP ratio
2.0
1.5
1.0
0.5
0.0
**
pNEX + PKM III K-R
KIBRA + PKM III K-R

Figure 4.5 KIBRA stabilizes inactive PKMs in isolated Aplysia sensory neurons.

A) Representative images of eGFP and mRFP-DN PKM Apl I (top), mRFP-DN PKM Apl II (middle), and mRFP-DN PKM Apl III (bottom) with either vector (pNEX3), KIBRA, or KIBRA-AAA in neurites of cultured *Aplysia* sensory neurons 24h after injection. **B**) Stabilization of DN PKMs is quantified as the average mRFP/eGFP ratio normalized within each experiment to the average mRFP/eGFP ratio of the pNEX group. KIBRA-AAA stabilizes DN PKM Apl I, while KIBRA stabilizes DN PKM Apl II and DN PKM Apl III. ANOVAs were performed separately for DN PKM Apl I (pNEX, n=39; KIBRA, n=66; $KIBRA_{RSR\text{-}AAA}$, n=70; $p<0.01$), DN PKM Apl II (pNEX, n=61; KIBRA, n=43; $KIBRA_{RSR\text{-}AAA}$, n=31; $p<0.01$), and DN PKM Apl III (pNEX, n=28; KIBRA, n=36; $KIBRA_{RSR\text{-}AAA}$, n=24; $p<0.01$). **C**) Representative images of eGFP and mRFP-PKM Apl III K-R with either pNEX or KIBRA in neurites of cultured *Aplysia* sensory neurons 24h after injection. **D**) Stabilization of PKM Apl III K-R is quantified as the average mRFP/eGFP ratio normalized as above. PKM Apl III K-R levels are higher in the presence of KIBRA compared to pNEX control. Unpaired t-test between KIBRA (n=31) and pNEX (n=24) groups ($p<0.01$).

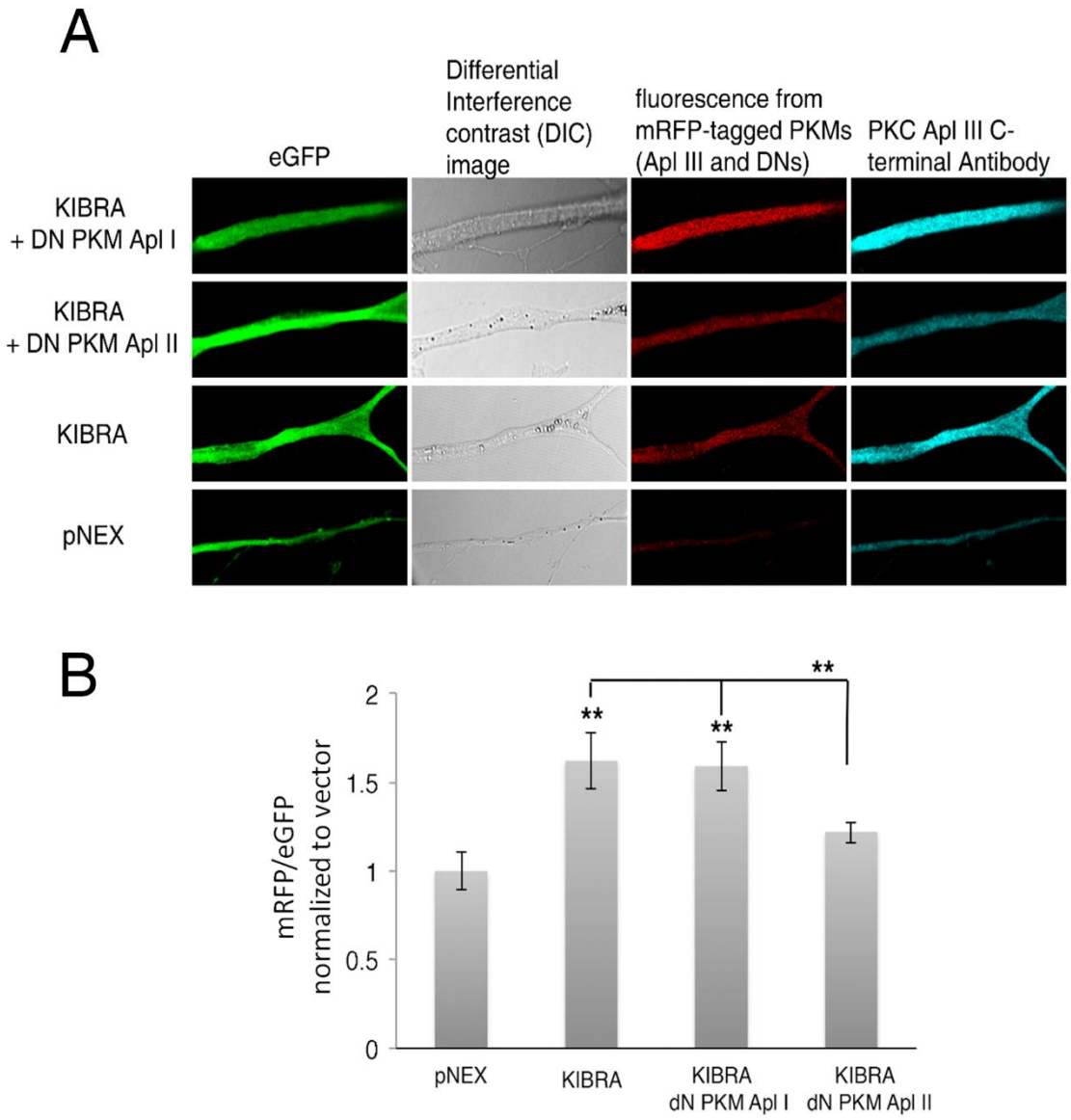


Figure 4.6 Overexpression of DN PKM Apl II interferes with KIBRA-mediated stabilization of overexpressed PKM Apl III.

A) Representative images of cultured *Aplysia* sensory neuron neurites co-expressing eGFP, mRFP PKM Apl III, mRFP DN PKM Apl I/II, and either KIBRA or empty vector (pNEX3). Cultures were fixed and stained with PKC Apl III C-terminal antibody followed by Alexa Fluor

647 donkey anti-rabbit (cyan) prior to imaging. **B**) PKM Apl III stabilization is quantified as the ratio between eGFP fluorescence and PKC Apl III C-terminal antibody staining (cyan) normalized to vector alone. One-way ANOVA shows that both KIBRA and KIBRA + DN PKM Apl I stabilized PKM Apl III compared to pNEX control and KIBRA + DN PKM Apl II ($p<0.01$). [pNEX, n=29; KIBRA, n=27; KIBRA + DN PKM Apl I, n=31; KIBRA + DN PKM Apl II, n=39]

B

```
ApKIBRA      ---MRYMNSLRSSTIKRSQTFSPACRQPPP--GVVCKLNRSDSDSSMPLYKK-TPFQRNT
ApKIBRAs     ---MRYMNSLRSSTIKRSQTFSPACRQPPP--GVVCKLNRSDSDSSMPLYKK-TPFQRNT
OcKIBRA      ---KRPTETVRKSTIRRSQTFSPADRQGS---AVVCKLNRSDSDSSMPLYKR-GPFQRNS
OcKIBRAs     --KKRPTETVRKSTIRRSQTFSPADRQGS---AVVCKLNRSDSDSSMPLYKR-GPFQRNS
DrKIBRA      -KSRGQSQLMDDRPVKRSQTFTPSEAFSK--NRYNCRLNRSDSDSAMHCGVAPHTFQRGA
DaKIBRA      -PFGVDGFHHRAILVKRSQTFSPSAAASR--AEYICRLNRSDSDSSMPLYRR-GPFQRNS
HoWWC2       --SSRQHPFVRSSVIVRSQTFSPGERN-----QYICRLNRSDSDSSTLAKK--SLFVRNS
HoWWC3       -RRARGSPFVRSGTIVRSQTFSPGARS-----QVVCRLYRSDSDSSTLPRK--SPFVRNT
HoKIBRA      VGTPSQGPFLRGSTIIRSKTFSPGPQS-----QVVCRLNRSDSDSSTLSKK--PPFVRNS
AmKIBRA      KGRAKRSDPLRNSTIVRSKTFSPGNRVNKQSGQVVCKLNRSDSDSSMPQYKK-GPFIRNS
NeKIBRA      -------PFQSFTCMEESLNNHPLRSKA-----YDINLNRSNSDCTARKTDR-SPFVRLS
AcKIBRA      -----PLGLFPGHGAEDEVPMMPLSRMP-----YDINLNRSNSDCTTRQTDV-SPFVRMS

                PKM
               binding

ApKIBRA      VSRRSLRWKRADGSVGFMPLTSHIPFR------TSLDLELDLQASHTKLSHLTDEISRLK
ApKIBRAs     VSRRSLRWKRADGSVGFMPLTSHIPFR------TSLDLELDLQASHTKLSHLTDEISRLK
OcKIBRA      TVRQSLRWKRLPGAAGSS--LRKMPIR------TSVDLELDLQASQRRLNHLRDDVYRLK
OcKIBRAs     TVRQSLRWKRLPGAAGSS--LRKMPIR------TSVDLELDLQASQRRLNHLRDDVYRLK
DrKIBRA      AERRSLRFHSKAPKSVTKLHH-------THIPRTSLDLELDLQAQHSKLYFLNDQIAKLQ
DaKIBRA      VERRSLRWRASSLAASAAAHRKSRGHPATLPPRTSVDLELDLLAQNKRLQELHEELSKLR
HoWWC2       TERRSLRVKRTVCQS---VLRRTTQEC---PVRTSLDLELDLQASLTRQSRLNDELQALR
HoWWC3       LERRTLRYKQSCRS--------SLAEL---MARTSLDLELDLQASRTRQRQLNEELCALR
HoKIBRA      LERRSVRMKRPSPPPQPSSVKSLRSER---LIRTSLDLELDLQATRTWHSQLTQEISVLK
AmKIBRA      MERRSMRWKKNMQQAAS---KAQKDKN---SKRTSLDLELDLQAFRTKQAQIEEEIVRLK
NeKIBRA      VERSSMRRKKRPVSWQGVQQYQHLQMMQSLPFDMFRYSDLENDQ-DIMPRQISGDIARLH
AcKIBRA      AERTSMRRKKRPVSWQGLQQYQQLSPLFGD-----GNSKSATVA-ELDVHQEASRAKQLS

                                                Splice

ApKIBRA      ELKYTLEESKNKGESELPSWLSENEKFHRLLSMAEKLQ-------TSSGIEKREYISKQ
ApKIBRAs     ELKYTLEESKNKGESELPSWLSENEKFHRLLSMAEKLATCVQARNTSSGIEKREYISKQ
OcKIBRA      ELKKGMEEAKARGANELPSWLTDDENFQRLLSEADKL---------AHKSENDPDNSKQ
OcKIBRAs     ELKKGMEEAKARGANELPSWLTDDENFQRLLSEADKLSI-VSSGKSAHKSENDPDNSKQ
DrKIBRA      NLKEVLQKACENKDPLVAAWAIENEEFQRLVARADPAKC-----------P---EERQL
DaKIBRA      ELKRRMEDARYLGNQDLPAWLSEDDRLQALLEHAEKRKE-----------EKTVEERKL
HoWWC2       DLRQKLEELKAQGETDLPPGVLEDERFQRLLKQAEKQAE-----------QSKEEQKQG
HoWWC3       ELRQRLEDAQLRGQTDLPPWVLRDERLRGLLREAERQTR-----------QTKLDYRHE
HoKIBRA      ELKEQLEQAKSHGEKELPQWLRDERFRLLLRMLEKRQ------------MDRAEHKGE
AmKIBRA      EIKQRLEEHQTRGDGRLPNWVSENELMQRLLVDAEKQLA-----------RRMGECAEE
NeKIBRA      RLKQWIEKALADGSTDVPKWLEDSEEYRSLVREIEGRQMRLPR-------VARRNHKAA
AcKIBRA      QLKKILEEAMDGGLTEVPKLLEENDEFQSLLRDVEGFRSWNLH-------GHRGRIPFS

ApKIBRA      DKRAEHLMKKVTKEVQRMRQ---GTQQSRMFTFREKMAFFTSANMDVPVIPSE—
ApKIBRAs     DKRAEHLMKKVTKEVQRMRQ---GTQQSRMFTFREKMAFFTSANMDVPVIPSE—
OcKIBRA      NKRAEQIMKKVTRDVEKIQK---NYFPNAFSCTHSCLWLIGCAPVDA--------
OcKIBRAs     NKRAEQIMKKVTRDVEKIQK---NYFPNAFSCTHSCLWLIGCAPVDA--------
DrKIBRA      ----QKLLMKTAKEIHKLRKTKVPKGCPDLVSFKEKITFFTRKGLSVPELPSE—
DaKIBRA      ----EKLVRRTSREIYRLRKSKAGKGQMDVISFKEKMAFFTKPNSSFSFLTQPFD
HoWWC2       -LNAEKLMRQVSKDVCRLRE-QSQKVPRQVQSFREKIAYFTRAKISIPSLPAD—
HoWWC3       -QAAEKMLKKASKEIYQLRG-QSHKEPIQVQTFREKIAFFTRPRINIPPLPAD—
HoKIBRA      -LQTDKMMRAAAKDVHRLRG-QSCKEPPEVQSFREKMAFFTRPRMNIPALSAD—
AmKIBRA      ERKRQDFLKRANKEMARLRK-QSSKDQPEKASFREKMAFFTSVNINVPLPTLPAD
NeKIBRA      DLKLVREKRASFSSSKSPKKPVYKDTYIEDKFNKEHKAWGGKDPCICTEPQLD—
AcKIBRA      RRNHKVLRERLNPPPSKPQKSSATPNGSEHWV-----------------------
```

C

Conservation of domains in ApKIBRA to other species percent identity/ percent similarity

Species	WW domains	C2 domain	KSD
Octopus	67%/84%	54%/74%	60%/76%
Drosophila	45%/66%	46%/61%	39%/58%
Human	47%/69%	41%/56%	43%/54%
Nematostella	38%/58%	42%/56%	15%/35%

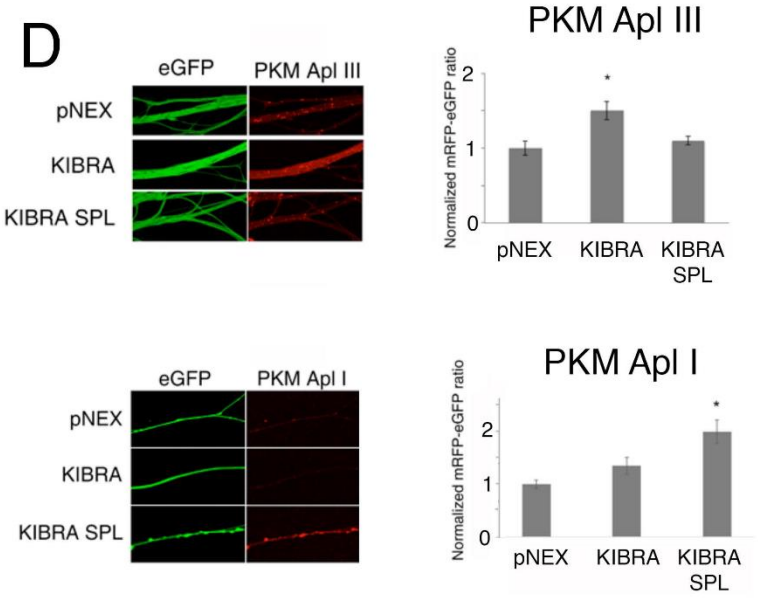


Figure 4.7 KIBRA Splice (SPL) stabilizes PKM Apl I, but not PKM Apl III.

A) Schematic of KIBRA sequence highlighting the three conserved domains and the placement of the putative PKM binding site and the splice. **B**) Alignment of the KSD domain from representative bilaterian and pre-bilaterian animals. Regions highlighted in purple represent alternative mRNA sequences identified by bioinformatics (accession numbers below). The three amino acids required for PKM binding are highlighted in red. Five regions of increased

homology are highlighted in green. In vertebrates, the KIBRA gene has two additional paralogues, termed WWC2 and WWC3 (KIBRA's alternative name is WWC1) and all three are included in the alignment. The primary and alternate sequences with splices were derived from the following sources: { Aplysia (Ap) XP_012936697.1 1288:1503; Aplysia splice gene tools: Octopus (Oc) XP_014775126.1 1038:1245; Octopus splice KOF84653.1 723:904; {Drosophila (Dr) NP_001034055.1 966:1156; Daphnia (Da) EFX86200.1 876:1090}; Amphioxus (Am) XP_019625123.1 995:1209; Human (Ho) WWC2 XP_024309993.1, 913:1116 Human WWC3 NP_056506.2 891:1090; Human KIBRA XP_016864767.1 821:1028l Human Kibra lacking splice PPQPS XP_005265907.1, Human KIBRA lacking Q XP_011532791.1; Nematostella (Anemone, Ne) ; XP_001629271.1 911:1121, Acropora (Coral; Ac) XP_015763151.1 950:1136. **C**) Table of homology between the *Aplysia* new region and representative species from the two other bilaterian and a prebilaterian (Nematostella). First number is percent identity and second is percent similarity from Prot blast at NCBI using *Aplysia* sequence as probe. **D**) Representative images of cultured *Aplysia* sensory neurons expressing eGFP, mRFP-tagged PKM Apl III (top) or PKM Apl I (bottom), and either KIBRA, KIBRA SPL, or empty vector (pNEX3). On left, quantification of stabilization of PKM Apl III (top) and PKM Apl I (bottom). All results are normalized within each experiment to the average mRFP/eGFP ratio of vector alone. KIBRA stabilized PKM Apl III compared to pNEX and KIBRA SPL groups (One-way ANOVA ($F_{2,116}$= 6.8. $p<0.01$) with Tukey's post-Hoc test *$p<0.05$ different from all other groups [pNEX, n=36 neurons; KIBRA, n=50 neurons; KIBRA SPL, n=32 neurons; neurons from three independent experiments]), while KIBRA SPL stabilized PKM Apl I compared to pNEX and KIBRA groups (One-way ANOVA $F_{2,68}$=6.2, $p<0.001$ with Tukey's post-Hoc test *$p<0.01$ compared to all groups [pNEX, n=21 neurons; KIBRA, n=25

neurons; KIBRA SPL, n=24 neurons; neurons from three independent experiments]). Error bars represent SEM.

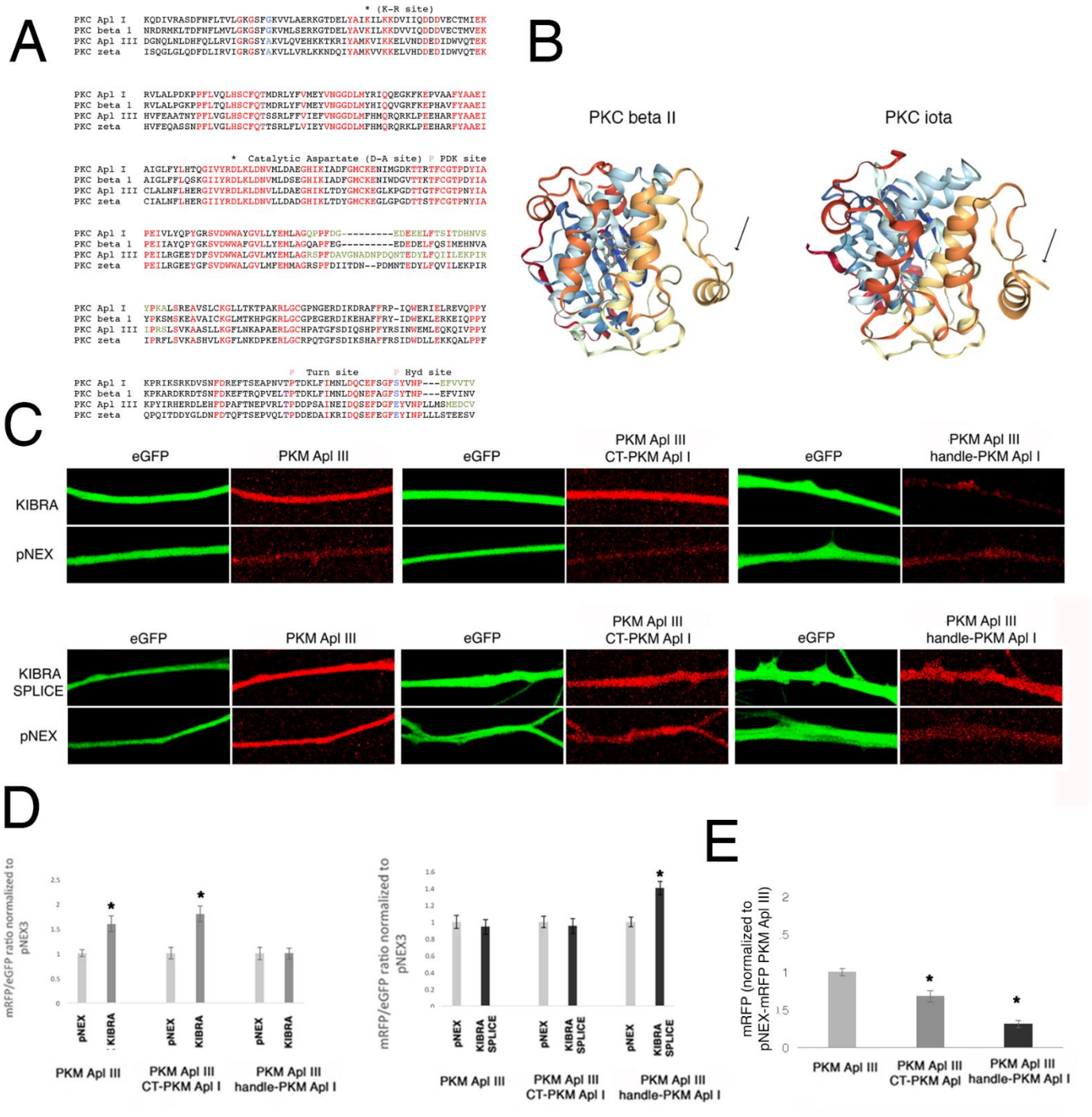

A
* (K-R site)
PKC Apl I KQDIVRASDFNFLTVLGKGSFGKVVLAERKGTDELYAIKILKKDVIIQDDDVECTMIEK
PKC beta 1 NRDRMKLTDFNFLMVLGKGSFGKVMLSERKGTDELYAVKILKKDVVIQDDDVECTMVEK
PKC Apl III DGNQLNLDHFQLLRVIGRGSYAKVLQVEHKKTKRIYAMKVIKKELVNDDEDIDWVQTEK
PKC zeta ISQGLGLQDFDLIRVIGRGSYAKVLLVRLKKNDQIYAMKVVKKELVHDDEDIDWVQTEK
PKC Apl I RVLALPDKPPFLVQLHSCFQTMDRLYFVMEYVNGGDLMYRIQQEGKFKEPVAAFYAAEI
PKC beta 1 RVLALPGKPPFLTQLHSCFQTMDRLYFVMEYVNGGDLMYHIQQVGRFKEPHAVFYAAEI
PKC Apl III HVFEAATNYPFLVGLHSCFQTSSRLFFVIEFVNGGDLMFHMQRQRKLPEEHARFYAAEI
PKC zeta HVFEQASSNPFLVGLHSCFQTTSRLFLVIEYVNGGDLMFHMQRQRKLPEEHARFYAAEI
* Catalytic Aspartate (D-A site) P PDK site
PKC Apl I AIGLFYLHTQGIVYRDLKLDNVMLDAEGHIKIADFGMCKENIMGDKTTRTFCGTPDYIA
PKC beta 1 AIGLFFLQSKGIIYRDLKLDNVMLDSEGHIKIADFGMCKENIWDGVTTKTFCGTPDYIA
PKC Apl III CLALNFLHERGIVYRDLKLDNVLLDAEGHIKLTDYGMCKEGLKPGDTTGTFCGTPNYIA
PKC zeta CIALNFLHERGIIYRDLKLDNVLLDADGHIKLTDYGMCKEGLGPGDTTSTFCGTPNYIA
PKC Apl I PEIVLYQPYGRSVDWWAYGVLLYEMLAGQPPFDG---------EDEEELFTSITDHNVS
PKC beta 1 PEIIAYQPYGKSVDWWAFGVLLYEMLAGQAPFEG---------EDEDELFQSIMEHNVA
PKC Apl III PEILRGEEYDFSVDWWALGVLMYEMLAGRSPFDAVGNADNPDQNTEDYLFQIILEKPIR
PKC zeta PEILRGEEYGFSVDWWALGVLMFEMMAGRSPFDIITDN--PDMNTEDYLFQVILEKPIR
P Turn site P Hyd site
PKC Apl I YPKALSREAVSLCKGLLTKTPAKRLGCGPNGERDIKDRAFFRP-IQWERIELREVQPPY
PKC beta 1 YPKSMSKEAVAICKGLMTKHPGKRLGCGPEGERDIKEHAFFRY-IDWEKLERKEIQPPY
PKC Apl III IPRSLSVKAASLLKGFLNKAPAERLGCHPATGFSDIQSHPFYRSINWEMLEQKQIVPPY
PKC zeta IPRFLSVKASHVLKGFLNKDPKERLGCRPQTGFSDIKSHAFFRSIDWDLLEKKQALPPF
PKC Apl I KPRIKSRKDVSNFDREFTSEAPNVTPTDKLFIMNLDQCEFSGFSYVNP---EFVVTV
PKC beta 1 KPKARDKRDTSNFDKEFTRQPVELTPTDKLFIMNLDQNEFAGFSYTNP---EFVINV
PKC Apl III KPYIRHERDLEHFDPAFTNEPVRLTPDDPSAINEIDQSEFDGFEYVNPLLMSMEDCV
PKC zeta QPQITDDYGLDNFDTQFTSEPVQLTPDDEDAIKRIDQSEFEGFEYINPLLLSTEESV
B
PKC beta II
PKC iota
C
eGFP
PKM Apl III
PKM Apl III CT-PKM Apl I
PKM Apl III handle-PKM Apl I
KIBRA
pNEX
KIBRA SPLICE
D
mRFP/eGFP ratio normalized to pNEX3
pNEX
KIBRA
KIBRA SPLICE
PKM Apl III
PKM Apl III CT-PKM Apl I
PKM Apl III handle-PKM Apl I
E
mRFP (normalized to pNEX-mRFP PKM Apl III)

Figure 4.8 The 'handle' domain of PKMs determines isoform specific stabilization by KIBRA.

A) Sequences of catalytic domains from classical and atypical PKCs from *Aplysia* and human are shown to illustrate regions of homology. Red amino acids are conserved between isoforms. Blue amino acids represent known important differences, a glycine in the ATP binding domain that partially explains the difference in ATP based inhibitors between atypical and classical PKCs and the carboxy-terminal hydrophobic (Hyd) phosphorylation site in classical PKCs that is a glutamic acid in atypical PKCs. The sites mutated to form catalytically inactive dominant negatives are illustrated. The green residues are the amino acids removed from PKM Apl III and replaced by the green residues in PKM Apl I in the chimeras. **B**) Rotated structures of the human atypical PKC iota isoform (Messerschmidt et al., 2005) and classical PKC beta II isoform (Grodsky et al., 2006) to illustrate the alpha helix switched in the chimera. From this orientation the helix appears as a 'handle'. **C**) Representative images of neurites from cultured *Aplysia* sensory neurons. All neurons expressing eGFP; Top panel, KIBRA or empty vector (pNEX3), Bottom panel, KIBRA SPLICE or empty vector (pNEX3); and from left to right, mRFP-tagged PKM Apl III, PKM Apl III-CT PKM Apl I, or PKM Apl III-handle PKM Apl I. **D**) The ability of KIBRA to stabilize the different PKMs was determined by a Student paired T-test between mRFP/eGFP ratios normalized to the pNEX control for each construct. Results were corrected by a Bonferroni test for multiple t-tests (6 for experiments in C) *$p<0.01$. [Top panel: PKM Apl III, pNEX n=30 neurons, KIBRA n=15 neurons; PKM Apl III-CT PKM Apl I, pNEX n=17 neurons, KIBRA, n=32 neurons; PKM Apl III-handle PKM Apl I, pNEX n=14 neurons, KIBRA n=33 neurons; results are from three independent experiments with all six groups; Bottom panel PKM Apl III, pNEX n=23 neurons, KIBRA SPL n=19 neurons; PKM Apl III-CT PKM Apl I, pNEX n=33 neurons, KIBRA SPL, n=23 neurons; PKM Apl III-handle PKM Apl I, pNEX n=20

neurons, KIBRA SPL n=29 neurons; results are from three independent experiments with all six groups). **E**) The levels of expression of the three constructs in the absence of KIBRA were quantified. The mRFP values are normalized to levels of PKM Apl III in each experiment. One-way ANOVA ($F_{2,135}$=24.5) with Tukey's post-Hoc test, *p<0.01 all groups different from each other [PKM Apl III, n=53 neurons; PKM Apl III-CT PKM Apl I, n=51 neurons; PKM Apl III-handle PKM Apl I, n=34 neurons; results are from twelve independent experiments with all three groups].

CHAPTER FIVE

Discussion

5.1 Overview

The work presented in this thesis has focussed on the upstream regulators of PKM activity and the downstream substrates of PKMs during memory maintenance using the *Aplysia* model system. In the introduction, I reviewed the literature surrounding the role of PKMs in memory maintenance in vertebrates and justified our use of the *Aplysia* model system in studying the role of PKMs at synapses. I introduced KIBRA as a possible stabilizer of PKMs in this process and presented the idea that KIBRA may be involved in determining isoform specificity of PKMs at synapses during different forms of memory.

In Chapter 3, I introduced the endocytic adaptor protein, Numb, as a potential substrate of PKMs involved in the regulation of AMPA receptor endocytosis during memory maintenance. In our model, unphosphorylated Numb interacts with AMPA receptors and the cell's endocytic machinery to internalize surface-bound AMPA receptors. This internalization is blocked by PKM-mediated phosphorylation of Numb. I present our data using pHluorin-tagged AMPA receptors showing that phosphorylation of Numb is required for the increased localization of AMPA receptors to the synapse following induction of m-ITF in *Aplysia* sensory-motor neuron cocultures.

In Chapter 4, I discuss the evidence in the literature supporting KIBRA as a possible stabilizing protein of PKMs during memory maintenance and introduce our model of how KIBRA upregulation may be the transcription-dependent step in PKM-dependent facilitation in *Aplysia*. I present our data showing that KIBRA stabilizes PKMs in an isoform specific manner,

supporting our hypothesis that selective stabilization by KIBRA mediates PKM isoform specificity at synapses. I introduce our KIBRA-AAA mutant and provide evidence that it acts as a dominant negative in regard to KIBRA-mediated stabilization of PKM Apl III. We show that stabilization by KIBRA does not require PKM catalytic activity. Our electrophysiology experiments show that KIBRA overexpression in the postsynaptic neuron is not sufficient to prolong m-ITF. We identify a splice site within KIBRA that determines the isoform-specific stabilization of PKMs, presenting a possible transcriptional mechanism through which different PKM isoforms may be upregulated during different forms of LTF. Our findings also indicate a potential region on PKMs that confers isoform identity, allowing KIBRA and KIBRA splice variants to differentially stabilize PKM isoforms.

5.2 Significance of this work and contribution to the field

5.2.1 Molecular mechanisms of memory maintenance

Our work has helped elucidate both the role of PKMs during memory maintenance as well as how the activity of PKMs is regulated. In our model, a facilitation-inducing event leads to an upregulation of KIBRA which interacts with and stabilizes PKMs at the affected synapse. PKMs maintain the facilitation-induced increase in synaptic strength by inhibiting AMPA receptor endocytosis through phosphorylation of the endocytic adaptor protein Numb. While we've presented evidence supporting this model, we don't discount the possibility that other molecular mechanisms play a role in memory maintenance as well.

The persistent activity of PKMs makes them ideally suited to our model of active memory maintenance, but other persistently active kinases may function in a similar manner. The unique

properties of CaMKII, for example, make it a unique alternative mechanism through which the AMPA receptor endocytosis may be prevented. The self-sustaining activity of CaMKII holoenzymes is due to their ability to autophosphorylate—additionally, CaMKII subunits are thought to undergo continuous replacement throughout the lifetime of the kinase, essentially allowing the kinase to remain in a perpetually active state and avoid degradation (Lisman and Goldring, 1988; Miller et al., 2005). It has been proposed that CaMKII is involved in the recruitment and stabilization of synaptic AMPA receptors (Lisman and Zhabotinsky, 2001), although there is controversy surrounding CaMKII's role in memory maintenance [see Smolen et al. (2019) for review]. However, CaMKII and PKMs may work in cooperation with one another—CaMKII can upregulate local protein synthesis via phosphorylation of the translation regulator CPEB (Atkins et al., 2005; Atkins et al., 2004), which may be important for the translation of PKMζ mRNA at vertebrate synapses [assuming PKMζ is synthesized locally at the synapse, which is still unknown]. Of course, there are other persistently active kinases that may also play a role in the long-term maintenance of synaptic strength [e.g. MAPK (Bhalla and Iyengar, 1999) and PKA (Hayer and Bhalla, 2005)]. Based on our findings concerning PKM isoform specificity at synapses, it is also possible that different persistently active kinases outside of the PKC/PKM family are involved in different forms of memory and/or in different brain regions.

The downstream substrates of persistently active kinases may also vary depending on the type of memory being maintained. While we identified Numb as a candidate substrate for PKMs in regulating AMPA receptor endocytosis, the phosphorylation of other AMPA receptor-associated proteins [e.g. PICK1 (Yao et al., 2008)] may have a similar effect on synaptic strength. A synthetic peptide modelled after the C-terminal tyrosine-rich binding motif of the

mammalian GluA2 AMPA receptor subunit ($GluA2_{3Y}$ peptide) is capable of preventing AMPA receptor endocytosis by competing for binding with proteins that bind this motif [e.g. BRAG2 (Scholz et al., 2010)]. This $GluA2_{3Y}$ competitive inhibitor is able to prevent the loss of synaptic strength caused by the PKMζ inhibitor ZIP (Migues et al., 2010), suggesting that PKMζ substrates endocytose AMPA receptors through this motif. However, it is not known whether Numb similarly binds this $GluA2_{3Y}$ peptide. Additionally, while we have concerned ourselves with glutamatergic synapses, changes in synaptic strength of non-glutamatergic synapses are likely maintained through entirely different mechanisms. While the work presented in this thesis has answered some essential questions regarding the role and regulation of PKMs during memory maintenance, there are still many holes remaining in our knowledge concerning how memories are maintained in the brain.

5.2.2 Elucidating the molecular memory trace—potential human applications

Despite the molecular focus of this thesis, potential benefits to humans can be extrapolated from the work we've presented. The ability to distinguish between different types of memory at a synaptic level may lead to further advances in techniques aimed at manipulating and potentially erasing damaging memories in humans (for example, memories associated with posttraumatic stress disorder).

Memory erasure through pharmacological inhibition of PKMs is not new, but targeted deletion of specific memories is a more nuanced process. With the application of novel optogenetic techniques made famous by Dr. Susumu Tonegawa's memory implantation experiments (Ramirez et al., 2013), erasure of memories encoded by specific neuronal ensembles is possible (Abdou et al., 2018; Lacagnina et al., 2019). However, while optogenetic techniques have allowed for memory ensembles to be distinguished at a circuit level, the underlying

molecular components that distinguish these ensembles from one another is still unclear. Our work on PKMs suggests possible molecular targets for further experiments aimed at erasing specific memories while leaving other memories intact. Our collaborators (led by Dr. Samuel Schacher at Columbia University) have already shown that different forms of plasticity on one postsynaptic neuron can be selectively erased by targeting specific PKM isoforms (Hu et al., 2017b). Replication of this result in the mammalian brain has not yet been achieved, but it presents a promising avenue for future study into possible memory erasure and manipulation in humans.

5.3 Future directions

5.3.1 How does KIBRA stabilize PKMs?

While we've shown that KIBRA stabilizes PKMs, we haven't addressed how this stabilization occurs. Does KIBRA-mediated stabilization of PKMs involve direct binding, or does this stabilization occur through an indirect method? There is still a lot to be learned about how KIBRA and PKMs interact, although hypotheses can be made based on known features of KIBRA's PKC binding domain.

The relationship between KIBRA and PKMs is not a typical kinase-substrate relationship. There are two PKMζ phosphorylation sites on KIBRA (positions S975 and S978 in human KIBRA) (Buther et al., 2004), but mutation of these residues does not affect KIBRA's ability to stabilize PKMζ (Vogt-Eisele et al., 2014). However, while not identified as a PKMζ phosphorylation site by Buther et al. (2004), S967 is a likely phospho-acceptor site for PKC iota (ι) (Soriano et al., 2016), and is one of the three sites identified by Vogt-Eisele et al. (2014) as

being crucial for PKMζ stabilization. Indeed, the regions flanking this serine display similar characteristics to other PKC substrates: the two highly charged arginine residues within two positions of the serine facilitate kinase interaction, and the nearby F-X-R motif is identical to the motif in Par3 that has been shown to act as an "affinity arm" in the interaction between Par3 and aPKC's catalytic domain (Soriano et al., 2016). Indeed, the pattern of interaction between Par3 and aPKC is believed to be very similar to that of KIBRA and aPKC due to the sequence similarity between Par3 and KIBRA in their PKC binding regions. A K-R motif present within Par3 facilitates interaction with aPKCs while simultaneously inhibiting catalytic activity. Interestingly, this K-R motif is also present in KIBRA, suggesting a possible inhibitory function between KIBRA and the PKMs it interacts with via this same region. All three of these sites are highly conserved and are present in *Aplysia* KIBRA.

Vogt-Eisele et al. (2014) have suggested that PKMζ retains its kinase activity while being stabilized by KIBRA. Using an *in vitro* phosphorylation assay, they showed that there was no difference in the kinase activity of PKMζ immunoprecipitated from cells expressing the kinase alone or from cells co-expressing KIBRA. However, this paradigm involves the dissociation of PKMζ from KIBRA before assessing kinase activity and is therefore unable to address whether direct binding of PKMζ to KIBRA may affect catalytic activity. Certainly, PKMζ catalytic activity is not permanently altered by KIBRA-mediated stabilization, but these results do not preclude a temporary inhibition of PKM activity conferred by interaction with KIBRA. Indeed, the kinase activity of purified PKCζ incubated with KIBRA is inhibited, while kinase activity of PKCζ incubated with KIBRA lacking the PKC/PKM binding region (region 919-978) is unaffected (Yoshihama et al., 2011). Incubation with a peptide derived from the same 919-978 region is sufficient to inhibit the catalytic activity of aPKCι *in vitro* (Soriano et al., 2016).

Specifically, it is the K-R motif within this region that seems to be important for this inhibition. It is possible that this motif confers a similar inhibitory effect to the kinase activity of PKMs during stabilization, assuming direct interaction between PKMs and KIBRA.

In the Par3-aPKC complex, inhibition of aPKC is reversed upon dissociation of the K-R inhibitory motif from the kinase (Soriano et al., 2016). Phosphorylation of Par3 by ROCK kinase at a site directly adjacent to the K-R motif (Nakayama et al., 2008) has been predicted to modulate removal of this K-R inhibitory arm (Soriano et al., 2016). It is interesting, therefore, that there are two known PKM phosphorylation sites within 3 amino acids of the K-R motif in KIBRA (S975 and S978, as mentioned above). It is possible that KIBRA binds to PKM and inhibits it through the same mechanism that Par3 inhibits PKCι, and that PKM regains its catalytic activity through a phosphorylation event on KIBRA that facilitates removal of the K-R inhibitory arm. This would, theoretically, restore PKM's catalytic activity and allow PKM to phosphorylate KIBRA at S967, leading to a full dissociation between the two proteins. Vogt-Eisele et al. (2014) determined that the K-R motif (position 971 and 972) was not essential for PKM co-immunoprecipitation, but that does not preclude its requirement for stabilization. Indeed, if transient kinase inactivation is a necessary component of KIBRA-mediated stabilization of PKMs, mutation of KIBRA's K-R motif might result in decreased stabilization of PKM Apl III in our stabilization assay.

Of course, there is also the possibility that KIBRA-mediated stabilization of PKMs does not require direct binding. A similar stabilization interaction that does not require binding has been shown between KIBRA and the large tumor suppressor kinase (Lats). While direct binding to Lats requires KIBRA's WW-domains, it has been shown that KIBRA-mediated stabilization of Lats does not (Xiao et al., 2011). An *in vitro* binding assay using a KIBRA fragment

containing the regions mentioned above will help clarify whether or not KIBRA directly interacts with PKMs.

5.3.2 Why does KIBRA-AAA act as a dominant negative?

We mutated the phosphorylatable serine and the two arginine residues mentioned above (*Aplysia* homologues of R965, S967, and R969) in our KIBRA-AAA mutant, while leaving the rest of the protein (including the F-X-R and K-R motifs) intact. This mutation led to an unexpected stabilization of PKM Apl I (which we have hypothesized is due to a conformational change that reveals a cryptic PKM Apl I binding site—see Chapter 4 discussion), but also caused KIBRA-AAA to act as a dominant negative in the stabilization of endogenously-cleaved PKM Apl III and in the expression of associative LTF (Hu et al., 2017b). Based on the hypothesis mentioned above—that KIBRA-mediated stabilization of PKM Apl III involves an inhibition of kinase activity that can be reversed through phosphorylation near KIBRA's K-R inhibitory motif—it is possible that KIBRA-AAA's dominant negative effect stems from an upregulation of the kinase responsible for phosphorylating KIBRA. Since we have shown that KIBRA-AAA stabilizes and leads to increased levels of PKM Apl I, it is possible that PKM Apl I is this unknown kinase.

PKM Apl I and PKM Apl III have been shown to be required for different forms of LTF (Hu et al., 2017a)—therefore, it is not unreasonable to predict that the stabilization of one may have an antagonistic effect towards the stabilization of the other. PKM Apl I may phosphorylate KIBRA to induce dissociation with PKM Apl III, ultimately resulting in decreased stabilization of PKM Apl III. The increased stabilization of endogenous PKM Apl I induced by overexpression of KIBRA-AAA may explain the observed decrease in PKM Apl III stabilization and the reversal of associative LTF. It is also possible that PKM Apl III has a similar effect on

the ability of KIBRA Splice to stabilize PKM Apl I. If so, one might expect KIBRA to act as a dominant negative in the stabilization of PKM Apl I and the expression of PKM Apl I-dependent non-associative LTF.

5.3.3 Investigating the role of other WWC proteins

In contrast to the singular WWC1 protein expressed in invertebrates, mammals express a family of WWC proteins. Human KIBRA (WWC1) contains approximately 40-50% sequence similarity with WWC2 and WWC3 (Wennmann et al., 2014), both of which are thought to have diverged from KIBRA. In addition to the WW and C2 domains, all three human WWC proteins contain an aPKC binding site as well as the phosphorylation sites shown to be important for regulating KIBRA activity (Wennmann et al., 2014; Yang et al., 2014). Neither WWC2 nor WWC3 contain the glutamic acid-rich region present in KIBRA, while WWC3 contains a unique arginine-proline-rich sequence N-terminal to the WW domain (Wennmann et al., 2014). The functions of WWC2 and WWC3 are not well characterized. Although *Aplysia* only expresses KIBRA/WWC1, we can clone some of the features of WWC2 and WWC3 into our *Aplysia* KIBRA and express these mutants in *Aplysia* neurons to assess the effect they have on PKM stabilization using our assay.

5.3.4 Differential localization of KIBRA and KIBRA Splice during different types of LTF

We've shown that KIBRA stabilizes PKM Apl III and that KIBRA-SPL stabilizes PKM Apl I, and presented this dichotomy as a possible means through which isoform specificity of PKMs is achieved during different forms of LTF. To investigate this hypothesis further, we can look at the localization of KIBRA and KIBRA Splice during associative and non-associative LTF. Following induction of PKM Apl III-dependent associative LTF, we would expect to see

an increased localization of KIBRA to synapses—conversely, induction of PKM Apl I-dependent non-associative LTF would result in increased synaptic localization of KIBRA-SPL. We expect this localization to be synapse-specific—KIBRA and KIBRA-SPL should localize to different synapses within a postsynaptic neuron receiving multiple presynaptic inputs. Characterization of the fluorescently tagged KIBRA and KIBRA-SPL constructs to be used in this experiment is currently underway.

We expect KIBRA to play a role in the localization of PKMs to their synaptic targets based on KIBRA's known role as a scaffolding protein in other cell processes. We can utilize our pHluorin-tagged AMPA receptor construct to investigate whether or not KIBRA colocalizes with AMPA receptors newly trafficked to the synapse following induction of LTF. Additionally, we can utilize the pHluorin assay to assess whether disruption of PKM stabilization affects AMPA receptor trafficking. What effect does overexpression of KIBRA have on the increase in pHluorin puncta fluorescence observed after induction of LTF? For example, disrupting the stabilization of PKM Apl III through overexpression of KIBRA-AAA would be expected to decrease the number of AMPA receptors in the synapse, resulting in decreased puncta fluorescence. Such an experiment would link the upstream regulation of PKM with the downstream effect on AMPA receptor trafficking, fleshing out our model for how PKM functions during memory maintenance.

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