The role of the atypical Protein Kinase C zeta orthologue, PKC Apl III, in synaptic plasticity and long term memory in *Aplysia californica*

A Thesis Submitted to McGill University in Partial Fulfillment of the Requirements of the Degree of Doctor of Philosophy in Psychology

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ABSTRACT

The search for how memories are formed and maintained over time has become a critical question in the scientific community. An important discovery in this field is the requirement for a persistently active kinase, called PKMz, to maintain long term synaptic plasticity and memory. In vertebrates, the atypical PKCz has a brain-specific transcript that produces this constitutively active kinase. The sensory-motor neuron synapse of *Aplysia* is a leading invertebrate model to study learning and memory. We have cloned the atypical PKC from *Aplysia*, which we call PKC Apl III. We do not find a transcript in *Aplysia* that forms a PKMz, and evolutionary analysis of atypical PKCs suggests formation of this transcript is restricted to vertebrates, despite the fact that inhibitors of PKMz erase memory in *Aplysia* in a fashion similar to rodents. However, overexpression of PKC Apl III in *Aplysia* sensory neurons leads to production of a PKM fragment of PKC Apl III, PKM Apl III, formed by calpain cleavage. We suggest that PKM forms of atypical PKCs play a conserved role in memory formation, but the mechanism of formation of these kinases has changed over evolution.

We further show that kinase activity is required for both nuclear export and efficient cleavage of PKC Apl III into PKM Apl III following overexpression. We used a FRET reporter to measure cleavage of PKC Apl III into PKM Apl III in live

Aplysia motor neurons. Our results show that 5-HT application induces cleavage of PKC Apl III in isolated motor neuron processes in a calpain- and protein synthesis-dependent manner. In summary, we provide evidence that PKC Apl III is cleaved into PKM Apl III during memory formation, and the requirements for cleavage are the same as the requirements for the plasticity. Our data supports the hypothesis that the PKM required for formation of the memory trace is formed by calpain-dependent cleavage in *Aplysia*.

RÉSUMÉ

La quête des mécanismes moléculaires sous-jacents à la formation de nos souvenirs et à la persistance de ceux-ci dans notre mémoire continue de préoccuper la communité scientifique. Parmi les découvertes importantes dans ce domaine, on compte celle d'une kinase active de façon constitutive, appelée la PKM zeta. Celle-ci est nécessaire pour le maintien de la plasticité synaptique à long terme et en conséquent de la mémoire. Chez les vertébrés, la PKM zeta est produite à partir d'un ARNm de la PKC atypique, qui est spécifique au cerveau, soit la PKC zeta. Chez les invertébrés, la synapse sensori-motrice de l'*Aplysie* constitue le modèle par excellence pour étudier l'apprentissage et la mémoire. Nous avons cloné la PKC atypique de l'*Aplysie* que nous avons surnommée la PKC Apl III. Nous n'avons pas trouvé d'ARNm chez l'*Aplysie* qui pourrait former une PKM zeta. Par ailleurs, l'analyse de l'évolution des PKC atypiques suggère que la formation de cet ARNm est restreinte aux vertébrés malgré le fait que les inhibiteurs de la PKM zeta peuvent effacer la mémoire chez l'*Aplysie* de façon similaire à leur effet chez les rongeurs. Cependant, la surexpression de la PKC Apl III dans les neurones sensoriels de l'*Aplysie* conduit à la formation d'un fragment PKM de la PKC Apl III, soit la PKM Apl III par un clivage dépendant de la calpaïne. Nous suggérons que le rôle des formes PKM des PKC atypiques

dans la formation de la mémoire est conservé mais que le mécanisme de formation de ces kinases a changé durant l'évolution.

Nous rapportons aussi que l'activité de la kinase est nécessaire pour son export nucléaire ainsi que pour un clivage efficace de la PKC Apl III en PKM Apl III suite à la surexpression de la protéine. Nous avons utilisé un reporter FRET pour mesurer le clivage de la PKC Apl III en PKM Apl III dans des neurones moteurs vivants. Nos résultats démontrent que la 5-HT induit le clivage de la PKC Apl III dans les prolongements du neurone moteur et que ce clivage dépend des calpaïnes et de la synthèse de protéines. En résumé, nous démontrons que la PKC Apl III est clivée en PKM Apl III pendant la formation de la mémoire et que les conditions requises pour le clivage sont les mêmes que celles qui sont requises pour la plasticité. Nos résultats appuient l'hypothèse que chez l'*Aplysie* , la PKM requise pour la formation d'une trace mnésique est formée par clivage dépendant de la calpaïne.

Publications arising from this work and contribution of authors

Chapter three and four of this thesis are based upon the texts and data that have been published or submitted (respectively) in the following manuscripts:

The atypical Protein Kinase C in *Aplysia* can form a Protein Kinase M by cleavage.

Joanna K. Bougie, Travis Lim, Carole Abi Farah, Varsha Manjunath, Ikue Nagakura, Gino B. Ferraro, and Wayne S. Sossin.

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Serotonin-induced cleavage of the atypical Protein Kinase C Apl III in *Aplysia*

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Contribution of Authors

Chapter Three

Gino Ferraro performed the PCR and initial cloning of PKC Apl III (Fig 3.1). Travis Lim performed the western blots characterizing the splice inserts in

PKC Apl III (Fig 3.3). Travis Lim also characterized the carboxy-terminal, N-terminal and splice antibodies on immunoblots. Carole Abi Farah performed the SF9 cell experiments testing the translocation ability of PKC Apl III (Fig 3.3) and helped to train me in the DNA microinjection technique. Varsha Manjunath conducted the calpain assays (Fig 3.7). Ikue Nagakura raised the phospho-specific antibody to PKC Apl III. I conducted the remaining experiments, drafted the figures, and wrote the manuscript. Wayne Sossin quantified the data from all experiments and edited the drafts of the manuscript.

Chapter Four

Margaret Hastings performed the calpain assays (Fig 4.2). Carole Abi Farah aided in conducting the FRET experiments and with overall guidance. Xiaotang Fan cloned the PKC Apl III mutant constructs. Patrick K. McCamphill performed part of the quantification for the D392-A mutant (Fig 4.4). I performed the remaining experiments, quantified most of the FRET experiments, drafted the figures, and wrote the manuscript. Wayne Sossin quantified the rest of the data, performed the kinase assays, and edited the drafts of the manuscript.

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List of Abbreviations

5-HT = Serotonin

Ab = Antibody

ACC = Anterior cingulate cortex

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR = AMPA receptor

aPKC = Atypical PKC

ATF = Activating transcription factor

Bis = Bisindolylmaleimide-1

CaMK = Calcium/calmodulin-dependent protein kinase

cAMP = Cyclic adenosine monophosphate

CFP = Cyan fluorescent protein

CIV = Calpain inhibitor V

cPKC = Conventional PKC

CREB = cAMP response element binding protein

C-terminal = Carboxy-terminal

DAG = Diacylglycerol

eIF = eukaryotic initiation factor

E-LTP = Early-long-term potentiation

fEPSP = Field excitatory postsynaptic potential

FRET = Förster resonance energy transfer

GFP = Green fluorescent protein

Glu-EP = Glutamate-evoked potential

HFS = High frequency stimulation

ITF = Intermediate term facilitation

I-LTP = Intermediate-long term potentiation

L-LTP = Late-long term potentiation

LTD = Long term depression

LTF = Long term facilitation

LTM = Long term memory

LTP = Long term potentiation

LTS = Long term sensitization

mRFP = Monomeric red fluorescent protein

NMDA = N-methyl D-aspartate

NMDAR = NMDA receptor

nPKC = Novel PKC

NSF = N-ethylmaleimide-sensitive factor

PAR = Partitioning-defective protein

PCR = Polymerase chain reaction

PDK = Phospho-inositide-dependent kinase

PI3K = Phosphoinositide 3-kinase

PKA = Protein kinase A

PKC = Protein kinase C

PKCz = PKC zeta

PKM = Protein kinase M

PKMz = Protein kinase M zeta

PRP = Plasticity related protein

PSD = Postsynaptic density

STF = Short term facilitation

STM = Short term memory

SWR = Siphon withdrawal reflex

TOR = Target of rapamycin

WT = Wild type

YFP = Yellow fluorescent protein

Chapter ONE

Introduction

Our memories define who we are: all of our experiences, thoughts, and emotions coalesce to provide us with a constantly changing, yet stable, sense of self. How is this possible? What is memory? How are our everyday experiences encoded in our brains? How are memories made and stored over time? What brain regions are important for memory? What are the cellular and molecular correlates of memory? These are only a sample of the questions which have plagued the scientific community for many years.

The cognitive and neural mechanisms of learning and memory are extremely complex, and have been the driving force behind many experimental research projects. It is surprising to note that despite the amount of research which has investigated the processes involved in learning and memory, whether these processes be at a behavioral or molecular level, very little is in fact known about the precise details of how human beings form and maintain memories.

1.1. The historical context of human memory: Consolidation theory

The traditional views of behavioral memory often place the complex entity of human memory into a few neat categories which are divided by somewhat

arbitrary time periods. For example, the process whereby a more volatile short-term memory (STM) is converted into stable long-term memory (LTM) is referred to as “consolidation theory”. Early work on consolidation theory dates back to 1878 when Hermann Ebbinghaus discovered that memorized lists of nonsense syllables could be disrupted by learning new information. He therefore suggested that there exists a time window for the stabilization of memory, and that forgetting could be interpreted as interference in this process (Ebbinghaus, 1913). Further support for this idea came in 1900 when Müller and Pilzecker conducted a similar experiment and theorized that memory required a time window of “consolidation” to solidify new memories and thus render them less susceptible to disruption from additional material (Müller, 1900).

1.2. The hippocampus and systems consolidation

The 1940s were also an important decade for the consolidation theory. Russell and Nathan (1946) were among the first to observe retrograde amnesia following head injuries damaging the hippocampal formation. Interestingly, they found that older memories were more resistant to damage while newer memories were more easily disrupted (Russell, 1946). These results eventually lead to the hypothesis that memories are initially formed and encoded in the hippocampus, and then are somehow slowly transferred to the cortex where they are stored

permanently and thus are resistant to interference. This processes is referred to as “systems consolidation” (McGaugh, 2000).

The importance of the hippocampus in memory formation, and further support for the theory of systems consolidation, came when Scolville and Milner published their study of the famous patient H.M. – now known to be Henry Molaison. In hopes of reducing the severity of his epilepsy, H.M. received a bilateral resection of the medial temporal lobe, including both hippocampi. Following the surgery, it was observed that H.M. could no longer form new long term memories, despite the fact that his ability to recall remote memories remained intact (Milner et al., 1968; Scoville and Milner, 2000).

1.2.1. Modifying the consolidation theory: Reconsolidation

Accordingly, the theories of consolidation and systems consolidation paint a picture of memory formation and storage as serial processes: a short-term memory is made and is then followed by consolidation into a long term memory which is stored temporarily in the hippocampus; over time the memory is no longer dependent on the hippocampus and is then transferred to a distributed network in certain areas of the neocortex. However, when we begin to examine how memories are formed and maintained at the cellular level, we quickly appreciate that this serial consolidation theory can be viewed as a greatly over-simplified paradigm. A primary example of this involves the relatively recent

addition of the theory of “reconsolidation” (Nader and Hardt, 2009). The traditional views of consolidation posit that this period of fixation occurs only once, thus rendering a long term memory relatively stable over time. However, reconsolidation is a theory which suggests that when previously consolidated memories are reactivated, they are rendered labile once more and then must undergo a second bout of consolidation – in other words, the memory must be reconsolidated (Hernandez and Abel, 2008; Nader and Hardt, 2009).

The study of consolidation, and later reconsolidation, was indeed a huge leap forward in the field of learning and memory. However, early researchers could not yet demonstrate the fundamental mechanisms underlying how memories were consolidated over time. How is a memory trace represented in the brain? Which neurons and which molecules are involved? How do they interact?

1.3. The cellular correlate of memory: Synaptic plasticity

One of the most seminal investigations into these fundamental questions examined the most elementary unit of learning and memory: the neuronal synapse. This theoretical work was postulated by D.O. Hebb, who was one of the first to suggest that lasting activity-dependent changes in synaptic connections were at the root of information storage in the nervous system (Hebb, 1949). Current understandings of cellular memory propose that the strength and number

of connections between neurons will determine whether a given memory will persist for an extended period of time. The particular strength of a given synapse depends on presynaptic and postsynaptic factors, including the amount of neurotransmitter released from a presynaptic neuron and the amount of receptors present to receive the neurotransmitters on the membrane of the postsynaptic neuron (Kandel 1979). Experience is also known to cause changes in the strength of synapses: this is referred to as synaptic plasticity, and is thought to be the cellular basis for behaviour learning and memory (Si et al., 2003). Thus, understanding the biochemical bases for synaptic plasticity is important for our understanding of learning and memory as a whole.

1.3.1. Molecular requirements of synaptic plasticity: Protein synthesis

One particularly well studied biochemical requirement for long term memory is new protein synthesis. It was Flexner and colleagues (1963) who were among the first to show that a protein synthesis inhibitor called puromycin could disrupt memory for a discriminative avoidance task in mice (Flexner et al., 1963). They were also able to show that injections of the inhibitor into the hippocampus affected newer memories, while injections into wider cortices were able to disrupt older memories ranging from 11 to 43 days old (Flexner et al., 1963). Reconsolidation has similarly been demonstrated to require protein synthesis. For example, Nader and colleagues (2000) injected anisomycin, a protein

synthesis inhibitor, directly in to the lateral and basal nuclei of the amygdala following reactivation of a previously consolidated long term auditory fear memory. Upon behavioral testing, these animals displayed intact post-reactivation STM but impaired post-reactivation LTM. Interestingly, animals who were given infusions of the inhibitor but were not subjected to memory reactivation were able to show intact LTM for the task. This suggests that it is indeed the reactivation of the previously consolidated memory which returns this memory into a labile state whereby an additional round of consolidation – reconsolidation – is required (Nader et al., 2000; Nader and Hardt, 2009).

1.3.2. The discovery of Long Term Potentiation

One cannot discuss the long term memory trace and synaptic plasticity without discussing long-term potentiation (LTP). The belief that LTP is *the* mechanism which underlies learning and memory has been coined the synaptic plasticity and memory hypothesis: “activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed” (Martin, 2000), p. 650).

What do we know about LTP? It is vitally important to begin with a specific definition. For simplicity’s sake, the following discussion will focus on N-methyl D-

aspartate (NMDA) -receptor dependent long-lasting strengthening of synaptic connections induced by synchronous and repeated activation of specific synapses in the hippocampus (Stevens, 1998; Martin, 2000; Lynch, 2004). It was the pioneering working of Bliss and Lomo in the 1970s which led to this definition: it was found that repetitive activation of excitatory synapses in the hippocampus, a brain area already known to be essential for learning and memory thanks to the previously discussed work of Scoville and Milner with patient H.M. (Scoville and Milner, 2000), results in an increase in synaptic strength that can persist for hours and even days (Bliss, 1973).

LTP is not a simple solitary process, it involves numerous overlapping temporally distinct phases that require different molecules: from early (E-LTP), to intermediate (I-LTP), to late LTP (L-LTP). To further complicate matters, it has also been demonstrated that these phases can occur in parallel to each other (Sossin, 2008b). While a great deal is understood about which molecules are required for which temporal phases of LTP, it is less clear whether LTP itself is *the* cellular correlate underlying long term memory. However, some recent experiments provide more direct and compelling evidence in support of the SPM hypothesis. Specifically, one study performed by Whitlock et al. (2006) examined an 'induction' strategy to investigate LTP as a potential substrate for behavioral memory, which states that learning should induce measurable changes in synaptic plasticity (Martin, 2000). In order to do this, the authors utilized an

inhibitory avoidance task that has shown to be rapidly acquired, very stable, and dependent on the hippocampus (Lorenzini et al., 1996). This task involves training rats in an apparatus where they are allowed to cross from an illuminated chamber to a darkened chamber where they receive a foot shock. Memory for this pairing is then measured as avoidance of the dark side of the chamber in subsequent trials (Whitlock et al., 2006). Following this training, the authors analyzed the hippocampi of these animals: it was found that the behavioral training directly mimicked the effect of high-frequency stimulation (HFS) in the CA1 region. Specifically, the authors observed the two following biochemical changes following training: an immediate NMDA receptor (NMDAR) dependent increase in phosphorylation of GluA1 containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and rapid delivery of GluA1 and GluA2 containing AMPA receptors to synapto-neurosome fractions. Electrophysiology also revealed a significant increase in the slope of evoked field excitatory postsynaptic potentials (fEPSP) following training. Furthermore, the authors found that learning induced increases in fEPSP partially occluded further CA1-LTP induced by HFS *in vivo* (Whitlock et al., 2006). Accordingly, it was concluded that the inhibitory avoidance learning directly induced LTP in the CA1 region of these rats. This is a powerful experiment, and one of the first studies to show the induction criteria for LTP as the memory trace: not only does a lack of LTP lead to memory deficits (in some tasks in some brain regions), but learning

and memory itself can generate the molecular changes which are believed to underlie the mechanisms of LTP.

1.3.3. Impaired LTP but intact memory?

An interesting finding that challenges the role of LTP as the cellular substrate underlying learning and memory is the GluA1 knock-out mice. These mutant mice displayed a strongly reduced AMPA receptor (AMPAR) current in CA1 pyramidal neurons, and consequently, failed to show LTP (Zamanillo et al., 1999). With this robust deficit in CA1 LTP in these mutant mice, it was strongly suspected that these animals would display a corresponding deficit in hippocampal forms of memory. In order to test this, the authors trained the animals in the Morris water maze. Surprisingly, it was found that the spatial memory of the mutant mice was equal to those the of wild type mice: both groups could acquire the hidden platform task, with escape latencies decreasing at similar rates. Furthermore, when the hidden platform was removed, both groups of animals spent significantly more time in the quadrant that had previously contained the platform, while also not differing in the number of crossings of the platform location (Zamanillo et al., 1999).

While this is an unexpected result, these mice are not without memory impairments: it has been shown that GluA1 deficient mice are indeed capable of reference memory hippocampal dependent tasks, like the water maze, but are

significantly impaired in certain hippocampal dependent tasks, such as the elevated T-maze task (Reisel et al., 2002; Schmitt et al., 2003). It is therefore possible that the conflicting results of a lack of LTP but intact memory in the water maze were simply due to differential requirements between brain area and behavioral test. These findings highlight that different forms of memory may in fact involve different mechanisms. There are multiple types of memory traces in the brain that require different molecular components; some are dependent on GluA1 while others are independent.

1.3.4. Molecular requirements of synaptic plasticity: Gene expression

Inhibiting gene transcription has also been shown to impair memory in a manner that is also dependent on a specific time window. Application of a transcriptional inhibitor, actinomycin D, at the time of training in a wide variety of species is able to impair long term memory retention (Brink, 1966; Squire, 1970; Pedreira, 1996). Not only has transcription been found to be important for consolidation, but also for reconsolidation: inhibitors of mRNA synthesis injected into the rodent hippocampus after memory reactivation impaired post-retrieval reconsolidation of this memory (Da Silva et al., 2008).

One particular transcription factor has been shown to be critical in long term memory: cyclic adenosine monophosphate (cAMP) response element binding protein (CREB). CREB is known to be activated by phosphorylation

following neuronal activity such as LTP, and is then able to induce CRE-dependent gene transcription of molecules which are involved in synaptic plasticity (Impey et al., 1998; Kandel, 2001). As such, CREB-dependent gene expression has been shown to be required for a variety of memory processes. For example, disrupting CREB in the hippocampus significantly impairs memory performance in the water maze (Bourtchuladze et al., 1994; Pittenger et al., 2002). CREB knock-out mice, which are lacking the two CREB isoforms that are most abundantly expressed in the mammalian brain, display various memory impairments in differing behavioral paradigms, brain regions, and species (Bourtchuladze et al., 1994; Guzowski and McGaugh, 1997; Josselyn et al., 2004). Not only is CREB necessary for long term memory, but it has also been found that CREB is sufficient for memory formation: increasing CREB levels in the dorsal hippocampus was found to induce robust spatial memory in paradigms that do not normally produce such strong spatial memories (Sekeres, 2010).

An additional constraint in this system is that CREB is repressed by the protein activating transcription factor 4 (ATF4), also known as CREB-2 (Gachon et al., 2001). This repressor is itself regulated downstream of CREB through translation by the eukaryotic initiation factor 2 (eIF2) –alpha. It has been demonstrated that eIF2-alpha directly stimulates the translation of ATF4, which then represses CREB activity (Vattem and Wek, 2004). Alternatively, decreasing eIF2-alpha phosphorylation decreases the levels of the CREB repressor: this

then lowers the threshold required to produce gene-expression dependent L-LTP, and consequently enhanced memory (Costa-Mattioli et al., 2007). Together, these results suggest that gene-expression is a critical rate-limiting step in the formation of long term memory.

1.4. The synaptic tagging and capture hypothesis

While the studies investigating the roles of gene-expression and LTP represent important steps towards understanding the relationship between behavioral memory and cellular memory, there are still many questions left unanswered. For example, it has been demonstrated that LTP, and other forms of synaptic plasticity, occur at a specific subset of synapses in a given brain region (Bliss, 1973). Accordingly, an important question concerns how LTP can demonstrate this synapse specificity. How do newly synthesized proteins and gene products become targeted at certain synapses that have undergone potentiation? The synaptic tagging and capture hypothesis is a model of how newly synthesized proteins and mRNA can be specifically targeted and "captured" at particular activated synapses without similarly tagging nearby synapses which have not received this stimulation. This form of synapse specificity is one of the criteria necessary for the synaptic plasticity and memory hypothesis to assert that LTP is the cellular substrate for learning and memory: synaptic tagging may therefore represent how this synapse specificity occurs

(Sossin, 1996; Frey and Morris, 1997; Frey et al., 2008; Redondo and Morris, 2011). The synaptic tagging theory can explain how a strong tetanus which induces a protein synthesis-dependent L-LTP in one particular pathway can simultaneously prolong the potentiation of an independent pathway that was only weakly stimulated and would only have produced E-LTP (Sajikumar et al., 2005). The details of what the tag itself consists of have yet to be completely elucidated, however, it has been shown that certain newly synthesized proteins called plasticity related proteins (PRPs) are required for the formation of the tag. The identity of potential PRP candidates will be discussed at a later point.

1.5. Physical changes in the brain: Morphological changes at synapses

As is often the case, these putative answers also raise new questions: is the tag purely constituted of a collection of proteins, or are specific morphological changes also involved? Does synaptic plasticity itself require structural plasticity? What factors are involved in morphological changes at pre-existing synapses as well as the formation of new synaptic connections? Does the formation of new synapses in fact underlie the long term memory trace? These are very important questions, as it is quite logical to assume that long term memories must also require some physical changes in the brain. Unfortunately, these are also not easy questions to answer: the complexity of the neuronal architecture can be appreciated by the fact that any given neuron can possess up to 100,000 spines,

each often forming a single synapse (Kopec and Malinow, 2006). Spines themselves can function as biochemical containers for signalling molecules that are activated by various forms of stimuli. Larger spines have been shown to represent the morphological substrate of strong synapses, whereas smaller spines conversely represent a substrate for weaker synapses. Larger spines also possess larger postsynaptic densities (PSD) that themselves contain more AMPA receptors and therefore display larger AMPA receptor (AMPAR) -mediated currents. These larger spines are also associated with higher probabilities of release from the presynaptic terminals that have more active zone area (Harris and Stevens, 1989; Ultanir et al., 2007). Furthermore, the number of synaptic AMPA receptors changes rapidly following LTP. As a consequence, this results in increases or decreases in the size of the corresponding spine. A direct correlation has also been shown between the magnitude of spine enlargement following LTP and the level of AMPAR-mediated synaptic currents (Matsuzaki et al., 2004). However, the particular reasons for these correlations between structure and function have yet to be fully explained and/or understood.

One particular protein found in abundance in the PSD that is well poised to link both structure and function of morphological changes at the synapse is PSD-95. PSD-95 is known to be involved in many aspects of synaptic transmission, to interact with both AMPA and NMDA-receptors (NMDAR), and to regulate the NMDAR-dependent changes in AMPAR number during processes such as LTP

(Ehrlich and Malinow, 2004; Nimchinsky et al., 2004). In a particular study, Steiner and colleagues (2008) examined spine morphology and PSD protein trafficking at CA1 dendritic spines. To do this, the authors delivered LTP-inducing stimuli consisting of glutamate pulses onto the spine heads; it was found that PSD-95 is required for the transient, as well as continuous, phases of activity-dependent spine growth. In response to this activity, it was observed that PSD-95 is rapidly trafficked out of the spines and that this process is dependent on calcium/calmodulin-dependent protein kinases (CaMKs). Specifically, this regulation was seen to occur through a CaMKII phosphorylation site on PSD-95: phosphorylation at this site inhibited LTP as well as LTP-induced spine growth. The authors therefore concluded that CaMKII and PSD-95 first act together to induce spine growth, and then the phosphorylation acts to terminate the process (Steiner et al., 2008).

While these results are important due to the experimental evidence that activity-induced processes such as LTP can induce morphological changes at synapses, the question still remains of whether the formation of new stabilized synapses similarly occurs. Furthermore, the more difficult question to answer is whether these synaptic spine changes and formation of new synapses can underlie the long term memory trace. In order to begin to investigate these questions, Holtmaat and colleagues (2008) examined the relationship between experienced induced structural and functional plasticity and synaptogenesis in

the mouse neocortex. To do this, they used transgenic mice expressing green fluorescent protein (GFP) or yellow fluorescent protein (YFP) in certain layers of the cortical pyramidal cells of the barrel cortex. The apical dendrites were then imaged through a glass window that was implanted over the primary somatosensory cortex skull. Simple manipulation of the whiskers served as sensory experience to induce changes in the spine and synapse dynamics in the cortex, and the spines were imaged for up to one month by imaging every four days. Accordingly, it was found that trimming of every other whisker resulted in the generation and loss of persistent spines (those present for 8 days or more). It was also found that new spines are able to form new synapses, but they do so slowly; the new synapses were primarily found on large, multi-synaptic boutons. Based on these data, it was suggested that these data demonstrate that spine growth is indeed followed by synapse formation, particularly on existing boutons (Holtmaat et al., 2008). While remodelling of synaptic connections often involves very quick turnover of newly created synapses, this previous result does suggest that experience can indeed produce longer term structural changes.

1.6. Conserved forms of memory in invertebrates: *Aplysia californica*

As outlined above, the study of reduced forms of memory in vertebrate animals does indeed pose some particular problems. While we have certainly been able to learn a great deal about the molecular, cellular, and neurological

correlates of the mammalian long term memory trace, the sheer complexity of the mammalian nervous system does nevertheless result in significant struggles in being able to determine what exactly may be going on at a specific synapse at a specific time point during memory formation and maintenance.

As such, it has been reasoned that if elementary forms of learning are common to animals with evolved nervous systems, then there must be conserved features of cellular and molecular learning that could be studied in simpler invertebrate animals (Kandel, 2001). In particular, studies of *Aplysia californica*, a marine mollusk, have yielded a great deal of physiological information about the biochemical mechanisms underlying learning and memory, and many of them have been shown to be also true of mammalian synaptic plasticity (Roberts and Glanzman, 2003). Using *Aplysia* to study the biochemical mechanisms of memory affords numerous advantages. The *Aplysia* nervous system is quite simple and is comprised of a small number of nerve cells (approximately 20,000) grouped into 10 major ganglia. In contrast, mammalian brains contain nearly one trillion central nerve cells. In addition, *Aplysia* neurons are in fact the largest known nerve cells in the animal kingdom and therefore lend themselves easily to various methods of biochemical manipulations (Kandel, 2001).

Similarly to mammals, *Aplysia* are also capable of certain forms of learning: the sensory to motor synapse model is known to be involved in the defensive withdrawal reflex of the animal's gill. This defensive withdrawal reflex

can be modified by habituation, sensitization, and classical conditioning (Hawkins et al., 1998). Kandel and colleagues were struck by the similarities of these forms of learning observed in *Aplysia* to the corresponding forms of mammalian learning. Evidence for the conservation of biochemical mechanisms was also established between *Aplysia* and vertebrates. Indeed, just like more complex mammalian learning, long-term memory for sensitization in *Aplysia* differed from short-term memory by the requirement of *de novo* protein synthesis (Kandel, 2001). While it appeared that higher vertebrate forms of memory were indeed conserved in *Aplysia*, it was still not precisely understood how learning could occur in a single specifically wired synapse. Experience induced changes in the strength of the synapse between cells, synaptic plasticity, thus emerged as the primary mechanism to explain how learning occurs at the neuronal level (Kandel, 2001; Roberts and Glanzman, 2003).

1.6.1. Synaptic plasticity in *Aplysia*: The role of serotonin

Additional research has sought to determine the molecular components that are responsible for the aforementioned forms of synaptic plasticity. It is known that experience induced release of serotonin (5-HT) in *Aplysia* causes an increase in synaptic strength, which is known as facilitation (Chitwood et al., 2001; Kandel, 2001; Marinesco and Carew, 2002). During facilitation, 5-HT is released from interneurons and acts upon receptors on the presynaptic sensory

neuron to enhance further neurotransmitter release (Kandel, 2001). Serotonin leads to an increase in presynaptic cAMP, which activates Protein Kinase A (PKA) and thus leads to synaptic strengthening through enhanced transmitter release produced by a combination of mechanisms. These include phosphorylation of potassium channels and the exocytosis machinery by PKA, leading to an enhanced excitability by allowing greater calcium influx into the presynaptic terminals (Klein, 1980; Klein, 1994; Kandel, 2001). It is this mechanism which is thought to underlie short-term presynaptic facilitation (STF), and thus STM, which is involved in behavioral sensitization to a stimulus. In addition, 5-HT also binds to receptors on postsynaptic motor neurons: a 10 minute application of 5-HT to cultured *Aplysia* motor neurons results in an activation of G-proteins which causes a rise in intracellular calcium. This rise in intracellular calcium consequently causes vesicles containing AMPA-type receptors to fuse and to be inserted into to the postsynaptic membrane (Chitwood et al., 2001). It is possible to block facilitation if 5-HT antagonists, such as cinanserin and cyproheptadine, are applied to *Aplysia* neuronal cultures (Brunelli et al., 1976; Mercer et al., 1991).

1.6.2. Molecular mechanisms of facilitation in *Aplysia*

Similarly to mammalian learning and memory ,which was discussed in the beginning of this introduction, facilitation can be dissociated into certain time-

dependent phases: STF lasting under 30 minutes, intermediate term facilitation (ITF) lasting 2 hours or more, and long term facilitation (LTF) lasting more than 24 hours (Ghirardi et al., 1995). These facilitatory phases are known to be mechanistically independent, as studies have shown differential requirements for each phase. For example, it is known that LTF requires new protein and RNA synthesis, ITF frequently, though not always, requires protein synthesis, and STF only requires modification of existing proteins that can occur through substrate phosphorylation by PKA (Müller and Carew, 1998). CREB-mediated gene transcription is also conserved in *Aplysia* and has shown to be required for gene-expression dependent LTF (Bartsch et al., 1998).

In *Aplysia*, ITF and LTF encompass multiple forms of plasticity that are also regulated by distinct molecular mechanisms (Sherff and Carew, 2002). For example, an activity-independent form of ITF can be induced by repeated pulses of 5-HT: this form of ITF thus requires protein synthesis for its induction, and activation of PKA for its maintenance (Müller and Carew, 1998; Sutton and Carew, 2000). Activity-dependent ITF, however, can be induced by a single pulse of 5-HT and thus does not require protein synthesis for its induction, but it does necessitate activation of protein kinase Cs (PKC) for its maintenance (Sutton and Carew, 2000). Interestingly, the previous state of the synapse is also important in determining the molecular requirements for various forms of plasticity. For example, at a naïve *Aplysia* synapse which has not previously undergone

facilitation, it is PKA phosphorylation which is required to increase the probability that synaptic vesicles will be released in order to increase synaptic strength (Ghirardi et al., 1992). However, it is PKC which is important to remove inhibition at a previously activated depressed synapse (a synapse which has previously undergone facilitation and is currently in a depressed state following this facilitation) (Ghirardi et al., 1992; Manseau et al., 2001; Zhao et al., 2006; Sossin, 2007).

1.6.3. Pre- and postsynaptic facilitation

Sensitizing stimuli, such as 5-HT application, are known to activate PKCs in *Aplysia* in both the presynaptic and postsynaptic neuron. Application of 5-HT to the presynaptic sensory neuron activates the calcium-independent PKC by translocating the kinase to the cell plasma membrane (Sacktor and Schwartz, 1990; Zhao et al., 2006). As previously mentioned, activity-dependent ITF requires PKC for its maintenance, and in fact depends on translocation of the calcium-dependent PKC to the plasma membrane (the different PKC isoforms will be discussed shortly) (Sutton and Carew, 2000; Zhao et al., 2006). However, activity independent ITF, which can be produced by a 10 minute application of 5-HT in the absence of neuronal activity, is dependent on numerous postsynaptic factors: elevation of calcium in the postsynaptic neuron, postsynaptic protein synthesis, and trafficking of postsynaptic AMPARs (Li et al., 2005; Villareal et al.,

2007). It has also recently been demonstrated that this form of ITF is the first form of facilitation that requires both presynaptic and postsynaptic mechanisms: specifically, it requires presynaptic PKC and postsynaptic calcium and CamKII, in addition to both presynaptic and postsynaptic protein synthesis (Jin, 2011). Strictly postsynaptic mechanisms have shown to be involved in application of 5-HT to isolated motor neurons. This was demonstrated to enhance the motor neuron's response to excitatory glutamate in a manner that also involves the activity of AMPARs (Trudeau and Castellucci, 1995; Chitwood et al., 2001).

1.7. Structure and activation patterns of PKCs

The activation of G-proteins by 5-HT as previously mentioned also leads to the activation of PKCs (Klein, 1994; Raymond et al., 2001). Owing to the many roles that PKCs play in various forms of plasticity, they have thus become the focus of a large number of experiments seeking to elucidate the molecular features of memory. As such, since the discovery of PKCs by Nishizuka (Takai et al., 1977; Takai et al., 1979; Castagna et al., 1982; Nakamura, 2010), a great deal of research has revealed the critical importance of PKCs.

PKC molecules consist of an amino terminal regulatory subunit (N-terminal) and a carboxy terminal catalytic subunit (C-terminal), which are both joined by a hinge domain linking region. PKCs consist of four isoform families: conventional PKCs (alpha, beta I and II, gamma), novel PKCs of the epsilon

family (epsilon and eta), which are also referred to as novel type I, novel PKCs of the delta family (delta and theta), or novel type II, and finally atypical PKCs (zeta, iota/lambda) (Newton, 2001; Sossin, 2007). *Aplysia* has only three identified PKC isoforms: one conventional, PKC Apl I, one novel type 1, PKC Apl II, and one atypical, PKC Apl III (Newton, 2001; Sossin, 2007). The PKC families differ in various manners, mainly in which cofactors are required for their activity. Conventional PKCs (cPKC) have two C1 domains which bind diacylglycerol (DAG), as well as a C2 domain that binds calcium. Novel PKCs (nPKC) have a 'novel' C2 domain that does not bind calcium, which is followed by two C1 domains that also bind DAG. Atypical PKCs (aPKC) are called such because they have a single 'atypical' C1 domain that does not bind DAG or other phorbol esters like the conventional and novel counterparts do. Furthermore, aPKCs lack a C2 domain and have instead a PB1 domain (see Fig. 1.1). Certain PKC isoforms are known to be enriched in the nervous system, such as PKC gamma, epsilon, and a truncated form of aPKC zeta (PKCz) called a PKM (which will be discussed below) (Tanaka, 1994; Hernandez et al., 2003).

1.7.1. PKC activation by lipid binding

These PKC isoforms are also different in their specific mechanisms of activation. It is known that PKCs are activated by a conformational change in the regulatory domain involving lipid binding: this removes the autoinhibitory

pseudosubstrate from the catalytic domain, thus allowing various substrates to be phosphorylated by the now active PKC (Newton, 1995b). For both cPKCs and nPKCs, lipid binding is greatly improved by DAG binding to the C1 domains. Lipid binding to the C2 domain of cPKCs and nPKCs occurs under different conditions and thus is a main distinguishing factor between these PKCs (Newton, 1995a). Conventional PKCs, such as PKC Apl I in *Aplysia*, require calcium to be associated with DAG upon binding in order for this kinase to be activated by translocation to the plasma membrane (Zhao et al., 2006). The C2 domain of the novel PKC Apl II in *Aplysia* lacks a residue for calcium binding, does not bind lipids well, and removal of the C2 domain in fact enhances the activation and translocation of this isoform (Pepio et al., 1998; Farah et al., 2008). In contrast, lipid binding to the C1 domain of aPKCs is also important for its activation, but the structure of the atypical C1 domain renders these kinases insensitive to DAG or calcium induced activation. Atypical PKCs also do not translocate to the plasma membrane following addition of a phorbol ester (Kazi, 2007). In addition, aPKCs have a PB1 domain on the N-terminal region of the enzyme which is not present in either cPKCs or nPKCs; this domain is known to be involved in protein-protein interactions which are important for activation of aPKCs (Moscat, 2000; Henrique and Schweisguth, 2003; Hirano et al., 2004).

1.7.2. PKC activation by phosphorylation

PKCs are also regulated by phosphorylation, and in fact all isoforms specifically require phosphorylation by phospho-inositide-dependent kinase (PDK) for their activity (Chou et al., 1998; Le Good et al., 1998). In the case of cPKCs and nPKCs, phosphorylation by PDK at a site in the kinase domain is required to retain stability and proper folding conformation of the kinase; these PKCs are also phosphorylated by PDK at a hydrophobic site in a C-terminal extension domain (Newton, 1995b; Balendran et al., 2000). While aPKCs are also phosphorylated by PDK at a site in the kinase domain, this phosphorylation is not specifically required for stability of the enzyme but is a mechanism for controlling activity of the kinase (Chou et al., 1998; Balendran et al., 2000). Atypical PKCs also do not possess a hydrophobic residue like the cPKCs and nPKCs, instead this site is replaced by a glutamic acid (Newton, 1995b).

1.7.3. PKC knock-out studies

The importance of PKCs can be further illustrated when examining the studies in which various PKC isoforms have been genetically removed. For example, PKC alpha knock-out mice have been shown to lack LTD in the cerebellum (Leitges et al., 2004). PKC beta knock-out mice display deficits in both cued and contextual fear conditioning (Weeber et al., 2000; Sossin, 2007). PKC gamma knock-out mice have been shown to exhibit some deficits in LTP,

and its counterpart long term depression (LTD), while also having mild to moderate impairments in the hidden-platform task of the Morris water maze as well as in context-dependent fear conditioning (Abeliovich et al., 1993). PKC epsilon knock-out mice have deficits in pain sensation, ethanol sensitivity, and ischemic preconditioning (Khasar et al., 1999; Aley et al., 2000; Dina et al., 2000). PKC theta knock-out mice display impaired activity-dependent synapse modification at the neuromuscular junction (Li et al., 2004). These studies clearly point to the important role for PKCs in many functions, especially in terms of synaptic plasticity, and thus learning and memory.

1.7.4. Additional roles for atypical PKCs

Atypical PKCs have been shown to be critical for a variety of functions. Cellular examples include embryo polarity, asymmetric cell division, and survival (Soloff et al., 2004). In vertebrates, two particular aPKC isoforms have been shown to be responsible for these functions, aPKCz and aPKC lambda: aPKCz mediates signal transduction in the immune system, whereas aPKC lambda is critical for early embryogenesis (Soloff et al., 2004). Furthermore, aPKCs are known to be linked to partitioning-defective proteins (PARs) which are essential for asymmetric cell division and polarized growth. It is also known that an additional compound Cdc42-GTP mediates the establishment of cell polarity (Joberty et al., 2000). The aPKC in *C. elegans*, PKC-3, co-localizes with PAR-3

and is also necessary for asymmetric cell division. In addition, PAR-6 also co-localizes with PAR-3 and PKC-3, and it is PAR-6 which binds to the PB1 domain of aPKCs to direct cell polarity (Ohno, 2001). Together, these three proteins are co-dependent upon each other as the loss of one of them leads to the mislocalization of the others. The interaction between PAR-6, PAR-3, aPKCs, and Cdc42 is required for the formation or maintenance of tight junctions in epithelial cells (Joberty et al., 2000).

1.8. Persistent activation of aPKCz in the long term memory trace

Persistent activation of kinases, such as aPKCz, is one mechanism which makes kinases important for cellular and behavioral forms of long term memory. By definition, long term memory must somehow persist long after the removal of the stimuli that initially gave rise to the learning. Therefore, persistent activation is an attractive candidate to explain how a given memory can persist over time. The mechanism through which persistent activation of kinases occurs is thought to be due to the relief of autoinhibition: a kinase can become constitutively active and relieved of autoinhibition if the regulatory domain is removed or cleaved off. This has been shown to be true of PKC, and it has been observed that maintenance of ITF induced by coupling 5-HT and activity requires persistent activity of a truncated kinase called PKM (Sutton et al., 2004).

One candidate constitutively active kinase is called PKM zeta (PKMz); a truncated and persistently active form of aPKCz that lacks the N-terminal regulatory domain and is therefore free of autoinhibition. PKMz has been widely implicated in memory processes: in rats, PKMz is the only PKC isoform whose levels specifically increase and remain elevated during the maintenance phase of L-LTP (Sacktor et al., 1993). In *Drosophila*, PKMz enhances performance in an odor avoidance task (Drier et al., 2002). Furthermore, it has been established that PKMz is both necessary and sufficient for the maintenance of LTP: Ling and colleagues (2002) demonstrated that PKMz inhibitors reverse previously established LTP, whereas other kinase inhibitors, such as CaMKII, do not (Ling et al., 2002). In order to determine which particular phase of LTP PKMz is involved in, a cell-permeable aPKC-selective inhibitor named ZIP has been used to examine how this drug affects synaptic potentiation in various systems. It was found that in the absence of ZIP, postsynaptic infusion of PKMz caused a significant potentiation of AMPA receptor responses in rat hippocampal slices. In addition, it was observed that infusions of ZIP did not block the early expression of LTP. However, ZIP did reverse L-LTP when applied one, three, and five hours following tetanization (Serrano et al., 2005). PKMz has also been shown to specifically establish and maintain long-term increases in the number of active postsynaptic AMPARs (Ling et al., 2006). These results suggest that PKMz is required for the late phase of LTP and not the induction or early to mid-phases; in

fact it is the only signalling molecule which has been demonstrated as both necessary and sufficient for maintaining this late phase of LTP.

Regulation of PKMz is also known to be bidirectional. A decrease in protein phosphorylation is implicated in LTD, a persistent depression of synaptic responses following low-frequency afferent stimulation in the rat hippocampus (Mulkey et al., 1993). Accordingly, LTD can be blocked and even reversed by infusions of phosphatase inhibitors, compounds which prevent dephosphorylation (Mulkey et al., 1993). Hrabetova and Sacktor (1996) therefore investigated the role of PKMz in LTP and LTD. It was found that PKMz is down-regulated in the maintenance of LTD, and that this was reversible by NMDA receptor antagonists (Hrabetova and Sacktor, 1996). These results suggest that PKMz plays bidirectional roles in both LTP and LTD.

In addition, PKMz is unique in that it is not generated by proteolysis like other truncated PKMs. Of the PKMs, PKMz is the only one expressed in the brain, with a relative profusion in the neocortex and hippocampus (Hernandez et al., 2003). PKMz is also expressed in the complete absence of PKCz (in PKCz knock-outs), suggesting that proteolysis is not the mechanism through which PKMz is formed. Instead, it has been determined that PKMz is translated from its own separate mRNA, which is transcribed from an internal promoter within the PKCz gene that is spared in the knock-out (Hernandez et al., 2003). As such, the formation of PKMz from its own mRNA allows for functional consequences not

possible through production by proteolysis. For example, Muslimov and colleagues (2004) have determined that PKMz mRNA is substantially present in the somatodendritic sections of rat hippocampal neurons. This dendritic localization of PKMz mRNA is important for various reasons. Firstly, it provides a potential explanation for the molecular bases of long term consolidation of information at a synapse: this could occur through a functional integration of local transduction pathways and the control translational mechanisms (Muslimov et al., 2004). Secondly, it suggests a possible role for aPKCs in postsynaptic plasticity.

1.8.1. The role of PKMz in vertebrate behavioral memory

In terms of behavioral memory, the past 10 years or so have yielded a great deal of information regarding the requirement for PKMz (PKMz) in maintaining the long term memory trace. One of the first, and most compelling, studies was conducted by Pastalkova et al. (2006): the authors utilized the experimental strategy of ‘erasure’ to directly asses whether PKMz may be a molecule essential to the long term memory trace. This strategy posits that targeted erasure of detectable learning-induced synaptic plasticity should subsequently induce forgetting (Martin, 2000). It was therefore reasoned that if PKMz activity is necessary for spatial long-term memory storage, inhibiting its activity a day after learning should cause retrograde amnesia. To examine this, the authors trained rats in a hippocampal dependent inhibitory avoidance task.

Twenty-two hours later the rats were injected with ZIP directly into both hippocampi. Two hours after the injections, the LTM of the animals was tested: rats who received ZIP failed to display intact long-term memory for the task. This observed forgetting was persistent, as the animals also did not display intact LTM even when tested one week following delivery of the drug. Furthermore, even remote memory measured at a time point of one month could be erased by ZIP (Pastalkova et al., 2006).

Follow-up studies from this group have also shown that other forms of LTM are maintained by PKMz, such as spatial memories tested in the water maze and the 8-arm radial maze, as well as amygdala dependent classically conditioned contextual memory and auditory fear conditioning (Serrano et al., 2008; Migues et al., 2010). Interestingly, this same study found that not all long term memories are maintained by PKMz: for example, procedural forms of memory, in addition to imprecise or coarse spatial information, were not erased by ZIP (Serrano et al., 2008).

To test the role of PKMz in memory storage in the neocortex, Shema et al. (2007) specifically assessed taste aversion memory, which is known to be stored in the insular cortex. In this paradigm, rats are presented with a novel taste which is then paired with an injection of lithium, thus rendering the animal ill. A single training trial is sufficient for the animals to learn to avoid this novel taste, producing a long-term memory that can last for up to several weeks. Injections of

ZIP into the insular cortex were similarly able to permanently erase the aversion memory, when the drug was injected from three to 25 days following training. Furthermore, the memory did not return up to one month following the injections, even when the authors attempted to reactivate the memory (Shema et al., 2007). A more recent study from this group has shown that ZIP can even erase the insular cortex memory at three months following training, but it does not affect the initial encoding of the memory trace or its initial short-term recall (Shema et al., 2009).

1.8.2. The role of PKMz in synaptic tagging

PKMz has also been implicated in synaptic tagging as a PRP. PKMz presents an interesting candidate as a PRP due to the previously discussed results in addition to the finding that it is only synthesized following strong stimulation and not weak tetanisation (Osten et al., 1996). Accordingly, the role of PKMz as a PRP in synaptic tagging has been studied. To do this, Sajikumar and colleagues (2005) positioned electrodes in the stratum radiatum of the CA1 region in rat hippocampal slice preparations for stimulation of two separate independent synaptic inputs, S1 and S2. The authors then induced L-LTP in synaptic input S1 by applying three stimulation trains of 100 pulses at 100 Hz, and E-LTP in synaptic input S2 by one 100 Hz train of 21 pulses: 30 minutes later the PKMz inhibitor ZIP was applied to both inputs. The induction protocols

displayed synaptic tagging: the L-LTP in S1 was able to transform the E-LTP in S2 to L-LTP, lasting up to 8 hours. However, following the application of ZIP, the L-LTP in S1 was reversed to baseline, and the potentiation in S2 also returned to pre-stimulation levels: ZIP was able to block the persistence of the potentiation following synaptic tagging (Sajikumar et al., 2005). The effect of PKMz was specific to the tagged synapses, because ZIP had no effect on an independent non-tetanized pathway. Furthermore, the effect of PKMz was also restricted to the late phase of LTP acquired by S2 following synaptic tagging due to the observation that application of ZIP 60 minutes after the E-LTP had no effect on its time course (Sajikumar et al., 2005).

It was also shown that PKMz is not involved in the maintenance of L-LTD; as such, the authors sought to examine the role of PKMz in cross-tagging: can elongation of a cross-tagged weakly depressed synapse by strong LTP transform it into an L-LTD mediated by PKMz? This did not seem to be the case, as ZIP specifically reversed L-LTP but was unable to prevent the long-lasting depression at the weakly stimulated, cross-tagged LTD synaptic input pathway (Sajikumar et al., 2005). This study therefore demonstrates that PKMz is indeed a PRP, which is LTP-specific and necessary for the transformation of early into late LTP during both synaptic tagging and cross-tagging (Sajikumar et al., 2005; Frey et al., 2008).

1.8.3. Additional roles for PKMz

More recent studies have investigated the role of PKMz in less traditional forms of memory: specifically in neuropathic pain and drug reward memory. Li et al. (2010) hypothesized that maintenance of synaptic plasticity may also underlie the persistence of pathological pain conditions. The anterior cingulate cortex (ACC) is a key brain region involved in chronic pain, and PKMz is a molecule implicated in the maintenance of long term memory, therefore they sought to investigate whether PKMz is involved in chronic pain in the ACC. Peripheral nerve injury was induced in mice and levels of PKMz were subsequently analyzed in the ACC, hippocampus, and spinal cord: it was found that PKM levels were increased only in the ACC three days after nerve injury. It was also found that PKMz levels were still elevated when nerve injury was measured seven to 14 days later. Furthermore, bilateral injections of ZIP into the ACC three and seven days following nerve injury produced analgesic effects, suggesting that neurons in the ACC are involved in neuropathic pain and these neurons undergo enhanced excitatory synaptic transmission that is maintained by PKMz activity (Li et al., 2010).

The role of PKMz in maintaining drug reward memory has also recently been investigated. It has been shown that Pavlovian conditioning plays a role in drug addiction, as drug cravings can be induced by environmental cues previously associated with intake of a certain drug (Ciccocioppo et al., 2004).

However, the mechanisms which underlie the maintenance of drug cue memories are presently unknown. In order to test whether PKMz is implicated in the persistence of drug reward memory, Li et al. (2011) trained rats in a drug conditioned place preference procedure where a specific context is paired with administration of either cocaine or morphine. The rats were then tested for preference of the context previously paired with the drug, and levels of PKMz activity were analyzed in the nucleus accumbens, a brain region previously implicated in cue-induced drug seeking in rats (Crombag et al., 2008). It was found that PKMz activity specifically in the nucleus accumbens core is required for the maintenance of the drug cue memories. Furthermore, the results also showed that PKMz maintained these memories by inhibiting GluA2-dependent AMPAR endocytosis (Li et al., 2011).

1.8.4. Overexpression of PKMz improves memory performance

Finally, one of the most compelling pieces of research which confirms the role of PKMz in long term memory maintenance utilized a technique that did not involve inhibiting the kinase and examining any resulting memory deficit; instead, the authors overexpressed PKMz in the rat neocortex using a lentivirus system (Shema et al., 2011). As previously discussed, this group has shown that long term memory of conditioned taste aversion is maintained by PKMz and can be erased by injections of ZIP into the insular cortex. Subsequently, the authors

sought to examine the effect of modulating the level of PKMz in the insular cortex by infecting this region with a lentivirus encoding either PKMz or a dominant negative PKMz. It was found that overexpression of PKMz was able to enhance long term memory for conditioned taste aversion, including memories that were formed before the enzyme was overexpressed. In contrast, overexpression of the dominant negative significantly disrupted the long term memory (Shema et al., 2011). These findings represent an important contribution supporting the role for PKMz as a molecular long term memory trace.

1.9. PKMz in *Aplysia*: PKM Apl III

While little is known about the role for PKMz in *Aplysia*, it has been recently implicated in maintaining LTM as well as long term synaptic plasticity in this system. As previously mentioned, a 10 minute application of 5-HT to the postsynaptic motor neuron produces a long term enhancement of the glutamate-evoked potential (Glu-EP) that is dependent on PKC for its induction (Chitwood et al., 2001; Villareal et al., 2007). Interestingly, it was found that chelerythrine, a kinase inhibitor that is specific to the atypical PKC Apl III at low concentrations, is able to block the long term maintenance of the Glu-EP. In contrast, applications of bisindolylmaleimide-1 (Bis), an inhibitor with greater selectivity for the conventional and novel isoforms of PKC, did not block the maintenance of the Glu-EP (Villareal et al., 2009). Maintenance of the Glu-EP was also found to be

dependent on the activity of calpain, an intracellular protease which cleaves PKC, but was not dependent on *de novo* protein synthesis (Villareal et al., 2009). Taken together, these results suggest that the long term maintenance of the Glu-EP is dependent upon a constitutively active PKM fragment that is formed by calpain cleavage of the full length atypical PKC Apl III.

Sensitization of the siphon-withdrawal reflex (SWR) in *Aplysia* is a form of LTM, referred to as long term sensitization (LTS), and this form of behavioral memory has also been linked to a persistently active PKM. As discussed earlier, the synaptic plasticity which underlies this behaviour is a LTF of the sensory to motor synapse mediated by 5-HT (Frost et al., 1985). Using similar inhibitor techniques as the mammalian studies, Cai et al. (2011) wanted to examine the role of PKMz in *Aplysia*, PKM Apl III, in maintaining both the behavioral LTS and synaptic LTF. It was found that inhibiting PKM Apl III with ZIP, as well as chelerythrine at low concentrations that are known to be specific for PKM Apl III (Villareal et al., 2009), was able to block both the maintenance of LTS as well as the maintenance of the LTF (Cai et al., 2011). These results not only support a conserved role for PKMs in *Aplysia*, but further demonstrate that mammalian and invertebrate systems share many of the same molecular and cellular components of the long term memory trace.

1.10. Main objectives of the current project

Accordingly, it can be appreciated that there exists a need to further elucidate the role of PKCz and PKMz in synaptic plasticity: PKMz is a strong candidate for a molecular mechanism underlying the long term memory trace, in both vertebrates and invertebrates. *Aplysia* represents a powerful model system in order to study the reduced but evolutionarily conserved forms of synaptic plasticity, in contrast to the significantly more complex mammalian model systems. As such, the first main objective of the current project was to clone and characterize the atypical PKCz orthologue in *Aplysia*, PKC Apl III, as very little was known about the role for this kinase in *Aplysia*. Furthermore, the second main objective was to determine if and how a PKM Apl III is formed in *Aplysia* and what role it may play in synaptic plasticity.

Figure 1.1.

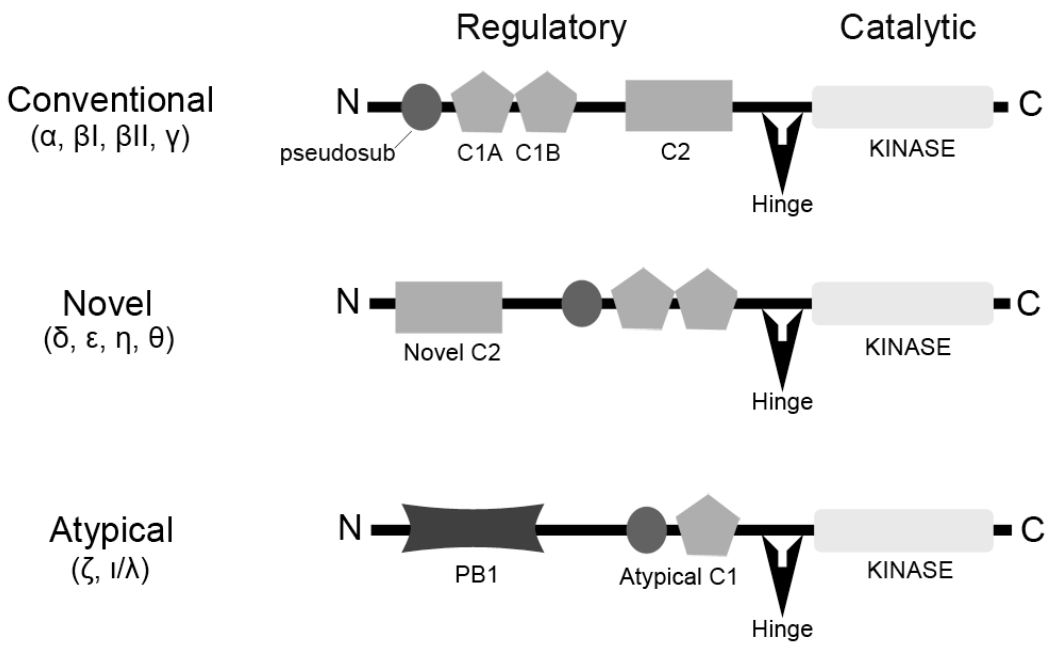

Regulatory
Catalytic
Conventional
(α, βI, βII, γ)
N
C
pseudosub
C1A
C1B
C2
KINASE
Hinge
Novel
(δ, ε, η, θ)
N
C
Novel C2
KINASE
Hinge
Atypical
(ζ, ι/λ)
N
C
PB1
Atypical C1
KINASE
Hinge

Figure Legends

Figure 1.1. Structure and activation patterns of PKCs. PKC molecules consist of an amino terminal regulatory subunit N-terminal (N) and a carboxy terminal catalytic subunit C-terminal (C), which are both joined by a hinge domain linking region. Conventional PKCs have two C1 domains which bind diacylglycerol (DAG), as well as a C2 domain that binds calcium. Novel PKCs have a 'novel' C2 domain that does not bind calcium, and two C1 domains that also bind DAG. Atypical PKCs have a single 'atypical' C1 domain that does not bind DAG or other phorbol esters. aPKCs lack a C2 domain and have instead a PB1 domain important for protein-protein interactions.

CHAPTER TWO

Methodology

This chapter describes the materials and methods used in the experiments throughout the following chapters three and four.

2.1. Animals

Aplysia californica (75-125g) were obtained from Marine Specimens Unlimited (Pacific Palisades, CA), and the Mariculture Facility of the University of Miami (Miami, FL). The animals were then maintained in a salt water aquarium until experimentation. The majority of the animals used in the following experiments were from the Miami facility.

2.2. Generation of DNA plasmid constructs

After sections of the sequence of PKC Apl III were elucidated by degenerate (polymerase chain reaction) PCR, 5' and 3' RACE, primers were generated with BamHI and EcoR1 sites at the end to amplify the full length sequence of PKC Apl III using PCR, and the amplified fragment was inserted into the BBACHis2 vector at BamHI and EcoR1 sites. Two independent PCRs were sequenced for the reference supplied to Genbank. A PKC Apl III (No Splice) was generated by amplifying a region surrounding the splice site from gill mRNA and

inserting it into the unique sites Nde I and Aat II in the BBACHis2-PKC Apl III construct. In this vector, a kinase dead form was made mutating lysine 297 to arginine using overlap PCR (K297-R). A PKM version of PKC Apl III was also constructed using a primer beginning in front of the splice sites. To generate mRFP –tagged PKC Apl IIIs in pNEX 3, all constructs were excised from the BBACHis2 vector with Bam HI and Hind III; the sites were then filled in with Klenow and inserted into pNEX3-mRFP cut with Sal I.

The kinase dead PKC Apl III D-A and PKM Apl III D-A were made by mutating the aspartic acid 392 in PKC Apl III to alanine using overlap PCR (D392-A). To make the CFP-PKC Apl III-YFP and CFP-PKC Apl III D392A-YFP constructs, cyan fluorescent protein (eCFP) was amplified by PCR using primers containing SphI and XhoI sites. The product of this amplification was then cut with SphI and XhoI and used to replace the mRFP from the aforementioned pNEX-3-mRFP-PKC Apl III construct, and cut with these same enzymes. Yellow fluorescent protein (eYFP) was then amplified by PCR using primers containing Nco and Blp1 sites with the nucleotides encoding a putative PDZ binding domain (MSMEDCV) added on at the 3'end. The product of this amplification was then cut with Nco and Esp1 and ligated to the pNEX-3-eCFP-PKC Apl III vector. Baculovirus expression constructs were generated using the Invitrogen Bac-to-Bac cloning system according to the manufacturer's instructions.

The pNEX-3-dsRED VAMP construct used in the synaptic co-culture experiments was made by cutting the VAMP protein sequence from a pNEX-3-dsred-VAMP (Houeland et al., 2007) with Nhe and Xma3, and then ligating this section into the pNEX-3-dsRED plasmid.

2.3. Protein Purification

SF9 cells in suspension were infected with baculovirus constructs. Three days after infection, His-tagged protein was purified using Invitrogen Pro- bond His-Affinity resin (Invitrogen), in modified purification buffer (20 mM HEPES pH 7.5, 10 mM MgCl2, 1mM DTT, 100mM KCl, 10% glycerol; For calpains: 20 mM HEPES pH 7.5, 1mM EDTA, 1mM DTT, 100mM KCl, 10% glycerol). Proteins were eluted in elution buffer (identical to purification buffer but with 0.25M Imidazole), DTT was added to a final concentration of 10mM, and the sample was concentrated and stored at -80 ^{o}C.

2.4. Antibodies

The following peptides were synthesized (Invitrogen) for antibody production and purification: N-terminal VTNTKNDVKYPDGFC-amide; C-terminal, FEYVNPLLMSEDCV-COOH; Splice, CEDHFVDAESFMTAK-amide (Research Genetics). For the phospho-specific antibody, the phospo KPGDTTG[pT]FC-amide and corresponding non-phosphorylated peptide were synthesized (Quality

controlled biochemicals; QCB). Peptides were coupled to BSA-Maleimide and Sulfo-link (Pierce) according to manufacturer's instruction. For the Splice antibody, cysteines was added to the sequence at the N-terminal, but for the other antibodies cysteines in the coding sequence were used. After conjugation to BSA-Maleimide, rabbits were injected with the adjuvant Titer-max and after three boosts the final serum was affinity purified on Sulfo-link columns. For the phospho-specific antibody, the serum was first absorbed on a column containing the non-phosphorylated protein and the eluate was affinity purified on the phospho-specific column. The antibodies that were used for the subsequent experiments are as follows: a C-terminal antibody (Ab) at a 1:1500 concentration, N-terminal Ab at a 1:1500 concentration, splice Ab at a 1:1500 concentration, and a phospho-specific Ab at a 1:400 concentration. In addition, a fluorescein isothiocyanate (FITC)-goat anti-rabbit green secondary antibody (Zymed), used at a 1:40 concentration was used in order to visualize the primary antibodies. In addition, an Alexa-647 anti-goat anti-rabbit far red secondary (Invitrogen) was used at a concentration of 1:200 in order to visualize the primary antibodies.

2.5. *In vitro* calpain assays

PKC Apl III was purified from baculovirus as above. Purified PKC Apl III was then incubated with purified calpain-1 (Calbiochem) at varying concentrations for 30 min at 30^{o}C, along with 5x Buffer (2M $CaCl_2$, 500mM

cystein, 1M imidazole). Samples were then loaded onto an SDS-Page gel and either stained with Coomassie or transferred to a nitrocellulose membrane for Western Blot analysis with antibodies to PKC Apl III.

For the experiments comparing the double tagged or untagged PKC Apl III calpain-1 purified from porcine erythrocytes (Calbiochem) was incubated with *Aplysia* PKC Apl III with or without CFP and YFP tags for 30 minutes at 30 degrees C in calpain activation buffer (final concentrations: 5mM L-cysteine, 100mM Imidazole, 5mM calcium). Reaction products were then subjected to SDS-PAGE followed by transfer to nitrocellulose membrane and probed with a mouse monoclonal antibody to the His-tag (Invitrogen). Cleavage was quantified as the loss of the full-length protein.

2.6. *Aplysia* cell culture and DNA plasmid microinjection

Aplysia dissociated sensory and motor neuron cultures were prepared according to the protocol outlined in (Zhao et al., 2006), with slight modifications. The ganglia were proteased and digested in a dispase containing solution for either 2 hours and 5 minutes at 37^{o}C, or at 19^{o}C for 18-19 hours.

Individual neurons were pulled from de-sheathed pleural (for sensory neurons) or abdominal ganglia (for LFS motor neurons) and isolated in L15 media (Sigma) containing 25-50% *Aplysia* hemolymph and plated on coverslips pretreated with poly-l-lysine (BD Biosciences). The cells were then left to attach

to the coverslips for a two day period prior to injection. The sensory to motor neuron co-cultures were prepared as outlined in (Montarolo et al., 1986): briefly, in order to promote synapse formation, the sensory neurons and motor neurons were plated with the processes of the sensory neuron in contact with the axon hillock of the motor neuron.

Solutions of the construct of interest at various concentrations were prepared in filtered double-distilled water and 0.5 % fast green dye. The solutions were loaded into glass micropipettes, and the tip of the micropipette was then inserted into the cell nucleus. Short pressure pulses were delivered until the nucleus became uniformly green. Following injection, the cells were kept at 19^{o}C and treatment with various reagents was performed one to three days post injection. The cells were then fixed and stained for immunocytochemistry or were imaged live for the Förster Resonance Energy Transfer (FRET) experiments.

2.7. Immuocytochemistry

Following treatment with the reagents, the cells were fixed for 30 minutes in 4% paraformaldehyde in 30% sucrose, 1 X PBS. In order to permeabilize the cells, they were washed in 0.1% Triton X-100 in 30% sucrose, 1 X PBS for 10-15 minutes. The cells were then washed three times in 1 X PBS, and washed again in NH_4Cl for 15 minutes to quench free aldehydes. Prior to addition of the antibodies, the cells were blocked for 30 minutes in 10% normal goat serum in

0.5% Triton X-100, 1 X PBS. Samples were then incubated with various primary antibodies diluted in the blocking solution for one hour, washed four times with 1 X PBS, and then treated in the dark with a secondary green fluorescent antibody; FITC-Goat Anti-Rabbit IgG (Zymed Laboratories) at a concentration of 1:40 diluted in the blocking solution. Cells were washed again in 1 X PBS, and finally mounted on slides using Dako fluorescent mounting media (DakoCytomation, Denmark).

2.8. SF9 cell culture

The SF9 cells were purchased from Sigma-Aldrich (Sigma-Aldrich, Oakville, Ontario, Canada). SF9 cells were grown in Grace's medium (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (Cansera, Etobicoke, Ontario, Canada) as a monolayer at 27°C. For transfection, cells were plated on MatTek glass bottom culture dishes (MatTek Corporation, Ashland, MA) with a glass surface of 14 mm and a coverslip thickness of 1.5 mm. Cells were transfected using the Cellfectin reagent (Invitrogen, Burlington, Ontario, Canada) following the recommendation of the manufacturer (Zhao et al., 2006).

2.9. Determination of specific activity

The CFP-YFP, wild-type (WT), and kinase dead kinases were purified from SF9 cells infected with baculovirus encoding the different PKC isoforms as described above. Kinase assays utilizing radioactive ^{32}P-ATP were performed as described (Lim and Sossin, 2006), with serial dilutions of the enzymes to assure the assay was in the linear range. All the constructs contained an HA tag and the relative amount of enzyme was quantified by immunoblotting a serial dilution of enzymes from the same aliquot of purified enzyme used in the enzyme assay with the HA antibody. All values were normalized to the WT PKC Apl III measured in that experiment. Specific activity was determined by dividing the activity by the amount of enzyme.

2.10. FRET imaging and quantification

Twenty-four hours following DNA microinjection, the cells were imaged using a Zeiss fluorescent microscope. Images of cells expressing CFP alone and YFP alone were taken as control measures for background subtraction. Each cell was imaged in the CFP, YFP, and FRET channels where exposure times were kept constant for all groups within each experiment. For the isolated motor neuron experiments where CFP-PKC Apl III-YFP was expressed at low levels, the FRET channel exposure times were doubled in order to yield visible FRET levels for quantification. While this strategy gives an inaccurate value for the

FRET ratio, it does not affect our ability to detect changes in the FRET signal over time. Axio-Vision Zeiss software was used to quantify the images, where CFP was assigned channel 1, YFP was assigned channel 2, and FRET was assigned channel 3. The FRET Xia formula was used in order to subtract spectral bleed-through from cyan and yellow channels (Xia and Liu, 2001). The end result is a FRET ratio ranging from 0-1, with a corresponding color coded FRET ratio map where lower levels of FRET are assigned cooler colors and higher levels of FRET are assigned warmer colors. The color coding scale can be changed within experiments to produce more visible maps and is marked on the left of each FRET map.

To determine changes induced by 5-HT, the FRET maps were converted to a grey scale image and coded so that the measurer was unaware of the treatment, or the time (pre versus post) for the image. NIH image was then used to outline a large region of interest in the process and this area was measured in both the pre and post image. The fold-change in signal was then calculated (post/pre). In most cases multiple pictures of each cell (2-3) and multiple regions of interest (2-3) were chosen and the average of the fold-changes was used to measure the 5-HT induced change in FRET for each treated cell.

2.11. Confocal microscopy and image quantification

Neurons expressing the mRFP, eCFP, or CFP-YFP constructs were imaged using a 510 or 710 Zeiss laser scanning confocal microscope, and pictures were captured using the 40X objective at the middle of the cell where the nucleus was as well defined as possible. Within each experiment the same laser power was used for all groups, unless otherwise described. Pictures were then opened in the IP Lab (BD Biosciences) analysis program, and the perimeter of the cell was either identified automatically (high expressing cells) or manually outlined (lower expressing cells). The density of concentric rings one pixel in width from the perimeter to the center of the cell body was measured as described (Zhao et al., 2006; Nagakura et al., 2008). The cytoplasm was defined as between 10-20 pixels inward from the perimeter while the last five inward pixels were defined as the nucleus. While the procedure was automated, visual inspection of all cells confirmed that these regions corresponded to the cytoplasm and nucleus of each cells. Fluorescence ratios were then calculated. For normalized ratios, the nuclear/cytoplasmic ratio was divided by the average nuclear/cytoplasmic ratio of control cells. When comparing mRFP expression or FRET levels between treatments, all values were normalized by dividing by the average fluorescence level (or FRET value) of the control cells from that experiment. These normalized values were then summated from the different experiments.

CHAPTER THREE

The atypical Protein Kinase C in *Aplysia* can form a Protein Kinase M by cleavage

3.1. Introduction

Experience induced changes in the strength of synapses are thought to be the biochemical mechanism underlying behavioral forms of learning and memory. At the cellular level, multiple memory traces are formed after experiences that last for different amounts of time (Sossin, 2008a). An attractive model to study these memory traces is the sensory-motor neuron synapse of *Aplysia californica*. Increases in the strength of this synaptic connection occur after learning and contribute to the memory of behavioral sensitization (Kandel, 2001). Moreover, these increases can be recapitulated in sensory-motor neuron cultures after addition of 5-HT (Montarolo et al., 1986), the same transmitter used in behavioral sensitization (Glanzman et al., 1989).

PKCs play major roles in both the induction and the maintenance of molecular traces (Sossin, 2007).The two phorbol ester-activated PKC isoforms, the conventional PKC Apl I and the novel PKC Apl II play different roles in different memory processes. 5-HT translocates PKC Apl II at sensory-motor neuron synapses and this activation is important for the ability of 5-HT to reverse

synaptic depression, a process linked to behavioral dishabituation (Manseau et al., 2001; Zhao et al., 2006). 5-HT alone does not translocate PKC Apl I in sensory neurons, however, coupling 5-HT and activity results in translocation of PKC Apl I in sensory neurons. Also, PKC Apl I activity, but not PKC Apl II, is important for the induction phase of activity dependent ITF in sensory neurons, which is a process linked to site-specific conditioning in *Aplysia* (Sutton et al., 2001; Zhao et al., 2006). PKC Apl I, but not PKC Apl II, has also been implicated in operant conditioning in B51 cells (Lorenzini et al., 1996).

Persistent activation of protein kinases is important for the maintenance of molecular traces that last for longer periods of time, in both *Aplysia* and vertebrates. In *Aplysia*, persistent activation of PKC is implicated in the ITF that is seen after coupling 5-HT and activity (Sutton et al., 2001). In vertebrates, a persistently active form of aPKC zeta (PKCz), PKMz, is necessary and sufficient for the maintenance phase of LTP, and an inhibitor of PKMz is able to reverses *in vivo* LTP and produce persistent loss of behavioral memory at time points where the memories are assumed to be consolidated (Ling et al., 2002; Serrano et al., 2005; Pastalkova et al., 2006; Shema et al., 2007; Sacktor et al., 2008; Serrano et al., 2008; Shema et al., 2011). Furthermore, PKMz is generated by translation of a unique mRNA whose transcription starts in an intron of PKCz (Hernandez et al., 2003).

3.2. Results

3.2.1. Cloning of PKC Apl III

We cloned the full-length sequence of the atypical PKC from *Aplysia*, using degenerate PCR and RACE, which will now be referred to as PKC Apl III, in order to study its role in synaptic plasticity. Examination of the trace archives of the *Aplysia* genome and available cDNA repositories did not indicate the presence of additional atypical PKCs. Like all atypical PKCs, PKC Apl III has a PB1 domain followed by the pseudosubstrate, an atypical C1 domain, a hinge domain and the kinase domain, and all of these domains are highly conserved (Fig. 3.1.A). Using the genome trace archive we have defined the exons of PKC Apl III, and the exon-intron usage is also highly conserved with vertebrate PKCs with a few exceptions (Fig. 3.1.B). Interestingly, one unique feature of PKC Apl III is the presence of two alternatively spliced exons in the hinge domain (Fig. 3.1.B).

3.2.2. Is there an alternative mRNA encoding PKMz in *Aplysia*?

In vertebrates, there is an alternative transcriptional start site between exons 4 and 5 that generates an mRNA that encodes PKMz. Using 5' RACE, we were unable to detect any mRNAs containing an alternative start site. In vertebrates, there are two forms of atypical PKC, PKCz and PKCi, but only PKCz

has a transcriptional start site between exons 4 and 5. A strong evolutionary constraint for the alternative transcript is the initiating methionine for PKMz. Indeed, the initiating methionine, present at the end of exon 5, is present in all vertebrate orthologues of PKCz, even in primitive fishes such as *Tetraodon*, but is not present in any PKCi (Fig. 3.1.C). Moreover, examining the atypical PKCs in the deuterostome lineages that diverged before vertebrates, where only one atypical PKC is present, the methionine is also absent, suggesting this methionine originated after the duplication into distinct PKCz and PKCi isoforms. The methionine is also absent in invertebrate atypical PKCs, including PKC Apl III (Fig. 3.1.C).

Another feature of the presence of the alternative start site is homology in the intron, both due to the transcriptional promoter and the sequence of the 5'UTR. Unlike the long intron present in vertebrate PKCs, the intron in *Aplysia* between exons 4 and 5 is only 440 base pairs long. We looked for homology in this intron from PKC Apl III to vertebrate PKCs and no significant homology was detected. We also examined the intron between exon 4 and exon 5 in the genomic region of the more-closely related mollusk *Lottia.* Again, no homologous region was found in the intron. In contrast, homology was seen between primitive fishes, mice and human in this region ((Hernandez et al., 2003); data not shown).

There has been some discussion of an atypical PKM in *Drosophila* (Drier et al., 2002).The intron/exon boundaries of the critical exons around the

transcriptional start site are conserved in *Drosophila*. Examination of the *Drosophila* database does show evidence for alternative transcriptional start sites in an intron of the *Drosophila* atypical PKC, and these are all in the intron preceding the equivalent of exon 4, not between the equivalent of exons 4 and 5. These transcripts encode proteins with an initiating methionine before the pseudosubstrate sequence, indicating that they may not encode PKMs. It is conceivable that these methionines are skipped and that these transcripts do encode an atypical PKM, but this would be an example of convergent evolution due to a new transcriptional start site and a new initiating methionine, not conservation of an ancient transcript.

While it is difficult to prove the absence of a molecule, based on bioinformatics analysis of a number of genomes, it is unlikely that the formation of a PKM form of the atypical PKC by an alternative start site is conserved in invertebrates. Instead, it appears likely that it arose during the duplication of atypical PKCs in the early vertebrate lineage.

3.2.3. PKC Apl III contains two alternatively spliced inserts in the hinge domain that are enriched in the nervous system

While cloning PKC Apl III we noted that there were two alternative exons not present in all transcripts (Fig. 3.2.A). To determine the abundance of messages that contained inserts we took advantage of restriction sites present in

each exon (Fig. 3.2). We amplified a fragment of PKC Apl III using PCR from either nervous system, gill, or ovotestis. We then cut these fragments with Taq I to identify fragments containing exon 8A, Fok I to identify fragments containing 8B and Bgl II to confirm the identity of the amplified fragment. All fragments cut completely with Bgl II to confirm amplification of PKC Apl III in all tissues. In the gill, there was no detectable cleavage with Fok, and Taq I cut only in the non-spliced region suggesting minimal inclusion of these exons in the gill. In ovotestis, there was no detectable cleavage with Fok I, but about 50% of the amplified fragments cut with Taq I, suggesting partial inclusion of exon 8A. In the nervous system, a high percentage of the fragments cut with both Fok I and Taq I signifying that most fragments contain both exons. This was confirmed by sequencing multiple amplifications demonstrating clones contain 8A alone, 8B alone or both 8A and 8B. The inclusion of exons specifically in the nervous system fragment can also be seen by the larger size of the lower Bgl II fragment in the nervous system digest (Fig. 3.2.B).

To confirm that the protein encoded by these exons was produced, we raised three antibodies to PKC Apl III: one to the Carboxy-terminal (C-terminal), one to the PB1 domain (N-terminal) and one specific to exon 8B (Splice), and compared immunoreactivity between the nervous system and the gill. The antibody to the C-terminal recognized a major band of approximately 70 kD in the nervous system, while the major immunoreactive band in the gill migrated faster

at approximately 67 kD. Similar bands were seen with the N-terminal antibody. The Splice antibody recognized only the higher molecular weight species and moreover, its relative intensity was much higher in the nervous system than the gill. This confirms the enrichment of the isoform with the splice sites in the nervous system. While the PCR results did not detect PKC Apl III with inserts in the gill, the antibody to the splice site did recognize a protein with the splice site. This may be due to better sensitivity of the immuoblots, or nervous system processes innervating the gill containing the larger isoform of PKC Apl III.

3.2.4. Overexpression of PKC Apl III in *Aplysia* sensory neurons induces cleavage of PKC Apl III

We generated a tagged form of PKC Apl III by creating a plasmid encoding monomeric Red Fluorescent Protein (mRFP) fused to the N-terminal of PKC Apl III, mRFP-PKC Apl III. We have previously generated similar fusions for PKC Apl I and PKC Apl II that retain biological activity (Manseau et al., 2001) . We initially expressed this construct in SF9 cells, and this construct was shown to be expressed in the cytoplasm, but was not translocated to membranes by DOG or phorbol esters (Fig. 3.3.A; data not shown). In similar experiments both PKC Apl I and PKC Apl II were translocated (Zhao et al., 2006; Farah et al., 2008). The lack of PKC Apl III translocation was expected as the atypical C1 domain of these PKCs does not bind DAG or phorbol esters (Chen, 1993).

mRFP-PKC Apl III was next overexpressed in cultured *Aplysia* sensory neurons. Unlike SF9 cells, confocal images revealed that mRFP is strongly expressed in the nucleus as well as the cytoplasm in sensory neurons (Fig. 3.3.B) This is a surprising result, as mRFP is not seen in the nucleus when similarly tagged versions of PKC Apl I or PKC Apl II are overexpressed (Zhao et al., 2006), and Fig. 3.3.B). Additionally, under live imaging conditions, mRFP-PKC Apl III still expresses in the nucleus of sensory neurons, signifying that the nuclear expression is not simply due to an effect of cell fixation (data not shown).

We then immunostained cells expressing mRFP-PKC Apl III using the antibody to the C-terminal. Surprisingly, staining with the antibody was not enriched in the nucleus similar to the mRFP, suggesting separation of the N-terminal containing mRFP and the C-terminal recognized by the antibody, presumably by endoproteolytic cleavage (Fig. 3.3.C & 3.3. D). The difference between mRFP staining and antibody staining was not due to recognition of the endogenous protein by the antibody, since at the laser power used for these images, no staining was observed in un-injected cells (Fig. 3.3.D). We were concerned that during cloning, we may have introduced a cleavage site between mRFP and PKC Apl III, and to rule this out, immunostained with the N-terminal antibody. This antibody showed significantly more staining in the nucleus, inconsistent with cleavage in the linker between mRFP and PKC Apl III, but consistent with cleavage somewhere after the PB1 domain (Fig. 3.3.E and 3.3.F).

We next examined endogenous staining of PKC Apl III using higher laser power. The staining resembled mRFP staining being distributed equally between the cytoplasm and the nucleus (Fig. 3.3.D). Thus, it appears that in *Aplysia* sensory neurons but not SF9 cells, PKC Apl III can localize to the nucleus. Moreover, when expressed at high levels, PKC Apl III is cleaved with the N-terminal continuing to localize to both the nucleus and the cytoplasm, but with the C-terminal restricted to the cytoplasm. This is consistent with the signals for nuclear shuttling being present in the N-terminal region. Thus, after cleavage the C-terminal fragment redistributes into the cytoplasm while the N-terminal fragment continues to shuttle between the cytoplasm and the nucleus (Fig. 3.4).

3.2.5. Cleavage is more efficient with the splice inserts

Upon further examination, we found that the putative cleavage is sensitive to levels of PKC Apl III: diluting the concentration of injected mRFP-PKC Apl III DNA revealed that the lower expressing cells show no differences between mRFP staining and staining with an antibody to the C-terminus (Fig. 3.4). Indeed, both are now equally distributed between the cytoplasm and the nucleus. To quantitate cleavage, we used the nuclear/cytoplasmic ratio of immunofluorescence with the C-terminal antibody, reasoning that after cleavage the cytoplasmic region no longer localizes to the nucleus (Fig. 3.4). At low levels of expression the staining with the antibody and mRFP were similar leading to a

ratio near to 1. At high levels of expression, immunostaining in the nucleus greatly decreased leading to a ratio close to 0.3 (Fig. 3.4). There is a fairly steep relationship between the level of overexpression and cleavage: no cleavage is observed at low levels of expression, and almost complete cleavage is seen as mRFP levels increase over a 2- 4 fold levels of expression (Fig. 3.4).

The splice inserts are in the hinge domain and may affect cleavage. Thus, we generated an mRFP-PKC Apl III lacking the splice inserts (No-Splice) and compared the amount of cleavage by comparing the nuclear/cytoplasmic ratio of the two constructs (Fig. 3.5.A, 3.5.B). Splicing does not affect expression of mRFP in the nucleus and is not required for cleavage as at high levels of mRFP-PKC Apl III expression: there is no difference in the nuclear/cytoplasmic ratio (Fig. 3.5.C). Similarly, at low levels of expression neither mRFP-PKC Apl III is cleaved. However, at intermediate levels of expression there is a significant difference in the nuclear/cytoplasmic ratio of mRFP-PKC Apl III and mRFP-PKC Apl III (No-Splice), suggesting that the splice sites increase the rate or efficiency of cleavage (Fig 3.5.B; values of individual cells in 3.5.A; overall data in 3.5.C). Importantly, in this intermediate stage, the levels of expression of the two constructs were not different (Fig. 3.5.D).

3.2.6. Cleavage is sensitive to inhibitors of calpain

Cleavage of PKC to a PKM is often mediated by calpain (Pontremoli et al., 1990; Sessoms, 1992).To determine if the cleavage induced by overexpression was also mediated by calpain, sensory neurons were injected with a high concentration (0.3 µg/µl) of the mRFP-PKC Apl III. One hour post injection, cells were treated with either Calpain Inhibitor V (100 µM), a cell-permeable, irreversible, non-specific inhibitor of calpains, or a vehicle solution. The cells were left to express over-night in their respective solutions, and were then fixed the next day for immunocytochemistry. As indicated in Figure 3.6, the calpain inhibitor was able to significantly block the overexpression induced cleavage of mRFP-PKC Apl III. This can be seen in the significantly higher green nuclear/cytoplasmic ratio when compared to the vehicle condition. Similar results were also seen using calpeptin, where the inhibitor was replenished in applications every hour as the drug is reversible (data not shown). In contrast, no inhibition of cleavage was seen with caspases inhibitors (data not shown). While levels of calcium are relatively low in resting sensory neurons, calpain activity has been detected in *Aplysia* neurons without stimulation in previous studies (Gitler and Spira, 1998; Khoutorsky and Spira, 2008).

3.2.7. PKC Apl III splice inserts provide a site for cleavage by calpain *in vitro*

To determine directly if PKC Apl III cleavage by calpain was regulated by the splice inserts, we purified PKC Apl III with the splice inserts (Wild-Type) and

PKC Apl III without the splice inserts (No-Splice) from SF9 cells infected with baculovirus encoding these isoforms, and subsequently performed *in vitro* cleavage reactions with purified calpain. While the PKC Apl III No-Splice is still cleaved by calpain, the splice inserts provide for more efficient cleavage (Fig. 3.7). This is consistent with the results in intact cells where the isoform without the splice sites is cleaved, but less efficiently. Notably, the pattern of cleavage seen by Coomassie staining indicated that the major cleavage site was different in the kinase with the splice inserts: the major band seen after cleaving PKC Apl III Wild-Type (arrow) migrated slightly below the 50 kD marker, at a lower molecular weight than the major band seen after cleavage of PKC Apl III No-Splice (squiggly arrow). There is also a minor band seen after cleaving PKC Apl III Wild-Type that migrated above the band seen after cleavage of PKC Apl III No-Splice, consistent with a fragment cut at the same site as PKC Apl III No-Splice but containing the splice inserts (arrowhead). All these fragments are immunoreactive with the C-terminal antibody and phospho-specific antibody (Fig. 3.7). Additionally, N-terminal fragments were not observed (N-terminal antibody; data not shown), presumably they were unstable under these conditions. The size of the major C-terminal fragment seen after cleavage of PKC Apl III Wild-Type is consistent with cleavage at or near the splice inserts (see schematic in Fig. 3.7). Supporting this idea, these fragments were not observed using the splice-specific PKC Apl III antibody; instead only the minor fragment, probably

cleaved at the same site as observed in the PKC Apl III No-Splice case was immunoreactive (arrowhead). The purified PKC Apl III No-Splice also does not react with the splice-specific PKC Apl III antibody, further confirming the specificity of the antibody itself.

3.2.8. Activity dependent cleavage of PKC Apl III can be induced by increasing intracellular levels of calcium

As cleavage by calpain is usually calcium-dependent, we examined whether cleavage could be induced in cells having a low amount of mRFP-PKC Apl III expression using the calcium ionophore, ionomycin. Initial experiments examining the nuclear/cytoplasmic ratio immediately after ionomycin revealed no effect (data not shown). However, if cleavage occurred in the cytoplasm, a change in the redistribution of mRFP-PKC Apl III would not be observed immediately; there would be a lag time until the remaining uncleaved mRFP-PKC Apl III redistributed into the cytoplasm, while the now cleaved cytoplasmic form of mRFP-PKC Apl III would not redistribute. Indeed, two hours after ionomycin treatment a small but highly significant decrease in the nuclear/cytoplasmic ratio was observed (Fig. 3.8). Interestingly, the ratio was not changed in the lowest expressing cells, suggesting that cleavage required both calcium influx and a moderate level of mRFP-PKC Apl III expression (Fig. 3.8).

3.2.9. PKC Apl III is phosphorylated downstream of 5-HT

Atypical PKCs are regulated by phosphorylation in the catalytic domain. In particular, phosphorylation of a site in the activation loop of atypical PKCs by a phosphoinositide-3 kinase (PI3K) -dependent PDK mechanism is known to control their activation (Chou et al., 1998). In *Aplysia*, there is indirect evidence that 5-HT activates PI3K, as a number of 5-HT-mediated processes including activation of target of rapamycin (TOR), synthesis of sensorin, and induction of morphological changes are blocked by inhibitors of PI3K (Khan et al., 2001; Udo et al., 2005; Hu et al., 2006).

To investigate regulation of PKC Apl III by PI3K and PDK, we raised a phospho-specific antibody to PKC Apl III at the PDK site. This antibody recognized expressed PKC Apl III, but not a GST-PKM Apl III fragment that should not be phosphorylated as it was isolated from bacteria (Fig. 3.9.A). While this antibody recognized multiple bands on immunoblots, it could be used to recognize the expressed PKC Apl III since, similar to the other antibodies, a laser power was used to detect the overexpressed protein where no immunoreactivity was detected in non-expressing cells. When mRFP-PKC Apl III was expressed at low levels in *Aplysia* sensory neurons, 5-HT increased phosphorylation at the PDK site (Fig. 3.9.B, 3.9.C). However, at higher levels of expression when mRFP-PKC Apl III was cleaved, there was no effect of 5-HT on the phosphorylation at the PDK site (Fig. 3.9.C). This result is in agreement with a

study on mammalian PKMz, where PDK phosphorylation of PKMz was constitutive due to the increased access of PDK to the phosphorylation site in the absence of the regulatory domain (Kelly et al., 2007). 5-HT phosphorylation of mRFP-PKC Apl III was also shown to be downstream of the PI3K-PDK pathway, as it was blocked by LY 294002, an inhibitor of PI3K (Fig. 3.9.D).

3.3. Discussion

3.3.1. Conservation of the role of PKMz in *Aplysia*

We cloned the full-length sequence of the atypical PKC from *Aplysia*, PKC Apl III, using degenerate PCR and RACE in order to study its role in synaptic plasticity. In vertebrates, there is an alternative transcriptional start site that generates an mRNA that encodes PKMz. However, we were unable to detect any mRNAs containing an alternative start site. It is unlikely that the formation of a PKM form of the atypical PKC by an alternative start site is conserved in invertebrates. Instead, it appears likely that it arose during the duplication of atypical PKCs in the early vertebrate lineage. Therefore, our results suggest that PKM forms of PKCs play a conserved role in memory formation and maintenance, but the mechanism of formation of these kinases has changed over evolution.

3.3.2. Overexpression of PKC Apl III in *Aplysia* sensory neurons induces cleavage of PKC Apl III

Using an overexpression paradigm, we have been able to detect cleavage of the atypical PKC Apl III into a PKM Apl III; however to be physiologically important, cleavage of the endogenous PKC Apl III is required. Cleavage is highly dependent on the level of expression, and this may explain the inability to detect the endogenous PKM form of PKC Apl III using immunoblots (data not shown). While increased cleavage is observed after increasing intracellular calcium in *Aplysia* sensory neurons with ionomycin, this still required a certain level of overexpression. Thus, if endogenous PKC Apl III is to be cleaved, it would have to be produced at high levels during the activation of calpain. Interestingly, this situation may occur in the motor neuron, where a PKC-dependent increase in the sensitivity to glutamate depends on both rapid protein synthesis and calcium (Chitwood et al., 2001; Khan et al., 2001; Udo et al., 2005; Hu et al., 2006; Villareal et al., 2007). Unfortunately, the nuclear/cytoplasmic assay to detect cleavage is slow and insensitive due to the requirement for nuclear shuttling. A more sensitive method of detection will be required to detect the local cleavage that is likely to by physiologically important.

Figure 3.1.

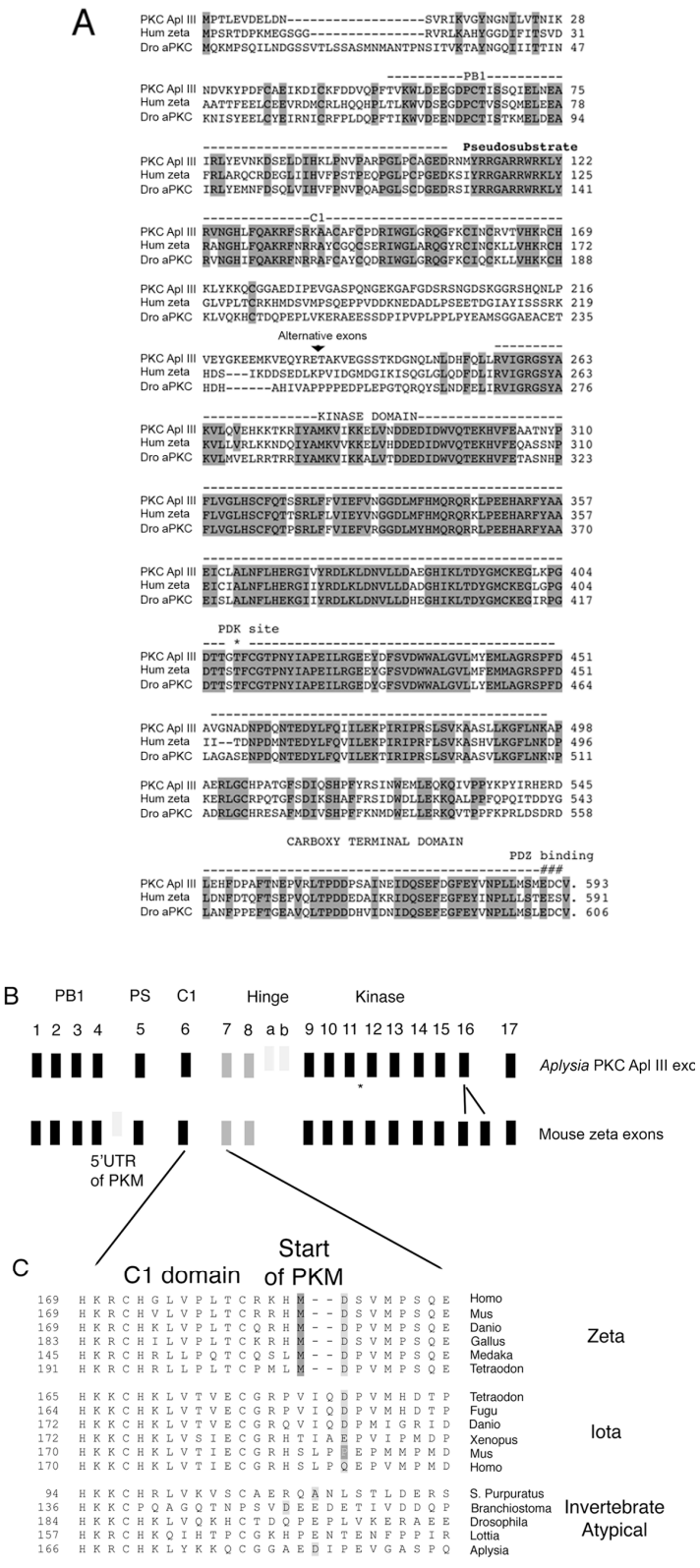

A
PKC Apl III
Hum zeta
Dro aPKC
Pseudosubstrate
C1
Alternative exons
KINASE DOMAIN
PDK site
CARBOXY TERMINAL DOMAIN
PDZ binding
B
PB1
PS
C1
Hinge
Kinase
1 2 3 4 5 6 7 8 a b 9 10 11 12 13 14 15 16 17
Aplysia PKC Apl III exc
Mouse zeta exons
5'UTR of PKM
C
C1 domain
Start of PKM
Homo
Mus
Danio
Gallus
Medaka
Tetraodon
Zeta
Tetraodon
Fugu
Danio
Xenopus
Mus
Homo
Iota
S. Purpuratus
Branchiostoma
Drosophila
Lottia
Aplysia
Invertebrate Atypical

Figure 3.2.

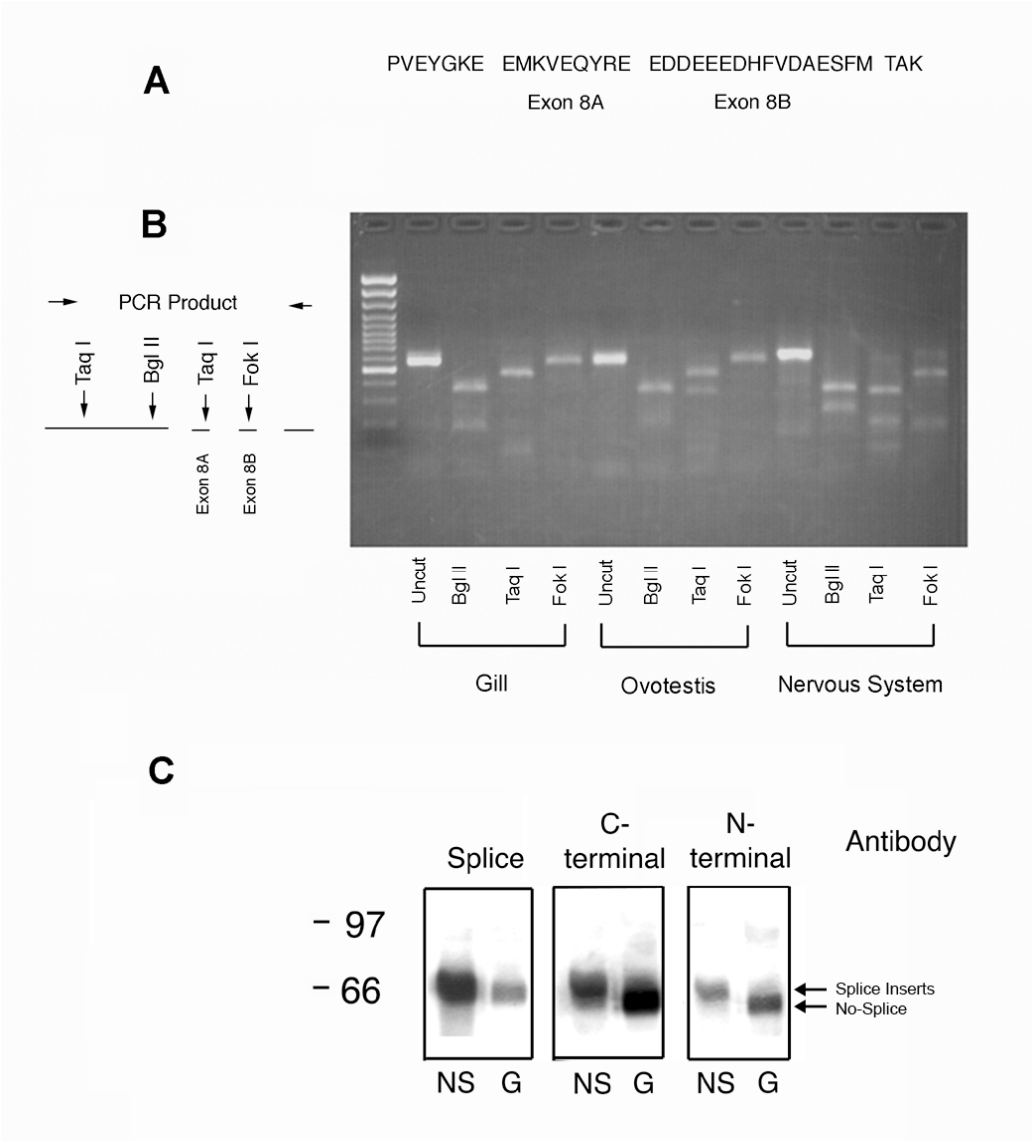

A
PVEYGKE EMKVEQYRE EDDEEEDHFVDAESFM TAK
Exon 8A
Exon 8B
B
PCR Product
Taq I
Bgl II
Taq I
Fok I
Exon 8A
Exon 8B
Uncut
Bgl II
Taq I
Fok I
Uncut
Bgl II
Taq I
Fok I
Uncut
Bgl II
Taq I
Fok I
Gill
Ovotestis
Nervous System
C
Splice
C-terminal
N-terminal
Antibody
97
66
Splice Inserts
No-Splice
NS G
NS G
NS G

Figure 3.3.

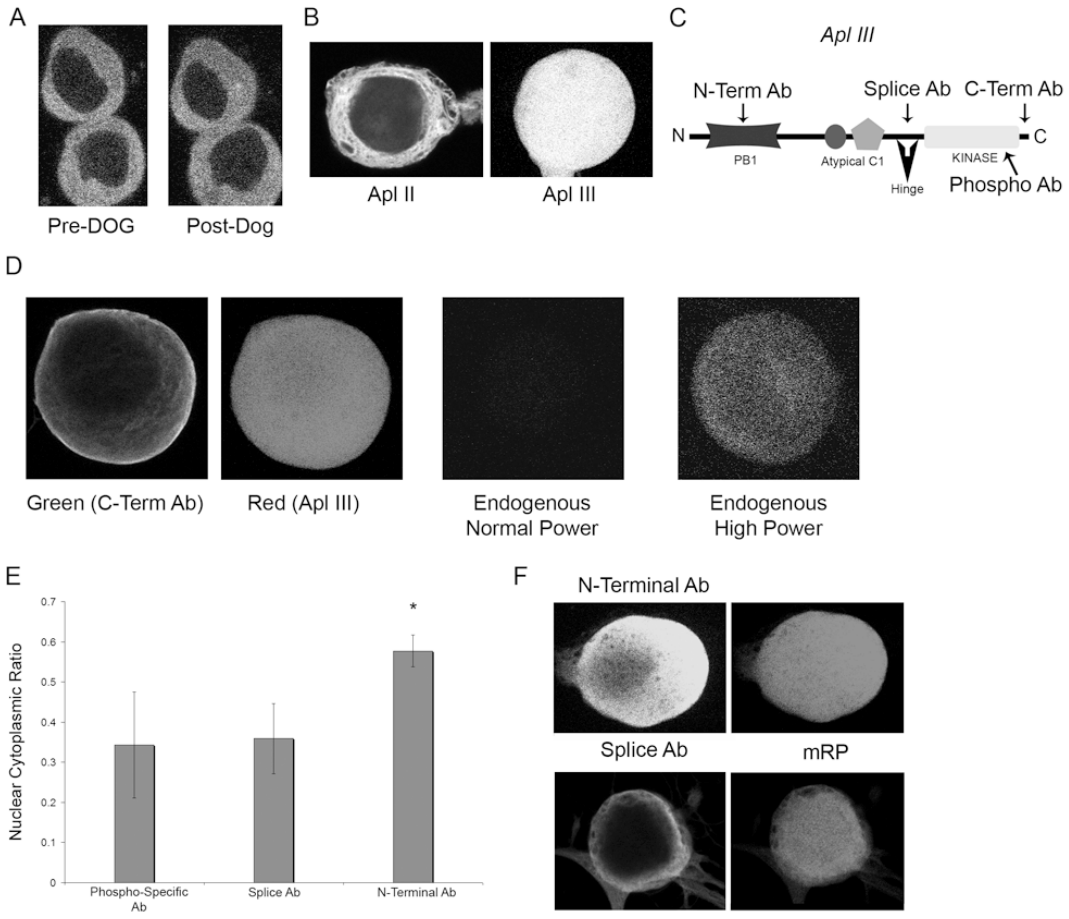

A
Pre-DOG
Post-Dog
B
Apl II
Apl III
C
Apl III
N-Term Ab
Splice Ab
C-Term Ab
N
C
PB1
Atypical C1
Hinge
KINASE
Phospho Ab
D
Green (C-Term Ab)
Red (Apl III)
Endogenous
Normal Power
Endogenous
High Power
E
Nuclear Cytoplasmic Ratio
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Phospho-Specific Ab
Splice Ab
N-Terminal Ab
F
N-Terminal Ab
Splice Ab
mRP

Figure 3.4.

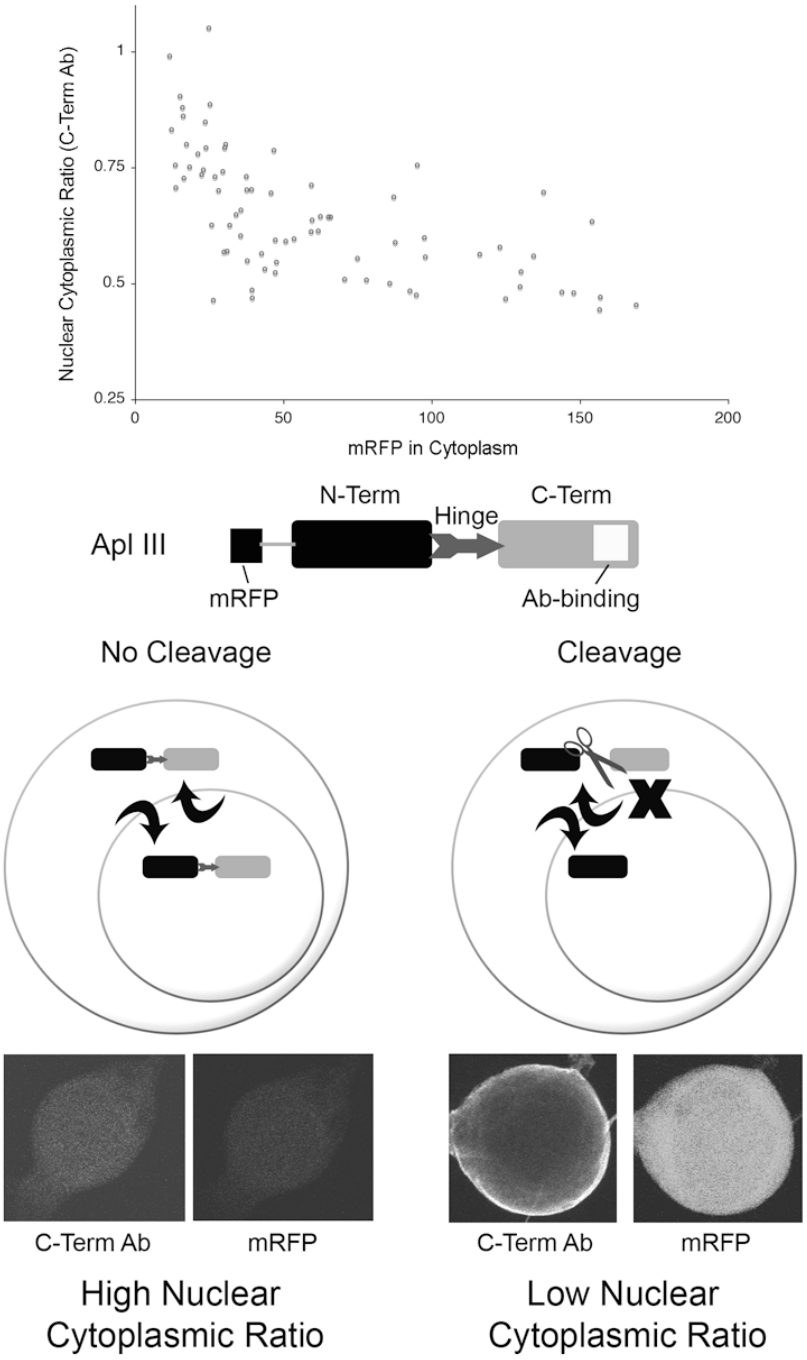

Nuclear Cytoplasmic Ratio (C-Term Ab)
1
0.75
0.5
0.25
0
50
100
150
200
mRFP in Cytoplasm
Apl III
N-Term
Hinge
C-Term
mRFP
Ab-binding
No Cleavage
Cleavage
C-Term Ab
mRFP
C-Term Ab
mRFP
High Nuclear Cytoplasmic Ratio
Low Nuclear Cytoplasmic Ratio

Figure 3.5.

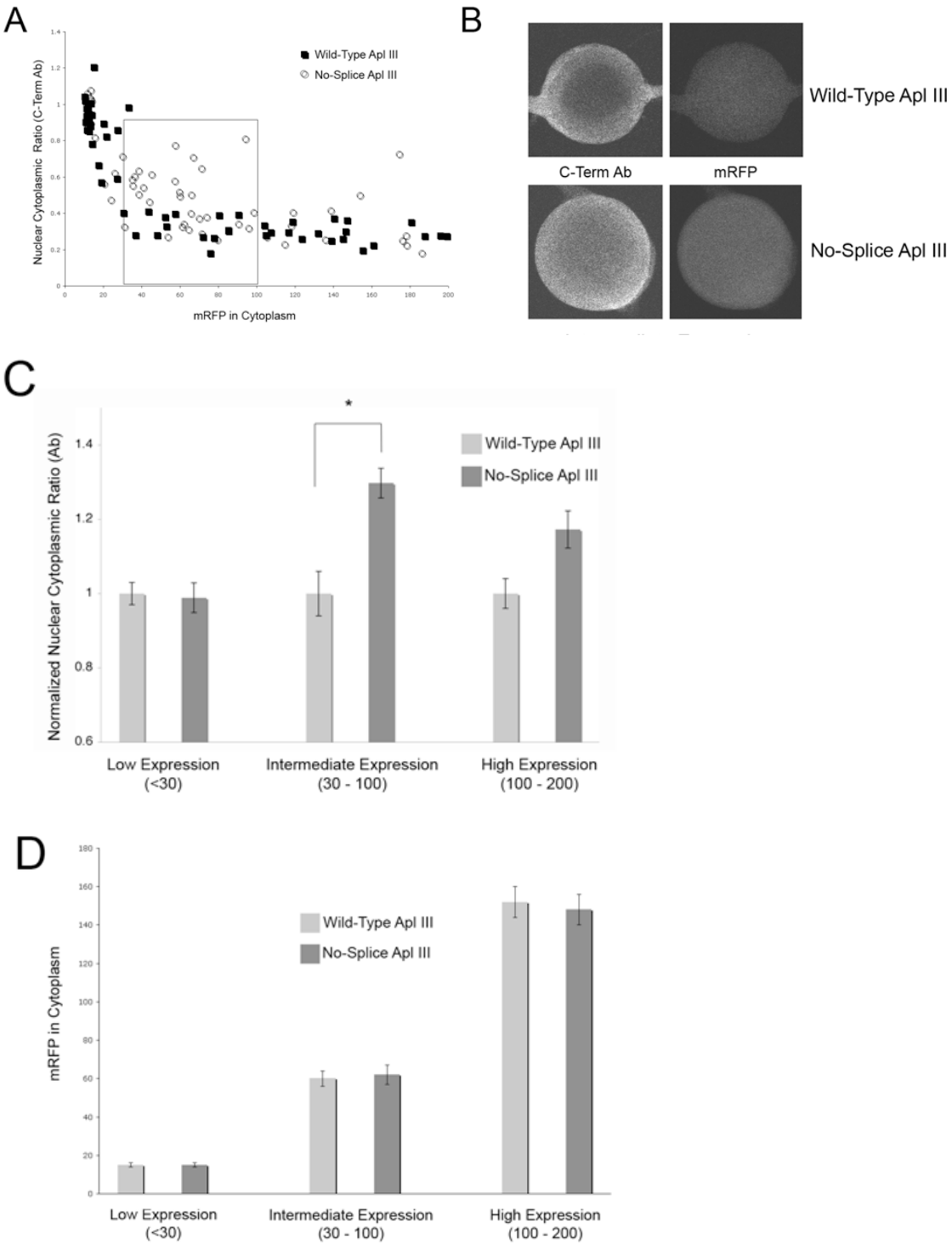

A
Wild-Type Apl III
No-Splice Apl III
Nuclear Cytoplasmic Ratio (C-Term Ab)
mRFP in Cytoplasm
B
Wild-Type Apl III
C-Term Ab
mRFP
No-Splice Apl III
C
*
Wild-Type Apl III
No-Splice Apl III
Normalized Nuclear Cytoplasmic Ratio (Ab)
Low Expression (<30)
Intermediate Expression (30 - 100)
High Expression (100 - 200)
D
Wild-Type Apl III
No-Splice Apl III
mRFP in Cytoplasm
Low Expression (<30)
Intermediate Expression (30 - 100)
High Expression (100 - 200)

Figure 3.6.

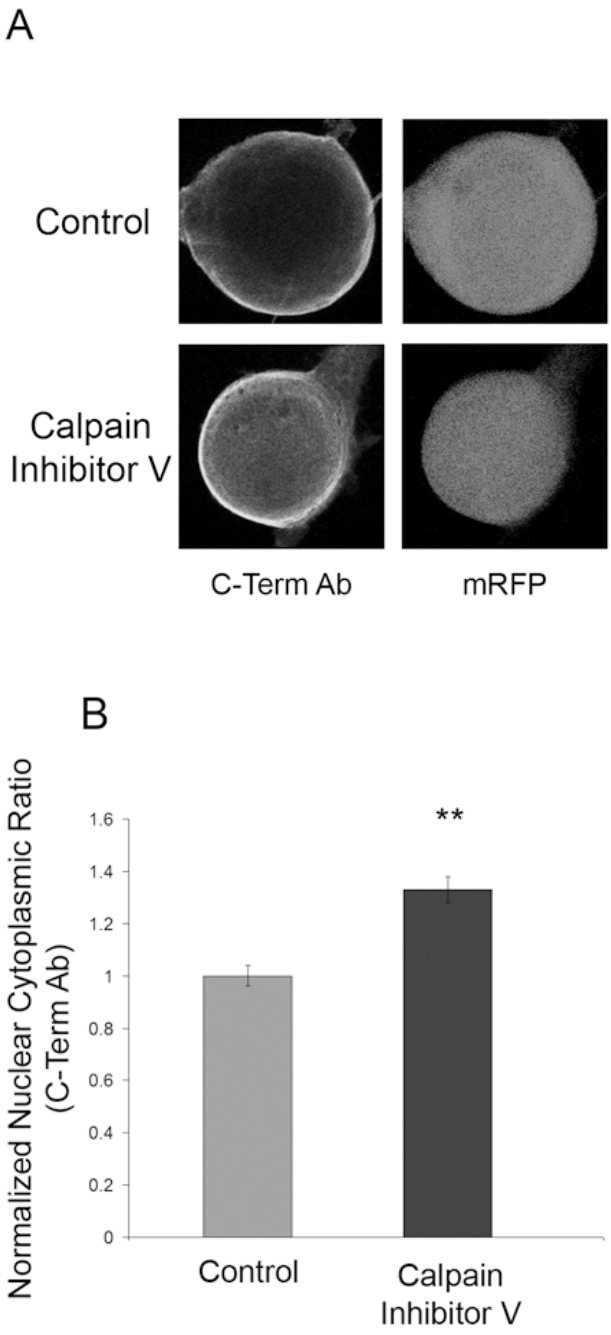
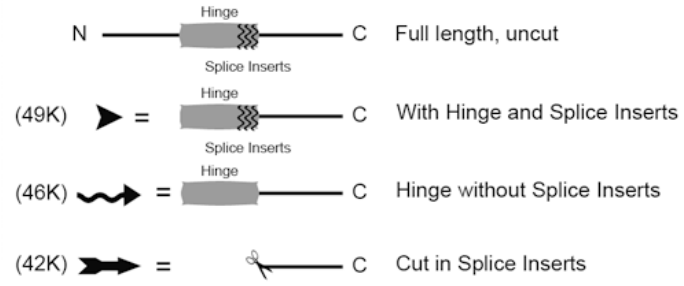
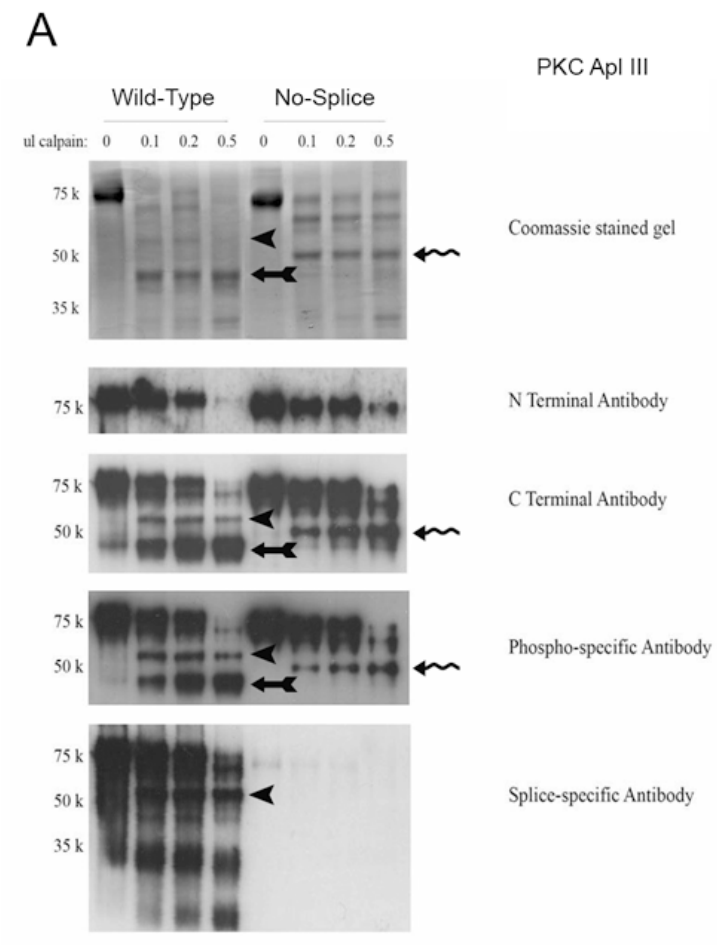

A
Control
Calpain Inhibitor V
C-Term Ab
mRFP
B
Normalized Nuclear Cytoplasmic Ratio (C-Term Ab)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
Control
Calpain Inhibitor V

Figure 3.7.


Hinge
N
C
Full length, uncut
Splice Inserts
(49K)
=
With Hinge and Splice Inserts
(46K)
Hinge without Splice Inserts
(42K)
Cut in Splice Inserts


A
PKC Apl III
Wild-Type
No-Splice
ul calpain: 0 0.1 0.2 0.5 0 0.1 0.2 0.5
75 k
50 k
35 k
Coomassie stained gel
N Terminal Antibody
C Terminal Antibody
Phospho-specific Antibody
Splice-specific Antibody

B

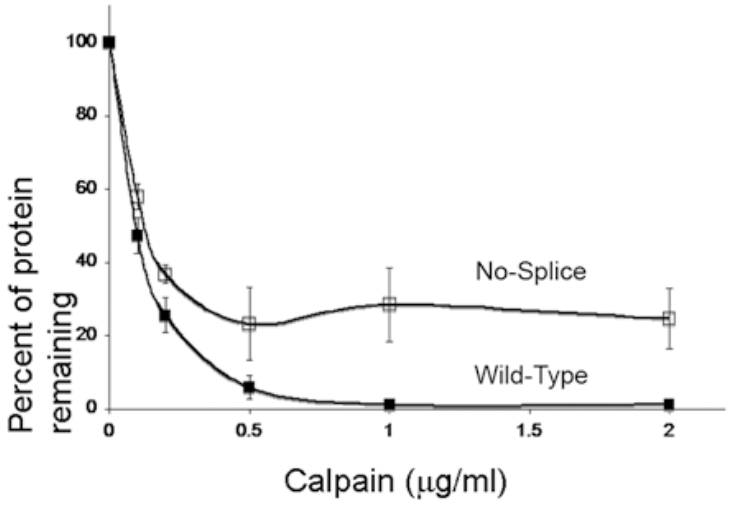

100
80
60
40
20
0
Percent of protein remaining
No-Splice
Wild-Type
0
0.5
1
1.5
2
Calpain (μg/ml)

Figure 3.8.

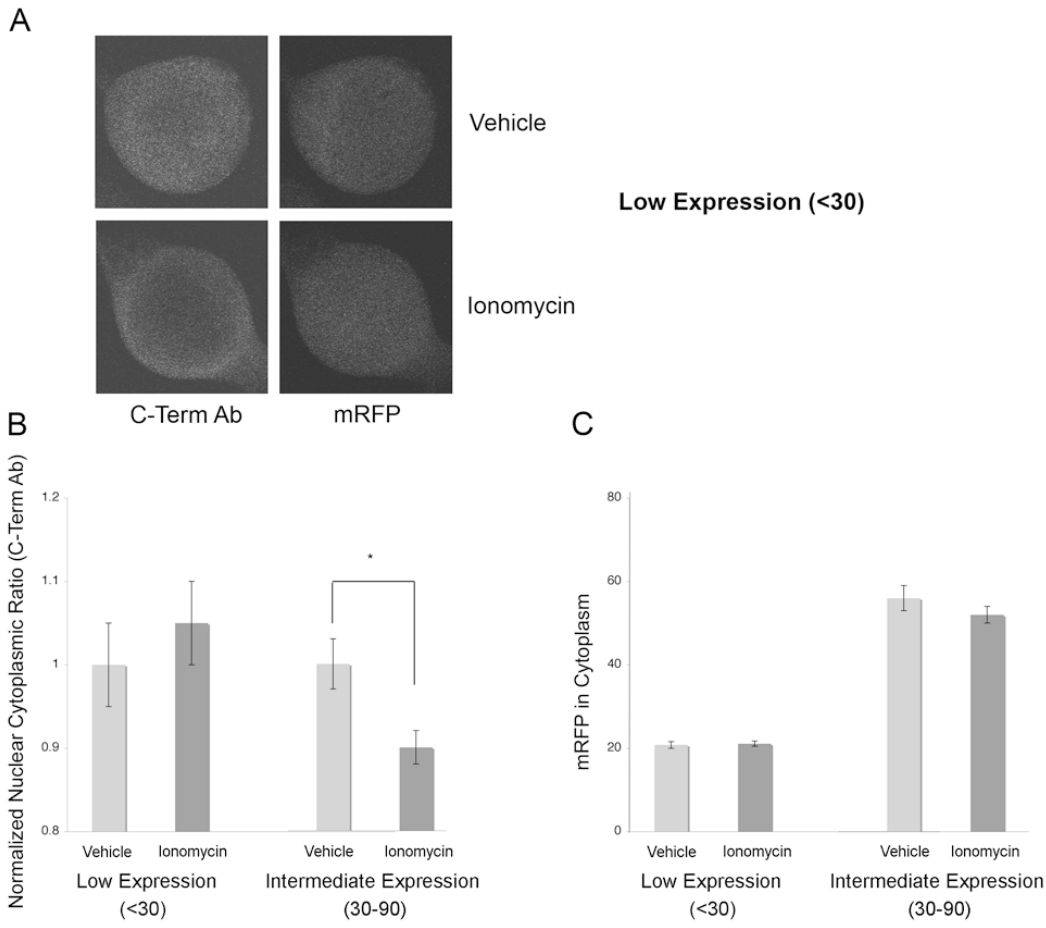

A
Vehicle
Ionomycin
Low Expression (<30)
C-Term Ab
mRFP
B
Normalized Nuclear Cytoplasmic Ratio (C-Term Ab)
1.2
1.1
1
0.9
0.8
*
Vehicle
Ionomycin
Vehicle
Ionomycin
Low Expression (<30)
Intermediate Expression (30-90)
C
mRFP in Cytoplasm
80
60
40
20
0
Vehicle
Ionomycin
Vehicle
Ionomycin
Low Expression (<30)
Intermediate Expression (30-90)

Figure 3.9.

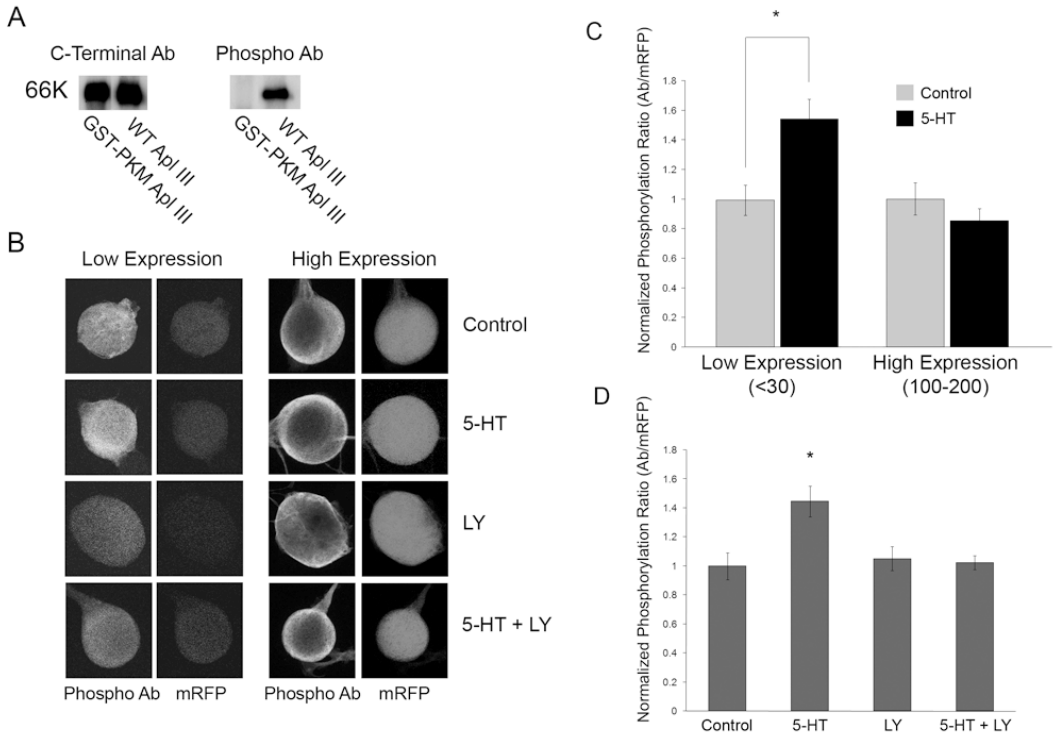

A
C-Terminal Ab
Phospho Ab
66K
WT Apl III
GST-PKM Apl III
WT Apl III
GST-PKM Apl III
B
Low Expression
High Expression
Control
5-HT
LY
5-HT + LY
Phospho Ab
mRFP
Phospho Ab
mRFP
C
Normalized Phosphorylation Ratio (Ab/mRFP)
Control
5-HT
Low Expression (<30)
High Expression (100-200)
D
Normalized Phosphorylation Ratio (Ab/mRFP)
Control
5-HT
LY
5-HT + LY

Figure Legends

Figure 3.1. Cloning of PKC Apl III and evolution of the alternative transcript encoding PKMz. **A:** Sequence alignment of Human PKC zeta (Hum zeta), *Aplysia* PKC Apl III (PKC Apl III) and Drosophila atypical PKC (Dro aPKC). Amino acids conserved in all three are shaded. The domains are indicated by dotted lines over the regions. The phosphorylation sites are starred (*), as is a putative PDZ binding domain (###) at the C-terminal. The arrow indicates where alternative exons are inserted in *Aplysia* PKC Apl III. **B:** Exon-intron structure of *Aplysia* PKC Apl III and Mouse zeta are shown. Black exons have conserved boundaries. The hinge exons 7 and 8 do not have conserved start and stop regions. In Mice, exon 16 is split into two exons. The positions of the alternative exons 8a and 8b in *Aplysia* and the position of the alternative transcriptional start site for PKMz are indicated. **C:** Alignment of the end of exon 6 and beginning of exon 7 in vertebrate and invertebrate sequences. All sequences from Genbank except for Lottia and Brachiostoma sequences that are from the Joint Genome Institute genome site (http://genome.jgi-psf.org). The methionine present in all vertebrate PKCz is the last amino acid in exon 6 and occurs immediately following the end of the C1 domain. This methionine is not present in any PKCi or invertebrate PKCs including primitive deuterostomes such as Branchiostoma that have a single atypical form.

Figure 3.2. Splice inserts of PKC Apl III are enriched in the nervous system. **A:** Exon sequence of the alternative splice inserts (exon 8a and exon 8b) in the hinge domain of PKC Apl III. **B:** Image of an agarose gel showing the results of digesting an amplified fragment of PKC Apl III cut with the restriction enzymes indicated. The product was amplified from cDNA isolated from Gill, Ovotestis or the Nervous System. A schematic of the placement of the restriction enzymes is shown on the left and the100 bp marker is run in the left lane. **C:** 20 µg of total protein extracted from either the Nervous System (NS) or Gill (G) were separated on SDS–PAGE gels, transferred to nitrocellulose and immunoblotted with the antibodies indicated.

Figure 3.3. PKC Apl III is localized in the nucleus and cleaved after overexpression in sensory neurons. **A:** SF9 cells were transfected with mRFP-PKC Apl III and live images are shown of the red signal either before or after treatment with 50 µg/mL of dioctanoylglycerol. **B:** Plasmids encoding either mRFP-PKC Apl II or mRFP-PKC Apl III were injected into sensory neurons, and the neurons were fixed one day later and imaged for mRFP. **C:** Schematic of mRFP-Apl III with sites of the peptides used for antibody generation. **D:** Plasmid encoding mRFP-PKC Apl III was injected into sensory neurons and neurons were fixed 1 day later and immunostained with the antibody to the C-terminal of PKC Apl III and simultaneously imaged for mRFP staining. The signal at the same

laser power is shown for a non-injected neuron from the same preparation, as well as another image of the same neuron after increasing the laser power to image endogenous expression. **E:** Quantification of sensory neurons expressing PKC Apl III immunostained with either the phospho-specific antibody (n = 2 experiments, 15 cells), the splice antibody (n = 4 experiments 17 cells), or the N-terminal antibody (n = 3 experiments, 24 cells). ANOVA revealed a significant difference between groups [$F(56,2) = 7.2$, $p < 0.01$] and Tukey's post-hoc test showed that the N-terminal antibody group was significantly different from both other groups (*, $p < 0.05$). **F:** A representative example of neurons expressing mRFP-PKC Apl III immunostained with either the N-terminal antibody or the anti-splice antibody. mRFP fluorescence is shown on the right.

Figure 3.4. Model of the cleavage of PKC Apl III. **Top:** The correlation between the nuclear/cytoplasmic ratio (Y axis) and the level of expression (mRFP fluorescence in cytoplasm on X axis) is shown for a representative experiment. A similar pattern has been observed in over six experiments. PKC Apl III construct with mRFP tag at the N-terminal and antibody site on C-Terminal, with hinge region linking the two. **Bottom:** At low expression levels, PKC Apl III is not cleaved and N-terminal (red) and C-terminal (green) remain linked shuttling together through the nucleus and cytoplasm. Sensory cells display a uniform pattern of red and green throughout the cell, resulting in a high

nuclear/cytoplasmic ratio. At high expression levels, cleavage of PKC Apl III takes place in the hinge domain, dissociating the N-terminal (red) from the C-Terminal (green). The N-terminal continues to shuttle in and out of the nucleus, while the C-terminal remains restricted to the cytoplasm. Sensory cells display a red throughout the cell while the green is limited to the cytoplasm, resulting in a low nuclear/cytoplasmic ratio.

Figure 3.5. PKC Apl III cleavage is regulated by splicing. **A:** The correlation between the nuclear/cytoplasmc ratio (Y axis) and the level of expression (mRFP fluorescence in cytoplasm on X axis) is shown for individual sensory neurons expressing mRFP-PKC Apl III Wild-Type (filled squares) or mRFP-PKC Apl III (No-Splice) (open circles). The region used to quantify differences in (c) is boxed. **B:** A representative example of sensory neurons expressing Wild-Type mRFP-PKC Apl III (above) or No-Splice mRFP-PKC Apl III (below) immunostained with the antibody to the C-terminal (left) and imaged for mRFP fluorescence (right). **C:** The average of the nuclear/cytoplasmic ratio stratified by the level of mRFP expression. Results are from three experiments: low expression, n = 21 (Wild-Type), 17 (No-Splice); intermediate expression, n = 15 (Wild-Type), 29 (No-Splice); and high expression, n = 12 (Wild-Type), 18 (No-Splice). *$p < 0.03$ two-tailed Student's t-test. **D:** Levels of mRFP expression in the three groups show no

differences between mRFP-PKC Apl III Wild-Type and mRFP-PKC Apl III No-Splice that could explain the difference in the ratio.

Figure 3.6. Cleavage is blocked by a calpain inhibitor. **A:** Sensory neurons expressing mRFP-PKC Apl III were treated one hour after plasmid injection with vehicle solution or Calpain Inhibitor V (100 μM), and then fixed one day later and immunostained with the carboxy-terminal antibody (left) or imaged for mRFP fluorescence (right). **B:** Quantified results of the normalized nuclear/cytoplasmic ratio from three experiments (control, n = 43 cells, Calpain Inhibitor, n = 74 cells), **$p < 0.001$ two tailed Student's t-test.

Figure 3.7. Calpain cleaves at the splice inserts *in vitro*. **A:** PKC Apl III, with (Wild-Type) and without (No-Splice) the splice inserts, was purified from baculovirus and incubated with concentrations of purified calpain indicated. A representative experiment with 90% of the protein separated on one SDS–PAGE gel and stained with Coomassie, and 10% transferred to nitrocellulose and sequentially stained with the antibodies shown on the right, with stripping between each antibody incubation. Molecular weight markers are indicated on the left. Three bands are labelled with arrows (see schematic for description). Schematic of predicted bands based on molecular weight of PKC Apl III. **B:**

Quantification of cleavage for PKC Apl III Wild-Type or PKC Apl III No-Splice with each point from a total of 5–10 experiments.

Figure 3.8. Ionomycin induced cleavage of PKC Apl III. **A:** Sensory neurons over-expressing PKC Apl III were treated with a calcium ionophone, ionomycin (1 μM for 10 min), or a vehicle solution, fixed two hours later and immunostained with the antibody to the carboxy-terminal (left) or imaged for mRFP (right). **B:** Quantification of the nuclear/ cytoplasmic ratio of control and ionomycin treated cells from three independent experiments (Cells > 30 and < 100 mRFP, Control, n = 63, ionomycin, n = 71; Cells < 30, Control, n = 33, ionomycin, n = 54) * $p < 0.01$ Student's t-test between control and ionomycin.

Figure 3.9. PKC Apl III phosphorylation at the PDK site is increased after 5-HT treatment. **A:** 10 ng of purified GST-catalytic domain and 50 ng of SF9 cell extract from cells infected with a baculovirus expressing PKM Apl III were separated on SDS–PAGE acrylamide gels, transferred to nitrocellulose, and immunostained with the antibody to the carboxyterminal (left) or the phospho-specific antibody to the PDK site (right). **B:** Sensory cells over-expressing PKC Apl III were treated with either a control solution, 5-HT (20 μM for 10 min), LY 294002 (10 μM for10 min), or both LY and 5-HT: LY (10 μM) was applied for 15 min prior to the 5-HT (10 min) treatment. Cells were immediately fixed and immunostained with the

phospho-specific antibody and imaged for mRFP. Examples are shown either at low levels of expression or high levels of expression. **C:** Quantification of normalized phosphorylation ratio for low and high expressing neurons: low expression data from three experiments, C, n = 14, 5-HT, n = 22, and high expression from two experiments, C, n = 7, 5-HT, n = 8. *, $p < 0.05$ Students two-tailed paired t-test using non-normalized data. **D:** Quantification of normalized phosphorylation after experimental treatments (C, five experiments, n = 18; 5-HT, four experiments, n = 44; LY three experiments, n = 12; 5-HT + LY three experiments, n = 26). ANOVA $F(96,3) = 8.9$, $p < 0.01$; Tukey's post-hoc test showed that 5-HT was different from all other groups, *, $p < 0.05$ and no other differences were seen.

CHAPTER FOUR

Serotonin-induced cleavage of the atypical Protein Kinase C zeta in *Aplysia* to generate a PKM.

4.1. Introduction

It has been recently demonstrated that, similarly to results in from rodent studies, inhibitors of PKMz reverse consolidated synaptic facilitation and long term memory in *Aplysia*, suggesting a conserved role for this memory trace (Cai et al., 2011). However, in rodents, PKMz is generated by translation of a unique mRNA whose transcription starts in an intron of PKCz (Hernandez et al., 2003). In the previous chapter, we demonstrated that this transcript does not exist in species below chordates (Bougie et al., 2009). Instead, we have postulated that overexpression of PKC Apl III in *Aplysia* neurons leads to constitutive expression of a PKM form of PKC Apl III that is formed by calpain cleavage. Together these data suggested that PKC Apl III is an attractive candidate for mediating synaptic plasticity in *Aplysia,* and that PKM forms of atypical PKCs play a conserved role in memory formation, but the mechanism of formation of these kinases has changed over evolution. However, it has not yet been shown that 5-HT can induce cleavage of PKC Apl III.

There are two forms of plasticity which potentially involve the formation of a PKM in *Aplysia*: the first involves a form of ITF, which was previously

discussed. This particular form of ITF has been shown to require combined action of 5-HT and firing of the sensory neuron which activated PKC Apl I, the conventional PKC in *Aplysia* (Zhao et al., 2006). Another form of ITM, site-specific ITM, was shown to involve PKC Apl I but did not require protein synthesis or activation of PKA either for its induction or maintenance. Instead, site-specific ITM was shown to depend upon calpain-dependent proteolysis of PKC Apl I, which subsequently produced a persistently active PKM (Sutton et al., 2004). The second paradigm involves PKC Apl III in LTF in isolated motor neurons. It has been found that it is PKM Apl III which mediates the long term maintenance of a 5-HT induced enhancement of synaptic strength in the motor neuron (Villareal et al., 2009).

The assay that was used to investigate cleavage of PKC Apl III and formation of PKM Apl III in *Aplysia* in the previous chapter, namely overexpression and immunocytochemistry, was slow and insensitive and unable to be used in order to examine similar processes for other PKCs such as PKC Apl I which do not show nuclear localization. Accordingly, a new assay was needed to examine formation of PKMs in *Aplysia*: Förster resonance energy transfer (FRET) was chosen. FRET is a tool utilized for examining spatial relationships between molecules, and which relies on the distance-dependent radiationless transfer of energy from a donor molecule to an acceptor molecule. The donor molecule is a chromophore that firstly absorbs energy emitted by

fluorescent light and the acceptor is the chromophore to which the energy is consequently transferred (Xia and Liu, 2001; Held, 2005). For the successful transfer of energy, the two chromophores must be within proximity of each other, anywhere from one to 10 nm and there must be spectral overlap of the donor emission spectrum and the acceptor absorption spectrum (Xia and Liu, 2001; Held, 2005). For the following experiments, we have opted to use Cyan Fluorescent Protein (CFP) as the donor chromophore and Yellow Fluorescent Protein (YFP) as the acceptor chromophore, as this is one of the most commonly used FRET pairs in which the spectral overlap exists. In all the following FRET experiments, we have used a double tagged PKC Apl III with CFP tagged to the N-terminal and YFP to the C-terminal, or a similar PKC Apl I construct (Fig. 4.1).

4.2. Results

4.2.1. Characterization of the FRET reporters

In order to monitor cleavage of PKCs during plasticity, we generated a construct predicted to show basal FRET between a CFP at the N-terminus and a YFP at the Carboxy-terminus: cleavage could then be measured by a loss of FRET. We first began by characterizing the CFP-YFP FRET constructs. To confirm that FRET was indeed being observed, we initially transfected either the CFP-PKC Apl III-YFP FRET construct or co-transfected CFP and YFP together in

SF9 cells. The cells were then imaged for FRET (see Chapter 2 for methods). The FRET maps presented in Figure 4.2.A display color coded images of the measured FRET signal, where warm colors represent higher levels of FRET and cooler colors represent lower levels of FRET (the grey color is unassigned and represents negative or undefined values sometimes seen in the periphery of expressing cells, or when no FRET is observed). It can be seen that the CFP-PKC Apl III-YFP construct has a measurable FRET signal, while there is no FRET that is observed even when both CFP and YFP are present in the same cell, indicating the requirement for close contact and proper conformation of the fluorochromes to observe FRET (Fig 4.2.A).

4.2.2. Calcium induced cleavage of PKC Apl I but not PKC Apl III

In order to determine if PKC Apl I, the conventional isoform in *Aplysia*, can also undergo cleavage in a PKM Apl I, we similarly expressed the CFP-PKC Apl I-YFP FRET reporter in SF9 cells. As this PKC is known to be activated by calcium (Newton, 2001; Sossin, 2007), we treated the SF9 cells expressing CFP-PKC Apl I-YFP with a calcium ionophore, ionomycin (2μM), to determine if increasing the levels of calcium within the cell could induce cleavage of PKC Apl I. It was found that treatment with ionomycin produced a significant decrease in FRET, indicating a calcium-induced cleavage of PKC Apl I into a PKM. Furthermore, when these cells were pre-treated for 10 minutes with a calpain

inhibitor, Calpain inhibitor V (100μM), prior to ionomycin treatment, this decrease in FRET was no longer seen. This result implies that the cleavage of PKC Apl I observed after treatment with ionomycin was in fact a calpain-dependent cleavage process (Fig. 4.3).

Next we wanted to test the similar CFP-PKC Apl III -YFP construct: SF9 cells expressing this PKC Apl III construct were treated with ionomycin in the same manner as above. Surprisingly, no significant loss in the FRET ratio for CFP-PKC Apl III-YFP was found following treatment with ionomycin. Accordingly, these results suggest that it is possible that PKC Apl III is simply less sensitive to calcium-induced cleavage than PKC Apl I (Fig 4.3).

4.2.3. Expressing the FRET reporters in *Aplysia* neurons

We have previously shown that overexpression of mRFP-PKC Apl III in neurons led to cleavage and differential localization of the N-terminal (nucleus and cytoplasm) and C-terminal (cytoplasm) of the protein (Bougie et al., 2009). This difference reflects a nuclear localization site in the N-terminal that leads to nuclear-cytoplasmic cycling of the N-terminus, and restriction of the C-terminal to the cytoplasm following cleavage due to the loss of the nuclear localization site (see Chapter 3). However, when the CFP-PKC Apl III-YFP was expressed in *Aplysia* neurons, this construct did not show the previously established pattern of fluorescent dissociation, our marker of cleavage. In the case of the CFP-PKC Apl

III-YFP, both the fluorescence from CFP and the fluorescence from YFP showed nuclear as well as cytoplasmic staining at all levels of expression (Fig. 4.2.B). One possible explanation was that the addition of the fluorescent reporter proteins blocked the cleavage of PKC Apl III by calpain.

To test this, we generated a baculovirus expressing the CFP-PKC Apl III-YFP with a His-tag at the N-terminus and purified the protein from baculovirus-infected SF9 cells. We compared the cleavage of this construct by mammalian calpains and found that the CFP-PKC Apl III-YFP was actually cleaved more efficiently than the WT PKC Apl III (Fig. 4.2.C), suggesting that a blockade of cleavage sites was not an explanation for the different distribution seen in neurons. We then assayed the purified kinases for kinase activity and found that the CFP-PKC Apl III-YFP had less specific activity than WT PKC Apl III (0.1 +/- 0.01, normalized to WT, n=3). This suggests that the addition of the YFP at the C-terminal of the protein lowered the specific activity of the kinase. It is known that PKC Apl III is activated by phosphorylation at the PDK site (Chou et al., 1998). To directly compare the impact of adding the YFP to the Carboxy-terminal side on PDK phosphorylation, we compared phosphorylation of an mRFP-PKC Apl III to the CFP-PKC Apl III-YFP. Consistent with a decrease in activity after addition of the YFP tag, there was much less staining of the CFP-PKC Apl III-YFP with the phospho-specific antibody (Fig. 4.2.B). To rule out that this difference was due to differences in expression, we compared staining with the

phospho-specific antibody to a CFP-PKC Apl III and even at comparable expression levels (visualized by CFP staining), there was much less staining with the phospho-specific antibody (Fig. 4.2.B). If the lack of cleavage of CFP-PKC Apl III-YFP was due to its decreased activity, it suggests that overexpression dependent cleavage of PKC Apl III might be due to kinase activation of a proteolytic event.

4.2.4. Kinase activity is required for overexpression induced cleavage of PKC Apl III

To test the hypothesis that kinase activity is required for cleavage of PKC Apl III, we generated a kinase-dead PKC in a distinct manner. A previously established problem that arises with kinase dead PKCs is that, due to a lack of phosphorylation at key sites, the kinases are not folded correctly. It has been shown that mutation of a conserved aspartate (D392 in PKC Apl III) to alanine leads to a kinase dead PKC that still maintains integrity of the ATP binding pocket but makes no side interactions with ATP. Thus, these mutants are kinase-dead but retain stability of the active conformer, and are still able to be phosphorylated and constitutively primed (Cameron et al., 2009). We made this mutation in the mRFP-PKC Apl III (mRFP-PKC Apl III D392A) and expressed it in neurons. Indeed, unlike the CFP-PKC Apl III-YFP, and a previous mutant we had generated (mRFP-PKC Apl III K297R), the mRFP-PKC Apl III D392A was highly

phosphorylated at the PDK site (Fig. 4.4.A). Despite this, the kinase activity of the purified mRFP-PKC Apl III D392A was extremely low (0.03 +/-0.03, normalized to WT, n=2).

Upon overexpression of this construct in *Aplysia* sensory neurons and subsequent antibody staining, we noticed a striking pattern of fluorescence: there was significant nuclear enrichment of mRFP-PKC Apl III D392A (Fig. 4.4.A). This was seen in both the mRFP signal and the antibody staining of the C-terminal using the phospho-specific antibody. Indeed, the nuclear/cytoplasmic ratio for both the mRFP fluorescence and the immunostaining of the phospho-specific antibody for mRFP-PKC Apl III D392A was significantly elevated compared to the WT mRFP-PKC Apl III (Fig. 4.4.A). Furthermore, this pattern of nuclear enrichment was also seen when a CFP-PKC Apl III D392A-YFP construct was expressed in SF9 cells (Fig. 4.5). This suggests that kinase activity is required for both nuclear export and cleavage and formation of a PKM Apl III, as mRFP-PKC Apl III D392A shows both decreased nuclear export and a lack of overexpression dependent redistribution of the C-terminus to the cytoplasm.

To further test this, we also treated cells injected with mRFP- PKC Apl III with a PKC inhibitor chelerythrine (10μM). When we added the inhibitor immediately following injection of the mRFP- PKC Apl III, we were able to replicate the staining pattern of the mRFP-PKC Apl III D392A (Fig. 4.4.B). Indeed, the nuclear/cytoplasmic ratio of the immunostaining with the phospho-

specific antibody shows the same increase as previously seen with mRFP-PKC Apl III D392A, consistent with the lack of export and lack of cleavage being due to kinase activity. However, chelerythrine treatment also greatly decreased the mRFP fluorescence making quantification of the N-terminal difficult in this case. This was not specific to the mRFP-PKC Apl III, as fluorescence of mRFP alone was also greatly reduced after treatment with chelerythrine (data not shown).

4.2.5. Kinase activity is also required for nuclear export of PKC Apl III

The strong nuclear localization of mRFP-PKC Apl III D392A implies that kinase activity is also required for efficient nuclear export. It is also possible that conformational changes induced by the mutation that affected nuclear import or export signals could explain this finding, as opposed to a loss of kinase activity. To distinguish between these possibilities, we generated an mRFP-PKM Apl III D392A construct lacking the regulatory domain that also lacks the nuclear import site and will thus be localized to the cytoplasm. In contrast, if the D-A mutation caused a conformational change we would expect this construct to instead be localized to the nucleus. Both mRFP-PKM Apl III and mRFP-PKM Apl III D392A were in fact cytoplasmic; there was no nuclear enrichment in either case (Fig 4.4.C). Thus, kinase activity is required for nuclear export as well as cleavage and formation of PKM Apl III.

4.2.6. Increasing kinase activity is sufficient to induce both nuclear export and cleavage

If overexpression of PKC Apl III activates cleavage through kinase activation of a proteolytic event, then it should be possible to rescue this deficit of cleavage of the inactive PKC by co-expressing an active kinase. To test this, we co-injected CFP-PKC Apl III-YFP with the constitutively active mRFP-PKM Apl III to determine if this could lead to cleavage, measured either by loss of FRET (Fig. 4.6.A) or by increased cytoplasmic localization (Fig. 4.6.C). As a control we used the kinase-inactive mRFP-PKM Apl III D392A. Using both assays, we observed a significant effect of expressing the kinase active mRFP-PKM Apl III WT compared to the kinase inactive mRFP-PKM Apl III D392A (Fig. 4.6). Results with mRFP-PKC Apl III D392A are similar to those seen with expression of mRFP alone (data not shown). These results indicate that increasing kinase activity within the cell by co-injecting PKM Apl III is sufficient to induce cleavage of PKC Apl III, although the amount of cleavage was still less than that seen with mRFP-PKC Apl III in which the C-terminal fragment becomes almost completely cytoplasmic.

4.2.7. 5-HT induces cleavage of PKC Apl III into PKM Apl III in *Aplysia* motor neuron processes in a calpain- and protein synthesis-dependent manner

Since the CFP-PKC Apl III-YFP is a substrate for cleavage, we could still use this construct to examine cleavage induced by 5-HT in live cells. In order to investigate the role of 5-HT-induced cleavage of PKC Apl III into PKM Apl III, we injected low levels of the CFP-PKC Apl III-YFP into isolated *Aplysia* motor neurons (of the LFS type). Low levels were used in order to minimize any possible dominant-negative effect of the construct, since it has diminished activity compared to the endogenous PKC Apl III. Following injection, the motor neurons were treated with 20μM of 5-HT for 10 min, a protocol which produces an enhancement of the postsynaptic Glu-EP that is thought to be dependent on PKM Apl III production (Villareal et al., 2009), or a vehicle solution. The FRET values were then measured in the motor neuron processes pre- and post-treatment. As can be seen by the FRET maps, a small but significant decrease in the FRET ratio was found in the cells treated with 5-HT, where no change was observed for the cells treated with the vehicle solution alone (Fig. 4.7.A; quantified in Fig. 4.7.B).

We then wished to test whether this 5-HT induced cleavage of PKC Apl III was dependent on calpain, as we have previously shown for the cleavage seen after overexpression (Bougie et al., 2009). It is also known that calpain is required for the enhancement of the postsynaptic Glu-EP (Villareal et al., 2009).

Accordingly, cells injected with low levels of the CFP-PKC Apl III-YFP were pre-treated with CIV, the general and irreversible inhibitor of calpains, at 100μM for 10 min prior to application of 5-HT, or a vehicle solution. Interestingly, the pre-treatment with the calpain inhibitor prevented the 5-HT induced loss of FRET, indicating that the 5-HT induced cleavage of PKC Apl III is dependent on calpain (Fig. 4.7.A; quantified in Fig. 4.7.B).

It has also been previously found that the 5-HT induced enhancement of the Glu-EP in *Aplysia* motor neurons is dependent on local protein synthesis and can be blocked with the protein synthesis inhibitor emetine (Villareal et al., 2007; Villareal et al., 2009). We used this inhibitor to test whether the 5-HT induced cleavage of PKC Apl III is also dependent on protein synthesis: again, the cells injected with CFP-PKC Apl III-YFP were either pretreated with emetine at 3μM for 10 min prior to 5-HT application, or pretreated with a vehicle solution alone. In this case it was found that inhibiting protein synthesis is also able to block the 5-HT induced cleavage of CFP-PKC Apl III-YFP (Fig. 4.7.A; quantified in Fig. 4.7.B).

Thus, 5-HT induces cleavage of the FRET construct in isolated motor neurons. Similar to the 5-HT induced enhancement of the Glu-EP, the loss of FRET was blocked by both calpain inhibitors and protein synthesis inhibitors. Our data suggests that the underlying mechanism for the enhancement of the glutamate response is cleavage of PKC Apl III into a PKM.

4.2.8. Does 5-HT induce cleavage of PKC Apl III in synaptic co-cultures?

If 5-HT can induce cleavage of PKC Apl III in isolated motor neurons, we also wished to determine if 5-HT can similarly induce cleavage of PKC Apl III in synaptic co-cultures. In order to investigate this possibility, *Aplysia* sensory neurons were plated together in culture with motor neurons (of the LFS type) to form synaptic co-cultures. In these experiments, it was the motor neuron which was injected with the CFP-PKC Apl III-YFP FRET construct. In some cases, the presynaptic sensory neuron was injected with a DNA plasmid encoding mRFP-VAMP, a presynaptic vesicle protein marker (Ahmari et al., 2000). Following injection, the co-cultures were treated with 20μM of 5-HT for 10 min as previously discussed, or a vehicle solution. The FRET values were then measured in the processes where expression of the presynaptic marker VAMP co-localized with the FRET reporter CFP-PKC Apl III-YFP (Fig. 4.8). Accordingly, it was found that the 5-HT treatment did produce a loss in FRET; however the change only approached significance ($p = 0.073$, Fig. 4.8). The number of data points was low for these sets of experiments due to the difficulty in preparing synaptic co-cultures. It is possible that the data may reach significance with additional replications.

4.3. Discussion

4.3.1. 5-HT induced formation of a PKM at the time of memory formation

In this chapter, we have provided evidence that PKC Apl III is cleaved into PKM Apl II by 5-HT at the time of memory formation, and the requirements for cleavage are the same as for the plasticity. Specifically, we have developed a FRET reporter to measure cleavage of PKC Apl III in living neurons and have applied it to demonstrate 5-HT mediated, calpain- and protein synthesis-dependent, cleavage of PKC Apl III in isolated *Aplysia* motor neurons, and potentially in synaptic co-cultures. Moreover, we have shown that cleavage of mRFP-PKC Apl III resulting from overexpression requires kinase activity, suggesting a putative positive feedback mechanism where initial calpain cleavage produces a persistently active PKM which then induces additional calpain activation.

Figure 4.1.

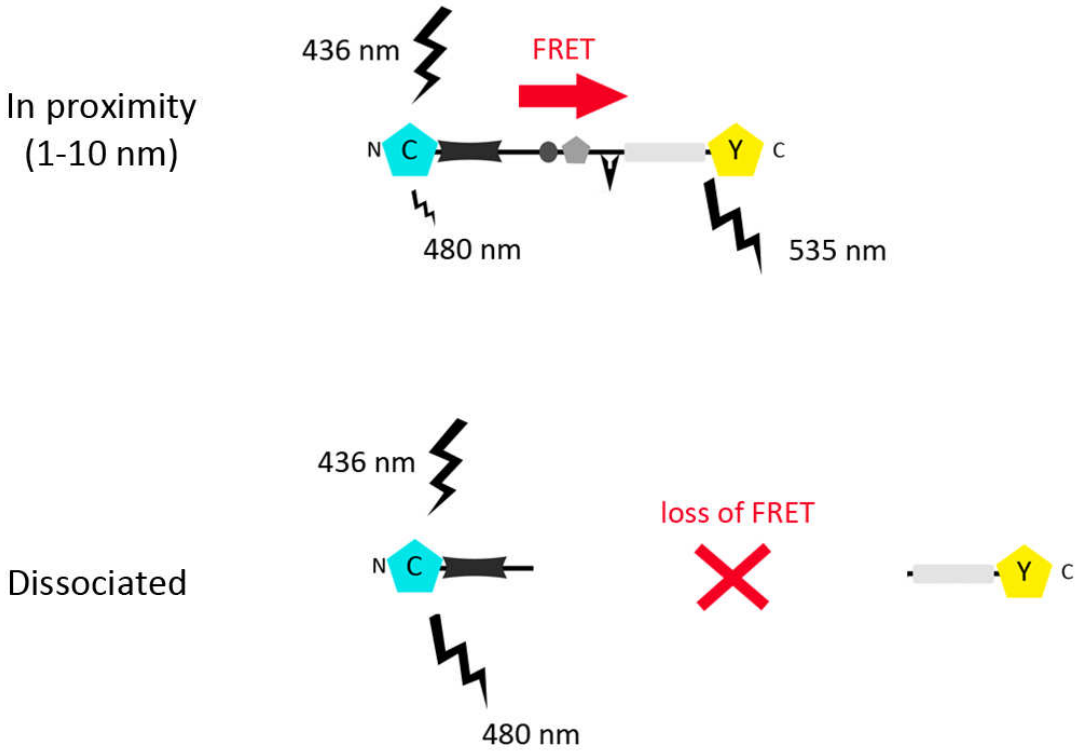
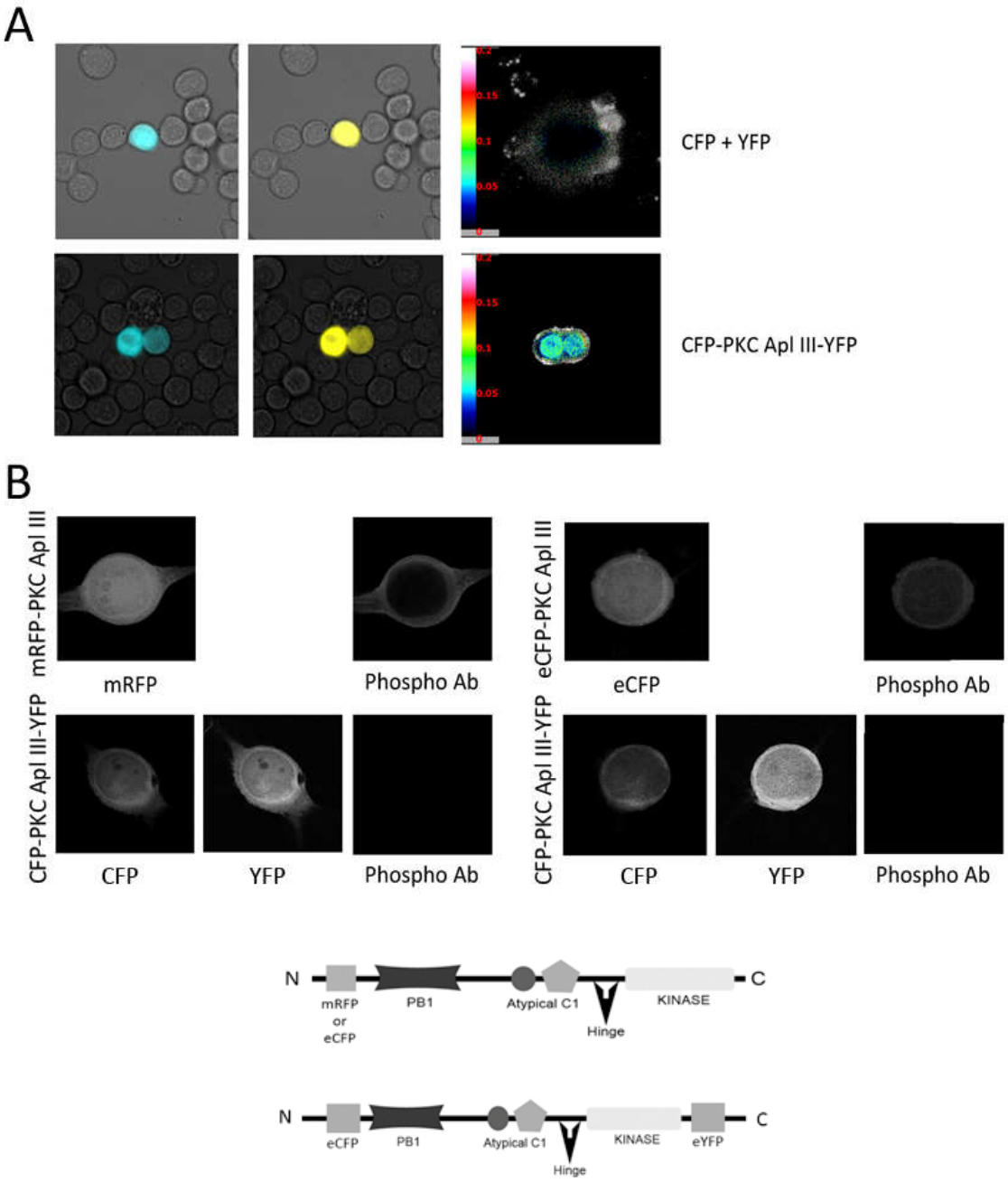

In proximity
(1-10 nm)
436 nm
FRET
N
C
Y
C
480 nm
535 nm
Dissociated
436 nm
loss of FRET
N
C
Y
C
480 nm

Figure 4.2.


A
CFP + YFP
CFP-PKC Apl III-YFP
B
mRFP-PKC Apl III
mRFP
Phospho Ab
CFP-PKC Apl III-YFP
CFP
YFP
Phospho Ab
eCFP-PKC Apl III
eCFP
Phospho Ab
CFP-PKC Apl III-YFP
CFP
YFP
Phospho Ab
N
mRFP or eCFP
PB1
Atypical C1
Hinge
KINASE
C
N
eCFP
PB1
Atypical C1
Hinge
KINASE
eYFP
C

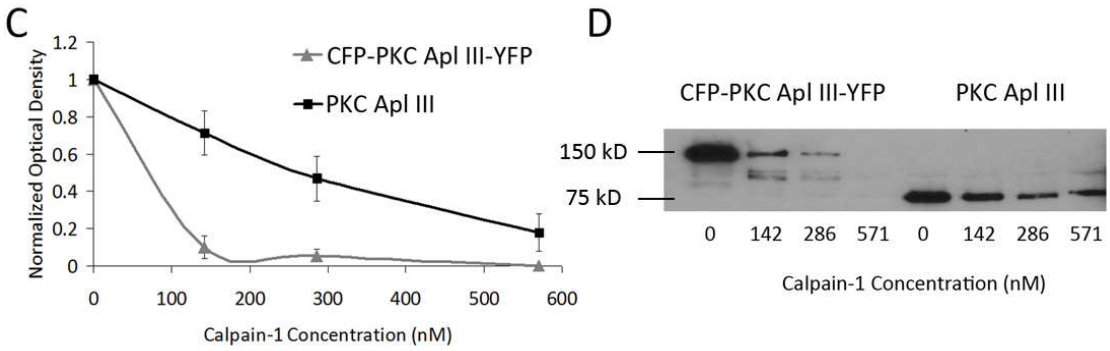

C
Normalized Optical Density
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
600
Calpain-1 Concentration (nM)
CFP-PKC Apl III-YFP
PKC Apl III
D
CFP-PKC Apl III-YFP
PKC Apl III
150 kD
75 kD
0
142
286
571
0
142
286
571
Calpain-1 Concentration (nM)

Figure 4.3.

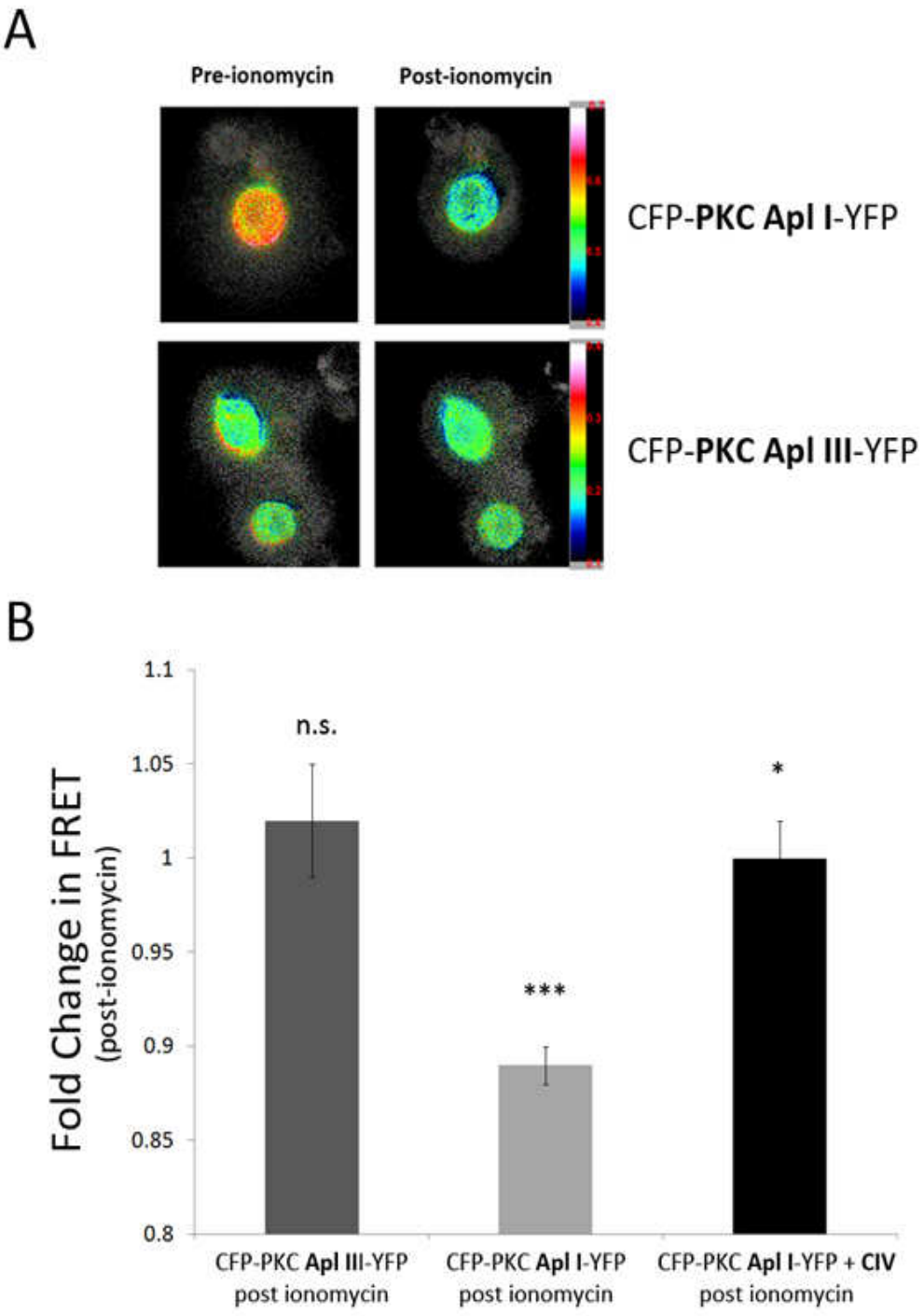

A
Pre-ionomycin
Post-ionomycin
CFP-PKC Apl I-YFP
CFP-PKC Apl III-YFP
B
Fold Change in FRET
(post-ionomycin)
1.1
1.05
1
0.95
0.9
0.85
0.8
n.s.
***
*
CFP-PKC Apl III-YFP post ionomycin
CFP-PKC Apl I-YFP post ionomycin
CFP-PKC Apl I-YFP + CIV post ionomycin

Figure 4.4.

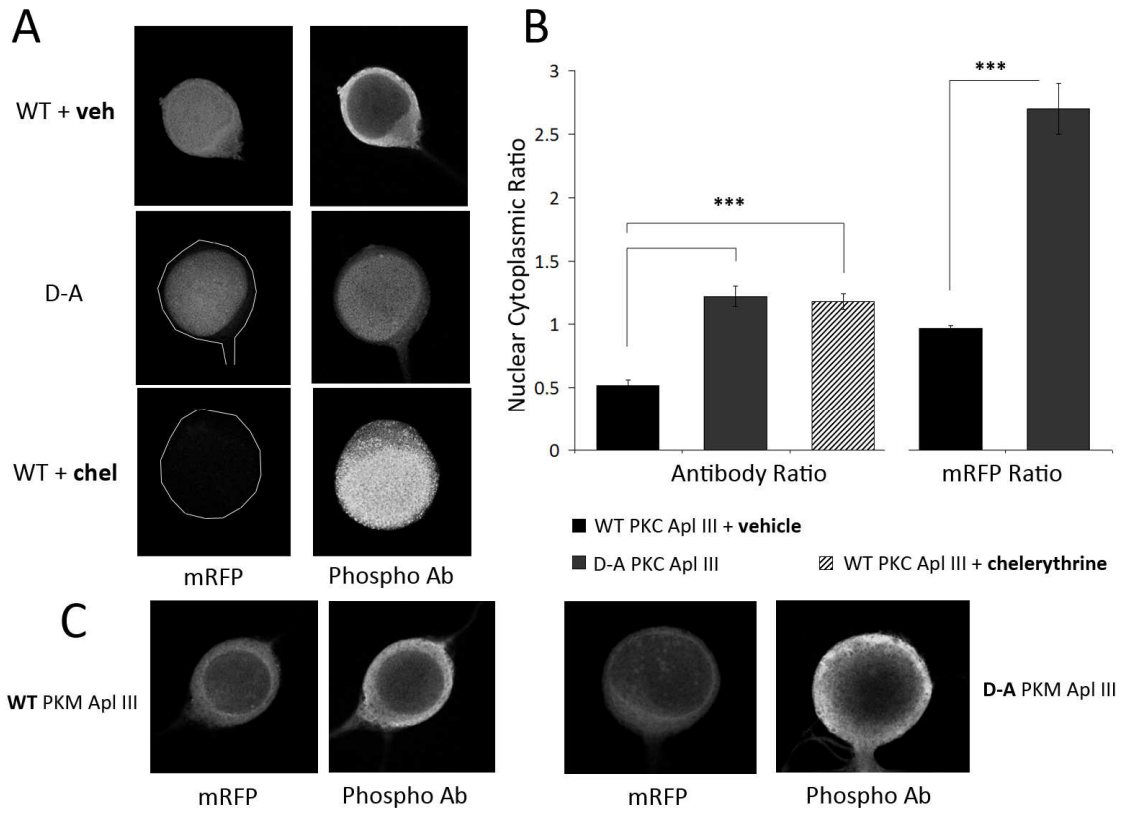

A
WT + veh
D-A
WT + chel
mRFP
Phospho Ab
B
Nuclear Cytoplasmic Ratio
3
2.5
2
1.5
1
0.5
0
***
***
Antibody Ratio
mRFP Ratio
WT PKC Apl III + vehicle
D-A PKC Apl III
WT PKC Apl III + chelerythrine
C
WT PKM Apl III
D-A PKM Apl III
mRFP
Phospho Ab
mRFP
Phospho Ab

Figure 4.5.

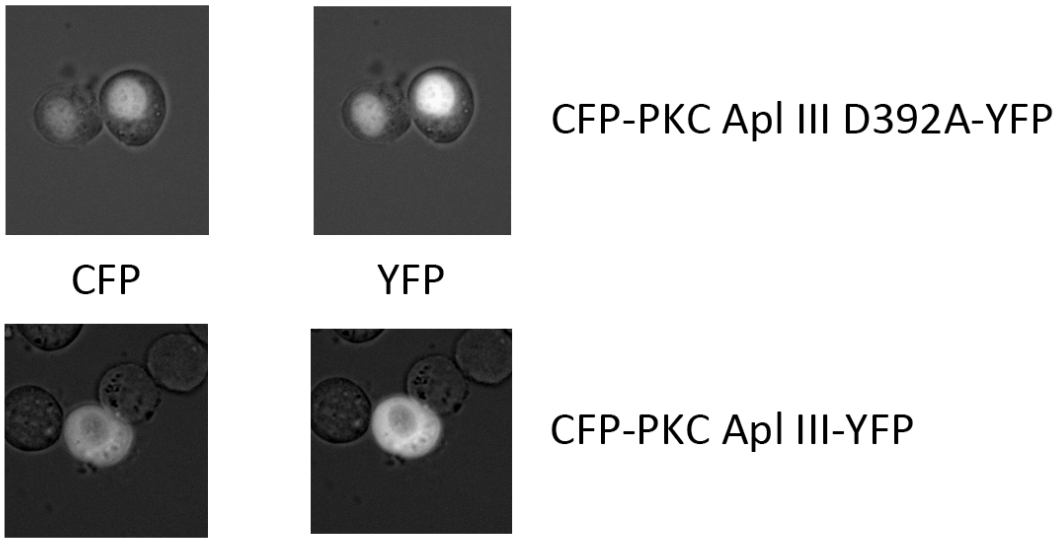


Figure 4.6.

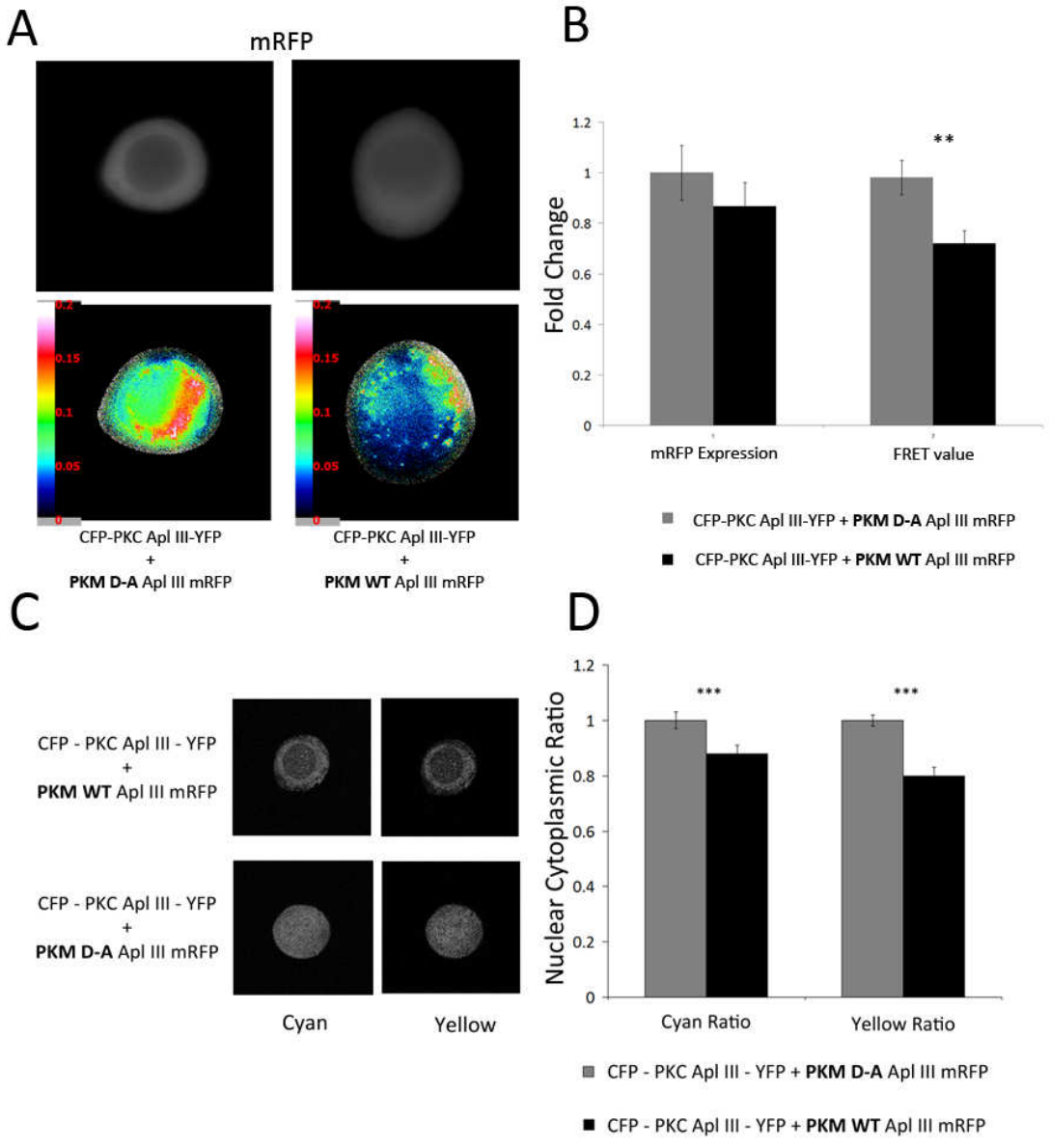

A
mRFP
0.2
0.15
0.1
0.05
0
CFP-PKC Apl III-YFP
+
PKM D-A Apl III mRFP
CFP-PKC Apl III-YFP
+
PKM WT Apl III mRFP
B
Fold Change
1.2
1
0.8
0.6
0.4
0.2
0
**
mRFP Expression
FRET value
CFP-PKC Apl III-YFP + PKM D-A Apl III mRFP
CFP-PKC Apl III-YFP + PKM WT Apl III mRFP
C
CFP - PKC Apl III - YFP
+
PKM WT Apl III mRFP
CFP - PKC Apl III - YFP
+
PKM D-A Apl III mRFP
Cyan
Yellow
D
Nuclear Cytoplasmic Ratio
1.2
1
0.8
0.6
0.4
0.2
0
***
***
Cyan Ratio
Yellow Ratio
CFP - PKC Apl III - YFP + PKM D-A Apl III mRFP
CFP - PKC Apl III - YFP + PKM WT Apl III mRFP

Figure 4.7.

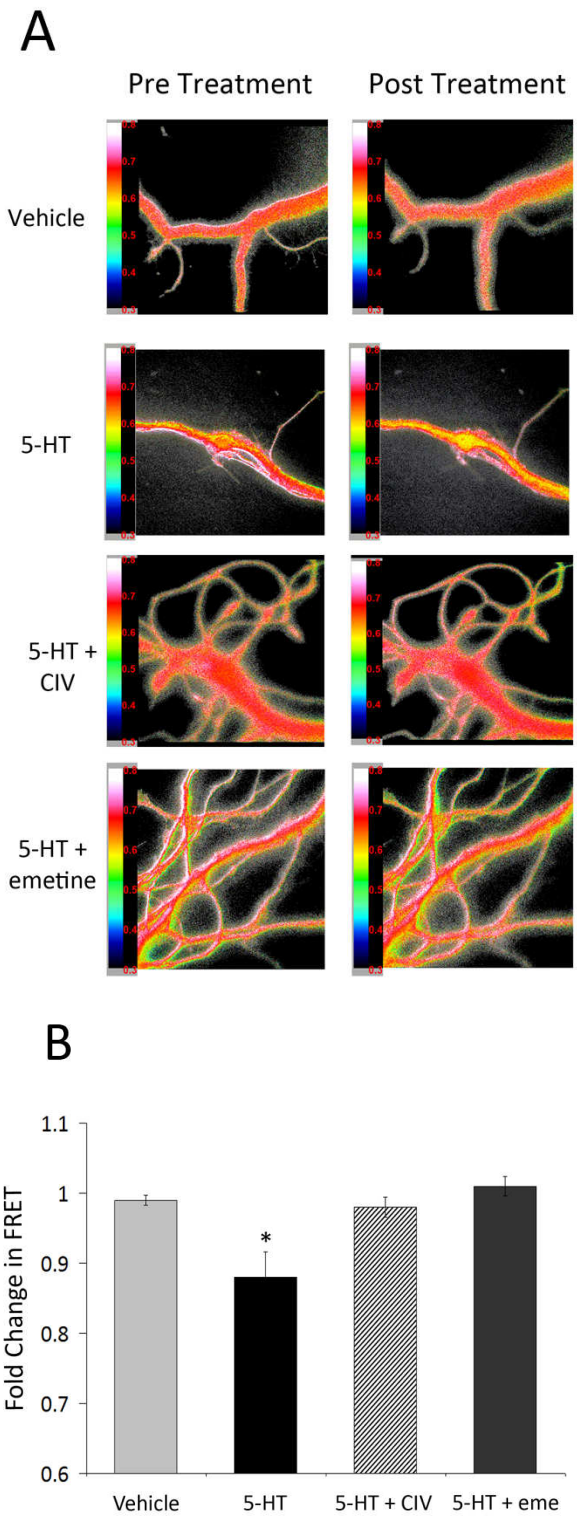

A
Pre Treatment
Post Treatment
Vehicle
5-HT
5-HT + CIV
5-HT + emetine
B
Fold Change in FRET
1.1
1
0.9
0.8
0.7
0.6
*
Vehicle
5-HT
5-HT + CIV
5-HT + eme

Figure 4.8.

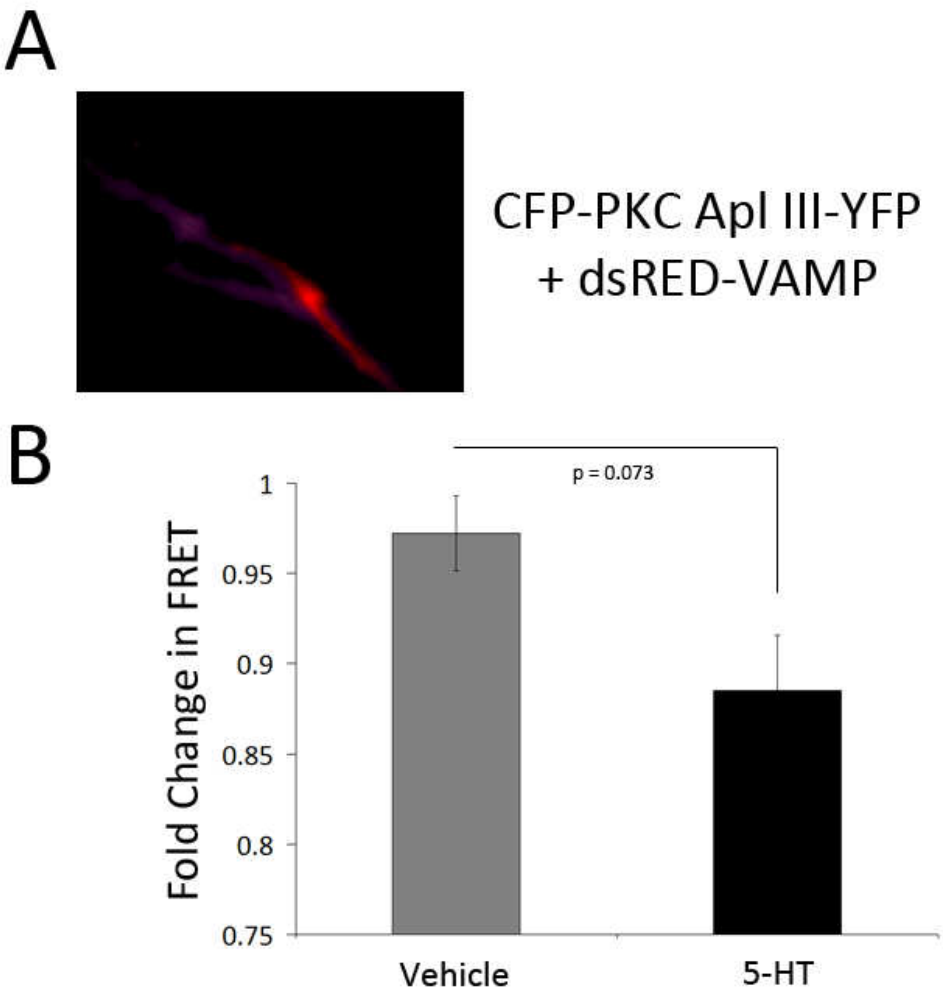

A
CFP-PKC Apl III-YFP
+ dsRED-VAMP
B
p = 0.073
Fold Change in FRET
1
0.95
0.9
0.85
0.8
0.75
Vehicle
5-HT

Figure Legends

Figure 4.1. Quantifying cleavage using the FRET assay. Förster resonance energy transfer (FRET) is a tool utilized for examining spatial relationships between molecules, and which relies on the distance-dependent radiationless transfer of energy from a donor molecule to an acceptor molecule. The donor molecule (CFP) is a chromophore that firstly absorbs energy emitted by fluorescent light and the acceptor (YFP) is the chromophore to which the energy is consequently transferred. For the successful transfer of energy, the two chromophores must be within proximity of each other, anywhere from 1 to 10 nm and there must be spectral overlap of the donor emission spectrum and the acceptor absorption spectrum (Xia and Liu, 2001).

Figure 4.2. Characterization of a FRET reporter, CFP-PKC Apl III-YFP. A: Transfected SF9 cells: top row, co-transfection of CFP and YFP in same cell; bottom row, CFP-PKC Apl III-YFP. Images were captured using a Zeiss fluorescent microscope. The FRET maps display color coded images of the measured FRET signal, where warm colors represent higher levels of FRET and cooler colors represent lower levels of FRET (the grey color is unassigned and represents negative or undefined values sometimes seen in the periphery of expressing cells, or when no FRET is observed). The FRET maps display detectable FRET for CFP-PKC Apl III-YFP (FRET = 0.0651 for cell on left, FRET

= 0.0638 cell on right) while no FRET for CFP and YFP in the same cell (FRET = 0.0000). FRET map scale ranges from 0.00 - 0.20. B: *Aplysia* sensory neurons expressing mRFP-PKC Apl III, CFP-PKC Apl III (top row) or CFP-PKC Apl III-YFP (bottom row) were stained with the phospho-specific antibody and images captured with a Zeiss confocal microscope, laser power was kept the same between all conditions. Representative diagrams of the constructs are shown, where the CFP or mRFP is located on the N-terminal, and YFP and/or antibody epitope is on the C-terminal. C: Calpain assay performed from purified proteins from baculovirus-infected SF9 cells expressing CFP-PKC Apl III-YFP or PKC Apl III with no fluorescent tags. The percentage of protein remaining was measured by optical density readings (Y-axis) and plotted against increasing concentrations of mammalian calpain-1 (X-axis). D: Representative blot of the calpain assay, purified CFP-PKC Apl III-YFP and PKC Apl III were probed with anti-His-antibody targeting His-tag at the N-terminus of each protein with increasing amounts of mammalian calpain-1 (0, 142, 286, and 571 nM respectively). CFP-PKC Apl III-YFP runs at a molecular weight of 150 kD whereas the untagged PKC has a molecular weight of 75 kD.

Figure 4.3. Calcium induced cleavage of PKC Apl I but not PKC Apl III. A: Transfected SF9 cells expressing CFP-PKC Apl I-YFP (top) and CFP-PKC Apl III-YFP (bottom). Color coded FRET maps are shown pre-ionomycin (2μM)

treatment, and five minutes post-treatment. Images captured with a Zeiss fluorescent microscope. FRET map scales ranges from 0.4-0.7 for top row, and 0.1-0.4 for bottom row. B: Quantification of A. Fold change in FRET following ionomycin is given for SF9 cells expressing CFP-PKC Apl III-YFP (first column) and CFP-PKC Apl I-YFP (second column). Third column displays cells expressing CFP-PKC Apl I-YFP that were pre-treated with calpain inhibitor V (CIV) (100μM) for 10 minutes prior to addition of ionomycin. Data is from three-six experiments, CFP-Apl III-YFP n = 81 cells, CFP-PKC Apl I-YFP n = 159 cells, and CFP-PKC Apl I-YFP + CIV n = 30 cells. Paired t-tests revealed a significant difference in FRET for CFP-PKC Apl I-YFP following ionomycin, *** $p < 0.001$, and not for CFP-PKC Apl III-YFP following ionomycin, $p > 0.05$. A non-paired student's t-test for the difference between cells expressing CFP-PKC Apl I-YFP treated with ionomycin in the presence or absence of CIV revealed a significant difference, * $p < 0.05$.

Figure 4.4. Kinase activity is required for cleavage and nuclear export of PKC Apl III. A: *Aplysia* sensory neurons expressing mRFP-PKC Apl III (top) were treated with a vehicle solution, mRFP-PKC Apl III D392-A (middle) treated with a vehicle solution, and mRFP-PKC Apl III treated with the PKC inhibitor chelerythrine immediately following injection of the plasmid DNA (10 μM; bottom). mRFP tags the N-terminal and Phospho Ab represents secondary antibody staining of the C-

terminal. All cells were stained with the phospho-specific antibody and visualized using a Zeiss laser scanning confocal microscope with laser power kept constant between conditions. Outlines are drawn around cells in order to visualize cell membrane. B: Quantification of conditions cited in Panel A. Nuclear/Cytoplasmic ratio was calculated for both antibody staining (C-terminal) and mRFP (N-terminal) for mRFP-PKC Apl III treated with vehicle (N=27 cells, three experiments), mRFP-PKC Apl III D392A treated with vehicle (N=31 cells, three experiments), and mRFP-PKC Apl III treated with chelerythrine (N=41 cells, three experiments). The mRFP nuclear/cytoplasmic ratio could not be measured for the chelerythrine condition. *** $p < 0.001$ Students T-test for all conditions. C: *Aplysia* sensory neurons expressing mRFP-PKM Apl III (left) and mRFP-PKM D392A Apl III (right), stained with phospho-specific antibody and imaged with a Zeiss laser scanning confocal microscope.

Figure 4.5. Nuclear enrichment of CFP-PKC Apl III D392A-YFP is also seen in SF9 cells. Transfected SF9 cells displaying CFP (left) and YFP (right) for cells expressing CFP-PKC Apl III D392A-YFP (top) and CFP-PKC Apl III-YFP (bottom).

Figure 4.6. Increasing kinase activity is sufficient to induce cleavage and nuclear export of PKC Apl III. A: *Aplysia* sensory neurons were co-injected with CFP-PKC

Apl III-YFP and either mRFP-PKM Apl III D392A (left) or mRFP-PKM Apl III (right) and FRET Xia levels were measured. Top row is representative expression of the mRFP-PKM, and bottom row is a color coded FRET map of the measured FRET ratio, scale 0.0 to 0.2. The FRET maps display color coded images of the measured FRET signal, where warm colors represent higher levels of FRET and cooler colors represent lower levels of FRET (the grey color is unassigned and represents negative or undefined values sometimes seen in the periphery of expressing cells, or when no FRET is observed). B: Quantification of A. mRFP-PKM Apl III expression levels and FRET values normalized to values for mRFP-PKM Apl IIID-A, **, P = 0.003, for mRFP-PKM Apl III D392A, n = 35 cells; three experiments, for mRFP-PKC Apl III, n = 32 cells; three experiments. C: *Aplysia* sensory neurons co-injected with CFP-PKC Apl III-YFP and either mRFP-PKM Apl III (top row) or mRFP-PKM D392A Apl III (bottom row). Images are of the CFP (N-terminal) and YFP (C-terminal) in both cases. D: Quantification of C. Normalized Nuclear/Cytoplasmic ratio calculated for both CFP and YFP. ***, P < 0.001 Students T-test for all conditions, n = 36 cells, two experiments for co-injection with mRFP-PKM Apl III and n = 38 cells, two experiments for co-injection with mRFP-PKM Apl III D392A.

Figure 4.7. 5-HT induces cleavage of CFP-PKC Apl III-YFP in motor neuron processes in a calpain- and protein synthesis-dependent manner. A: Color coded

FRET maps of *Aplysia* motor neuron processes expressing low levels of CFP-PKC Apl III-YFP, pre and post treatment. Scale from 0.3 – 0.8, The FRET maps display color coded images of the measured FRET signal, where warm colors represent higher levels of FRET and cooler colors represent lower levels of FRET (the grey color is unassigned and represents negative or undefined values sometimes seen in the periphery of expressing cells, or when no FRET is observed). FRET exposure time was doubled for these experiments to optimally view FRET levels in the processes. Cleavage is indicated as a loss of FRET. B: Quantification of A): Fold change in FRET was calculated for cells treated with a vehicle solution, 5-HT (N=22 cells, nine experiments, 5-HT + calpain inhibitor V (N=10 cells, three experiments, or 5-HT + emetine (a protein synthesis inhibitor) (N=9 cells, three experiments). * $P < 0.05$, Students t-test with unequal variance using the Bonferroni correction for multiple tests.

Figure 4.8. Trend towards 5-HT induced cleavage of CFP=PKC Apl III-YFP in synaptic co-cultures. A: *Aplysia* synaptic co-culture, example of synaptic varicosities between the motor neuron expressing CFP-PKC Apl III-YFP (shown in purple) and the sensory neuron expressing dsRED-VAMP (red). B: Quantification of fold change in FRET following 10 minute treatment with 5-HT (20μM). Student's t-test did not reveal a significant difference, $p = 0.073$, $n = 4$

SN-MN pairs for vehicle condition and n = 6 SN-MN pairs for 5-HT condition, data pooled from three separate experiments.

CHAPTER FIVE

Conclusion

5.1. Overview

In chapter one, a comprehensive review of the literature was presented: the history of the search for the long term memory trace was discussed, beginning with seminal investigations into human memory consolidation by Ebbinghaus in 1878. The evolution of the search for the long term memory trace was then examined by exploring how the traditional consolidation theory was refined to include the discovery of the importance of the hippocampus, systems consolidation, as well as reconsolidation. Subsequently, I discussed early investigations by Hebb into synaptic plasticity, the cellular correlate of the long term memory trace. The influential discovery of LTP was then reviewed, with a focus upon the requirement for *de novo* protein synthesis and gene transcription. The synaptic tagging and capture hypothesis was then examined, in addition to the relationship with morphological changes at synapses following the induction and maintenance of long term plasticity.

Importantly, the ability to study conserved forms of memory in lower invertebrates was then considered. Due to the complexity of the mammalian nervous system, I argued for the significance of studying reduced forms of the long term memory trace in the marine mollusk *Aplysia californica*. The distinct

time-dependent forms of plasticity in *Aplysia* were examined, including STF, ITF, and LTF, and the role of various isoforms of PKCs in these forms of plasticity. Finally, the role of a persistently active truncated kinase called PKMz in maintaining the long term memory trace was evaluated in both rodents as well as *Aplysia*.

In chapter three, I presented the cloning and characterization of the atypical PKCz orthologue in *Aplysia*, PKC Apl III, and the search for how PKMz is formed in *Aplysia*. In mammals, PKMz is translated from a unique alternative mRNA transcript: however, in *Aplysia*, we were unable to find any such alternative transcript. Accordingly, our evolutionary analysis suggested that PKM forms of PKCs play a conserved role in memory formation and maintenance, but the mechanism of formation of these kinases has changed during evolution. In addition, through the use of an overexpression paradigm we have been able to detect cleavage of the atypical PKC Apl III into a PKM Apl III. The presence of alternative splice inserts in the hinge domain of PKC Apl III provide a site for calpain cleavage: thus, PKC Apl III is cleaved into a PKM Apl III by calpain. Furthermore, it was found that 5-HT treatment was able to activate PKC Apl III through phosphorylation at the PDK site that is downstream of the PI3K-PDK pathway.

In chapter four, I presented the development of a FRET reporter which we used to measure cleavage of PKC Apl III in live SF9 cells or *Aplysia* neurons.

Using these reporters we demonstrated that, in SF9 cells, a calcium ionophore was able to induce a cleavage of PKC Apl I but not PKC Apl III. In neurons, we found that the CFP-PKC Apl III-YFP construct did not display the typical dissociated pattern of fluorescence that indicates a cleavage event, and that this construct displays lower overall kinase activity compared to the untagged or single tagged PKC Apl III. This led us to investigate whether kinase activity is in fact required for cleavage of PKC Apl III into PKM Apl III. As such, we found that both cleavage and nuclear export are dependent on kinase activity, and that increasing kinase activity is sufficient to induce both nuclear export of PKC Apl III as well as cleavage. Importantly, we also demonstrated that facilitating stimuli such as 5-HT application are able to induce cleavage of PKC Apl III into PKM Apl III in *Aplysia* motor neuron processes in a calpain- and protein synthesis-dependent manner.

5.2. Contribution and significance of the studies

The main objectives of the previously discussed studies were to clone and characterize the atypical PKC zeta in *Aplysia*, PKC Apl III, as very little was known about the role for this kinase in *Aplysia* (see Chapter Three). The second main objective was to determine if and how a PKM Apl III is formed in *Aplysia* and what role it may play in synaptic plasticity (see Chapters Three and Four). The following discussions will examine how the aforementioned studies

investigated these objectives and what contribution and significance they provided in the field of the search for the long term memory trace.

5.2.1. Conservation of the role of PKMz in maintaining the long term memory trace

It has been widely demonstrated that mammalian PKMz is required for maintaining the long term memory trace, and inhibition of its activity permanently erases long term memory at time points when a given trace is expected to be firmly consolidated (Pastalkova et al., 2006; Shema et al., 2007; Shema et al., 2011). The evidence that inhibiting PKMz erases memory is based on two inhibitors of PKMz (ZIP and chelerythrine) (see (Sacktor, 2011) for a review) and an inhibitor that blocks the downstream action of PKMz on AMPA receptors (Yao et al., 2008). Memory is also blocked by the expression of a dominant negative PKMz (Ling et al., 2002; Shema et al., 2011), and moreover, overexpression of PKMz can enhance long term memory in both *Drosophila* and rodents (Drier et al., 2002; Shema et al., 2011). Thus, the evidence is strong that PKMz forms a long term memory trace. There is also evidence for increased translation of the alternative transcript during memory formation (Hernandez et al., 2003); however, it has not yet been shown that specific blockade of translation of the PKMz message blocks either the induction or the maintenance of memory, and thus a role for calpain-mediated cleavage in vertebrates cannot be ruled out at

this point. In chapter three, we showed that while the mechanism of formation of the atypical PKM may have changed during evolution, the presence of an atypical PKM is conserved. It has also been recently shown that the role of PKMz is conserved in *Aplysia*, as inhibiting this kinase using either ZIP or chelerythrine erases the long term memory of behavioral sensitization, in addition to erasing the long term enhancement of synaptic plasticity which is the cellular correlate of this behavioral memory (Cai et al., 2011).

5.2.2. Evolutionary change in mechanism of formation of PKMz

While the role of PKMz has shown to be conserved, we have demonstrated that the mechanism of formation is not. We have been unable to detect an alternative transcriptional start site for PKMz in *Aplysia* (Fig. 3.1), despite thorough investigation. Our evolutionary analysis strongly argues for the absence of an initiating methionine which would encode PKMz in *Aplysia*. However, it must be stated that absence of evidence is not evidence of absence. It is possible that there may indeed exist an alternative transcript, perhaps in another region of the PKCz gene such as the 5' untranslated region, but our attempts to locate this transcript have been unsuccessful. In addition, the ability to generate a separate transcriptional form of PKMz may have required gene duplication, since the full length atypical PKC has major conserved roles in polarity, including axon determination in the nervous system (Banker, 2003).

Once the gene had been duplicated, one isoform could be devoted to cell polarity, and the other optimized for PKM formation.

Furthermore, it is possible that the evolutionarily more ancient mechanism of calpain cleavage has not been lost in vertebrates. For example, there is evidence for a role for calpains in the induction of LTP, as inhibitors of calpain have been shown to block the induction phase of LTP (Denny et al., 1990). Calpains are involved in the remodelling of dendritic spines following tetanizing stimulation such as LTP, and they are known to have a wide variety of substrates in addition to PKC, including NMDA and AMPA receptors (Oliver et al., 1989; Denny et al., 1990; Vanderklish et al., 1996; Liu et al., 2008; Zadran et al., 2010). Owing to these important roles for calpain in long term synaptic plasticity, it is reasonable to suggest that PKMz can also be formed by calpain cleavage in mammals. While PKMz can be expressed in complete absence of the full length PKCz gene (Hernandez et al., 2003), it is possible that PKMz is concurrently formed by calpain cleavage of PKCz in addition to its translation from the alternative transcript. There is currently no evidence that completely excludes this possibility. Furthermore, a recent finding has shown that BDNF maintains L-LTP by regulating levels of PKMz in the absence of protein synthesis (Mei, 2011). The authors postulate that this is due to BDNF preventing the degradation of PKMz; however, it is also likely that this may be due to increased calpain-mediated cleavage of the full length PKCz.

5.2.3. The role of PKMz in regulating AMPA receptor trafficking

It will be interesting to determine if the evolutionary role of PKMz in regulating AMPA receptor trafficking is also conserved. In mammals, it has been shown that PKMz maintains the potentiated state of synapses by persistently increasing the amount of AMPARs in the postsynaptic membrane: this occurs specifically through the trafficking protein N-ethylmaleimide-sensitive factor (NSF), which maintains the number of GluR2 containing AMPARs (Ling et al., 2002; Ling et al., 2006; Yao et al., 2008). The model of how PKMz maintains long term memory postulates that persistent kinase activity is required to maintain this potentiated state of synapses due to an opposing force which drives AMPARs out of the postsynaptic compartment and returns the synapse to its pre-potentiated state (Sacktor, 2011). This opposing force involves disrupting the interaction between NSF and GluR2, which consequently removes GluR2 receptors from postsynaptic sites (Yao et al., 2008).

Indeed, recent work suggests that, in *Aplysia*, PKM Apl III is important for insertion of AMPA receptors (Villareal et al., 2009). It was found that general inhibitors of PKC, chelerythrine and Bis, both blocked the induction of 5-HT-dependent enhancement of the postsynaptic Glu-EP, which is dependent on insertion of AMPARs (Villareal et al., 2009). However, only chelerythrine, at doses specific for PKM Apl III, and an inhibitor of calpain, were able to block the maintenance of the Glu-EP (Villareal et al., 2009). Accordingly, maintenance of

the Glu-EP through insertion of AMPA receptors may be mediated by a PKM-type fragment generated by calpain-dependent proteolysis of atypical PKC. Thus, PKM forms of atypical PKCs may also have a conserved role in regulating AMPA receptor trafficking.

However, there is some conflicting data concerning the relationship between PKMz and AMPAR trafficking. In *Aplysia*, it has been shown that inhibiting exocytosis with botulinum toxin type B (Botox B) similarly blocks the 5-HT induced enhancement of the Glu-EP (Chitwood et al., 2001). However, rodent studies have shown that while Botox B does indeed depress AMPAR-mediated transmission in hippocampal slices, the toxin did not inhibit AMPAR potentiation induced by perfusion of PKMz (Yao et al., 2008). It is possible that these results can be resolved by the finding that most AMPARs are localized to the synapse during LTP through lateral diffusion from an extra-synaptic pool, and not necessarily exocytosis (Makino and Malinow, 2009). In a situation where there exists a large extra-synaptic pool of AMPARs, it is possible that we would not see an effect of Botox B if AMPARs are incorporated in the synapse through lateral diffusion and not insertion of new receptors. Accordingly, it is clear that this subject warrants further investigation.

5.2.4. Is the role of PKMz conserved in synaptic tagging?

As previously discussed in the introduction, PKMz has been identified as a plasticity related protein (PRP) in the process of synaptic tagging in vertebrates. Specifically, PKMz was shown to be a specific PRP for LTP (Sajikumar et al., 2005; Frey et al., 2008). Thus, PKMz produced by a strong LTP-inducing stimulus at one synapse could be captured at another synapse that had been tagged by receiving a weak stimulus: this weak stimulus which would have only produced E-LTP now produces L-LTP (Sajikumar et al., 2005). Synapse specific forms of plasticity have also been demonstrated in *Aplysia*: when one bifurcated sensory neuron was connected to two separate motor neurons it was found that application of 5-HT to one connection induced plasticity specifically at that connection, in a CREB-dependent manner that produced growth of new synaptic connections (Martin et al., 1997). While it is known that five pulses of 5-HT is normally required to induce LTF, it was found that when one connection received five pulses of 5-HT the other connection consequently only required one pulse of 5-HT for LTF and to induce growth of new synaptic connections (Martin et al., 1997). This suggests that synaptic tagging has occurred in *Aplsyia* SN-MN synapses, as the non-stimulated connection is able to capture the PRPs formed after 5-HT was applied to the first connection.

Since the process of synaptic tagging seems to be conserved in *Aplysia*, and PKMz is important in synaptic tagging in vertebrates, it would be interesting to determine if the role of PKM Apl III is also conserved in this process. Our

findings do not specifically address the role of PKM Apl III in synaptic tagging in *Aplysia*, but the finding that PKM Apl III is formed by cleavage raises some interesting possibilities. It can be reasoned that activated PKC Apl III may act as a synaptic tag to subsequently recruit calpain to the required synapse; once calpain is activated at the synapse, it can then cleave PKC Apl III into PKM Apl III which produces long lasting synaptic plasticity. It is also possible that an additional unknown protein may be required to activate or recruit calpain and thus serve as a PRP. No PRPs have currently been identified in *Aplysia*; therefore PKC Apl III represents an interesting candidate for further study.

5.2.5. Reconciling two theories of the long term memory trace

If the long term memory trace is truly stored in the persistent activation of a kinase, do morphological changes at synapses still play a role in maintaining long term memory? Can these two models be reconciled? PKMz maintains long term memory by actively regulating AMPARs at the postsynaptic membrane (Yao et al., 2008), larger PSDs on dendritic spines result from increased numbers of AMPARs (Harris and Stevens, 1989; Ultanir et al., 2007), and spine growth can be followed by synapse formation (Holtmaat et al., 2008). If we examine these findings as a whole, we can appreciate how these two models do not necessarily cancel each other out. For example, it is possible that persistent activity of PKMz functions to increase and maintain the number of active postsynaptic AMPARs

which subsequently results in morphological changes at existing synapses, and potentially addition of new synapses. This possibility would therefore theoretically allow roles for both persistent enzymatic activity in addition to morphological changes at synapses. Currently, there is no data to suggest a direct link between these two processes, but this possibility is not necessarily ruled out by either experimental model.

5.2.6. Is there endogenous PKM Apl III in *Aplysia*?

Our studies also raise further experimental questions. Using an overexpression paradigm and a FRET assay, we have been able to detect calpain mediated cleavage of the atypical PKC Apl III into a PKM Apl III; however we have not yet been able to detect cleavage of the endogenous PKC Apl III. Our results suggest that cleavage is highly dependent on the level of expression, and this may explain the inability to detect the endogenous PKM form of PKC Apl III using immunoblots (data not shown). While increased cleavage is observed after increasing intracellular calcium in *Aplysia* sensory neurons with ionomycin, this still required a certain level of overexpression. Thus, if endogenous PKC Apl III is to be cleaved, it would have to be produced at high levels during the activation of calpain. Interestingly, this situation may occur in the motor neuron, where a PKC-dependent increase in the sensitivity to glutamate depends on both rapid protein synthesis and calcium (Chitwood et al., 2001; Khan et al., 2001; Udo et al., 2005;

Hu et al., 2006; Villareal et al., 2007; Villareal et al., 2009). Importantly, the maintenance of this increased sensitivity is blocked by low concentrations of the kinase inhibitor chelerythrine, as is PKC Apl III (Villareal et al., 2009). While our current methodological tools are unable detect an endogenous PKM, the 5-HT induced formation of PKM Apl III in motor neurons indicates an important physiological role for PKMz at the time of memory formation in *Aplysia*.

5.2.7. Is PKM formation specific for PKC Apl III?

Both PKC Apl I and PKC Apl II can be cleaved *in vitro* by calpain to form a PKM (Sutton et al., 2004), similarly to PKC Apl III. Since PKC Apl I and PKC Apl II do not shuttle through the nucleus, the immunocytochemistry assay to detect cleavage for PKC Apl III is not valid for PKC Apl I and PKC Apl II. However, the normal translocation of eGFP-PKC Apl I and eGFP-PKC Apl II suggest that they are not cleaved to form PKMs by overexpression (Zhao et al., 2006). However, ITF induced by coupling 5-HT and activity is thought to be maintained by a PKM (Sutton et al., 2004). This kinase is inhibited by Bis, but PKC Apl III is not (Sutton et al., 2001; Villareal et al., 2009) suggesting that other isoforms of PKC can also generate PKMs. Indeed, dominant negative forms of PKC Apl I block this form of facilitation (Zhao et al., 2006). Furthermore, we demonstrated that increasing intracellular levels of calcium could induce a cleavage of PKC Apl I into PKM Apl I, but not PKC Apl III into PKM Apl III, in SF9 cells. Thus, it is likely that PKMs

may be formed from distinct isoforms of PKC depending on the type of stimulation.

5.2.8. Kinase activity is required for cleavage of PKC Apl III into PKM Apl III

We have shown that kinase activity is required for cleavage of mRFP-PKC Apl III after overexpression using two distinct methods: producing a kinase-dead mutant to directly assess the requirement of kinase activity as well as using pharmacological agents to inhibit kinase activity. In addition, we also found that the FRET reporter construct CFP-PKC Apl III-YFP was not cleaved after overexpression, presumably due to its reduced kinase activity. However, since the CFP-PKC Apl III-YFP can still be cleaved by calpain *in vitro*, kinase activity is not required for cleavage *in vitro* with an activated calpain. Thus, the kinase activity is likely required to activate the calpain in neurons.

5.2.9. Is the kinase-dependence for cleavage downstream of a kinase-dependence for nuclear export?

The inactive mRFP-PKC Apl III D392A was enriched in the nucleus, suggesting that kinase activity is also required for nuclear export of the protein. Ideally, we could mutate the nuclear localization sequence in this context and then determine whether the kinase dead cytoplasmic protein was still cleaved. However, the sites for nuclear import and export of PKC Apl III are not well

understood. Putative nuclear import and export sites were identified for vertebrate PKC iota (Perander et al., 2001) but we did not observe differences in nuclear localization after mutating these sites in PKC Apl III (data not shown). Expression of mRFP-PKC Apl III in SF9 cells did not lead to nuclear localization (see Fig 3.3 and (Bougie et al., 2009)), suggesting specific factors in *Aplysia* neurons may be required for nuclear import; however, expression of kinase dead mRFP-PKC Apl III D392A in SF9 cells did in fact lead to nuclear retention (Fig 4.5), suggesting that this may be more of a quantitative than qualitative difference. PKC Apl III may 'piggy-back' into the nucleus through binding to another protein that has a nuclear localization sequence, and this binding would involve an N-terminal sequence. Perhaps this binding is removed by PKC Apl III phosphorylation of this protein, allowing the kinase to shuttle back to the cytoplasm. The identity of these potential binding proteins is currently unknown, but may present an interesting opportunity for further investigation.

5.2.10. Is kinase activity required to induce calpain mediated cleavage and formation of a PKM?

We have shown that kinase activity is sufficient for cleavage of PKC Apl III through co-expression of the less active CFP-PKC Apl III-YFP with the constitutively active WT PKM Apl III. The presence of the active PKM is sufficient to both increase export of PKC Apl III (as seen by the decrease in nuclear

localization) and to induce cleavage of the CFP-PKC Apl III-YFP (as measured by a loss of FRET) (Fig. 4.6). It is possible that kinase activity is required to recruit calpain in order for the cleavage to occur. Similarly, it is also possible that the kinase must phosphorylate an additional target that subsequently activates or recruits calpain. However, it should be noted that the amount of cleavage after co-expressing the PKM was considerably less than seen after overexpression, where it appeared that most of the overexpressed kinase is cleaved. It may be that normally, activation occurs in a complex where the kinase to be cleaved directly activates the calpain that cleaves it, and thus the trans-effect of adding PKM is not as strong. It is also possible that protein-protein interactions involving the full-length PKC are important for targeting the calpain or associated protein for phosphorylation, again explaining the decreased ability of PKM to stimulate cleavage in trans.

The fact that activity appears to be involved in recruitment of the calpain suggests a positive feedback pathway that may be important for the maintenance of PKM activation. Following calpain mediated cleavage of PKC Apl III into PKM Apl III, there is accordingly increased kinase activity which then acts in turn to again recruit further calpain to induce additional cleavage. This is a putative positive feed back loop, as we presently have no direct evidence for its existence.

5.2.11. PKM Apl III is formed following facilitating stimuli

We have shown that 5-HT increases PKC Apl III phosphorylation in sensory cells through the PI3K pathway, and 5-HT is also able to induce cleavage of PKC Apl III into PKM Apl III in isolated *Aplysia* motor neuron processes (Fig. 4.7). It has been previously established that a 10 min treatment of isolated motor neurons with 5-HT produces an enhancement of the postsynaptic glutamate response that is thought to be dependent on PKM Apl III production (Villareal et al., 2009). Furthermore, it has recently been shown that both the cellular long-term facilitation as well as behavioral long-term memory in *Aplysia* is maintained by PKM Apl III-dependent protein phosphorylation (Cai et al., 2011). We have contributed further to this finding by demonstrating that in *Aplysia*, 5-HT, and potentially other facilitating stimuli such as behavioral learning, induce cleavage of PKC Apl III into PKM Apl III in a calpain- and protein synthesis dependent-mechanism. While it is known that 5-HT induces protein translation in *Aplysia* neuron processes (Villareal et al., 2007), it is not known which proteins are synthesized in order to induce cleavage of PKC Apl III. It is possible that it is in fact calpain which needs to be synthesized *de novo* in order to cleave PKC Apl III into PKM Apl III. Alternatively, it may be a protein that is required to recruit calpain to cleave PKC Apl III. Since PKC Apl III is cleaved when expressed at high levels, an attractive hypothesis is that the protein translated is PKC Apl III itself and the increase in the levels of the protein activated the cleavage. Our experiments argue against this, since even when

CFP-PKC Apl III-YFP is overexpressed cleavage is still protein-synthesis dependent.

5.3. Future Directions

5.3.1. Cloning the calpains in *Aplysia*

We are currently working towards cloning and characterizing *Aplysia* calpains in order to identify the calpain involved in cleaving PKCs into PKMs and determining their mechanism for activation. As previously mentioned, similarly to PKCs, calpains are also subdivided into various isoform families, with approximately 14 different isoforms overall. Of these isoforms, it is calpain-1 and calpain-2 which are most abundant in the central nervous system (Croall and Ersfeld, 2007). In *Aplysia* to date, our lab has cloned four calpains and is currently elucidating the identity, role, and functions of these calpains. Future experiments will seek to co-overexpress calpain with PKC Apl III in *Aplysia* neurons to determine if these calpains can induce cleavage of PKC Apl III into PKM Apl III. Furthermore, this can also be applied to PKC Apl I and PKC Apl II to investigate formation of additional PKMs in *Aplysia*.

5.3.2. Plasticity at the soma or the synapse

We have provided evidence for the role of cleavage of PKC Apl III into PKM Apl III following 5-HT treatment in isolated motor neurons, and potentially at the synapse. It is clear that future experiments should continue to investigate this important question in synaptic co-cultures, and it will also be important to produce replications in order to increase the sample size of these experiments. In addition, it will be interesting to see if it is only overexpression of PKC Apl III in the postsynaptic cell which is required for the 5-HT induced formation of a PKM, or if overexpressing PKC Apl III in the presynaptic cell could also lead to a 5-HT induced formation of PKM Apl III. Similarly, we did do some preliminary studies examining 5-HT induced cleavage of PKC Apl III in isolated sensory neurons, but the data was inconclusive due to various methodological reasons. Future experiments can utilize the FRET assay to answer these questions.

5.3.3. Additional plasticity paradigms

Moreover, we have not yet investigated the role of cleavage of PKC Apl III into PKM Apl III using alternative plasticity paradigms. For example, future studies could utilize the FRET assay to examine cleavage of PKC Apl III in synaptic co-cultures following five spaced applications of 5-HT; a protocol that induces LTF in *Aplysia* (Kandel, 2001). It would also be interesting to test cleavage of PKC Apl III in an activity-inducing paradigm which pairs 5-HT with

sensory neuron firing: this protocol leads to an ITF that is dependent on persistent activity of PKC (Sutton and Carew, 2000).

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