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The role of PKMζ in the maintenance of long-term memory: a review

Abstract: Long-term memories are thought to be stored in neurones and synapses that undergo physical changes, such as long-term potentiation (LTP), and these changes can be maintained for long periods of time. A candidate enzyme for the maintenance of LTP is protein kinase M zeta (PKMζ), a constitutively active protein kinase C isoform that is elevated during LTP and long-term memory maintenance. This paper reviews the evidence and controversies surrounding the role of PKMζ in the maintenance of long-term memory. PKMζ maintains synaptic potentiation by preventing AMPA receptor endocytosis and promoting stabilisation of dendritic spine growth. Inhibition of PKMζ, with zeta-inhibitory peptide (ZIP), can reverse LTP and impair established long-term memories. However, a deficit of memory retrieval cannot be ruled out. Furthermore, ZIP, and in high enough doses the control peptide scrambled ZIP, was recently shown to be neurotoxic, which may explain some of the effects of ZIP on memory impairment. PKMζ knockout mice show normal learning and memory. However, this is likely due to compensation by protein-kinase C iota/lambda (PKCɩ/λ), which is normally responsible for induction of LTP. It is not clear how, or if, this compensatory mechanism is activated under normal conditions. Future research should utilise inducible PKMζ knockdown in adult rodents to investigate whether PKMζ maintains memory in specific parts of the brain, or if it represents a global memory maintenance molecule. These insights may inform future therapeutic targets for disorders of memory loss.

Keywords: long-term potentiation; long-term memory; memory erasure; PKM zeta; protein kinase; zeta inhibitory peptide.

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Introduction

Understanding the neurobiological basis of learning and memory is a major goal of modern neuroscience. The main processes involved in memory formation are encoding, consolidation, storage, and retrieval (Brem et al. 2013). Encoding is the acquisition of the memory in response to a perceived stimulus. Consolidation involves converting the newly acquired temporary and unstable memory, to a more stable and long-lasting memory. Storage can be classified into short-term memory, which is characterised by a small storage capacity and a rapid temporal decay, and long-term memory, which has a larger storage capacity and slower decay, allowing information to be retained for longer periods. Lastly, retrieval is described as the process of bringing information to the focus of attention (McElree 2006).

In 1921, Richard Semon proposed that memories are stored in physical memory traces within the brain, which he termed the memory engram (Semon 1921). The engram theory hypothesised that learning activates a specific set of neurones causing permanent physical and chemical changes in those neurones. The idea that learning induces physical changes to neurones was strongly supported by the discovery of long-term potentiation (LTP) (Bliss and Lømo 1973). LTP is the long-lasting increase in synaptic efficacy between nerve cells, and is the putative cellular mechanism underlying memory (Lynch 2004). This increased synaptic efficacy is a result of the both pre-synaptic and post-synaptic changes, for example the upregulation of post-synaptic glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Lisman 2009; Shi et al. 1999).

LTP consists of two phases, specifically early- and late-LTP. Early-LTP is triggered by single tetanised stimulation, lasts 2–3 h, and involves modification of pre-existing proteins; this is thought to contribute to short-term memory (Pang and Lu 2004). Late-LTP, is triggered by repeated high frequency tetanised stimulation, lasts hours to weeks, and involves new protein synthesis; this is thought to contribute to long-term memory (Baltaci et al. 2019; Pang and Lu 2004). Given the long-lasting nature of late-LTP and long-term memory, yet transient nature of action potentials and ion channel activity, it can be assumed that there must

be changes to gene expression and protein synthesis in order to maintain both LTP and long-term memory. Indeed, protein synthesis inhibitors block long-term memories but leave short-term memories unaffected (Kelleher et al. 2004; Lynch 2004). Furthermore, LTP is dependent on glutamatergic N-methyl-D-aspartate (NMDA) receptors, activation of which leads to an influx of calcium ions (Ca^{2+}) into the post-synaptic cell (Bliss and Collingridge 1993). This Ca^{2+} influx activates downstream signalling molecules, such as protein kinases, which are responsible for regulating gene expression and protein synthesis (Kandel 2012).

Protein kinase C (PKC) is a family of kinase enzymes that have previously been implicated in the maintenance of LTP and long-term memory (Vianna et al. 2000). The PKC family contribute to many cellular functions through their role in phosphorylating, and thus activating or deactivating, various intracellular signalling proteins (Gould and Newton 2008). The PKC isoforms can be divided into three classes based on their mode of regulation by secondary messengers: (1) conventional (α, β, γ) which respond to Ca^{2+} and diacylglycerol, (2) novel (δ, ε, η, θ) which respond to diacylglycerol only, and (3) atypical (ζ, ι/λ) which are insensitive to Ca^{2+} and diacylglycerol and respond to phospholipids (see Table 1) (Gould and Newton 2008).

The generic structure of conventional, novel, and atypical PKC consists of an active catalytic C-terminal domain, a hinge region connecting the C- and N-terminus, and a regulatory N-terminal domain. The regulatory N-terminal domain binds to and blocks the catalytic C-terminal domain (see Figure 1), a form of autoinhibition that prevents excess activity (Hernandez et al. 2003). Therefore, under basal conditions, PKC isoforms are inactive. Secondary messengers bind to the regulatory domain, inducing a conformation change, which releases the autoinhibition (Hernandez et al. 2003). The secondary messengers that activate the PKC isoforms are rapidly

chelated, and therefore the activation of PKC is transient (Sacktor 2012). However, it has previously been shown that persistent PKC activity is associated with LTP maintenance (Klann et al. 1991). This discrepancy led to the discovery of a constitutively active isoform of protein kinase C-zeta (PKCζ), termed protein kinase M-zeta (PKMζ) (Sacktor et al. 1993).

PKMζ lacks the regulatory N-terminal domain that is typically found in PKC isoforms and is therefore persistently active (Hernandez et al. 2003). This is because PKMζ is synthesised by an internal promoter within the PKCζ gene that only transcribes the catalytic C-terminus (Hernandez et al. 2003). PKMζ can be rendered inactive by zeta inhibitory peptide (ZIP), a cell-permeable synthetic chemical that mimics the regulatory N-terminal domain (see Figure 1) (Ling et al. 2002).

PKMζ is thought to be both necessary and sufficient for the maintenance of LTP (Ling et al. 2002). Furthermore, inhibition of PKMζ, even one month after training, can reverse LTP and 'erase' established long-term memory (Pastalkova et al. 2006). Therefore, PKMζ may represent the substrate for long-term memory maintenance. This may be of importance in disorders of memory impairment such as Alzheimer's disease. In fact, PKMζ has shown to aggregate in the brains of patients with Alzheimer's disease, the significance of which is not yet understood (Crary et al. 2006). Furthermore, increasing levels of PKMζ can enhance already established long-term memories (Shema et al. 2011). These findings may inform new therapeutic targets for Alzheimer's disease, particularly since currently offered treatments are not always effective, and where they are, provide only modest improvements (Herrmann et al. 2011). However, progress in this field relies on the validity of the claims made about the role of PKMζ in the maintenance of memory.

Table 1: classification of PKC isoforms. PKC isoforms are divided into conventional, novel, and atypical subfamilies based on the cofactor(s) that activate them.

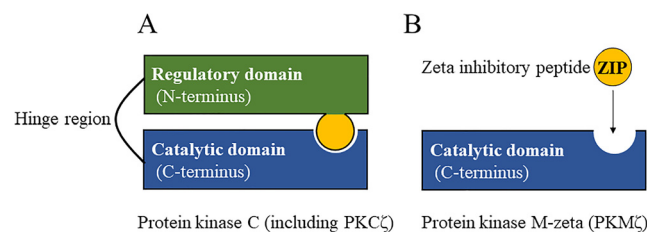| PKC subfamily	PKC isoforms	Cofactor(s)
Conventional	PKC α	Ca^{2+}
	PKC β	Diacylglycerol
	PKC γ	
Novel	PKC δ	Ca^{2+}
	PKC ε	
	PKC η	
	PKC θ	
Atypical	PKC ι/λ	Phospholipids
	PKC ζ	

Figure 1: PKMζ is a constitutively active isoform of the atypical protein kinase PKCζ.
(A) Conventional, novel, and atypical protein kinase C isoforms, e.g. PKCζ, consist of a regulatory N-terminal domain, a hinge region, and a catalytic C-terminal domain. (B) PKMζ is a constitutively active isoform of PKCζ that consists of the catalytic domain only. Zeta inhibitory peptide (ZIP) can mimic the regulatory N-terminal domain and inactivate PKMζ.

The aim of this review is to identify the mechanism by which PKMζ maintains LTP and review the current evidence pertaining to the role of PKMζ in long-term memory maintenance. This will be achieved through a literature review and critical appraisal of primary research papers of *in vitro* and *in vivo* studies involving PKMζ and its inhibition.

PKMζ maintains late-long-term potentiation

If PKMζ has a role in long-term memory, it must also have a corresponding role in LTP. Ling et al. (2002), showed that perfusion of PKMζ to tetanised hippocampal slices enhances synaptic potentiation, which occludes further LTP. However, perfusion of a heat inactivated PKMζ blocks LTP. These findings suggest that PKMζ and LTP enhance synaptic transmission through the same mechanism (Ling et al. 2002). They then went on to show that inhibition of PKMζ, with ZIP, reversed established LTP (Ling et al. 2002). However, ZIP had no effect on a non-tetanised pathway, suggesting that ZIP does not affect baseline transmission (Ling et al. 2002). *In vivo* data show that LTP that was maintained for 22 h after tetanisation in the hippocampus returned to baseline following ZIP injection (Pastalkova et al. 2006). Analysis of the time course of the effectiveness of ZIP revealed that ZIP only reverses LTP when applied hours after tetanisation (Serrano et al. 2005), suggesting that ZIP is only effective in the late phase of LTP. These findings suggest that PKMζ is necessary for the maintenance of late-LTP, since inhibition of PKMζ causes reversal of already established late-LTP (see Figure 2). This section will explore the mechanisms by which PKMζ is thought to maintain late-LTP.

Synthesis and persistence of PKMζ

Under basal conditions, PKMζ mRNA is synthesised from a region of the PKCζ gene, PRKCζ, transcribing only the catalytic domain (Hernandez et al. 2003). The PKMζ mRNA is rapidly transported to the dendrite of neurones (Muslimov et al. 2004), and its translation to PKMζ is inhibited by protein interacting with NIMA 1 (PIN1) (Westmark et al. 2010). On the induction of LTP, NMDA receptor activation and subsequent Ca^{2+} entry activates several protein kinases including protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinase (CAMKII), and mitogen-activated protein kinase (MAPK) (Kelly et al.
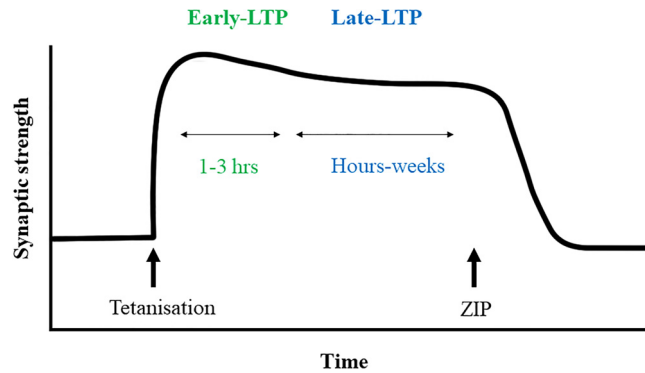

Figure 2: ZIP reverses established LTP.
Schematic showing LTP induced by tetanisation of cultured neurones that can last for hours to weeks. Inhibition of PKMζ with ZIP can reverse established late-LTP. This suggests that PKMζ is necessary for the maintenance of late-LTP.

2007). Activation of these kinases release the translational block on PKMζ mRNA (Kelly et al. 2007). The newly synthesised PKMζ is constitutively active, but requires phosphorylation by phosphoinositide-dependent protein kinase-1 (PDK1) to achieve maximal activation (Kelly et al. 2007).

Once synthesised, PKMζ levels are maintained by a positive feedback mechanism where PKMζ phosphorylates and inhibits PIN1, thus removing the translational block on PKMζ mRNA (see Figure 3) (Westmark et al. 2010).

PKMζ prevents GluA2-dependent AMPA receptor endocytosis and lateral diffusion

PKMζ is thought to maintain LTP by upregulating the number and persistence of post-synaptic AMPA receptors (Ling et al. 2006), an important mechanism in memory and learning (Kessels and Malinow 2009).

Newly synthesised PKMζ is localised to synapses that have recently been activated by a process called synaptic tag and capture (Sajikumar et al. 2005), where a 'tag' is expressed at recently activated synapses that can bind and 'capture' proteins that are essential for maintaining late-LTP (Frey and Morris 1997). N-ethylmaleimide-sensitive fusion protein (NSF) is an ATPase that stabilises GluA2-subunit containing AMPA receptors at the post-synaptic membrane (Nishimune et al. 1998). PKMζ has shown to interact with NSF, since blocking the interaction between NSF and GluA2 receptors disrupts PKMζ-induced synaptic potentiation (Yao et al. 2008). A crucial mechanism of NSF is via its interaction with protein interacting with C kinase-1 (PICK1) (Hanley et al. 2002). PICK1
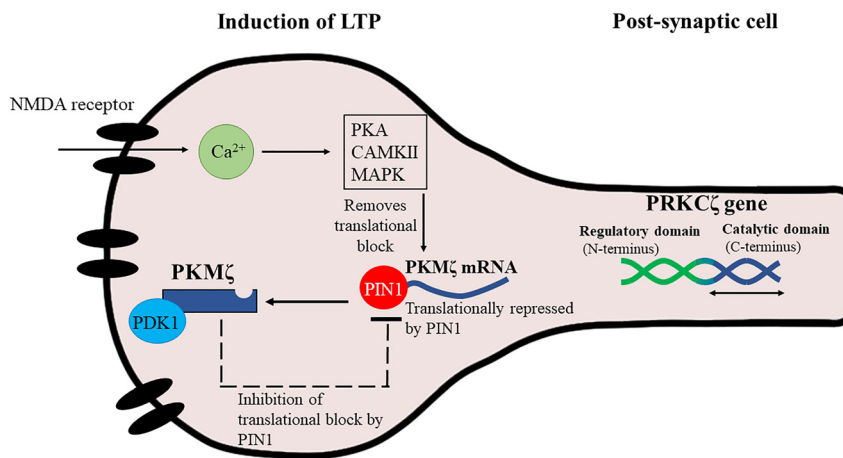
Figure 3: Synthesis of PKMζ during LTP. On the induction of LTP, Ca^{2+} ions flow into the post-synaptic cell, subsequently activating various kinases. The kinases remove the PIN1 mediated translational block on PKMζ mRNA. Newly synthesised PKMζ is phosphorylated by PDK1 to achieve maximum activation. PKMζ levels are maintained via a positive feedback loop. Adapted from Sacktor (2011).

promotes GluA2 receptor endocytosis and stores a pool of extra-synaptic GluA2 receptors (Iwakura et al. 2001). NSF blocks the PICK1-GluA2 interaction, thus preventing endocytosis (Hanley et al. 2002). Together these findings suggest a mechanism whereby PKMζ binds to NSF which promotes its interaction with PICK1 and thus prevents endocytosis of GluA2 receptors (see Figure 4). This is supported by data from Migues et al. (2010), who showed that blocking the endocytosis of GluA2 receptors blocks the ability of ZIP to reverse LTP. Since AMPA endocytosis is an active process (Dong et al. 2015), PKMζ provides a constant opposition and thus shifts baseline AMPA transmission to a more potentiated state. However, the exact nature of the interaction between PKMζ, NSF, and PICK1 cannot be determined solely on these inhibition studies since PKMζ may activate multiple intracellular mechanisms, one of which eventually binds to NSF.

A recent study using quantum-dot imaging, which allows for live tracking of AMPA motility, demonstrated that over-expression of PKMζ in cultured hippocampal neurones increases the number of immobile GluA2 AMPA receptors, suggesting that PKMζ prevents the lateral diffusion of GluA2 receptors (Yu et al. 2017). Lateral diffusion of AMPA receptors is another mechanism by which neurones mediate synaptic plasticity, alongside endocytosis and exocytosis (Borgdorff and Choquet 2002). One mechanism by which PKMζ anchors GluA2 receptors to the post-synaptic membrane may be via its interaction with postsynaptic density protein 95 (PSD95) (Shao et al. 2012). PSD95 is a highly abundant scaffolding protein that stabilises GluA2 receptors at the post-synaptic membrane (Yudowski et al. 2013). PKMζ over-expression increases the amount of PSD95 and this effect is reversed by inhibition of PKMζ (Shao et al. 2012).
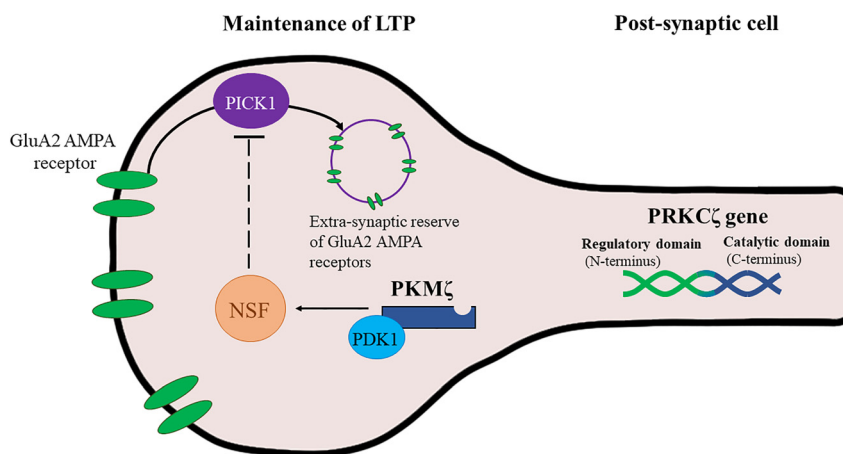

Figure 4: PKMζ inhibits GluA2 AMPA receptor endocytosis. PKMζ binds to NSF which inhibits PICK1 mediated endocytosis of GluA2 AMPA receptors.

PKMζ changes the morphology of dendritic spines

Alongside changes at the synaptic level, structural changes to dendrites are thought to contribute to long-term memory (Basu and Lamprecht 2018). Learning and LTP are associated with morphological changes to the dendritic spine, which are divided into three types: (1) thin, (2) mushroom, and (3) stubby (Bourne and Harris 2008). PKMζ promotes development of stubby spines, which are considered to represent more stable dendritic spines (Ron et al. 2012).

In summary, PKMζ maintains LTP by preventing endocytosis and lateral diffusion of GluA2 AMPA receptors, and by stabilising dendritic spine length. Since PKMζ primarily maintains late-LTP, which is thought to correlate with long-term, rather than short-term, memory (Pang and Lu 2004), it is important to consider if the *in vitro* effects of PKMζ translate to *in vivo* effects on long-term memory.

The role of PKMζ in the maintenance of long-term memory

This section will review studies demonstrating memory 'erasure' by PKMζ inhibition. Although many studies have been carried out investigating the role of PKMζ in different types of memory, this section will focus on spatial memory, conditioned taste aversion, conditioned fear memory, and sensorimotor memory, since these have been most widely studied.

Studies investigating PKMζ use a variety of behavioural assays to measure memory and learning. Behavioural assays discussed in this review are summarised in Table 2.

Spatial memory

Long-term spatial memory is thought to be stored in the hippocampus (Ramos 2000). Pastalkova et al. (2006) studied the role of PKMζ in the maintenance of hippocampal spatial memory in the active place avoidance task (see Table 2). Rats were trained to a level that was maintained for at least one month, and then given bilateral ZIP injections into the hippocampus 2 h before testing. ZIP-injected animals actively explored the shock zone as if naïve, suggesting impairment of one-month old spatial memories. This impairment can be seen up to one month after ZIP injection. Furthermore, injection of staurosporine, an inhibitor of conventional and novel PKC, but not PKMζ

(Ling et al. 2002), had no effect on long-term spatial memory. This suggests that the impairment is not due to inhibition of other PKC isoforms. The rats were able to relearn the same task and cellular staining revealed normal hippocampi suggesting a normally functioning hippocampus. In this study, the rats were not tested in an open field to assess for hyperactivity which may confound the results of the active place avoidance task (Bahník and Stuchlík 2015). However, a follow up study by Serrano et al. (2008) found no effects of intra-hippocampal ZIP injections on locomotor activity. Furthermore, they corroborated the findings of Pastalkova and colleagues by demonstrating ZIP-induced impairment of learned spatial memory in the eight arm radial maze and Morris water maze task (Serrano et al. 2008). Similar findings can be seen in the object location test, a memory assay that does not require conditioning (see Table 2). Hardt et al. (2010) found that intra-hippocampal ZIP injection impaired both 1-day and 6-day old object location memory, with ZIP-injected rats exploring the novel location for the same amount of time as the familiar location, a finding expected of untrained rats (Hardt et al. 2010).

A recent study found higher levels of PKMζ in the hippocampi of rats trained in a long-term memory task, but not in a short-term memory task (Hsieh et al. 2017). Furthermore, when repeated training produced spatial memories that lasted for one month, levels of PKMζ remained persistently elevated for one month (Hsieh et al. 2017). In contrast, when training produced memories that last for one day, but decay by one month, levels of PKMζ persisted for one day and returned to baseline by one month (Hsieh et al. 2017). Therefore, the persistence of PKMζ correlates with the duration of memory retention, further supporting the notion that PKMζ maintains stored memory. Together, these studies demonstrate a role of PKMζ in the maintenance of spatial memory in the hippocampus.

Conditioned taste aversion

Conditioned taste aversion (CTA) is a long-lasting learned association between a particular taste and concurrent illness (see Table 2), that is thought to be stored in the insular cortex (Rosenblum et al. 1993). Shema and colleagues investigated the role of PKMζ in CTA memory (Shema et al. 2007, 2009). They first showed that inhibition of PKMζ via intra-insular cortex ZIP injections impaired established CTA memory, with ZIP-injected rats consuming the conditioned stimulus as if untrained (Shema et al. 2007). This impairment is evident even when tested up to 1 month later, suggesting that the memory impairment is

Table 2: summary of behavioural assays used to measure memory and learning. Spatial memory can be assessed using active place avoidance, eight-arm radial maze, Morris-water maze, and object location. Conditioned memory can be assessed by conditioned taste aversion and contextual/auditory fear conditioning. Procedural memory can be assessed in a reach-to-grasp task.

Assay	Memory modality	Method	Interpretation
Active place avoidance (Pastalkova et al. 2006)	Spatial memory	Rodent placed on a rotating platform that has a fixed region where a mild shock is delivered. This is accompanied by surrounding visual cues. As a shock is delivered the rodent quickly moves to a shock-free zone. After several rounds of repetition, the rodent learns to avoid the shock zone.	Number of entries and time taken to enter the shock zone is measured. Normal spatial memory results in a progressive decrease in the number of entries and a progressive increase in latency of first entry into the shock zone. Can be confounded by locomotor changes.
Eight-arm radial maze (Serrano et al. 2008)	Spatial memory	Rodent placed in an apparatus with 8 arms. 4 arms contain a food pellet and 4 arms do not each, these remain consistent. After training, the rodent learns the location of which arms contain the food pellet and which do not.	Number of times the rodent explores an unbaited arm is measured, termed errors. Normal spatial memory results in a progressive reduction in errors. Can be confounded by locomotor changes.
Morris-water maze (Serrano et al. 2008)	Spatial memory	Rodent has a natural aversion to water. Rodent placed in a chamber containing water with a hidden platform that would render the rodent above water. After training, rodent is quicker at finding the platform.	Time to find platform, path length, and direction are measured. Normal spatial memory results in a progressive decrease in these parameters with repeated training. Can be confounded by loco-motor changes.
Object location test (Hardt et al. 2010)	Spatial memory	Based on rodent's tendency to explore objects in novel locations. Rodent placed in a chamber with 4 objects and allowed to explore and habituate. On testing day, the location of two objects are switched.	Time spent exploring the objects in novel and familiar locations measured. Normal spatial memory results in a longer time spent exploring the novel location compared to familiar location. Can be confounded changes in scent, noise, and anxiety.
Conditioned taste aversion (Shema et al. 2007)	Conditioned memory	Rodent given a neutral food, typically saccharin (conditioned stimulus), which is followed by gastric malaise, typically induced by LiCl (unconditioned stimulus). The rodent forms an association between saccharin consumption and subsequent illness and develops an aversion.	Amount of saccharin consumed is measured. Normal conditioned memory results in reduced consumption of saccharin compared to controls. Can be confounded by motivational changes.
Fear conditioning (Kwapis et al. 2009)	Conditioned memory	Rodent given a cue, such as neutral context or neutral tone, paired with an aversive outcome, typically foot shock. Rodent forms an association between the context/tone and shock. Rodent displays freezing behaviour in response to the context/tone.	Freezing behaviour in response to cue is measured. Normal conditioned memory results in freezing behaviour in response to context/tone. Can be confounded by anxiety.
Reach to grasp task (Kraus et al. 2010)	Procedural memory	Rodent is food restricted and then trained to reach a food pellet through a narrow gap. This requires the rodent to learn to pronate, grasp, supinate, and then eat the food pellet. Rodents are trained in this task until they reach a >80% success rate.	Number of successful reaches measured. Normal procedural memory results in a progressively higher success rate with continued training. Can be confounded by motor impairments.

not transient (Shema et al. 2007). Furthermore, a single application of ZIP, even 3 months after training, can abolish CTA memory, indicating disruption of an enduring mechanism (Shema et al. 2009). On the other hand, when PKMζ is over-expressed, already established CTA memory is strengthened as suggested by a higher aversive index in rats with over-expressed PKMζ compared to those with physiological levels (Shema et al. 2011).

When ZIP is injected into the insular cortex before or directly after CTA training, there is no effect on subsequent

performance in the CTA test (Shema et al. 2007, 2009). Furthermore, Levitan et al. (2016) demonstrated that ZIP is most effective in impairing CTA memory when injected 48 h after training, a period when consolidation of memory is considered to be complete. These findings suggest that PKMζ has a role in memory storage but not acquisition or consolidation. It is interesting to note that, contrary to nearly all other studies on PKMζ, Levitan et al. (2016) used female rats in their experiment. Although the use of female rodents in experiments is necessary, given the sex bias in neuroscience research (Beery and Zucker 2011), some evidence suggests that PKMζ levels vary between male and female rats following long-term spatial training (Sebastian et al. 2013). Whether similar findings are seen in CTA training remains to be investigated.

These findings suggest that PKMζ maintains CTA memory in the insular cortex. However, the encoding of CTA memory requires other brain regions, such as the basolateral amygdala (BLA) (Molero-Chamizo 2017). Gamiz and Gallo (2011) found that ZIP injected into the BLA inhibits CTA acquisition but has no effect on established CTA memory. This suggests that PKMζ may also play a role in the encoding of memory in certain brain regions such as the amygdala. Alternatively, the results may reflect the non-specific effects of ZIP on other PKC isoforms that are critical for memory encoding (Wang et al. 2016), which will be discussed in a later section.

Conditioned fear memory

Encoding, storage, and retrieval of fear conditioned memory, in which neutral cues are paired with aversive outcomes such as a foot shock, is dependent on multiple brain regions including the BLA, hippocampus, and medial prefrontal cortex (Maren et al. 2013).

Serrano et al. (2008) showed that intra-BLA ZIP injections, one day following contextual and auditory conditioning, significantly reduced freezing behaviour compared to control, suggesting impairment of the conditioned fear memory (see Table 2). However, ZIP injection into the hippocampi had no effect on conditioned freezing responses (Serrano et al. 2008). This finding was corroborated by Kwapis et al. (2009), who showed that inhibition of PKMζ with ZIP disrupts one day old auditory fear memory when injected into the BLA but not when injected into the hippocampus. Furthermore, contextual fear conditioning increases levels of PKMζ in the amygdala but not the hippocampus (Oliver et al. 2016). These results suggest that PKMζ maintains conditioned fear memory in some brain regions, such as the amygdala, but not in others, such as

the hippocampus. However, an alternative explanation may be that the hippocampus is involved in the encoding of fear memory which is then stored in the BLA.

The idea that PKMζ maintains conditioned fear memory in the BLA was challenged by Parsons and Davis (2011), who showed that ZIP only impairs memory if tested a few days after injection. Testing fear memory 10 or 15 days after ZIP injection had no effect on memory with rats showing normal freezing behaviour. These findings suggest that the memory loss induced by ZIP is temporary. This contrasts a subsequent study that found impairments of conditioned fear memory 1 week after ZIP injection into the BLA; however, this study used twice the dose of that used by Parsons and colleagues (Gámiz and Gallo 2011). Furthermore, previously described studies have demonstrated memory impairment up to 1 month after ZIP injection; however, these studies used twice the volume of that used by Parsons and colleagues (Pastalkova et al. 2006; Shema et al. 2007). Given these discrepancies, future studies should identify the minimum effective dose of ZIP and the appropriate volume for various brain regions, since the same volume in a relatively larger structure, such as the hippocampus, may not be appropriate in a relatively smaller structure, such as the BLA.

Procedural motor memory

Procedural motor memory is the memory of the skills required to perform a specific task that often works at an unconscious level (Camina and Güell 2017), and is thought to be stored in the motor cortex (Luft et al. 2004). One method of assessing motor learning is a reaching task which involves grasping a food object and placing it in the mouth (see Table 2).

von Kraus et al. (2010) trained rats in the reaching task until maximum proficiency. Subsequent bilateral ZIP injection into the sensorimotor cortex resulted in a success rate similar to that of untrained rats, suggesting disruption of skilled motor memory (von Kraus et al. 2010). Retraining revealed a similar learning curve to that of untrained rats, indicating a lack of memory savings. Memory savings is the more rapid rate of re-learning a skill compared to the original rate of learning (Krakauer and Shadmehr 2006). Given the lack of memory savings, the authors suggest that the skilled motor memory following ZIP injection was 'erased' and since the learning curve of retraining was unaffected, these findings are unlikely to represent cortical damage.

These findings were replicated by Gao et al. (2018), and the authors went on to show that training in the task

resulted in a corresponding increase in PKMζ levels, which peaked as proficiency in the task reached a peak (Gao et al. 2018). Trained rats that were injected with an antisense oligonucleotide directed against PKMζ mRNA showed a more rapid rate of forgetting, with no effect seen with an inactive antisense oligonucleotide. This suggests that the inhibition of PKMζ synthesis disrupted the maintenance of the skilled memory for the task, a form of memory that is stable even without regular training (Gao et al. 2018).

In these studies, memory impairment following ZIP injection was measured by a reduction in the success rate of the learned task. However, in the study by von Kraus et al. (2010), ZIP injected rats were unable to grasp the food despite being able to pronate and accurately reach for food. Therefore, future studies should assess for any potential effects of ZIP on reflex or muscle function.

Long-term memory maintenance by PKMζ

In summary, these studies demonstrate that PKMζ is involved in the maintenance of long-term spatial memory in the hippocampus, CTA memory in the insular cortex, fear memory in the amygdala, and procedural memory in the sensorimotor cortex. This is since inhibition of PKMζ results in impairment of established long-term memory, an effect that is long-lasting, but inhibition of other PKC isoforms has no effect on long-term memory. Furthermore, most studies demonstrate no effect on memory impairment if ZIP is injected prior to training, suggesting that the effects seen are not due to encoding impairments.

Many of the studies conclude that the results do not reflect retrieval difficulties since the impairments in memory can be seen up to one month after ZIP injection, despite ZIP being fully cleared from the brain within 24 h (Kwapis et al. 2012). However, differentiating storage versus retrieval has been a facet of considerable debate in experimental amnesia (Squire 2006). Furthermore, a recent seminal paper has argued that LTP is a function of memory retrieval rather than storage, and that the memory impairments seen with protein synthesis inhibitors are due to a disruption of memory retrieval and not storage. Ryan and colleagues identified and tagged the neural correlates, or engram, associated with contextual fear memory (Ryan et al. 2015). As expected, introduction of a protein synthesis inhibitor into the hippocampus following contextual fear conditioning disrupted the memory. However, optogenetic activation of the engram retrieved the contextual fear memory, suggesting that the amnesia induced by protein synthesis inhibitors is due to a retrieval rather than storage problem (see Ryan and Tonegwa 2016, for a review). This is

further supported by a study by Gisquet-Verrier et al. (2015). In their study, injection of the protein synthesis inhibitor cycloheximide into the hippocampus impaired inhibitory avoidance memory. However, re-administration of cycloheximide, prior to training, reversed the memory impairment. Similar findings were demonstrated with the non-amnestic agent Lithium Chloride. These findings suggest that the injection of the amnestic agent and subsequent retraining caused the memory to become less accessible, due to state dependent recall, i.e. a retrieval problem, rather than a direct effect of the protein synthesis inhibitor on memory storage.

Although experimental protocols exist for differentiating storage versus retrieval in complex memory tasks, such as the Morris-water maze (Hoz et al. 2004), such protocols are not currently available for more simple memory tasks such as CTA. Therefore, it remains to be determined if the memory impairment induced by inhibition of PKMζ with ZIP represents a storage or a retrieval problem in these memory studies. Nevertheless, the studies involving memory erasure with ZIP raise several other key questions.

Firstly, since ZIP is cleared from the brain within 24 h, and therefore the inhibition of PKMζ is temporary, why is the disruption of memory is permanent? Following the conventional theory that the disruption is of memory storage rather than retrieval, one possible explanation is that the initial inhibition of PKMζ disrupts the positive feedback loop on its own synthesis (see Figure 4). When PKMζ levels eventually recover, the synaptic 'tag and capture' that localised PKMζ to that synapse at the induction of LTP is lost (Sacktor 2011). Therefore, the recovered PKMζ is no longer able to potentiate the specific synapses encoding a memory. This is supported by a study by Xue and colleagues who showed that extinction training, to partially extinguish conditioned place preference memories associated with drug cues, results in a subsequent decrease in PKMζ levels in the basolateral amygdala (Xue et al. 2012). This model should be further clarified at the synaptic level using live fluorescent imaging of anti-PKMζ antibodies demonstrating PKMζ levels and PKMζ trafficking following ZIP infusion. Secondly, why does inhibition of PKMζ not impair all established memories? ZIP has shown to reverse established LTP (Ling et al. 2002) but have no impact on long-term depression (LTD) (Sajikumar et al. 2005). In the hippocampus, encoding of spatial memory is thought to occur via an LTP-dependent mechanism (Kemp and Manahan-Vaughan 2007). However, contextual information such as features and orientation in space may be encoded and stored via a LTD-dependent mechanism (Kemp and Manahan-Vaughan 2007). Since

ZIP does not affect LTD, it may only impair memories that are dependent on LTP. This is supported by data from Serrano et al. (2008), which showed that intra-hippocampal ZIP injection impairs place memory but not contextual memory. However, it remains to be determined if an LTD-dependent mechanism encodes certain forms of memory in other brain regions, such as the insular cortex, amygdala, and sensorimotor cortex, to explain the very specific memory impairments seen with ZIP injection into these regions. Lastly, what role does PKMζ play in the modification of memories? Memories are updated during a process called reconsolidation where they become transiently amenable to modification (Lee et al. 2017). Fear memories that have recently been activated are resistant to the effects of ZIP injected into the prelimbic cortex, but not the broad-spectrum PKC inhibitor chelerythrine (da Silva et al. 2020). The effects of ZIP are re-established after 6 h. This suggests that other PKC isoforms are involved in the memory reconsolidation process and PKMζ in the maintenance of the reconsolidated memory. However, Levitan and colleagues found that the effect of ZIP in the gustatory cortex is only re-established 45 h after re-activation of conditioned taste aversion memory (Levitan et al. 2016). Furthermore, both of these studies are not consistent with the findings of Crespo and colleagues, who showed that ZIP injected into the nucleus accumbens does inhibit drug memory reconsolidation (Crespo et al. 2012). Therefore, the precise role of PKMζ in memory reconsolidation is not clear and this may differ between different brain regions and different types of memory.

The studies demonstrating reversal of LTP and 'erasure' of memory using ZIP make a convincing case for the role of PKMζ in the maintenance of long-term memory in certain brain regions. However, the use of ZIP has recently been called into question.

The use of ZIP to study PKMζ

Many studies investigating the role of PKMζ have relied heavily on the use of ZIP as a specific inhibitor of PKMζ. These studies demonstrated the specificity of ZIP by comparing its effects to that of chelerythrine, a benzophenanthridine alkaloid that strongly inhibits PKMζ both *in vitro* and *in vivo* (Ling et al. 2002; Serrano et al. 2008). However, emerging evidence suggests that ZIP may be both non-specific and neurotoxic.

Wu-Zhang et al. (2012) used light microscopy techniques to detect the protein–protein interactions between PKMζ and ZIP. PKMζ was virally over-expressed in cell lines and hippocampal brain slices in which ZIP failed to inhibit

PKMζ, even at doses well above that used in most studies (Wu-Zhang et al. 2012). Therefore, the authors conclude that ZIP is not an inhibitor of PKMζ and that the effects of ZIP on LTP and long-term memory are likely due to effects on other cellular substrates. However, in this study, the authors did not quantify the level of over-expression of PKMζ compared to endogenous levels. Furthermore, in an experiment replicating the methodology of Wu-Zhang and colleagues, Yao et al. (2013) found that the protocol used to over-express PKMζ produced a 30-fold increase in PKMζ relative to endogenous levels, a level at which the dose of ZIP used would have little effect on PKMζ. However, at physiological levels of PKMζ, they found ZIP to be a potent inhibitor of PKMζ (Yao et al. 2013).

In contrast to the study by Yao and colleagues, Bogard and Tavalin found that ZIP, at the same dose that was used in the previous studies, binds non-selectively to several PKC isoforms including PKMζ, PKCζ, and PKC-iota/lambda (PKCι/λ) (Bogard and Tavalin 2015). They found that ZIP has a higher affinity for PKMζ compared to other PKC isoforms; however, this is likely since ZIP must compete with the endogenous regulatory domain that is found in full length PKC isoforms but not in PKMζ.

The findings that ZIP is not an inhibitor of PKMζ (Wu-Zhang et al. 2012) or that ZIP binds non-specifically to multiple PKC isoforms (Bogard and Tavalin 2015) conflicts with previously described data demonstrating the impairment of memory with ZIP but not with the broad-spectrum PKC inhibitor staurosporine (Pastalkova et al. 2006).

One potential explanation may be ZIP related neurotoxicity. Sadeh et al. (2015) showed that typical doses of ZIP induce excitotoxic cell death in hippocampal cultures. These effects were even seen, albeit at higher doses, for scrambled ZIP (Sadeh et al. 2015), an inactive form of ZIP that is universally used as a negative control in most of the *in vitro* and *in vivo* studies described. In addition, LeBlancq et al. (2016) demonstrated that ZIP reduced neural activity in a similar pattern to that of the anaesthetic agent lidocaine, suggesting neural silencing effects of ZIP. This may be secondary to the cell death described by Sadeh et al. (2015). Together, these findings suggest that some of the memory impairment seen with ZIP may be due to neurotoxic effects resulting in a reduction in neural activity and this is consistent with findings that show that ZIP can reduce baseline synaptic transmission (Pastalkova et al. 2006; Volk et al. 2013). However, several studies have shown that animals are able to relearn tasks, possibly suggesting a lack of neurotoxicity (Pastalkova et al. 2006; von Kraus et al. 2010). Furthermore, they do not explain the amnesia seen 24 h to 1 month after ZIP injection, a point at

which ZIP is considered to be fully cleared from the brain (Kwapis et al. 2012).

In summary, studies investigating the role of PKMζ may be confounded by the non-specific and neurotoxic effects of ZIP. Therefore, the use of transgenic mice to specifically disrupt PKMζ synthesis may provide more insights into the role of PKMζ in long-term memory.

PKMζ knockout mice

Two independent studies using transgenic mice have suggested a less significant role for PKMζ. Lee et al. (2013) and Volk et al. (2013) developed PKMζ knockout mice by targeting the catalytic domain of the PRKCζ gene (see Figure 4). Western blot analysis of PKMζ confirmed the deletion. In these studies, PKMζ knockout mice showed no abnormal morphological changes, unusual behaviours, or locomotor activity changes compared to wild-type mice.

Volk et al. (2013) demonstrated that knockout mice showed normal induction and maintenance of LTP (Volk et al. 2013). Furthermore, the knockout mice were able to learn and remember both auditory fear conditioning and the Morris water maze task (Volk et al. 2013). Lee et al. (2013) demonstrated that PKMζ knockout mice showed no difference in learning conditioned fear, object recognition, rotarod motor skill memory, and conditioned place preference tasks compared to wild-type mice (Lee et al. 2013). These results suggest normal synaptic plasticity, memory and learning in mice lacking PKMζ, and conflict with previous findings that PKMζ is an essential memory maintenance molecule. Furthermore, ZIP reversed established LTP and long-term memory in both PKMζ knockout mice and wild-type mice. These findings further support emerging evidence that ZIP may have non-specific effects on targets other than PKMζ.

However, in these studies, the use of transgenic mice disrupts PKMζ expression before the induction of LTP, the point at which PKMζ is typically synthesised. Therefore, compensatory mechanisms may develop in these mice. Indeed, Tsokas et al. (2016) showed elevated levels of another PKC isoform, PKCι/λ, in PKMζ knockout mice (Tsokas et al. 2016). Inhibiting PKCι/λ reverses LTP and impairs established spatial memory in PKMζ knockout mice but not in wild type mice. Furthermore, specifically inhibiting PKMζ synthesis using RNA oligonucleotides, blocks late-LTP induction and spatial long-term memory in wild-type mice but not PKMζ knockout mice (see Figure 5). Together, these findings suggest that in wild-type mice,
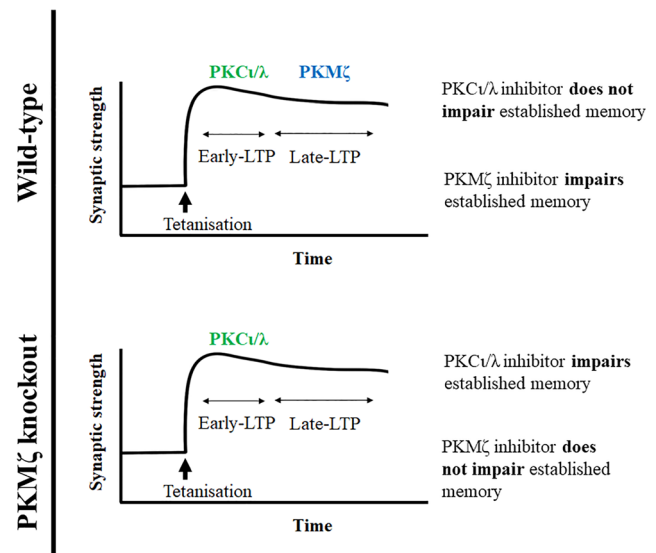

Figure 5: Compensation by PKCι/λ in PKMζ knockout mice. In wild-type mice, inhibition of PKMζ, but not PKCι/λ, reverses established memory; this is because in wild-type mice PKCι/λ is involved in early-LTP only. In PKMζ knockout mice, inhibition of PKCι/λ reverses established memory; this is because in knockout mice PKCι/λ is involved in both early and late-LTP due to a compensatory mechanism. This model is based on the studies by Lee et al. (2013), Tsokas et al. (2016), and Volk et al. (2013) described above.

PKMζ maintains LTP and long-term memory; however, in PKMζ knockout mice, there is a compensatory mechanism by PKCι/λ.

Under normal conditions, PKCι/λ is necessary for the induction of LTP by upregulating GluA1 AMPA receptors at the post-synaptic membrane (Ren et al. 2013). PKCι/λ levels transiently rise during induction of LTP and knockdown of PKCι/λ disrupts LTP formation (Wang et al. 2016). As described previously, PKMζ is necessary for the maintenance of LTP by upregulating GluA2 AMPA receptors at the post-synaptic membrane. Therefore, under normal conditions, PKCι/λ is necessary for LTP induction, and PKMζ is necessary for LTP maintenance (Wang et al. 2016). In PKMζ knockout mice, PKCι/λ can compensate (see Figure 5), and vice versa (Sheng et al. 2017).

This model would explain the findings that ZIP impairs LTP and long-term memory in PKMζ knockout mice since ZIP can also bind to and inhibit PKCι/λ (Bogard and Tavalin 2015). However, since ZIP has shown to be non-specific and neurotoxic to cultured hippocampal cells, the use of ZIP to specifically study PKMζ should be limited. Instead, studies should make use of short hairpin RNA (shRNA) to specifically disrupt PKMζ synthesis without affecting other PKC isoforms (Dong et al. 2015). Indeed, Wang and colleagues have shown that shRNA knockdown of PKMζ disrupts established hippocampal long-term memory (Wang et al.

2016). A similar approach should be applied to future studies on other forms of long-term memory.

Conclusion

Maintenance of LTP is considered to be the substrate of long-term memory. PKMζ, a constitutively active isoform of the PKC family, is thought to maintain LTP and long-term memory by preventing AMPA receptor endocytosis, thus potentiating synaptic efficacy. In this capacity, PKMζ has shown to maintain memory in certain brain regions such as the hippocampus, amygdala, and neocortex. However, this evidence is largely based on the use of ZIP, a synthetic chemical based on the regulatory domain of full-length PKC isoforms, that is considered to be a specific inhibitor of PKMζ. Emerging evidence suggests that ZIP may be non-specific and neurotoxic, particularly since ZIP can impair memory in PKMζ knockout mice. Future research on PKMζ should consider using shRNA technology to specifically disrupt synthesis of PKMζ whilst leaving other PKC isoforms unaffected.

Under normal conditions, induction of LTP leads to a transient rise in PKCι/λ followed by a more permanent rise in PKMζ to maintain LTP. In PKMζ knockout mice, the rise in PKCι/λ is sustained. This may explain findings that PKMζ knockout mice show normal synaptic plasticity, learning, and memory. Future research should attempt to identify if, and how, this compensatory mechanism becomes activated under normal conditions.

A significant difficulty in experimental amnesia is differentiating between storage and retrieval deficits. Many studies conclude that the effects seen are not due to retrieval deficits since memory impairments can be seen even after ZIP has been fully cleared. However, given that ZIP has shown to have neurotoxic effects, a long-lasting retrieval deficit cannot be ruled out. The development of experimental protocols that can differentiate between storage and retrieval deficits will be an invaluable tool in the future of memory research. Alternatively, future studies could implement *in vivo* electrophysiology techniques to determine the neural correlates of memory acquisition, for example if memory acquisition results in particular neural oscillations, or brainwaves. In animals where stored memory is considered to be 'erased', the presence of a particular 'memory acquisition oscillation' upon retraining may suggest that the animal is learning anew, and therefore that the memory was erased.

Understanding the molecular mechanisms of memory is an ongoing challenge for neuroscientists. PKMζ has received particular attention as a candidate molecule for the maintenance of long-term memory. Furthermore, some studies have shown enhancement of memory when PKMζ is over-expressed. Future research should identify if this memory enhancement can be seen in animal models of dementia, such as Alzheimer's disease, to inform on future therapies for the memory deficits seen in these disorders.

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